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Foreword

Located in Ames, IA, the National Animal Disease Center (NADC) is the largest US federal animal health research facility focused on high-impact endemic diseases of livestock and wildlife. In 2009, the Center moved into new $470M state-of-the-art facilities that conduct high-level bio-containment research in large animal livestock and wildlife species. The newly constructed facilities are among the most extensive and advanced high-containment large animal disease research facilities in the world; there are probably fewer than five comparable facilities worldwide. These state of the art facilities combined with concurrent advances in the scientific fields of genomics, microbial ecology, immunology, and systems biology are converging to create unprecedented opportunities for NADC scientists to build upon their strong tradition of leadership in animal health research to create a new center that can once again define innovation and global leadership in animal health and food safety research.

The NADC's historic mission has been to conduct basic and applied research on selected endemic diseases of economic importance to the U.S. livestock and poultry industries. Research from NADC has impacted nearly every major animal disease control or management effort in the US since 1961. For example, diagnostic tests and vaccines developed at NADC to detect and prevent Hog Cholera Virus were integral for ultimately eradicating this costly disease of swine from the U.S. Most major veterinary vaccines for critical diseases such as Brucellosis in cattle, Leptospirosis in cattle, Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), and Pseudorabies Virus in swine had their research origins or were developed and tested at the NADC.

The NADC's current business focuses around four strategic research themes that include ruminant diseases and immunology; emerging diseases (most notably viral and prion diseases); zoonotic diseases in wildlife and livestock species; and, microbial ecology in food safety and animal health. NADC researchers across all four of these strategic themes are pioneering early development and integration of high-throughput genomics and systems biology platforms to yield new and exciting breakthroughs in potential molecular-based detection and diagnostic technologies. For example, NADC scientists have sequenced and analyzed the genomes and expressed proteins from several strains of the causative bacteria for Johnes disease. Using this knowledge, these scientists recently identified highly specific protein markers that show promise in detecting early stage pre-clinical infected animals so they can be isolated and culled before shedding the organism or developing clinical disease. Johnes disease costs the US dairy industry over $1B annually, and having a reliable pre-clinical diagnostic test would be a major breakthrough for better controlling and minimizing the impact of this disease. NADC researchers led the national agricultural research response to the novel 2009 pandemic H1N1 influenza virus. Since 2009, NADC's influenza research team has conducted successful proof-of-principle research demonstrating the potential power and validity of a comparative genome sequencing-based approach to early diagnosis and analysis of newly emergent strains of influenza virus.

NADC Research Units (2012)

Food Safety and Enteric Diseases Unit –
Dr. Thad Stanton, Research Leader

Virus and Prion Diseases Research Unit –
Dr. Marcus Kehrli, Research Leader

Infectious Bacterial Diseases Research Unit –
Dr. Steve Olsen, Research Leader

Ruminant Diseases and Immunology Research Unit –
Dr. Eduardo Casas, Research Leader

We hope that you enjoy reviewing this compilation of the NADC's published research for 2010.

Best Regards,

Kurt A. Zuelke, DVM, Ph.D.
Director, USDA ARS NADC

2010 Research Highlights

- Change in predominance of circulating bovine viral diarrhea virus over a 20-year time span. BVDV strains isolated in years 1988, 1998, and 2008 were characterized and the prevalence of BVDV1a, BVDV1b, and BVDV2a strains were determined. The proportion of field strains identified as BVDV1a showed a steady decrease over the observed time span while the proportion of BVDV1b strains showed a steady increase. These findings contribute to a growing body of evidence that suggests BVDV1b strains need to be added to vaccines for improved control.

- Determined that bovine immune cells could convert vitamin D into a form that will alter gene expression in bovine immune cells. The goal of this work is to determine the role of vitamin D in the health and well-being of a dairy cow and to determine that level of vitamin D necessary for full immune cell function.

- Demonstrated that the live attenuated vaccine Mycobacterium bovis Calmette-Guerin persists in tissues of White-Tailed Deer for up to 9 months after vaccination. The attenuated live vaccine was primarily detected in lymphoid tissues without evidence of colonization of muscle (i.e., meat potentially consumed by humans); thus, the risk of transmission to humans is minimal. These findings underscore the necessity of detailed safety studies for use of vaccines intended for wildlife that may be consumed by humans.

- Found that cattle inoculated with the Brucella abortus strain RB51 vaccine were not protected against infection with Brucella suis. Cattle infected with Brucella suis remain negative on some serologic tests used for Brucella abortus diagnosis, whereas other serologic tests were sensitive for detecting infection. This data will be beneficial for state and federal regulatory officials in their efforts to eradicate brucellosis from cattle.

- Identified a novel antigen expressed uniquely by Mycobacterium avium subspecies paratuberculosis. This gene is expressed and protein produced from it only with Mycobacterium avium subspecies paratuberculosis strains tested thus far.

- An experimental homologous 2009 A/H1N1 vaccine provided optimal protection with no shedding or clinical disease. Full licensure was granted to a commercial company from the USDA for this vaccine to help protect pigs from the 2009 pandemic H1N1 strain of influenza.

- Identified a strain of Haemophilus parasuis that doesn't cause disease but protects against infection with disease causing strains. Strains of H. parasuis that don't cause disease can be used as a vaccine to protect pigs against disease causing strains, and DNA sequencing can now be used to compare the genomes of the different isolates for identification of genes contributing to the ability of H. parasuis to cause disease.

- Inoculation of domestic and European bovine spongiform encephalopathy (BSE) isolates into cattle. To date, no side-by-side comparison of domestic BSE has been made with European BSE isolates. Between February 16-18, 2010, ARS researchers at Ames, Iowa, inoculated cattle with domestic and European BSE isolates. This study is expected to last at least 2 years before all animals will show signs of clinical disease and will provide the first direct comparison of these isolates and provide sufficient material for future studies of BSE. The start of research efforts on 24 milestones characterizing atypical versus classical BSE are dependent on tissues obtained at the completion of this animal study.

- Production of cattle containing the rare 211K PRNP allele associated with genetic bovine spongiform encephalopathy (BSE). The 211K PRNP allele identified as being associated with genetic BSE had been identified in only 1 living animal, limiting the ability to actively study the impact of this allele on BSE. ARS researchers at Ames, Iowa, with cooperators at Iowa State University, produced 13 calves to date (about half containing this allele) using superovulation and embryo transfer. Production of these calves initiates a long-term animal study testing the hypothesis that this rare, naturally-occurring allele is a cause of genetic BSE in older cattle. This also enables ARS scientists to expand and preserve a unique

scientific resource for the study of BSE as some of these calves are now being used to expand the pool of infectious BSE material from the Alabama 2006 BSE case, which is another resource in limited supply, and will ultimately allow proof of the existence of genetic BSE.

- Preruminant calf model for studying the effects of vitamin D status in the neonate. Effects of vitamin D status on the growth and health of the preruminant, milk-fed dairy calf are not well described. Historically, vitamin D deficiency has been associated with disruption of calcium homeostasis resulting in osteomalcia, osteopenia, osteoporosis, and muscle weakness. Recent evidence suggests low plasma vitamin D concentrations not previously considered representative of deficiency are linked to altered immune function and increased infectious disease susceptibility. In addition, justification for the current National Research Council (NRC) recommendation of 600 IU of vitamin D/kg dry matter of milk replacer or the substantially higher concentrations typically found in commercial milk replacers is limited. To enhance our understanding of the impact of vitamin D on the health of the neonatal calf, ARS researchers at Ames, Iowa, are developing methods to manipulate predictably vitamin D status from birth to weaning. The objective of our most recent study was to determine if vitamin D status could be controlled experimentally so as to establish three groups of calves representing low vitamin D status, current NRC recommendation for large breed dairy calves, and the highest dietary level used in the field. All dairy calves were fed the same milk replacer with treatment groups defined by the amount of vitamin D present in the milk replacer. Immunological and metabolic data from this study are being summarized and analyzed. A group of calves from this study were aerosol challenged with bovine respiratory syncytial virus (BRSV), a significant cause of respiratory disease in young calves. This unique approach demonstrated that it is feasible to produce clinical disease in the lung of preruminant calves, providing a potential challenge model for examining the effects of endocrines/micronutrients on infectious diseases susceptibility.

- Effect of plane of nutrition on endocrine and mineral status of preruminant calves. Improvements in growth performance resulting from feeding greater amounts of milk replacer with higher protein concentrations have provided support for intensified or accelerated feeding program during the preruminant phase of development. These programs are thought to increase the plane of nutrition to more “natural” levels and support more “biologically appropriate” early growth. Potential effects of intensified nutrition programs and the associated increased growth rate on endrocrine/mineral levels in preruminant calves are poorly understood. ARS scientists have recently demonstrated that serum insulin concentrations are influenced by nutritionally modulated growth rate during the preruminant period suggesting the preruminant calf may provide a unique tool to investigate the effects of nutrient status on metabolism and appetite regulation in the neonate. A second study considered the effects of neonatal growth rate on endocrine and mineral status of dairy calves fed to achieve no-growth, low-growth, and high-growth rates during the period. Plasma concentrations of vitamins A, E, and D as well as mineral concentrations for all groups remained within ranges considered normal for milk-fed calves; however, plasma vitamin E (RRR-alpha-tocopherol) concentrations were significantly lower in high-growth calves relative to levels in no- and low-growth groups suggesting that the increased growth rate associated with feeding at a high plane of nutrition promotes increased utilization of vitamin E. Further research is necessary to determine if additional dietary vitamin E may be necessary to optimize vitamin E status of calves fed to achieve high-growth rates.

- Metabolic and immunologic responses of the preruminant dairy calf to sustained environmental cold. In the northern United States, calves born in the late winter and early spring often experience sustained periods of cold during the first weeks of life. ARS scientists evaluated the effects of sustained exposure to cold on the growth, health, and select metabolic and immunologic responses of preruminant calves. Results demonstrated when provided with adequate nutrition preruminant calves exhibit a remarkable degree

of adaptability to sustained cold. When compared to warm environment calves, cold environment calves had comparable growth rates likely sustained by their increased intake of starter grain. Immune response variables (i.e., antibody responses to vaccination as well as blood cytokine and inflammatory response proteins) also were unaffected by cold. With the exception of a modest increase in respiratory scores, health of cold-stressed calves was comparable to that of warm environment calves. These results indicate that the successful adaptation of the preweaned dairy calf to sustained cold is linked to the availability of adequate nutrition.

- Stimulation of lung (i.e., alveolar) immune cells from neonatal calves by bacterial components enhances the expression of proinflammatory cytokines in response to bovine respiratory syncytial virus infection. Estimated economic losses to the U.S. cattle industry due to bovine respiratory disease complex (BRDC) may approach $1 billion. The BRDC involves the interplay of viruses, such as bovine respiratory syncytial virus (BRSV) and bacterial pathogens. ARS researchers at Ames, Iowa, evaluated the potential for bacterial derived components (i.e., lipopolysaccharide) to compromise the protective role of alveolar immune cells known as macrophages. Data indicate that when lung macrophages from young calves are co-stimulated with bacterial lipopolysaccharide and BRSV, there is at least a two-fold increase in gene expression levels of proinflammatory cytokines, IL-1beta, IL-8, and IL-23p19, over levels seen with BRSV alone. This suggests a potential mechanism whereby bacterial co-infection could contribute to an enhancement in respiratory lesions induced by BRSV in young calves. Further deciphering of the complex interplay between bacteria and viruses will be critical in developing treatment modalities for BRDC.

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Fluorescence Spectroscopy of the Retina for Diagnosis of Transmissible Spongiform Encephalopathies

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The feasibility of exploiting fluorescence spectra of the eye for diagnosis of transmissible spongiform encephalopathies (TSEs) was examined. Retinas from scrapie-positive sheep were compared with scrapie-negative sheep using fluorescence spectroscopy, and distinct differences in the fluorescence intensity and spectroscopic signatures were observed. The characteristic fluorescent signatures are thought to be the result of an accumulation of lipofuscin in the retina. It appears that the eye, in particular the retina, is a useful tissue for noninvasive examination of some neurological pathologies such as scrapie. The development of procedures based on examinations of the eye that permit the detection of neurological disorders in animals holds great promise.

Neurological disorders of the central and peripheral nervous system are caused by a disease, trauma, or injury to the nervous system. They can be difficult to treat and often are debilitating. Transmissible spongiform encephalopathies (TSEs) are one of many other well-known neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, systematic amyloidosis, and maturity-onset diabetes that involve accumulation of extracellular aggregates leading to tissue damage and disease.[1−4] TSEs are slowly progressive, insidiously degenerative diseases that affect the central nervous system (CNS) of both humans and animals and are usually accompanied by the production of "spongiform" changes in the brain. TSEs are believed to be transmitted by abnormal proteins, which are resistant to enzymatic degradation, called prions and in the case of scrapie, designated as PrP^{sc}.[5]

The TSE of most concern for the food supply is bovine spongiform encephalopathy (BSE), a fatal neurodegenerative transmissible disease in cattle. It is thought to be associated with variant Creutzfeldt−Jakob disease (vCJD) in humans.[6] The oral route of infection is considered to be the most probable path for transmission of BSE to humans.[7,8] In order to reduce the risk of human exposure, specified risk material (SRM, e.g., brain and spinal cord) from cattle is removed during slaughter and processing. Prohibition of SRM in the human food chain is considered of critical importance for protection of consumers from BSE.[9,10] Regulations regarding SRM have been promulgated by the European Commission (Annex V Commission Regulation (EC) No. 999/2001) and for the United States by the Food Safety and Inspection Service (99 CFR 310.22). Many other countries have also banned bovine CNS tissues from meat products, and its presence is cause for import rejection and international trade disputes. Developing technology for monitoring CNS tissue in meat and other food products as well as for diagnosing animals for TSEs will be increasingly important in securing the safety of the world's food supply.

Fluorescence spectroscopy has been commonly used in a variety of biological applications, and its feasibility for the detection of fecal contamination by exploiting fluorescent chlorophyll metabolites on meat during slaughter has been previously reported.[11] Instruments developed for this application are currently being used to screen carcasses in real time at beef processing plants in the United States and France. A similar approach can be applied to CNS detection by exploiting lipofuscin, a highly fluorescent, heterogeneous, high-molecular weight material that has been shown to accumulate in high concentrations in neural

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tissues.[12−17] Our previous work has shown that brain and spinal cord samples display highly conserved spectral characteristics, and these characteristics have potential for use in meat processing applications.[18−20] We have investigated the spectra and spectral intensities of the two most important SRM tissues (brain and spinal cord) and compared the data to non-CNS tissues. On the basis of a study of the spectral signatures of CNS tissue, we have previously suggested the development of devices for detecting CNS tissue in meat products by the rapid monitoring of fluorescence spectra.[19] It is known that the amount of fluorescent lipofuscin in CNS tissue increases as a result of spontaneous and experimentally induced Creutzfeldt−Jakob's disease (CJD). Boellaard et al. have demonstrated a relationship between lipofuscin production, a decline in autophagocytosis activity, and the experimental induction of CJD in mice.[13] Furthermore, there is substantial documentation linking neurological disease produced by TSEs to eye damage and the accumulation of lipofuscin.[13,21−28]

In this work, we investigate the use of fluorescence spectroscopy of the retina to identify scrapie, which we use as a model for TSEs. Scrapie is the most widespread TSE affecting sheep and goats worldwide. It is characterized by a gradual onset; and advanced cases show typical disease characteristics including unthriftiness, compulsive itching, balance and ambulatory abnormalities, convulsions, and eventual death. The disease has been observed for centuries. At present, it is incurable and the most common form of control is quarantine, euthanasia, and proper disposal of the carcass. Scrapie is not considered to be infectious to humans.

While several research groups are actively involved in developing spectral examinations of the eye to assess the extent of macular degeneration and other abnormalities,[27−35] to our knowledge, few have reported on the use of the retinal scans for this particular pathological application.[25,26,36,37] The objective of this work is to find spectral signatures of intrinsic fluorescent markers of eye that can provide a nonlethal and noninvasive means of determining if a living animal is infected with a TSE. Our experiments were designed to address the following questions: (1) Can spectra obtained from the eye be used for identification of neurological disease? (2) Can the effects of neurological disease observed in the eye be distinguished from those associated with normal aging? (3) Can images of the eye be obtained that report on neurological disease? With these questions in mind we have now extended previous work and investigated fluorescence spectroscopy of the retina for potential diagnosis of neurological disease. Our investigations, which have focused on scrapie-positive and scrapie-negative sheep, suggest that the retina is a most promising part of the eye for revealing spectroscopic signatures indicative of neurological disease.

MATERIALS AND METHODS

Materials. DAKO Target Retrieval Solution and biotinylated antimouse IgG (made in horse) were obtained from DAKO Corp., Carpinteria, CA, and Vector Laboratories, Burlingame, CA, respectively. A Basic Alkaline Phosphatase Red Detection Kit and NexES IHC modules were acquired from Ventana Medical Systems, Inc., Tucson, AZ. All other chemicals used for pathological procedures were obtained from Sigma Aldrich.

Animal Tissue Samples. Tissue samples from both scrapie-positive and scrapie-negative sheep were obtained from several sources. Samples from 73 sheep were collected for the study for a total of 140 eyeballs. In some cases only one eye was collected from the animals sampled. Both positive and negative tissue samples were harvested according to a standardized necropsy procedures from sheep identified through the National Scrapie Surveillance Plan administered by the Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture (USDA).[38] Samples were also obtained from the Caine Veterinary Teaching Center, University of Idaho, Caldwell, ID. The identity of individual producers if known has been withheld. Scrapie-negative tissue samples were also obtained locally from sheep at the National Animal Disease Center (NADC), Ames, IA. All procedures relating to the care and experimental use of live sheep used on site at NADC were approved by the NADC Institutional Animal Care and Use Committee. Information regarding breed, age, and sex was also obtained for each tissue sample used in this study (see Table S-1 in the Supporting Information).

Disease Diagnosis. All animals were diagnosed for scrapie infection status using conventional pathological methods. To

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obtain brain tissue, the disarticulated heads of sheep were split near the sagittal midline with a bandsaw. Each half of the brain was carefully removed from the calvarium and one of the halves was immersion fixed in 10% neutral buffered formalin for 2−3 weeks. The formalin-fixed brain was cut into 2−4 mm wide coronal sections at four levels: (1) brainstem at the obex, (2) cerebellum at midlevel lateral lobe, (3) midbrain at the rostral colliculus, and (4) hippocampus and adjacent cerebral cortex. The tissue blocks were placed in histopathology cassettes, dehydrated, and embedded in paraffin wax. Paraffin-embedded tissues were sectioned to 5 μm thickness, affixed to charged glass slides, and stained with hematoxylin and eosin (HE) for examination by light microscopy or left unstained for immunohistochemistry (IHC) processing.

An automated IHC method for detection of infectious prion protein (PrP^{sc}) was used as described previously.[39] In brief detail, following deparaffinization in xylene and rehydration through gradations of alcohol to water, tissue sections were autoclaved (121 °C, 1.38×10^5 Pa) for 30 min in an antigen retrieval solution and labeled with an indirect avidin−biotin system designed for an automated immunostainer. The primary antibody was a cocktail of two monoclonal antibodies, F89/160.1.5[40] and F99/97.6.1,[41] each used at a concentration of 5 μg/mL, and incubation was carried out at 37 °C for 32 min. The secondary antibody was biotinylated antimouse, diluted 1:200. The preparation was incubated for 8 min at 37 °C.

Sample Preparation. The eyeballs collected were stored frozen until use. After thawing, the eyeballs were dissected; and the retina or other eye tissue was removed and placed on a plain glass microscope slide (25 mm × 75 mm × 1 mm). The retina, in particular, was oriented as a thin layer on the microscope slide enabling the entire area of the sample to be exposed to the excitation light.

Steady-State Measurements. Steady-state fluorescence spectra were obtained on a SPEX Fluoromax-2 (ISA Jobin-Yvon/SPEX, Edison, NJ) with a 5 nm band-pass unless otherwise specified and corrected for lamp spectral intensity and detector response. Fluorescence spectra were collected in the front-faced orientation. For the experiments reported in this study, the samples were excited at λ_{ex} = 470 nm with an interference filter on the excitation side, and emission was collected at $\lambda_{em} \geq$ 505 nm using a cutoff filter before the detector to eliminate scattered light. Polarized fluorescence spectra were obtained using two polarizers, with one on the excitation side and another on the emission side with the appropriate excitation interference and cutoff emission filters. Fluorescence spectra were obtained using horizontal−horizontal (HH) and horizontal−vertical (HV) orientations of the polarizers.

Hyperspectral Fluorescence Imaging Microscopy. The hyperspectral images were captured on a system located in the Roy J. Carver Laboratory for Ultrahigh Resolution Biological Microscopy, Iowa Sate University. This system was based on a NIKON ECLIPSE TE 2000-E microscope. The illumination source was an X-Cite 120 PC from EXFO. Samples were excited at 470 nm using an interference filter, and fluorescence was collected at ≥500 nm with a long-pass filter and a Nikon Plan Fluor 10×/0.30 PH1-DL objective. The collected fluorescence was dispersed using a Spectral-DV spectrometer from Optical Insights and captured by a Photometrics Cascade 512 B CCD camera (Roper Scientific). The software used for image capture was Mélange V3.7.

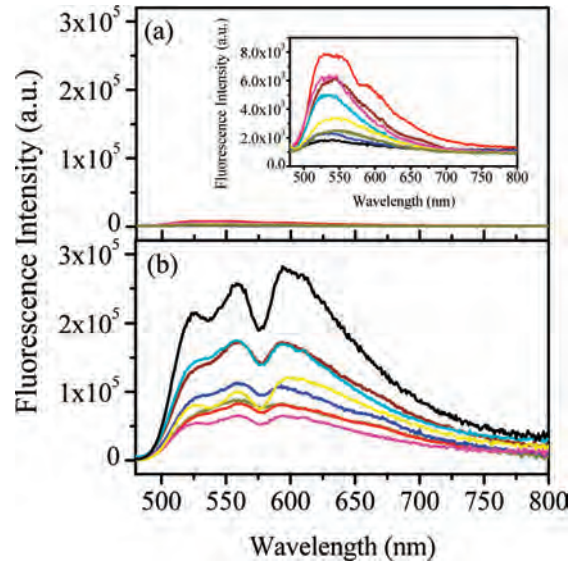

Figure 1. Fluorescence spectra of the retina from scrapie-negative and scrapie-positive sheep. Comparison of (a) scrapie-negative and (b) scrapie-positive sheep retinas at λ_{ex} = 470 nm. Representative, different, front-faced fluorescence spectra from eight individual retinas are shown for each scrapie-negative and scrapie-positive animal. Note that the intensity values are in the same range for both data sets on the primary graphs. The inset graph for the scrapie-negative data uses an expanded ordinate. Significant differences of fluorescent intensity exist between the two data sets.

RESULTS AND DISCUSSION

We studied a total of 140 sheep eyeballs from 73 animals obtained from several sources. A total of 35 of the animals were scrapie-positive and 38 of the animals were scrapie-negative. In the sheep identified as scrapie-negative, there was no evidence of spongiform change or other significant lesions, as assessed by microscopic examination of hemotoxylin and eosin stained brain sections. Likewise, there was no labeling indicative of PrP^{sc} seen in brain tissue sections examined following IHC (immunohistochemical) processing. Conversely, in sheep diagnosed as scrapie-positive, the observation of both lesions and IHC labeling confirmed a positive diagnosis for scrapie.

Fluorescence spectra from various parts of the sheep eye (cornea, iris, lens layer, lens gel, lens, vitreous humor, retina, tapetum, optic nerve, and sclera) were obtained at different excitation wavelengths using a fluorometer in a front-faced geometry. The retinas (and the sclera, see Figure S-1 in the Supporting Information) show large differences in spectral features when comparisons are made between scrapie-negative and scrapie-positive samples. The sclera is the protective tissue that covers the entire eyeball except the cornea. The retina is the preferred part of eye for noninvasive diagnosis of TSE affected animal because it can be directly accessed by light to excite the pigments, in contrast with the sclera. Front-faced fluorescence spectra of solid retina samples from scrapie-negative and scrapie-positive

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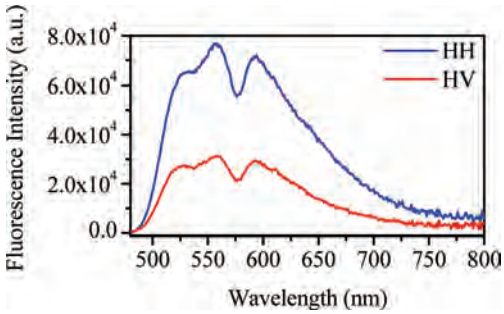

Figure 2. Polarized fluorescence spectra from a scrapie-positive sheep. Spectra were collected in a front-faced orientation. Samples were excited at 470 nm with an interference filter in the excitation light path, and fluorescence was collected after 505 nm with a long pass filter. In order to determine whether the observed structure is due to scattered light, the experiments were conducted with two polarizers in either of two positions: HH (horizontal excitation−horizontal emission) or HV (horizontal excitation−vertical emission). The data indicate that the spectral structure observed is authentic and not an artifact.

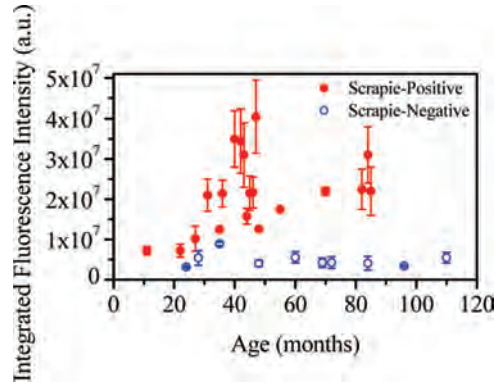

Figure 3. Plot of the integrated fluorescence intensity from scrapie-positive and scrapie-negative sheep retinas as a function of age. All points represent multiple spectroscopic determinations with standard error bars. Those with visible error bars are an average of 4−12 experimental replications. The remaining data points with error bars smaller than the symbol are with $N = 2$. Fluorescence spectra were collected as described in a front-faced orientation. For all experiments, samples were excited at 470 nm with an interference filter in the excitation light path, and fluorescence was collected at wavelengths greater than 505 nm to eliminate scattering. Note that the differences in total fluorescence due to disease status greatly exceed any differences that result from age.

sheep excited at 470 nm are presented in Figure 1. The data indicate that fluorescence spectra from eyes (especially the retina) are very rich and vary considerably with excitation wavelength (λ_{ex}). When $\lambda_{ex} = 470$ nm, the fluorescence intensity of the scrapie-positive retinas is significantly greater than that of scrapie-negative sheep. The spectra from scrapie-positive samples were also more structured and displayed two intense peaks at ~560 and ~600 nm with a shoulder at ~525 nm. The scrapie-positive samples also displayed a minimum at ~575 nm. The literature indicates that TSE is caused by an accumulation of protease-resistant prions in central nervous system tissues such as brain, spinal cord, and eye.[5] It is also known that lipofuscin accumulation occurs in the diseased eye (e.g., in retinal pigment epithelium).[42−48] The spectral differences in scrapie-positive retinas compared to scrapie-negative retinas can be attributed to the result of altered or increased lipofuscin owing to prion infection.

Given the pronounced structural differences observed in the spectra of the scrapie-positive tissue, additional control experiments were undertaken to ensure that these data were not an experimental artifact, since spectra were collected from solid samples, which may have contributed to excessive light scattering. Although spectral structure was not pronounced in the scrapie-negative samples, it was necessary to determine that the results were independent of the polarization of the excitation radiation. The data in Figure 2 demonstrate that the spectra observed were, indeed, independent of polarization. We suggest that these spectral differences are the result of neurological disease or in this case, scrapie.

It has been reported that the intracellular fluorescent pigment lipofuscin accumulates in the retina with increasing age.[49−51] It is important to distinguish the spectral differences arising from scrapie with respect to those arising from normal aging. A plot of integrated fluorescence intensity obtained from both scrapie-positive and scrapie-negative sheep retinas as a function of age is shown in Figure 3. Samples were obtained from sheep up to 9 years of age (see Table S-1 in the Supporting Information). The data indicate clear differences, with the scrapie-positive retinas being more fluorescent than those from the scrapie-negative retinas. The differences in total fluorescence due to disease status greatly exceed any differences that result from other factors, such as age.

In addition, hyperspectral fluorescence microscopy was used to compare images of retinas with respect to disease status. Representative images from different regions of sex- and age-matched retinas for scrapie-negative and scrapie-positive sheep are displayed in Figure 4. The scrapie-positive retina shows significantly higher fluorescence intensity as opposed to the scrapie-negative retina, as indicated by the grayscale intensity gradient of the images. This result is also consistent with the data presented in Figures 1 and 3 indicating that the scrapie-positive retinas are more fluorescent than those of scrapie-negative retinas. The scrapie-positive images also contain aggregates of fluorescent material, as indicated by the arrows in Figure 4 and are more heterogeneously fluorescent. In contrast, the scrapie-negative

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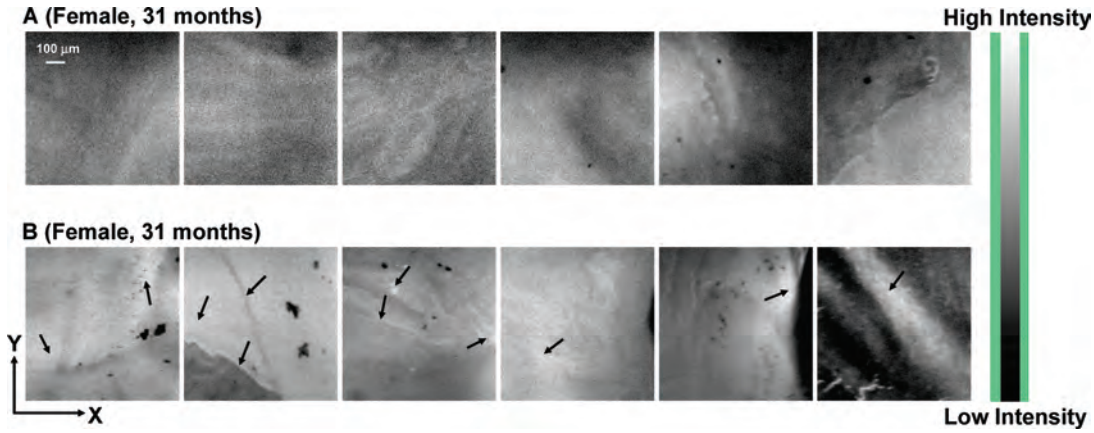

Figure 4. Hyperspectral fluorescence images of the retina of sheep. Representative images from different regions of sex- and age-matched (31 months) (A) scrapie-negative and (B) scrapie-positive sheep retinas. The samples were excited at 470 nm using an interference filter, and fluorescence was collected at wavelengths greater than 500 nm using a long-pass filter. The images shown are 800 μm by 800 μm. All images were captured under identical conditions. High intensity is represented by white; low intensity by black on a grayscale gradient. Note the more intense fluorescence and aggregation of fluorescent material in the scrapie-positive images.

tissue is more homogeneous and is weakly fluorescent. This further supports our observations from front-faced fluorescence spectra that scrapie-positive retinas show higher fluorescence with prominent structure. From this comparison of age-matched retinas, we conclude that the scrapie-positive retinas contain very bright regions of highly fluorescent material, which is suggestive of neurological damage as a result of disease.

Others have reported observations that support this current research. Recently Smith et al. have shown that the function and morphology of retinas are altered in TSE-infected cattle and sheep infected with scrapie.[25,26] Hortells et al. concluded that PrP^{sc} in the retina was highly correlated with the occurrence of scrapie[27] whereas Rubenstein et al. used ultraviolet fluorescent spectroscopy to detect different forms of PrP^{sc} in the eye.[28] Fundus autofluorescence has also been investigated for possible diagnostic use in a large number of retinal diseases[33,35] including the "conformational diseases" caused by accumulation of proteinaceous aggregates.[34] Autofluorescence has also been used to map lipofuscin distribution in the retina of humans.[33] In this study we have not determined the specific structures that are responsible for the fluorescence but others have suggested that lipofuscin and other macromolecular aggregates are responsible.[27,34]

CONCLUSIONS

Given our results, we suggest that the distinct differences in the spectral signatures of the retina are diagnostic of animals naturally infected with scrapie. We further suggest that the eye, and in particular the retina, will be a useful tissue for noninvasive determination of neurological pathologies such as scrapie.

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SUPPORTING INFORMATION AVAILABLE

Fluorescence spectra of sclera and additional information mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Call of the wild: antibiotic resistance genes in natural environments

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Abstract | Antibiotic-resistant pathogens are profoundly important to human health, but the environmental reservoirs of resistance determinants are poorly understood. The origins of antibiotic resistance in the environment is relevant to human health because of the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens. This Review explores the presence and spread of antibiotic resistance in non-agricultural, non-clinical environments and demonstrates the need for more intensive investigation on this subject.

Antibiotic resistance genes in human pathogens such as methicillin-resistant *Staphylococcus aureus*[1] have become notorious because they confound the tools that are used to treat disease (FIG. 1). In particular, resistance determinants in pathogens are commonly encountered after the introduction of an antibiotic to clinical use, and treating human pathogens with antibiotics directly affects the frequency of resistance to those antibiotics in these pathogens[1–4].

The presence of antibiotic resistance elements in pathogenic bacteria is made all the more problematic because of the prevalence of horizontal gene transfer, the process by which bacteria acquire genes from the environment[5]. Many of the known antibiotic resistance genes are found on transposons, integrons or plasmids, which can be mobilized and transferred to other bacteria of the same or different species. There is evidence of the transfer of resistance elements to known human commensal bacteria and pathogens[6,7], and gene transfer in the human intestinal microbiome is extensive[8].

What are the sources and reservoirs of these transferable genes? A full understanding of the pressures and circumstances that lead to the evolution and dissemination of antibiotic resistance genes in pathogens is impossible without a detailed examination of the origin and role of resistance genes in natural environments. This Review discusses the environmental sources of antibiotic resistance, the functions and roles of resistance genes in microbial ecology and the ways by which those genes may be disseminated in response to human antibiotic use.

Selection pressures in the environment

Antibiotics are essential for the treatment of bacterial infections in humans and animals; it is therefore a top priority to preserve their efficacy. For decades, clinicians and scientists have called for the prudent use of antibiotics, in an effort to slow the development and epidemic spread of resistance[9–11]. Prudent use of antibiotics in humans demands that physicians establish that a bacterial infection is responsible for the patient's symptoms before an antibiotic prescription is written. By contrast, in agriculture antibiotics are used in the absence of acute infection. Some of the same antibiotics that are used to treat human pathogens, such as amoxicillin and erythromycin, are also used to treat disease, promote growth and improve feed efficiency in animals[12] (BOX 1). Just as in hospital settings, the agricultural use of antibiotics selects for antibiotic resistance, arguably in a more widely disseminated fashion owing to the farm-wide administration of prophylactic antibiotics in feed and water. Antibiotics from both urban and agricultural sources persist in soil and aquatic environments, and the selective pressure imposed by these compounds may affect the treatment of human diseases[13,14]. As another example, the prophylactic use of antibiotics in fish farms has led to a rise in the number of resistant bacteria[15]. Strikingly, these resistant bacteria can transfer the resistance genes to human pathogens[16]. The selection pressure applied by the antibiotics that are used in clinical and agricultural settings has promoted the evolution and spread of genes that confer resistance, regardless of their origins (FIG. 2).

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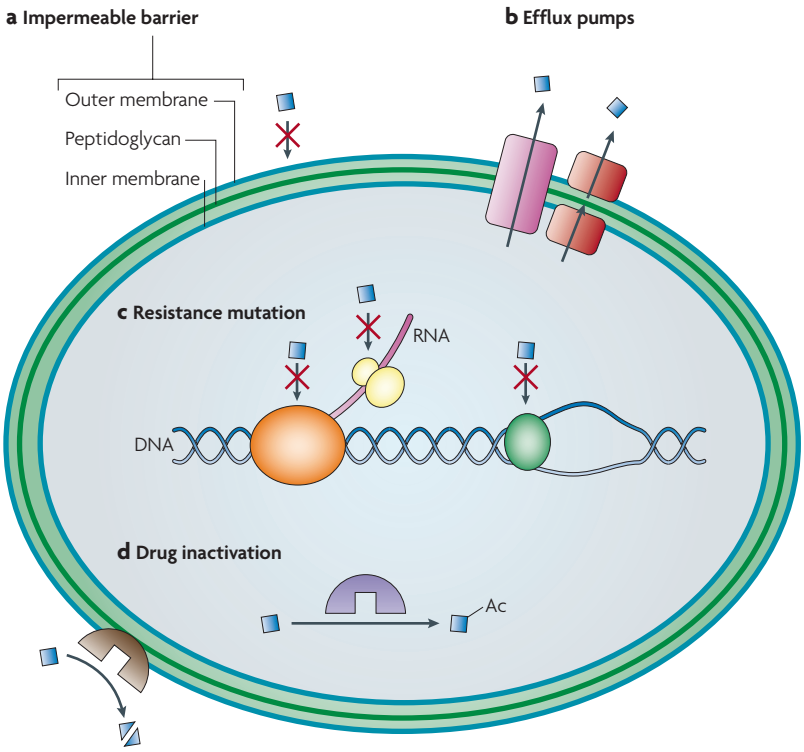

Figure 1 | **Mechanisms of antibiotic resistance in a Gram-negative bacterium.** **a** | Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics (blue squares) simply because they have an impermeable membrane or lack the target of the antibiotic. **b** | Multidrug resistance efflux pumps. These pumps secrete antibiotics from the cell. Some transporters, such as those of the resistance–nodulation–cell division family (pink), can pump antibiotics directly outside the cell, whereas others, such as those of the major facilitator superfamily (red), secrete them into the periplasm. **c** | Resistance mutations. These mutations modify the target protein, for example by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (green), which cause resistance to floroquinolones, in RNA polymerase subunit B (orange), which cause resistance to rifampicin, and in the 30S ribosomal subunit protein S12 (encoded by *rpsL*) (yellow), which cause resistance to streptomycin. **d** | Inactivation of the antibiotic. Inactivation can occur by covalent modification of the antibiotic, such as that catalysed by acetyltransferases (purple) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by β-lactamases (brown) acting on β-lactam antibiotics. Ac, acetyl group.

The use (and misuse) of antibiotics by humans is probably not the only selective pressure for antibiotic resistance in natural microbial communities: compounds and conditions that occur in these communities may provide additional selection pressures. Indeed, most antibiotics are produced by strains of fungi and bacteria that occur naturally in all environments, including soil[17] (FIG. 2). Most antibiotic-producing strains carry genes encoding resistance to the antibiotics that they produce[18,19], and these genes are usually found in the same gene cluster as the antibiotic biosynthesis pathway genes[17,20]. Antibiotics produced in the environment may exert selective pressure on neighbouring organisms. However, it is difficult to determine the natural concentrations of antibiotics in soil microcosms or the extent of the selective pressure that they may pose.

Accidental resistance genes. The presence of various potentially offensive compounds and conditions in nature might select for specific or nonspecific mechanisms of antibiotic resistance (FIGS 1,2). Bacteria cultured from the marine air–water interface were shown to be more highly resistant to antibiotics than bacteria cultured from the bulk water[21], and numerous conditions, including radiation and pollution, that may select for antibiotic resistance in this habitat have been suggested, although the mechanisms of cross resistance to antibiotics are unknown. Some genes confer antibiotic resistance but are likely to have other primary roles in the environment[22]. Certain classes of efflux pumps, for example, offer general mechanisms of resistance, because they pump various toxins, such as heavy metals and other toxic molecules, out of cells[23,24]. For some chromosomally encoded multidrug resistance pumps, such as those of the resistance–nodulation–division family, antibiotic resistance is now thought to be an associated function of the primary role that they serve in the environment[24], which might be, for example, to provide tolerance to toxic compounds. The microbial communities of insect guts that had no known exposure to antibiotics contain efflux pumps that confer resistance to antibiotics when transferred to *Escherichia coli*[25,26]. Both oil fly larvae and gypsy moth larvae ingest compounds that might stress microorganisms; oil fly larvae eat organic solvents, and gypsy moth larvae feed on diverse plants that produce various toxic compounds. In *Shewanella oneidensis*, a bacterium that lives in sediment, the multidrug efflux transporter gene *mexF* enhances fitness in the environment and confers resistance to chloramphenicol and tetracycline in the laboratory[27]. Further information on the roles of efflux pumps in bacteria can be found in REF. 28.

The movement of antibiotic resistance genes

Physical forces. Physical forces, such as those created by wind and watershed, are important drivers of the spread of antibiotic resistance genes (FIG. 2). Antibiotics and their resistance genes have been widely distributed in the environment since before the introduction of antibiotic chemotherapies, but human activities have probably increased the prevalence of resistant bacteria in the air and water. As a result, antibiotic resistance is more common in *E. coli* and *S. aureus* isolates from air inside the home than in isolates from outside[29,30], although a study of sulphonamide resistance in *E. coli* detected more antibiotic-resistant isolates in dust outside homes than in dust inside homes in Mexico[31]. Marine and freshwater ecosystems also contain bacteria from many sources, including antibiotic-resistant bacteria from anthropogenic sources[32]. Even bacteria from environments that are thought to be stationary, such as soil, can be moved by the forces of nature; one example is the intercontinental transport of bacteria on desert dust[33].

Animals. Wild animals provide a biological mechanism for the spread of antibiotic resistance genes (FIG. 2). Proximity to human activities influences the antibiotic resistance profiles of the gut bacteria of wild

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Resistotype
The antibiotic resistance genotype and phenotype of a bacterium.

mammals, which live in densely populated microbial habitats in which antibiotics select for resistance. Ninety percent of the bacterial isolates from mice and voles captured in rural England were resistant to β-lactam antibiotics[34]. By contrast, the faecal enterobacteria of wild elk, deer and voles in Finland have almost no resistance[35]. For the most part, Finland is less densely populated than England; therefore, these findings might suggest that human activities influence antibiotic resistance in bacterial communities in wild animals, although other influences that affect the frequency of antibiotic resistance cannot be eliminated (including differences in the testing methodologies used or variation in the intrinsic antibiotic resistance of the isolate populations). Likewise, a study of *E. coli* isolates from wild animals in Mexico and in Australia found a higher frequency of antibiotic resistance in the isolates from Mexico[36]. It was suggested that this difference may be due to widespread human settlement and use of antibiotics in Mexico along with possible selection pressures from the host animals[36]. Other studies demonstrate a similar association — African baboons and apes that are in contact with humans harbour more antibiotic-resistant enteric bacteria than those that dwell in areas that are remote from human activity[37,38].

Wild birds carry a reservoir of antibiotic-resistant bacteria with the potential for long-distance dissemination. Birds, and migratory waterfowl in particular, can travel great distances and inhabit a wide variety of environments, from agricultural lagoons to remote mountain lakes, and can potentially spread resistance genes along the way. Proximity to human activity increases the number of the antibiotic-resistant bacteria that are associated with wild birds. Gulls and geese nesting near waste or agricultural water harbour more antibiotic-resistant *E. coli* than do birds associated with unpolluted water[39,40]. Antibiotics also seem to affect resistance in remote bird populations: in arctic birds, 8% of *E. coli* isolates were recently found to be resistant to at least 1 of 17 antibiotics tested, and 4 were resistant to 4 or more antibiotics[41]. One isolate was resistant to cefadroxil, cefuroxime and cefpodoxime, a pattern that is common in clinical isolates[41]. Many birds breed in the arctic and migrate to up to six continents. They probably acquire antibiotic-resistant bacteria either from environments that are under human influence or from other birds that contact those environments, illustrating the great geographical distances that can be travelled by resistance genes that are associated with human selective pressures.

One study looking at the effect of the proximity of human activity on the presence of antibiotic resistance genes found no antibiotic-resistant *E. coli* from remote animal populations but notable populations of antibiotic-resistant *E. coli* in animals that were proximal to anthropogenic activity[42]. Animals that live with humans, including pets such as cats and dogs, are reservoirs of antibiotic-resistant bacteria as a result of both antibiotic treatment for disease and the transfer of resistant bacteria from humans[43]. The data suggest that exposure to antibiotics has affected antibiotic resistance in the enteric bacteria of wild animals, although many more studies using standardized methods are required to define this impact. More complete profiles of antibiotic resistance in wild animals will contribute to our understanding of the origins and roles of antibiotic resistance genes in natural intestinal microbial communities, which, in turn, will help us manage emerging zoonotic diseases.

Humans. Antibiotic-resistant bacteria have been found in even the most secluded communities, although proximity to dense human populations affects the antibiotic resistotypes that are found. For example, although antibiotic-resistant *Salmonella* and *Shigella* species were isolated from humans living far from Kathmandu, Nepal, there were far fewer than were isolated from humans living near to the city[44]. High levels of antibiotic resistance were also found in *E. coli* from an isolated human population in Bolivia[45], and the resistance genes in the remote community (such as β-lactamase TEM (*blaTEM*)-like genes and the aminoglycosyl adenyltransferase gene *aadA1*) closely matched genes from antibiotic-exposed environments[46]. As this population has little access to modern health care, and contact with people outside the community is minimal, the results show that antibiotic resistance in this remote community is entirely due to a diverse array of resistance genes that had immigrated from elsewhere. Despite the barriers, antibiotic resistance genes have been transmitted to the most isolated human populations, where they exist even in the absence of an obvious selection pressure.

Box 1 | **Antibiotics in food production**

Antibiotics are used in diverse settings for food production. Animals are treated with antibiotics for both curing disease and promoting growth[7], fruit trees are often treated prophylactically with antibiotics to control bacterial infections[98], and aquaculture relies on antibiotics to manage infectious disease[15]. In each of these situations, the effects of the antibiotics extend beyond the site of use. Antibiotics applied in animal farming operations leach into waterways and groundwater; in many aquaculture settings, antibiotics diffuse into the water surrounding the pens; and antibiotics sprayed on plants can drift aerially.

The Food and Agriculture Organization (FAO) of the United Nations has the task of monitoring and compiling global statistics about the international regulation of pesticide use. The philosophy and legality of the administration of antibiotics in agricultural settings vary among countries. In 1985, Sweden enacted the Feeding Stuffs Act, which outlawed the administration of antibiotics to livestock for growth promotion[99], in stark contrast to the many industrial countries that use vast quantities of antibiotics for this purpose. Even countries that have adopted similar overall patterns of regulation vary in the specifics. For example, the United Kingdom, the United States, Norway, Mexico, India and Indonesia have approved the use of oxytetracycline in aquaculture, whereas among these same countries only Mexico and Indonesia permit the use of enrofloxacin[100].

Some governments have taken action in response to public concern about the use of antibiotics in agriculture. Consumers are also directly shaping farm practices, leading McDonald's to purchase only antibiotic-free beef for its globally sourced restaurants[101]. As more large corporations and governments follow suit, antibiotic application practices in agriculture might be reduced and standardized globally.

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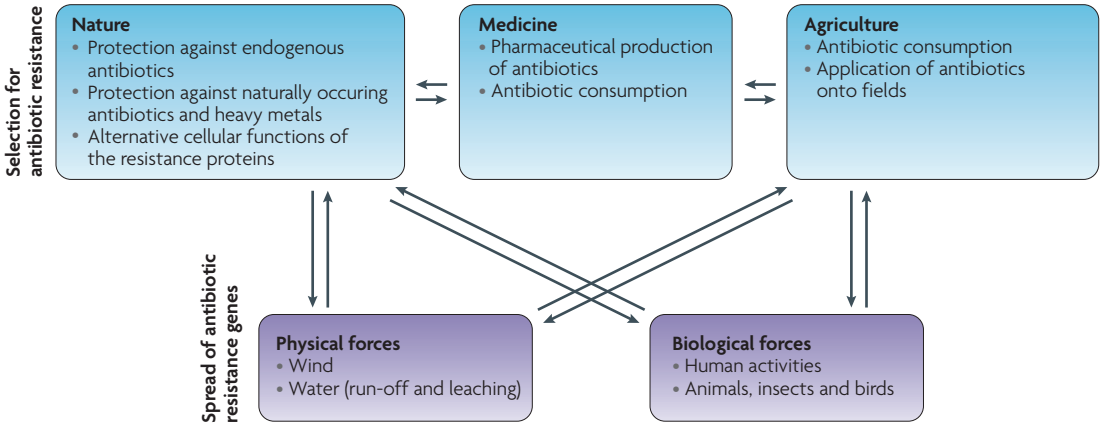

Figure 2 | **Sources and movement of antibiotic resistance genes in the environment.** Resistance genes exist naturally in the environment owing to a range of selective pressures in nature. Humans have applied additional selective pressure for antibiotic resistance genes because of the large quantities of antibiotics that we produce, consume and apply in medicine and agriculture. Physical and biological forces also cause widespread dissemination of resistance genes throughout many environments.

Antibiotic resistance in natural communities

Little is known about the selection pressures on antibiotic resistance genes in the era before antibiotics were turned into pharmaceuticals or in remote environments with little direct human contact. A more comprehensive understanding of the natural roles of putative antibiotic resistance genes will provide important information on their origins and functions.

The pre-antibiotic era. The only environments that are truly exempt from the influence of human antibiotic use existed before the antibiotic era. The time before the introduction of sulphonamides, which occurred in the late 1930s, can be considered 'antibiotic naive', in the sense that no industrial production of antibiotics took place. However, heavy metals were used for disease treatment for centuries prior to the use of antibiotics, and this may have selected for genes encoding both heavy-metal and antibiotic resistance[47]. Retrospective studies show that resistance genes were present in bacteria that did not produce antibiotics before the widespread dissemination of the drugs. Out of 30 *E. coli* strains that were lyophilized before 1950, 4 were resistant (to various degrees) to the 8 tested antibiotics, and each resistance element could conjugate into *E. coli*[48]. In another analysis of 433 enterobacterial strains collected from around the world between 1917 and 1952 (known as the Murray collection), 24% could transfer plasmids and 11 strains were resistant to ampicillin or tetracycline, although the resistance was not conjugative[49]. More recent analysis of antibiotic resistance profiles from enteric bacteria collected before and after the introduction of antibiotics mirrors these findings[50]. Thus, determinants of antibiotic resistance existed naturally and were probably subject to horizontal transfer long before the extreme selection pressure that was imposed in the antibiotic era. This predisposition for the genetic exchange of resistance elements is certain to have facilitated the rapid outgrowth of antibiotic resistance in pathogenic bacteria.

Soil. Owing to the movement of antibiotics and resistance genes on the wind and on feathers, it is unlikely that any environment can be considered truly pristine. However, despite the movement of soil particles by physical forces, soil itself is a stationary complex, and some soils are far removed from human influences. Thus, studies of antibiotic resistance in soil show that environmental bacteria harbour antibiotic resistance genes independently of human activities.

Culturable bacteria in soil harbour genes encoding enzymes that degrade or otherwise inactivate antibiotics. Bacteria that grow on antibiotics as the sole carbon and nitrogen sources include: *Pseudomonas fluorescens* grown on streptomycin[51]; *P. fluorescens*[52], *Burkholderia cepacia*[53] and eight unidentified strains[54] grown on penicillin; an unidentified *Streptomyces* sp. isolate and *Streptomyces venezuelae* grown on chloramphenicol[55,56]; a *Flavobacterium* sp. isolate grown on chloramphenicol[57]; and various bacteria, mostly of the phylum Proteobacteria, grown on various antibiotics[58]. In addition to antibiotic degradation, culturable soil bacteria use many mechanisms of resistance to antibiotics. In the most comprehensive study to date on antibiotic resistance in one soil species, over 400 actinomycetes cultured from forest, agricultural and urban soils were found to have highly varied resistance profiles; moreover, some exhibited resistotypes that had not been seen before, such as inactivation of telithromycin by a novel structural modification[59].

The roles of antibiotics and resistance in nature

The discovery of the great therapeutic potential of microbial compounds in both the laboratory and the clinic led to the preconception that antibiotic activity must be important and widespread in nature. This led to an almost complete disregard of the other potential functions of natural products from microorganisms. On the basis of limited genomic studies, it is thought that microbial populations are capable of

Pristine
Unspoiled or unpolluted by human activities.

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producing a wide range of bioactive small molecules (the parvome[60]), only a handful of which have been isolated, identified and used as antibiotics and other types of therapeutics. Bacteria of the phylum Actinobacteria[61], a huge taxonomic group that is characterized by a high genomic GC content and that comprises diverse genera, all produce complex bioactive small molecules. It can be estimated that actinobacteria make millions of such molecules. Most of these molecules cannot be detected under laboratory conditions, and the few that have been identified include the most important antibiotics. It has been suggested that antibiotics have been produced for over 500 million years, dating back to the Cambrian period and the emergence of vertebrate fish[62]. Antibiotic-like molecules, or at least their component parts, are likely to be even older than this — the non-protein amino acids that are found as components of peptide antibiotics have been detected in meteorites and other primordial sources[63].

The roles of antibiotics in microbial communities. Using war as a metaphor for the interaction of microorganisms, the activities of antibiotics in the laboratory and in clinical applications led to the assumption that these molecules have hostile roles in nature. The number of such bioactive molecules in any given environment must provide a considerable armamentarium. However, the *in situ* concentrations of the compounds with antibiotic activity have never been measured, and there are few ecological examples of probable antibiotic functions for microbial products in nature. One example is the fungus-growing ant system, in which ants carry an antibiotic-producing actinomycete (a *Pseudonocardia* sp.) on their cuticle and use this bacterium specifically for biocontrol of the fungal garden parasite, *Escovopsis* sp.[64,65]. A second example is in the biocontrol of the causative agent of potato scab, *Streptomyces scabies* str. RB4, by the antibiotic-producing suppressive strain, *Streptomyces diastatochromogenes* str. PonSSII[66]. Many factors may contribute to disease suppression, but the fact that antibiotic-resistant strains of the pathogen are restored in their ability to cause disease is evidence for a direct role for antibiosis[66]. Because of the sparse evidence for widespread antibiotic-mediated killing in nature, it is important to investigate the role of sublethal doses of antibiotics in microbial communities.

The demonstration of quorum-sensing reactions in a range of microbial species opened up a profitable field of investigation that has had implications for research into various aspects of bacterial lifestyles, such as pathogenesis, community structure and biofilm formation[67]. These all involve the production of specific bioactive compounds that, at low concentrations, activate biochemical pathways in one or more target organisms. Interestingly, some autoinducers used in quorum sensing have antibiotic activity at higher concentrations and may also provoke changes in eukaryotic host organisms or tissues[68]. These studies have led to the realization that bioactive small molecules (other than amino acids, sugars and nucleic-acid bases) have important roles in microbial biology[69]. For example, quinolones, phenazines and pheromones are bioactive small molecules that exist widely in nature, and they each possess a range of biological functions.

In recent years, several antibiotics and other bioactive molecules (such as bacteriocins) have been tested for activity at concentrations below those needed for the inhibition of cell growth. Almost all of the tested compounds exhibit hormesis[70,71]. This suggests that the compounds constitute a new form of signalling network, in which the receptors for the small molecules are cytoplasmic macromolecular structures such as ribosomes and the DNA replication, RNA replication and cell wall synthesis complexes[72]. Note that many of these receptors were previously identified as the inhibitory targets for the bioactive molecules. The binding of the ligands to their receptors at low concentrations initiates a profusion of different transcription patterns, depending on the nature of the ligand and the target. Modulation of host transcription leads to metabolic and behavioural changes in the microorganisms, as has been described in several reports[70,73]. These changes can be assumed to be signalling responses that adjust metabolism in mixed microbial communities.

The role of antibiotic resistance genes in microbial communities. As mentioned above, the production of an antibiotic is associated with the presence of genes encoding one or more self-protection processes; antibiotic biosynthesis gene clusters always encode one or more potential resistance proteins that are either specific to the compound being made (for example, they modify the compound or target) or multifunctional (for example, efflux systems). In addition to the so-called self-resistance function, the resistance genes that are contiguous with the biosynthesis genes could be involved in regulation of the biosynthesis pathway. One study that supports this concept found genes encoding export proteins embedded in the actinorhodin biosynthesis pathway of *Streptomyces coelicolor*[19], but is this yet another example of anthropocentric reasoning? Evidence shows that antibiotic resistance genes are common in natural environments and existed, even on plasmids, before the use of antibiotics. Phylogenetic analyses date the origin of serine β-lactamases at over 2 billion years ago and suggest that many of these enzymes have been plasmid encoded for millions of years[74].

Another form of resistance in isolates that do not produce antibiotics is mutation of the target gene product, which reduces or prevents inhibition by antibiotic binding. However, antibiotic resistance may not be the only consequence of these mutations, as they are often pleiotropic. These types of mutations have been found frequently in environmental bacteria, which are therefore, presumably, unresponsive to a specific small molecule signal in the environment. When soil bacteria were screened for isolates that were resistant to known antimicrobials such as the fluoroquinolones, it was found that the isolates had independent alleles of the DNA gyrase subunit A gene (*gyrA*) with different

Parvome
The range of biologically active, low-molecular-mass (< 5 kDa) compounds that are produced by defined biosynthetic pathways in bacteria, yeast, plants and other organisms.

Antibiosis
An interaction between microorganisms involving a small molecule that is produced by one organism and detrimental to the other.

Hormesis
A dose-dependent response phenomenon shown by bioactive compounds and drugs, such that they have contrasting activities at low (subinhibitory) and high (inhibitory) concentrations.

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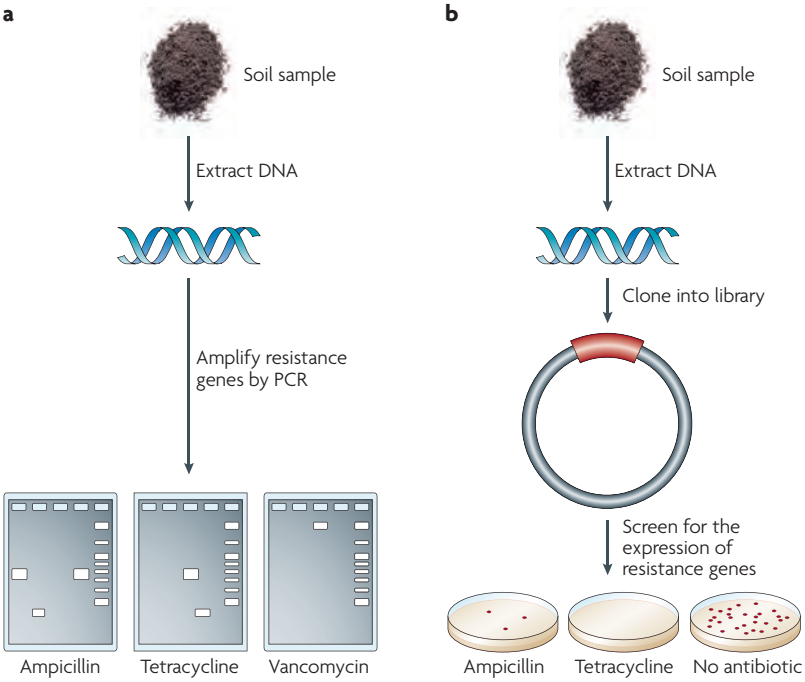

Figure 3 | **Detecting antibiotic genes in natural samples. a** | A PCR-based approach. DNA is extracted from bacteria in a soil sample and resistance genes are amplified with resistance gene-specific primers and detected by gel electrophoresis. **b** | Functional metagenomics. DNA is extracted from soil samples and cloned into libraries. These libraries are introduced into *Escherichia coli* or another amenable host, and the transformants are tested for drug resistance.

levels of resistance[75]. Subsequent studies have shown that spontaneous mutations causing resistance often lead to a range of metabolic phenotypes, including variations in the ability to use different carbon, nitrogen or phosphate sources for growth[76]. In addition, mutations in ribosomal proteins that conferred resistance to streptomycin, spectinomycin or macrolides were found to cause a range of altered phenotypes, as shown by phenotypic array studies (H. H. Wang and J. Davies, unpublished observations). Bacteria carrying these mutations were selected under laboratory conditions in the presence of an antibiotic, but in the environment the selection could alternatively be the ability to grow on an available peptide or the presence of a particular carbon or phosphorus source. Thus, the widespread presence of antibiotic-resistant strains in the environment may have arisen in response to various selective pressures. It is clear that more extensive studies of the roles of natural resistant strains and their diverse phenotypes are needed.

In addition to the alternative cellular and environmental roles of known antibiotic resistance genes, other types of genes that contribute to resistance have been recognized. Studies undertaken at the species level revealed an extensive set of genes that contribute to the antibiotic resistance phenotype but that primarily encode proteins with other functions in the cell (the so-called intrinsic resistome). In *E. coli*, for example, 4,000 random single-gene knockouts were screened for hypersensitivity to antibiotics[77,78]. Of these, 140 knockouts were identified that increased the sensitivity to at least 1 of 7 antibiotics[77]. Some genes were implicated in resistance to many types of antibiotics (for example, an insertion in the gene encoding the DNA helicase exonuclease V subunit-γ (*recC*) increases susceptibility to ciprofloxacin, rifampin, sulphamethoxazole and metronizadole) and others were involved in resistance to a specific antibiotic (for example, an insertion in the transcriptional regulatory gene *phoP* increases susceptibility to ampicillin)[77]. Similar studies were performed in *Pseudomonas aeruginosa*[79,80] and *Acinetobacter baylyi*[81], revealing that there is little overlap between the intrinsic resistomes of different organisms. These results implicate possible targets for antibiotic potentiators and illustrate the breadth of the genes, even in a single organism, that can contribute to the overall environmental antibiotic resistome.

Potentiator
A compound or molecule that augments the activity of an antibiotic.

Challenges in studying natural resistance

Detecting antibiotic resistance genes. The application of culture-independent approaches, such as PCR and metagenomics, to the study of antibiotic resistance in the environment has uncovered the vast diversity of antibiotic resistance genes in soil bacteria (FIG. 3). A prairie soil was analysed by PCR for the presence of genes encoding TEM-type β-lactamases. Numerous polymorphisms of the TEM sequence were discovered in this prairie soil as well as in soil containing transgenic plants[82]. However, certain *Taq* polymerases have been shown to be contaminated with DNA that encodes TEM-type β-lactamases[83] (B. Converse and J. Handelsman., unpublished observations), indicating that precautions need to be taken when using PCR methods to detect TEM-related β-lactamases. PCR has also been used to detect vancomycin[84] and gentamicin[85] resistance genes in unamended soils, revealing sequences that are closely related to known genes. Primers for *tetM*, one of the known tetracycline resistance genes, were used to analyse unamended garden soils by PCR but detected no related tetracycline resistance genes[86]. PCR is therefore a powerful tool to detect potential antibiotic resistance genes in soil environments.

In contrast to PCR, functional metagenomics can be used to query an environment for genes that are not closely related to known resistance genes. Functional metagenomics is the study of the collective genome of a group of organisms by cloning DNA directly from an environmental sample into a host organism (often *E. coli*) followed by screening or selecting for the desired function[87]. When applied to a Wisconsin remnant oak savannah soil site, functional metagenomics yielded diverse and new antibiotic resistance genes, including nine aminoglycoside resistance genes and one tetracycline resistance gene[88]. Six of the aminoglycoside resistance genes encoded 6′-*N*-acetyltransferases, and a phylogenetic analysis of these enzymes at the amino acid level showed that they cluster together and are divergent from previously known 6′-*N*-acetyltransferases. The same method was applied to the metagenome of an Alaskan soil that was distant from anthropogenic activities, in

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Box 2 | **Human exposure to antibiotic-resistant bacteria in wildlife**

Potential routes for human contact with wild animals and their microbiota, which may contain antibiotic-resistant strains, include:

- Translocation of wildlife into suburban areas owing to game release, habitat destruction, pollution and changes to water storage, irrigation or the climate.
- Ecotourism, hunting and camping.
- Exotic foods, wet markets, bushmeat and game farms.
- Exotic pets and the long-distance transport of live animals.
- Zoos, aquaria, wildlife safari parks and circuses.
- Trapping or rearing of fur-bearing animals.

search of resistance to β-lactam antibiotics. This study revealed 13 β-lactamases (including 1 bifunctional enzyme) that represent each of the 4 known classes of β-lactamases but that are deeply divergent from and ancestrally related to the β-lactamases found in clinical isolates[89]. Interestingly, none of the functional β-lactamases from the Alaskan soil metagenome were related to TEM-type β-lactamases, which is in contrast to the results of the PCR-based investigation of prairie soil. Functionally characterizing the metagenomes of bacterial communities from a variety of environments will expand our knowledge of the potential sources and novel alleles of antibiotic resistance genes.

Part of the challenge of addressing and reviewing the big questions surrounding the ecology of antibiotic resistance genes in the environment is that inconsistent methods have been used to monitor the resistance properties of environmental bacteria. Most studies of resistance in bacteria from the environment are based on culturing followed by selection. However, the method of resistance determination is not standardized; therefore, the data on antibiotic resistance in the environment come from studies that have used a range of media types, antibiotic concentrations and incubation periods. This makes it difficult to compare results between environments. For example, Antarctic marine waters contain copious antibiotic-resistant bacteria, including many isolates that are resistant to ampicillin[90]. However, resistance was determined by growth on 50 μg ml^{-1} ampicillin, which is not consistent with other studies of antibiotic resistance in aquatic environments or with how resistance is defined for clinical isolates, which are tested for minimum inhibitory concentration according to standard protocols[91]. The absence of guidelines for resistance studies of environmental bacteria makes it difficult to draw conclusions about a single environment or to make comparisons between environments.

The inconsistencies in the methodologies are further aggravated by the infrequent and incomplete monitoring of antibiotic resistance in natural environments. The intestinal microbiota of wild animals, for example, have rarely been examined for carriage of antibiotic resistance genes. Little is known about these bacteria more generally[92], despite the alarming statistic that in the past 2 decades approximately 75% of all types of emerging human diseases came from wildlife[93]. As potential routes for human contact with wild animals expand (for example, through translocation of wildlife into suburban areas owing to habitat destruction and an increase in the exotic food and pet trades), the potential for bacterial transfer also increases (BOX 2). The data on antibiotic resistance in wild animals also show the need for multiple strategies to be used to fill our knowledge gaps. For example, less than 1% of the bacteria in the environment are culturable by standard techniques, although for some environments this estimate is conservative. Despite the known limitations of culturing, nearly all studies of antibiotic resistance in wild animals are based on culturing for enteric bacteria. However, considering that the spread of antibiotic resistance genes by horizontal gene transfer occurs between diverse bacteria with ease and that resistance genes are maintained even in the absence of selection[8,94], it is important to characterize the resistance genes in the entire community, including both culturable and unculturable strains. In addition, culture-independent techniques have the potential to carry microbial ecologists beyond gene definition and into gene expression studies. Techniques such as reverse transcription PCR, when coupled with metagenomics, enrich studies by investigating which genes are expressed across the entire community. Both culture-dependent and culture-independent approaches have their limitations, so combining these approaches will develop the most comprehensive portrait of the resistance profile of a microbial community, which will form the basis for understanding the effects of environmental resistance genes on human pathogens and the role of antibiotic resistance genes in unperturbed communities.

Conclusions

Little is known about the antibiotic resistomes[59] of the vast majority of environmental bacteria, although there have been calls for a greater understanding of the environmental reservoirs of antibiotic resistance and their potential impacts on clinically important bacteria[95,96]. The data on antibiotic resistance before the antibiotic era and in soil highlight how little we know about the ecology of antibiotic resistance genes in the wild. We do not have a complete picture for any environment of all of the types of resistance genes in both the cultured and uncultured community. Soil is particularly challenging to assess, because of its chemical and physical heterogeneity (soil displays variation on a scale of 1 metre or less)[97]. Despite the gaps in our knowledge, it is clear that some organisms and some environments harbour antibiotic resistance genes irrespective of the human use of antibiotics. The prevalence and diversity of resistance genes in the environment inspire hypotheses about the native roles of so-called resistance genes in natural microbial communities. Considering that antibiotic treatment is our primary, and in many cases only, method of treating infectious diseases, we conclude that more detailed studies of environmental reservoirs of resistance are crucial to our future ability to fight infection.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
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Entrez Genome Project: http://www.ncbi.nlm.nih.gov/genomeprj
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10 Proteome and Antigens of *Mycobacterium avium* subsp. *paratuberculosis*

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10.1 Introduction

In spite of new molecular and immunological methods for diagnosis and vaccine discovery, Johne's disease (JD) remains a major problem, with increasing prevalence worldwide. An important tool in Johne's control would be a simple, rapid and inexpensive assay to detected infected animals or herds. Although enzyme-linked immunosorbent assays (ELISAs) present many of these attributes, currently available tests suffer from limitations in sensitivity and specificity that undermine their potential utility in disease control. Despite this, the inherent benefits of ELISA tests have led to enormous efforts being made for discovery of antigens from *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) that could be used in such a test format. In fact, much of the field of *MAP* proteomics has been driven by the premise that the existing platforms can be readily adapted, using *MAP*-specific antigens, to offer newer and more accurate assays.

This chapter reviews what is known about the proteome of this significant veterinary pathogen by discussing the use of various whole-cell preparations and fractionated components thereof. With improvements in technology, many new modifications to the traditional ELISA assay have been introduced,

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including flow cytometry and assays based on surface-enhanced Raman scattering (SERS). Also, new antigen extracts have been created that appear to improve on the current ELISA test, which has historically used a whole-cell sonicated extract as the antigen. Particularly promising is the EV-ELISA extract, with potential sensitivities above 90%. Recombinant protein expression strategies have gained new momentum as well. The construction and use of the first protein array for *M. avium* subsp. *paratuberculosis* (*MAP*) is one such application that has flowed from this strategy and will be discussed at the end of this chapter.

10.2 Study of *MAP* Proteins

Proteins are generally studied in one of two ways. In function studies, proteins are assessed for a particular property, such as the kinetics of an enzyme or the role it performs in a metabolic pathway. In applied studies, proteins are evaluated for their ability to elicit protection in the host or their utility as a specific antigen in diagnostics. For the ruminant pathogen *MAP*, very few studies have been performed that address the former, yet several studies have been published which fulfil the latter. Because a well-defined and strongly immunogenic antigen can serve both diagnostic needs and vaccination, tremendous efforts have been invested in order to identify the antigen that best serves these purposes for any given pathogen. A survey of the literature shows that no less effort has been put forth to identify antigens of *MAP*, which causes JD. Approximately 250 *MAP* proteins have been evaluated immunologically in some manner. This chapter focuses on applied studies of *MAP* proteins.

Ideally, proteomics is the study of all the proteins produced by an organism. However, in practice, only a subset of these proteins can truly be analysed at any one time, owing to current methodological limitations. Therefore, investigators must divide or fractionate the proteome into more manageable subsets to enable the mass spectrometer to 'see' all the proteins present or enable a polyacrylamide gel to resolve all the proteins in the fractionated sample. From the published genome sequence of *MAP* strain K-10, we now know that there are 4350 open reading frames (Li *et al.*, 2005). If we exclude the 45 tRNA genes and one rRNA sequence, which do not encode proteins, we are left with just over 4300 coding sequences. Therefore, in order to analyse the entire proteome, this is the target number of proteins that investigators should strive to resolve. In more practical terms, technology has not advanced enough to make this ideal a reality. As a result, this chapter will focus on the fractions of the proteome that have undergone analysis and how this information may aid in antigen-based detection of *MAP*.

10.3 Antigen Preparations

Several types of *MAP* antigen preparations have been evaluated and used for serodiagnosis of JD for over two decades (Nielsen and Toft, 2008). These unpurified mycobacterial extracts or fractionated preparations naturally contain multiple antigens. This poses a specificity problem for current serological tests (see Nielsen, Chapter 24, this volume), as crude extracts contain mixtures of *MAP* proteins along with abundant polysaccharide antigens, consisting predominantly of arabinomannan and arabinogalactan. Since these antigenic components are often conserved across the genus, the resulting assays suffer from cross-reactivity. The protoplasmic antigen preparation (PPA) and lipoarabinomannan (LAM), each of which will be described in more detail below, have been adapted to commercial ELISAs (Yokomizo *et al.*, 1983; Sugden *et al.*, 1987). Diagnostic sensitivities of the commercial ELISAs were reported to be around 60% in the early 1990s, but more recent studies report sensitivity as low as 15–20% (Whitlock *et al.*, 2000; Nielsen and Toft, 2008; Shin *et al.*, 2008). Of note, the sensitivity of ELISAs in most of the published reports was evaluated against a positive faecal culture as the reference test, and this apparent decrease in ELISA sensitivity may instead reflect improved sensitivity of faecal culture in the 1990s (Whitlock and Rosenberger,

1990). The true sensitivity of these ELISA-based tests using PPA and LAM remains an issue for further evaluation.

10.4 Protoplasmic Antigen (PPA)

PPA is a crude antigen mixture prepared by thorough physical disruption of mycobacteria followed by removal of cell debris and cell wall components (Beam *et al.*, 1969). The PPA preparation method described by Beam *et al.* (1969) included pressure disruption, ultracentrifugation, filtration and delipidation steps. Other investigators have prepared PPA by simply disrupting the bacilli with prolonged sonication and removing large, insoluble particles by centrifugation. For this reason, PPA has also been called sonicate antigen (Waters *et al.*, 2003; Bannantine *et al.*, 2008a) or cell extract (Cho and Collins, 2006). Although PPA preparation is commercially available from Allied Monitor and has been used in several reports on JD diagnosis, the preparation protocol is proprietary. Regardless of the differences in PPA preparation protocols, it is likely that PPA contains mostly soluble cell-associated proteins.

In 1983, Yokomizo *et al.* (1983) developed an ELISA test for JD by using PPA, and since then this antigen preparation has been used in commercial ELISA kits and reported widely in the literature (Nielsen and Toft, 2008). In follow-up studies, Yokomizo reported that the ELISA test had false-positive results caused by cross-reactive antibodies and that pre-absorption of serum samples with *Mycobacterium phlei* reduced the false positives (Yokomizo *et al.*, 1985). This pre-absorption step is still being used in the current ELISA tests (Shin *et al.*, 2008). However, a careful and comprehensive search for a better absorbent has not been reported and may be a goal for future research. The cross-reaction of bovine antibodies to PPA seems unavoidable since the preparation contains numerous cytosolic proteins that are involved in well-conserved biochemical processes, such as transcription and lipid biosynthesis.

Some attempts have been made to purify specific antigens in PPA. Bech-Nielsen and colleagues partially purified PPA using SDS-polyacrylamide gel electrophoresis (PAGE), and they observed a major protein band of approximately 34–38 kDa (Bech-Nielsen *et al.*, 1992). An ELISA of the purified PPA showed a higher sensitivity (83%) than that of crude PPA, suggesting that the remaining components of this protein preparation may actually be more antigenic than the intact preparation itself. Also, Abbas *et al.* (1983) isolated an abundant PPA protein that constituted approximately 8% of the total protein in PPA and tested it for antibody binding in an ELISA format. In 44 JD-negative serum samples tested in the study, the ELISA did not show any false-positive reactions; however, the number of tested samples was too small to conclude the antigen was not cross-reactive. Although the identities of the antigenic proteins have not been discovered in any of these studies, they do suggest that PPA contains strong antigens for the development of an accurate diagnostic test.

10.5 Culture Filtrate Preparations

A well-known antigen preparation derived from the filtrate of a spent culture, termed purified protein derivative (PPD), has been a critical reagent for testing cell-mediated immune responses to mycobacterial infections. It is the antigen of choice for the gamma interferon test (Stabel and Whitlock, 2001; Stabel *et al.*, 2007) as well as skin testing (Robbe-Austerman *et al.*, 2007). Historically, the tuberculin form of PPD was made from autolysed *Mycobacterium tuberculosis* cultured for extended periods of time (Lachmann, 1988). The intact bacilli were removed by centrifugation and the culture supernatant was heated, filtered and precipitated with trichloroacetic acid. This PPD preparation was supplied by the Veterinary Laboratories Agency in Weybridge, UK for many years and is still produced by a number of commercial organisations.

In the case of *MAP*, PPD preparations are difficult to standardize and hence their immunological potency varies widely. In fact, laboratories around the world use different strains

of *M. avium* subspecies for producing PPD, and this confusion has resulted in an effort to characterize the genetic diversity of these strains as an initial step towards standardization (Semret *et al.*, 2006). This study found that some of the Johnin production strains contain a large deletion encompassing just over seven genes, which may result in the poor immunological potency observed with those preparations.

To prepare Johnin PPD, Reid's media is used to culture the bacilli to form pellicles on the surface of the media. The cultures are incubated without shaking and must be handled very gently to prevent the surface pellicle from sinking into the media, which would theoretically render the preparation unusable as an immunological reagent. While this fact is widely acknowledged among producers of PPD, even tuberculin PPD, there is no documented evidence for this conclusion to our knowledge. After a secondary culture has been inoculated into 500 ml Reid's media, it is incubated without shaking for 10 weeks. The cultures are then autoclaved and the cell pellicle is removed with a sterile nylon gauze. The remaining culture filtrate is precipitated with the addition of 40% tricholoroacetic acid, harvested by centrifugation, washed in salt buffer, dissolved in a sodium phosphate buffer, quantitated and stored. Immunological potency is tested in sensitized guinea pigs (Steadham *et al.*, 2002).

It is important to note that the method by Cho and Collins (2006) to prepare culture filtrate antigens is different from the PPD preparation. The culture filtrate, which is also reported to contain strong antigenic components (Cho and Collins, 2006), seems easier to prepare. A culture of Reid's media is harvested by centrifugation to remove the bacilli and the media is filtered and concentrated by size exclusion before undergoing dialysis as the final step.

There is tremendous variability in the potency of PPD preparations. Not only is the starting material different for PPD production but the method of preparation is also believed to be a major cause of its variability, begging the question of why Johnin PPD is not prepared using the same method as that used to make tuberculin PPD. There have been some recent, although unpublished, efforts to define the components of PPD through proteomic approaches. One of these studies employed isobaric protein tags to determine quantitative differences between a highly potent preparation of PPD and a preparation of low potency, both produced at the National Veterinary Services Laboratory in Ames, Iowa, USA (Bannantine, Robbe-Austerman and Paustian, unpublished results). A few studies have evaluated recombinant proteins for performance in the gamma interferon test when compared with Johnin PPD (Griffin *et al.*, 2005; Nagata *et al.*, 2005; Shin *et al.*, 2005). Koets *et al.* (2001) used a PPD-ELISA for detection of 54 *MAP*-infected and 50 uninfected samples and found that an IgG2 response in the infected group, but not an IgG1 response, was significantly higher than in the uninfected group. Caution should be used for vaccine trials, as immunized animals may yield positive reactions in skin tests with either *MAP* PPD (Hines *et al.*, 2007) or *Mycobacterium bovis* PPD (Nedrow *et al.*, 2007).

Two independent research groups recently made major progress in comprehensive proteomic analysis of culture filtrate (CF) proteins and their potential as diagnostic antigens. Leroy *et al.* (2007) were the first to systematically analyse CF proteins of *MAP* cultured in Sauton media. The CF proteins were first separated by 1-D and 2-D SDS-PAGE, and 320 protein spots were excised from these gels and analysed by tandem mass spectrometry. They subsequently identified 25 putative *MAP*-specific proteins through comparative genomic analysis (Leroy *et al.*, 2007). Five of the 25 *MAP*-specific proteins were combined and tested for reactivity with antibodies in sera of *MAP*-infected and uninfected cattle. Based on the results obtained with 19 *MAP*-infected and 48 uninfected serum samples, they reported that the five-protein cocktail had a similar level of diagnostic sensitivity to that of a commercial ELISA test. In a second study, Cho and Collins (2006) prepared both a cellular extract (i.e. PPA) and CF from the JTC303 strain of *MAP* cultured in modified Watson–Reid broth and tested each protein preparation for reactivity with antibodies in sera of *MAP*-infected and uninfected cattle. They found that an ELISA prepared

using CF (JTC-ELISA) was more sensitive than that prepared with a culture extract. In a subsequent study, the diagnostic sensitivity of the JTC-ELISA was compared with that of five commercial ELISAs using 856 bovine sera (Shin *et al.*, 2008). The sensitivity of the JTC-ELISA (56.3%) was significantly higher than that of five current ELISAs (28.0–44.5%). Importantly, the JTC-ELISA showed dramatically higher sensitivity (40%) than that of other ELISAs (20%) in samples from cattle shedding low levels of *MAP* in their faeces. Collectively, these data suggest that secreted proteins of *MAP* appear to contain the stronger antigens for antibody-based tests.

Cho *et al.* (2006) went on to define some of the antigens present in the culture filtrate. They successfully identified 14 proteins, including MAP1569 (ModD), MAP3527 (PepA) and MAP3531c (antigen 85C) among others. Interestingly, ModD was shown to be the most antigenic of the 14 proteins and this same protein was previously identified as a fibronectin attachment protein that mediates uptake by M cells (Secott *et al.*, 2001, 2002, 2004).

10.6 Ethanol Extract

In 1995, Eda *et al.* (2005) used flow cytometry to demonstrate that antibodies in sera of *MAP*-infected cattle bound to the surface of *MAP* but not to that of other mycobacterial species. This observation led to the inference that *MAP* has unique antigens on its outer surface. Furthermore, the antibody–*MAP* binding complexes were detected in natural bovine infections several months earlier than the faecal culture test or commercial ELISA test. The empirical diagnostic sensitivity and specificity of this novel flow cytometric assay were estimated to be 95.2 and 96.7%, respectively. These data suggested that by detecting antibodies to the surface antigens of *MAP* one could develop a diagnostic test to detect early *MAP* infection. In a subsequent study, the same investigators found that an 80% ethanol solution was the most effective solvent for extracting surface antigens that specifically reacted with sera of *MAP*-infected cattle (Eda *et al.*, 2006). An in-house ELISA was developed using the ethanol-extracted preparation and named EVELISA for Ethanol Vortex ELISA. This test showed an improvement over the flow cytometric assay, with an empirical diagnostic sensitivity and specificity of 100 and 96.9%, respectively. The ethanol-extracted mycobacterial antigens were further fractionated by the Folch wash method and acetone precipitation into three fractions: aqueous, interface and chloroform. Each fraction showed reactivity to antibodies in sera of *MAP*-infected cattle; however, empirical sensitivity of the fractions was lower than the EVELISA (unpublished observations), indicating that the complete repertoire of antigens in the ethanol extract was required to maintain the high sensitivity of the EVELISA. Efforts are currently underway to define the components in the ethanol extract preparation. Preliminary data suggest the preparation contains predominantly carbohydrate and lipid components with a small, but important, protein component.

10.7 Lipid Antigens

Members of the genus *Mycobacterium* produce complex lipid components in their cell wall. Unfortunately, lipidomic studies of *MAP* are still in the initial stages, with very few published reports. Glycopeptidolipids (GPLs) are surface lipids that consist of a lipopeptide core that is N-linked to long chain fatty acids. O-linked to this same lipopeptide core are mono- and oligosaccharides. These sugar moieties are antigenic and form the basis for the 28 different serovars of the *M. avium* complex (Chatterjee and Khoo, 2001). While GPLs can make up more than 70% of the lipids exposed at the surface of *M. avium* isolates (Etienne *et al.*, 2002), *MAP* are deficient in GPL biosynthesis genes, as revealed by inspection of the K-10 genome sequence (Biet *et al.*, 2008). Interestingly, sheep strains of *MAP* have the genes necessary to synthesize GPL (Paustian *et al.*, 2008), although it remains to be determined if these strains actually do produce GPLs.

Lipoarabinomannan (LAM) is a cell wall lipoglycan found in several species of mycobacteria (Nigou *et al.*, 2003, 2004). It is composed

of a lipid moiety, a highly branched 30–35 mannopyranose core, and around 60 units of arabinopyranose (Nigou *et al.*, 2003). The structures of LAM differ slightly among species of mycobacteria, with the most important difference, in terms of biological activity, being capping motifs attached to the non-reducing end of the arabinan chain (Briken *et al.*, 2004). For instance, LAM of slow-growing *Mycobacterium* species, such as *M. tuberculosis* (Chatterjee *et al.*, 1992), *Mycobacterium leprae* (Khoo *et al.*, 1995) *and M. avium* subsp. *homissuis* (Khoo *et al.*, 2001) are modified with a capping structure consisting of one to three mannose residues (ManLAM). The fast-growing *Mycobacterium* species *Mycobacterium smegmatis* and *Mycobacterium fortuitum* have been shown to express LAM modified with inositol phosphate caps (PILAM) (Gilleron *et al.*, 1997) or, in the case of *Mycobacterium chelonae*, uncapped LAM (AraLAM) (Guerardel *et al.*, 2002). Although the cap structure of LAM has not been biochemically characterized in *MAP*, its genome sequence is known to contain genes involved in mannose cap synthesis (Li *et al.*, 2005; Dinadayala *et al.*, 2006). It is therefore likely that LAM from *MAP* is decorated by mannosides similar to that observed for other subspecies of *M. avium*.

LAM is capable of inducing strong humoral immune responses (Waters *et al.*, 2003) and, similar to PPA, has been used as an antigen in commercial diagnostic tests for JD (Jark *et al.*, 1997). The drawback is that LAM is conserved among the mycobacteria. Its preparation involves harvesting the mycobacteria, resuspending them in distilled water, passing them through a French press, followed by centrifugation, at which point the supernatant is subject to proteinase K treatment and filtration (Jark *et al.*, 1997). As reviewed by Nielsen and colleagues, reported sensitivities of ELISA tests developed using LAM (LAM-ELISA) were comparable to those of PPA-ELISAs (Nielsen and Toft, 2008). In 1997, Sugden and colleagues tested serum samples using LAM-ELISA and PPA-ELISA, and both tests detected at least 11 of 15 positive *MAP* samples (Sugden *et al.*, 1997). However, LAM does have an advantage over PPA, in that it is a purified, well-characterized antigen and therefore lends itself more readily to production quality control. Large-scale cultures for production of LAM would be needed for commercial development and this could be a drawback. None the less, LAM may still be a good antigen candidate for the development of a *MAP* ELISA when used in combination with other, more specific, *MAP* antigens.

A major cell wall lipopeptide for *MAP*, termed Para-LP-01, has been reported (Eckstein *et al.*, 2006). These investigators were able to show that this lipid is specific and immunoreactive for *MAP*, based on an ELISA assay developed using the lipid as a coating antigen. This same laboratory is separating and analysing a complete set of lipids by thin layer chromatography using solvents with a wide range of polarity, to identify additional diagnostic and vaccine targets. Unfortunately, Para-LP-01 was not synthesized and produced in large enough quantities to enable further testing and validation. A second *MAP* lipid, termed lipopentapeptide, was successfully synthesized and shown to be unique to *MAP* strains (Biet *et al.*, 2008). This group confirmed that only *MAP* and not other mycobacteria produce this lipopentapeptide. They compared this lipid to *MAP* PPD in an ELISA format and demonstrated similar sensitivity but higher specificity for the lipid. When the lipopentapeptide moieties were synthesized separately, it was determined that the peptidyl moiety was highly immunogenic whereas the lipid moiety was poorly recognized by cattle sera (Biet *et al.*, 2008).

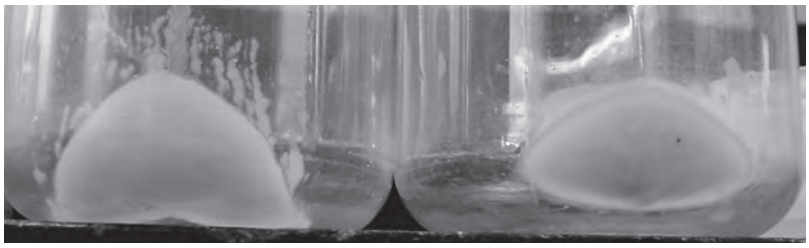
10.8 Recombinant Antigens

There are two important advantages of recombinant proteins in serodiagnostic testing of hosts with *MAP* infection. First, traditional techniques to detect antibodies in animals with JD have used crude extract mixtures, with substantial variability between extracts. Secondly, extracts such as PPD or PPA are prone to cross-reactivity with antibodies generated against different bacterial exposures. The availability of genome sequence data has now made cloning and expression of any coding sequence from this organism straightforward. Furthermore, long culture

periods can be bypassed if antigen production is performed in a faster-growing bacterium. As a side benefit, when *Escherichia coli* is used as the surrogate expression host, striking morphological or physiological changes in the bacterium can sometimes be readily observed, as shown in Fig. 10.1. These changes may supply telling clues as to the function of the protein.

Previous studies with other mycobacteria such as *M. tuberculosis* have provided a foundation such that antigen selection is no longer random but instead based on experimental data. In fact, several *MAP* recombinant proteins have been analysed based on promising immunological studies with *M. tuberculosis* or *M. leprae* (Mullerad *et al.*, 2002; Rosseels *et al.*, 2006; Sechi *et al.*, 2006). One study has shown that recombinant proteins can actually detect a higher percentage of infected deer than PPD or PPA antigens when used in an ELISA format (Griffin *et al.*, 2005). These data suggest that recombinant proteins are better than native proteins, which are not amenable to purification. However, in certain cases, the native protein is more immunogenic than the recombinant one, presumably because of post-translational modifications that only *MAP* can perform. A decrease in immunogenicity has been shown for the MAP1569 (ModD) recombinant when compared with the native protein (Cho *et al.*, 2007). In such cases, unless there is a very easy way to purify that particular protein, it may not be practical as an antigen for a routine diagnostic test.

Nearly all recombinant antigens have been produced using *E. coli* as the host for expression. In only a few studies were *M. smegmatis* (Dupont *et al.*, 2005; Bach *et al.*, 2006; Sechi *et al.*, 2006), a viral vector (Bull *et al.*, 2007) or cell-free methods (Li *et al.*, 2007) used to produce the recombinant proteins. For most of the studies, a single protein was produced and characterized in isolation. More recently, the strategy of analysing more than one protein has emerged. Some investigators have addressed the need to evaluate multiple mycobacterial antigens in parallel by recombinant expression combined with immunological assays via multi-antigen print immunoassays (MAPIA) (Lyashchenko *et al.*, 2000; Waters *et al.*, 2004), immunoblot analysis (Paustian *et al.*, 2004) or ELISA (Cho *et al.*, 2007; Shin *et al.*, 2004; Olsen *et al.*, 2001; Willemsen *et al.*, 2006; Bannantine *et al.*, 2008c). It would be even more informative if dozens of proteins were not only compared with each other but also with known mycobacterial antigens and other *MAP* proteins. A comprehensive analysis of all *MAP* antigens at a whole-genome level in an unbiased assay system is needed to fully determine the antigenic basis of host protective and pathogenic responses to *MAP* infection. The development of a *MAP* protein array (discussed below) aims to address these issues.



MAP3532c **MAP2226c**

Fig. 10.1. Pellets of *Escherichia coli* at the bottom of a 500-ml centrifuge bottle, which were harvested 2.5 h after induction with IPTG. The pellet on the left was induced to express MAP3532c, which is a membrane protein that contains an ABC type 2 transporter domain, and the pellet on the right was induced to express MAP2226c, a hypothetical protein with a DNA binding domain. Notice that the MAP3532c recombinant *E. coli* has a slimy texture that cannot stick to the side of the centrifuge bottle and therefore is sliding down to the bottom. The *E. coli* on the right, producing MAP2226c, is more typical of a discreet, defined pellet observed for wild-type *E. coli* and most recombinant *E. coli*.

In the gamma interferon (IFN-γ) test, fresh whole blood is collected from the animal and stimulated with antigen, to measure IFN-γ release from sensitized lymphocytes via ELISA. Traditionally, the stimulating antigen used is Johnin PPD. The use of this antigen has contributed to the variable specificities obtained with this test (Kalis *et al.*, 2003). Therefore investigators have examined recombinant proteins in an effort to increase specificity while maintaining the test's high sensitivity. The HSP 70 protein appears to be a promising candidate for eliciting a measurable cell-mediated immune response (Koets *et al.*, 2006), although several other recombinant proteins have been evaluated in the gamma interferon test as one measure of cell-mediated responses (Nagata *et al.*, 2005; Shin *et al.*, 2005).

Unfortunately it is difficult to compare these studies with the goal of determining whether there is a single best stimulating antigen. It would be ideal if investigators would include at least one previously reported antigen in their study design to evaluate whether the antigen they are reporting is better or worse, in terms of sensitivity and specificity, than that previously reported. In this manner, the field can advance incrementally until the best set of antigens for specific tests is established and put into use.

10.9 Proteomic Studies of *M. avium* subsp. *paratuberculosis*

The proteome is the set of proteins expressed by *MAP* under a given condition. By cataloguing the different sets of proteins present in changing conditions, we gain insight into the biology of the organism and initiate large-scale antigen discovery.

Early proteomic efforts to characterize the PPA, PPD and secreted fractions were done using SDS-PAGE gel electrophoresis and agar gel immunodiffusion. However, use of these methods only characterized the sizes of protein and detected the most abundant proteins. For example, in 1985, Bech-Nielsen *et al.* (1985) reported that there are 27 proteins present in the sonicated cell extract of *MAP*. Using the latest proteomic technology, including fractionation methods and two-dimensional gel separation, we now know that there are several hundred proteins present in these types of cellular extracts (Egan *et al.*, 2008). Subsequent proteomic efforts focused on immunoscreening genomic expression libraries for *MAP* antigens using sera from infected cows or other hosts (Bannantine and Stabel, 2001; Gioffre *et al.*, 2006; Willemsen *et al.*, 2006). These studies enabled the first proteome-wide antigen screens of *MAP*, assuming the genomic expression library was representative. Antigens that have been identified from these studies include three secreted antigens (Gioffre *et al.*, 2006; Willemsen *et al.*, 2006) and the major membrane protein encoded by MAP2121c (Bannantine and Stabel, 2001). Thus far no antigen identified from these expression library immunoscreens has been incorporated into a routine diagnostic test.

As an initial foray into modern proteomic studies of *MAP*, two physical disruption methods were compared for their ability to represent the proteome. The results suggested that sonication and bead beating worked equally well, with a similar numbers of spots visualized on silver-stained, two-dimensional gels. The bead-beating procedure may be more advantageous since the tube remains closed during the procedure (Lanigan *et al.*, 2004). *MAP* proteomes have subsequently been compared with other subspecies under the same conditions (Hughes *et al.*, 2007, 2008; Radosevich *et al.*, 2007), or *in vitro* and *in vivo* conditions have been compared for the same strain (Egan *et al.*, 2008; Hughes *et al.*, 2007). The study by Hughes and colleagues examined the proteome of terminal ileum scrapings from sheep, as compared with *MAP* cultured in Middlebrook 7H9 broth, and was able to resolve approximately 1000 protein spots on two-dimensional gels for each preparation. They successfully identified ten of the proteins present in higher abundance in the sheep-extracted proteome compared with the Middlebrook-extracted proteome. Although they did not use quantitative proteomics in this study, several proteins with similar abundance between the two preparations were used to normalize the data.

10.10 Protein Arrays

Because JD is a multi-stage disease and, unlike *M. tuberculosis* or *M. leprae*, *MAP* survives longer in the environment outside the host, it is critical to understand how protein expression is regulated or modified in the widely variant conditions experience by the pathogen. From a vaccine or diagnostic standpoint, it is important to know which antigens are expressed at which stage of the disease or life cycle; these types of questions can be addressed using protein array technology.

The power of protein arrays is that equivalent amounts of many proteins are analysed in parallel, thus enabling direct comparison of known antigens with newly identified antigens spotted on the array. Furthermore, this systematic approach enables more comprehensive profiles of the host immune response to the pathogen at any stage of JD. The construction of a protein array for *MAP* was recently undertaken in our laboratories. A 48-spot protein array was initially built to determine the utility of this method as an antigen discovery tool (Bannantine *et al.*, 2008d), and this array was then expanded to include 92 recombinant *MAP* proteins (Bannantine *et al.*, 2008b).

Although protein arrays have only recently been developed, they are already at the forefront of proteomic research (Hoeben *et al.*, 2006; Coleman *et al.*, 2007; Engert *et al.*, 2007; Lee *et al.*, 2007; Bannantine *et al.*, 2008b; Spurrier *et al.*, 2008). High-throughput protein purification and spotting is the most common method of protein array production and is the method used to generate the *MAP* protein arrays. However, some investigators have used cell-free translation systems to avoid the labour-intensive process of purifying large sets of proteins, to reduce the storage time and to prevent loss of protein stability or activity (He and Taussig, 2008). We have also investigated this method (Li *et al.*, 2007), but found more consistent expression of *MAP* proteins using the traditional recombinant protein production approach (Bannantine and Paustian, 2006). Purified proteins have the added benefit of ensuring that immunological reactivity is associated with that protein and not due to contaminating proteins from the expression host.

While protein arrays can be used to study protein–protein interactions or quantify antibody levels (Kersten *et al.*, 2003; Coleman *et al.*, 2007), the *MAP* protein array was developed specifically for immune profiling of cattle. This profiling could probably lead to a new diagnostic, but it may be even more valuable in the search for a subunit vaccine. In vaccine strategies, it may be better if one can identify which proteins invoke an immune response within cattle and use only those proteins in a vaccine formulation as opposed to simply immunizing with a whole-cell lysate or killed bacteria.

These studies have shown that single recombinant proteins were much more readily detected than the PPA also spotted on the same array. This suggests that ELISA tests incorporating PPA as the plate-coating antigen might be improved if the assay used one or more recombinant proteins. Why do single antigens react stronger than the PPA? This is most likely due to the heterogeneous nature of the PPA preparation, as described earlier in this chapter. Because so many proteins are present in the preparation and each protein is represented in small quantities, no single antigen can stand out. However, once a dominant antigen is isolated and spotted in a pure form, it should theoretically elicit potent recognition. This observation recalls the work of Bech-Nielsen *et al.* (1992), where a partially purified PPA resulted in a higher ELISA sensitivity than did the crude PPA.

Although only a few cattle could be tested using the protein array, the antibody profiles demonstrated consistency between animals (Bannantine *et al.*, 2008b). In contrast, recombinant proteins tested in sheep showed frustratingly inconsistent results, as no single protein emerged as strongly detected in all sheep (Bannantine *et al.*, 2008c). As there are too many variables between these two studies, it is not possible to make any general conclusions about sheep and cattle humoral immune responses.

10.11 Concluding Remarks and Future Directions

No single method of proteomic analysis of *MAP* antigens is ideal. It would take many

research dollars and several years to assemble a protein array that represents all proteins produced by this ruminant pathogen. Even if that were possible, recombinant production of these proteins will probably affect the antigenicity of at least a subset of them, due to mycobacteria-specific, post-translational modifications. One study has already indicated that certain recombinant proteins are not as antigenically potent as the native proteins (Cho *et al.*, 2007), although this does not appear to be the case for all recombinant proteins (Bannantine *et al.*, 2004, 2008d; Griffin *et al.*, 2005). Traditional proteomic strategies using two-dimensional separation are not without their limitations either. The current state-of-the-art technology remains incapable of separating and resolving a complete proteome. Therefore, only subfractions of the proteome are analysed in any single experiment. Additionally, none of the proteins in the gel are immediately known or consistently located among different experiments. Only through laborious excision of spots and analysis by mass spectrometry can selected proteins be identified. As a result of these and other limitations with each method, the researcher must carefully consider the question to be answered before selecting the method to address a stated hypothesis.

New ideas and methods for the preparation of antigenic proteins have yielded recent breakthroughs that may translate into better diagnostic products to control Johne's disease. This is especially true for the ethanol vortex preparation that has been incorporated into an ELISA test (Eda *et al.*, 2006), and other ELISA tests also show promise (Shin *et al.*, 2008). In addition, the genome sequence has enabled new proteins to be tested in a high-throughput manner (Bannantine *et al.*, 2008a,b). This is encouraging in that the discovery pipeline for *MAP* antigens is now flowing.

Surprisingly little research has applied *MAP* antigens to Crohn's disease studies. In fact, cell-mediated immune responses from Crohn's disease patients have not been reported against *MAP* antigens. This situation may soon change, as a focused research direction is proposed in a new American Academy for Microbiology report on the link between Crohn's disease and *MAP* (Nacy and Buckley, 2008). The report calls for several research priorities, among them improved immunodetection of *MAP*.

As data accrue, it may be worthwhile to conduct a systematic review of the scientific literature and determine whether useful antigens have been described but their potential has not yet been fully exploited. It seems many described antigens of *MAP* have not been followed up for a variety of reasons. Because detection of antibody or a cell-mediated immune response is simple, rapid and relatively inexpensive, activities leading to the discovery of new antigens with immunodiagnostic potential could easily be intensified.

Although development of antigen-based diagnostics has been the driving force behind many of the advances described, proteomic technologies will be critical in understanding the biology and pathogenesis of *MAP*. For example, blue native gel technology along with the protein array will be instrumental in identifying protein–protein interactions on a genome-wide scale. The blue native technique is excellent at resolving membrane protein complexes (Schagger and von Jagow, 1991), and proteins that participate in these complexes can then be identified by mass spectrometry. Finally, specific interactions of the newly identified membrane protein(s) can be confirmed using the protein array. The protein array can also be used to identify pathogen proteins that may initially interact with the bovine epithelial cells lining the intestine. From these types of experiments, we may finally obtain molecular details surrounding the initial events during infection, beyond what has recently been discovered with the oxidoreductase gene (Alonso-Hearn *et al.*, 2008) and the major membrane protein (Bannantine *et al.*, 2003).

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Characteristics of an extensive *Mycobacterium avium* subspecies *paratuberculosis* recombinant protein set

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ABSTRACT

In the first step of a comprehensive large-scale antigen discovery project, 651 genes of *Mycobacterium avium* subspecies *paratuberculosis* were expressed in *Escherichia coli*. All of these were purified by affinity chromatography, dialyzed in phosphate buffered saline, and analyzed on SDS–PAGE gels. Collectively, these purified recombinant proteins represent 14.9% of the total *M. avium* subsp. *paratuberculosis* proteome. This volume of protein expression and purification has yielded unique observations that may be missed in smaller scale expression and purification projects. For example, the 252 putative membrane proteins predicted by PSORTb analysis, resulted in lower average expression yields (3.51 mg/l culture) than the 176 predicted cytoplasmic proteins (7.27 mg/l culture). A few proteins (MAP0107c, MAP3169c and MAP3640) appear to promote lysis of *E. coli* since there was a drop in optical density of the growth culture minutes after the inducing agent was added. Certain *M. avium* subsp. *paratuberculosis* proteins, when expressed in *E. coli* changed the color of the column resin or appearance of harvested cell pellets. Finally, 19 proteins showed an absorbance maximum at 260 nm rather than 280 nm that was attributed to binding of nucleic acid during purification. This extensive recombinant protein repository provides a powerful tool for proteome- and genome-scale research of this organism.

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Introduction

High-throughput protein production projects are important to obtain milligram quantities of pure protein from large numbers of coding sequences to perform structural studies, build protein arrays, define protein interactions, generate antibodies, etc. This approach is currently being used to develop the human protein atlas [1] and our laboratory has taken a conceptually similar approach with *Mycobacterium avium* subsp. *paratuberculosis*, a bacterium the causes Johne's disease in cattle, goats and other ruminant animals [2]. Johne's disease is a chronic gastrointestinal inflammatory disease prevalent in areas of the United States with high concentrations of dairy cattle. Diarrhea, reduced feed intake, weight loss, and eventually death characterize the late stage of this intestinal disorder. The disease has had considerable impact on the global agricultural economy [3,4]. This well established veterinary pathogen has also been implicated as an etiologic agent of Crohn's disease [5], leading researchers to speculate on a potential pathogenic role for humans.

We initially attempted a high-throughput production of *M. avium* subsp. *paratuberculosis* recombinant proteins using an in vitro transcription and translation system [6]. This approach eliminated cloning and host expression, but resulted in low yields with no expression in a high proportion of the genes tested. Subsequently, *Escherichia coli* has been used as the cloning host of choice due to its convenient handling and long history of successful recombinant protein expression. This, combined with the high yield and solubility of the maltose binding protein (MBP[1]) fusion system, has enabled the development of a 96-spot protein array for initial antigen screening [7]. This work clearly showed the importance of expanding the protein array to enable a greater number of direct protein comparisons within a given experiment.

The identification of *M. avium* subsp. *paratuberculosis* antigens that could allow early, sensitive, and specific detection of infection is critically needed to implement sound disease control measures. However, the current practice of using complex *M. avium* subsp. *paratuberculosis* protein mixtures as antigens, including Johnin purified protein derivative or whole cell extracts, compromises assay specificity due to the presence of conserved epitopes in those mixtures. Therefore, immunoassays specific for the diagnosis of Johne's disease requires purified antigens unique to *M. avium* subsp. *paratuberculosis*.

In the current study, we took inventory of the purified recombinant proteins on hand and discovered some interesting trends that

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[1] *Abbreviation used*: MBP, maltose binding protein.

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might only be appreciated in a collection of this size. The findings provide a guide to help future investigators with their protein purification studies on this bacterium and are more broadly applicable to other mycobacteria.

Materials and methods

Cloning of M. avium subsp. paratuberculosis coding sequences

Annotated coding sequences selected from the *M. avium* subsp. *paratuberculosis* K-10 genome project [8] were amplified from strain K-10 genomic DNA prepared as described previously [9]. Because the DNA has an average GC content of 69% [8], Dimethyl sulfoxide (up to 5%) or Advantage GC-2 PCR kit (Clontech, USA) were used to aid in amplification of GC-rich sequences. In most cases, primers were designed to amplify the entire coding sequence; however, partial sections were cloned if the entire coding sequence was either too large or gave poor expression in *E. coli*. The 5′ primer was designed with the *XbaI* restriction site and contained 21 gene-specific nucleotides whereas the 3′ primer was designed with the *HindIII* site for directional cloning into the pMAL-c2x expression vector, an early generation plasmid originally obtained from New England Biolabs, USA. This plasmid has an inducible lacZ promoter, an exact deletion of the *malE* signal sequence and *XbaI* and *HindIII* restriction recognition sequences within the MCS. PCR products were purified using Gene Clean Turbo kit (Q-Biogene, USA) and set up for overnight ligation at 16 °C. The ligation was used to transform *E. coli* DH5-α frozen competent cells and plated on LB media supplemented with glucose (0.2%) and ampicillin (100 μg/ml). Ligated constructs resulted in an in-frame fusion between the *malE* gene in the vector, which encodes MBP, and the mycobacterial reading frame of interest. In all cases, MBP is the N-terminal located affinity tag.

Protein expression and purification

The methods for expression and purification of *M. avium* subsp. *paratuberculosis* recombinant proteins are described in detail elsewhere [10]. Briefly, *E. coli* DH5-α harboring the expression plasmid were cultured by rotary shaking at 37 °C in 1-liter LB media supplemented with glucose (0.2%) and ampicillin (100 μg/ml) and induced with 250 μl of 1 M IPTG when the $O.D_{600nm}$ measured between 0.4 and 0.6. After a 2.5-h induction at 37 °C, cells were harvested by centrifugation and stored at −20 °C overnight. The harvested cells were then thawed followed by brief sonication (3 × 1 min bursts at 30 watts with a 2-min incubation on ice between each burst). The induced lysate was clarified by centrifugation and the fusion protein was then affinity purified using amylose resin column chromatography. The fusion protein was eluted with column buffer containing 10 mM maltose. Collected fractions were analyzed by SDS–PAGE gels stained with GelCode Blue (ThermoScientific) and spectrophotometrically using a NanoDrop spectrophotometer. The most concentrated samples were pooled and dialyzed (Pierce dialysis cassettes) in 1-liter PBS at 4 °C with three exchanges. In rare instances, mass spectrometry was used to confirm proteins that had unexpected migration patterns on SDS–PAGE gels.

Enzyme treatment of proteins at 260 nm

Proteins with an absorbance peak maximum at 260 nm were exposed to Turbo DNase (Applied Biosystems-Ambion), RNaseA (Qiagen) and Proteinase K (Qiagen) as follows. An aliquot of the recombinant protein was diluted to 1–3 mg/ml in PBS buffer and treated with either Turbo DNase (1 unit/mg protein) and RNaseA (3.5 units/mg protein) or Proteinase K (2 mAU/mg protein). Nanodrop spectrometry was used to observe the recombinant protein spectra immediately before and after enzyme addition. The protein–enzyme mixture was then added to a dialysis cassette with a 10,000 molecular weight cutoff (Thermo Scientific) and incubated at 37 °C for 3 h. Spectral analysis was again performed after incubation with the enzyme. Untreated recombinant protein was used as the negative control and a recombinant protein with an absorbance peak maximum at 280 nm was used as the positive control. All treated and untreated samples were also analyzed by GelCode Blue stained SDS–PAGE and ethidium bromide stained agarose gels.

Electron microscopy

All fixation and staining procedures were conducted at room temperature. *E. coli* cells were fixed for 2–4 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed cells were washed in the same buffer three times and were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.4, for 2 h. After washing in the same buffer 3×, cells were incubated with 30% ethanol for 10 min. The cells were further dehydrated with a graded series of ethanol, then propylene oxide and embedded in epoxy resin (Embed 812). All ultrathin sections were double stained with uranyl acetate and Reynolds lead citrate and then observed under a Tecnai 12 BioTWIN transmission electron microscope.

Bioinformatic and statistical analysis

Signal sequence predictions were determined using SignalP software (http://www.cbs.dtu.dk/services/SignalP). PSORTb analysis was used to predict protein localization based on a number of factors including transmembrane helices, signal peptide, motif search, similarity to proteins with known subcellular location, etc. [11] (http://www.psort.org/psortb/). BLAST similarity searches were performed locally on coding sequences by comparison to the GenBank nonredundant protein database (Oct 24, 2009 release). SAS software was used for the Wilcoxon test on the lognormal distributions of protein location versus yield.

Results

Characteristics of the M. avium subsp. paratuberculosis recombinant protein set

A total of 707 expression clones were confirmed to be correct and in-frame by DNA sequencing. Of these cloned genes, 651 produced a fusion protein consisting of the *M. avium* subsp. *paratuberculosis* derived protein of interest with an N-terminal MBP affinity tag, giving a 92% successful expression rate as assessed by UV spectrometry and SDS–PAGE analysis (Fig. 1). A complete list of these recombinant proteins, including yield, length, location prediction and functional description, is presented in Supplementary Table 1. By comparison, 55,000 cloned bacterial genes in the target database maintained by the Protein Data Bank resulted in a successful expression rate of only 30% [12]. The extent that our collection of 651 proteins represents the total proteome is as follows. The total amino acids predicted by the annotated *M. avium* subsp. *paratuberculosis* genome are 1469,392 contained among 4350 genes. The total amino acids present in this recombinant protein collection are 191,496. Therefore, 14.9% of the *M. avium* subsp. *paratuberculosis* proteins and 13.5% of all amino acids are represented in this repository making it the largest known mycobacterial recombinant protein set.

The length of recombinant mycobacterial proteins ranged from 37 amino acids (50S ribosomal protein L36) to 1108 amino acids

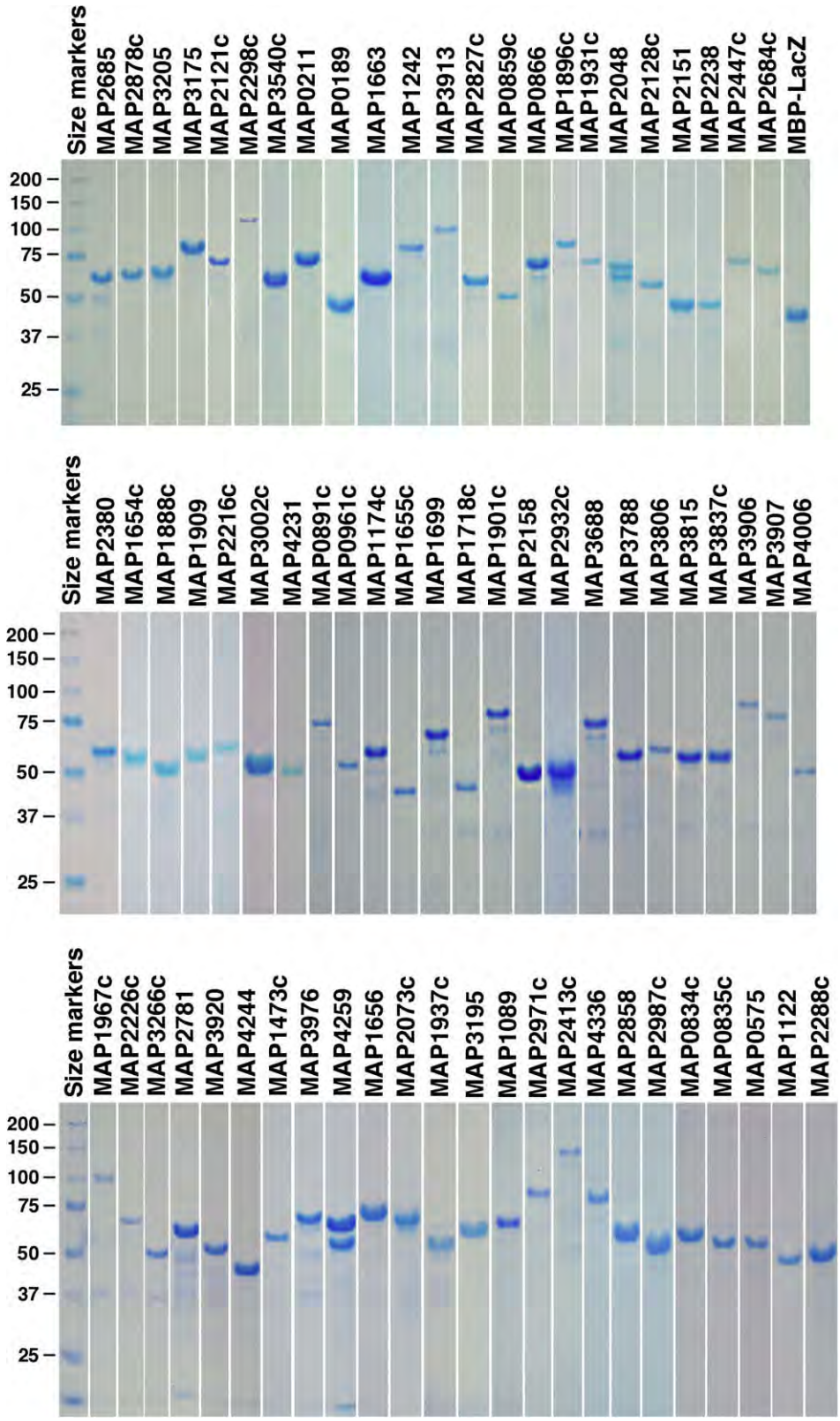

Fig. 1. SDS–PAGE analysis of 72 *M. avium* subsp. *paratuberculosis* recombinant proteins purified by affinity chromatography. The unique identifier for each *M. avium* subsp. *paratuberculosis* recombinant protein is indicated above the lane. The gels were loaded with identical volumes of eluted protein irrespective of the concentration. Protein size standards are shown in kilodaltons at the left of each row.

(cell wall arabinan synthesis protein). The average length is 298 amino acids. The distribution of recombinant protein length is shown in Fig. 2A. A majority of the proteins were produced at full-length from the annotated translational start to the stop codon. However, when truncated versions of specific proteins were necessary to perform experiments, they were engineered with the N-terminal and C-terminal halves expressed independently. This was the case for MAP1272c, MAP1643, MAP2121c, MAP2239,

MAP3638c, MAP3840, MAP4321c and MAP4336 (data not shown). Another gene was so large (MAP1242 at 12 kb) that it was divided and cloned into seven segments with each segment expressed independently [13]. A truncated recombinant protein is represented in Supplementary Table 1 by the number of amino acids present in the fusion protein along with the total amino acids encoded by the gene (i.e. 230/245). A full-length recombinant protein is indicated by the word "all" (i.e. all 245).

The largest functional category of proteins in the collection included the hypothetical proteins, totaling 155 and representing 24%. Proteins in this category were specifically selected for future screening of these little-known proteins to identify which might be the best antigens for use in diagnostic tests or as vaccine candidates. Functional studies are also planned for proteins in this category. All but two of the 51 annotated lipoproteins encoded in the genome are represented in this repository. A unique and consistent characteristic of the lipoproteins was that they created excessive foaming of the buffers used during the purification process. This trait was especially noticeable during resuspension of harvested IPTG-induced *E. coli* cells.

Protein yield

Of the 651 proteins, 324 were produced in a yield of greater than 4.0 mg whereas only 50 resulted in less than 1 mg from a 1-liter culture. The average yield of protein was 5.28 mg/l of culture. In general, the yield of protein declined with increasing numbers of mycobacterial amino acids participating in the fusion protein (Fig. 2B). The highest yield obtained was 33.9 mg from 1-liter of culture expressing MAP0810, a cold shock DNA-binding protein. In contrast, 57 genes did not produce protein under the conditions described (Supplemental Table 2). A few of these proteins even appeared toxic to *E. coli* as shown by a sharp drop in the optical density of the culture within minutes of induction with IPTG (Fig. 3). This phenomenon has been also been observed previously with MAP0176 and MAP0243c [14].

Protein yield as a function of subcellular location

According to PSORTb prediction, the recombinant protein set contains 176 cytoplasmic proteins (9.5% of all predicted cytoplasmic proteins in *M. avium* subsp. *paratuberculosis*), one cell wall protein, 252 cytoplasmic membrane proteins (30% of predicted membrane proteins in *M. avium* subsp. *paratuberculosis*), 37 extracellular proteins (100% of predicted extracellular proteins in *M. avium* subsp. *paratuberculosis*) and 185 proteins with uncertain localization predictions (Supplemental Table 1). SignalP analysis on the full set of proteins shows that 183 have a signal peptide and the remaining 528 have no recognizable signal peptide (Supplemental Table 1). Analysis of the yield among these proteins

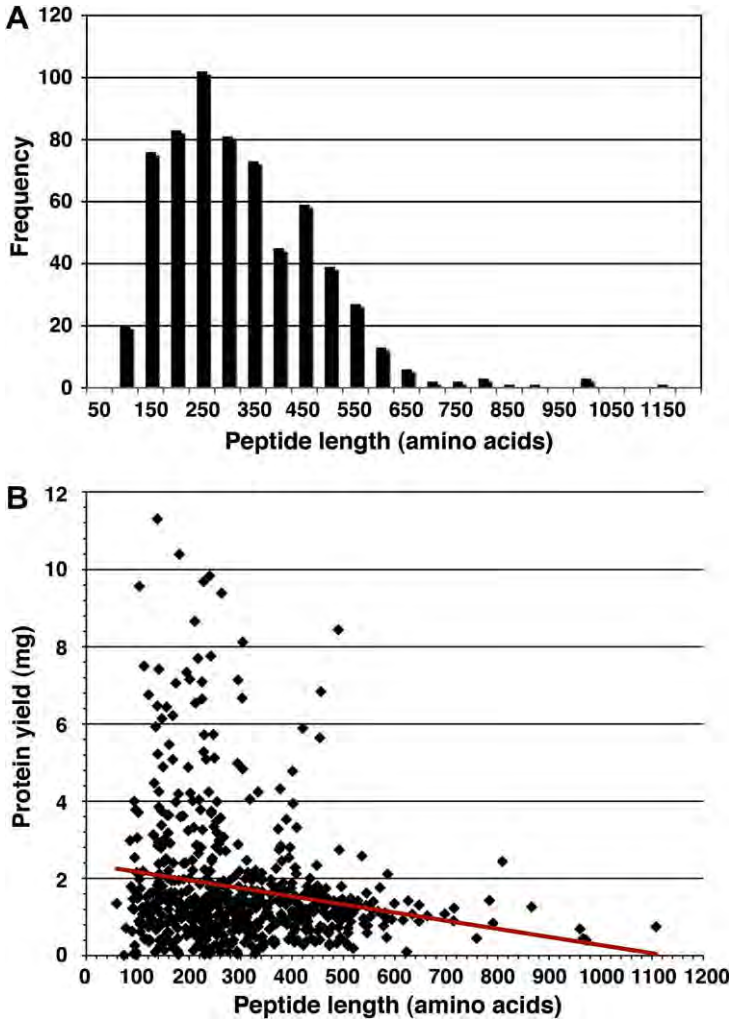

Fig. 2. Distribution of size and yield for recombinant *M. avium* subsp. *paratuberculosis* proteins. (A) Frequency of peptide length in the recombinant protein set. Peptides from 200–250 amino acids were the highest at just over 100. The average peptide length was 298 amino acids. (B) Scatter plot showing the relationship between peptide yield and amino acid length. Trend line indicates decreasing yields as peptide length increases. Yields above 10 mg/l were only observed with peptides of 500 amino acids or less.

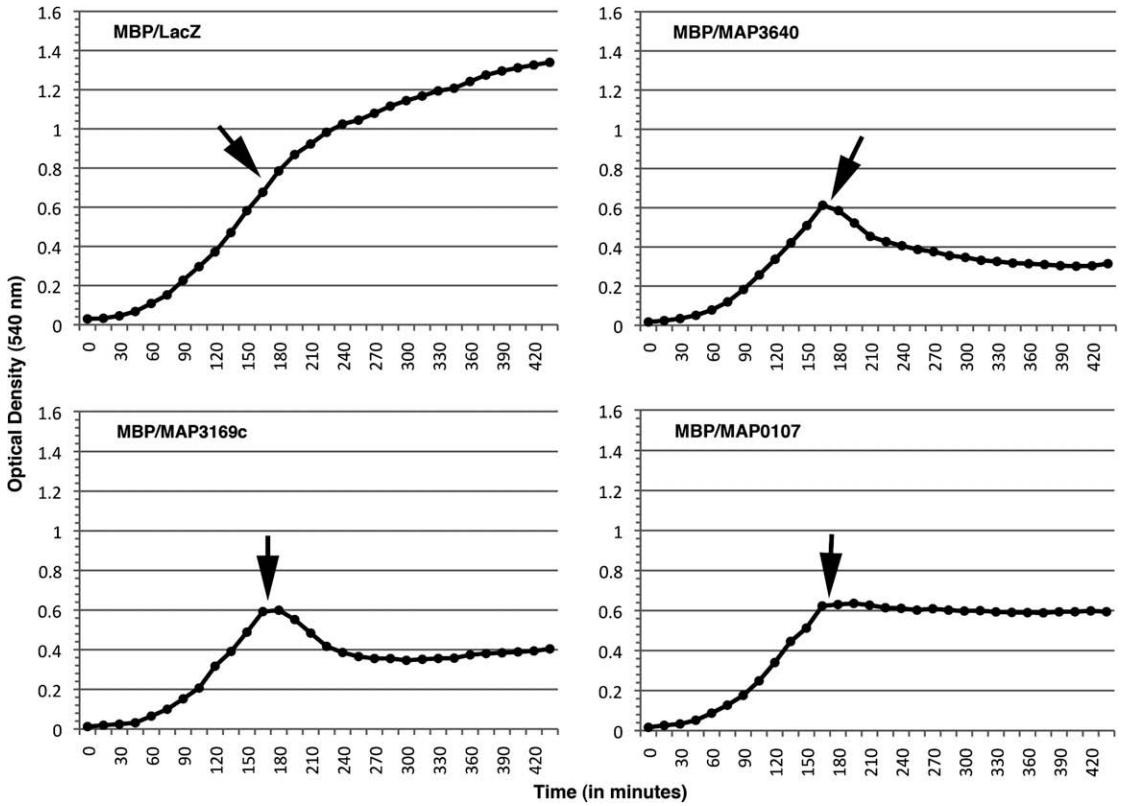

Fig. 3. Three *M. avium* subsp. *paratuberculosis* fusion proteins are toxic to *E. coli*. Shown are four growth curves of *E. coli* harboring recombinant expression plasmids with the fusion protein indicated on each graph. Optical density measurements of cultures were taken every 15 min. The MBP/LacZ growth curve is typical of that seen for wild-type *E. coli* and many *M. avium* subsp. *paratuberculosis* genes. However, for the cultures induced to express, MAP0107 (transporter that is part of the mce2 operon), MAP3169c (hypothetical protein) and MAP3640 (membrane protein), there was an immediate drop or leveling off of the optical density indicating either lysis or bacteriostasis. Arrows identify the point in the growth curve were the IPTG inducing agent was added.

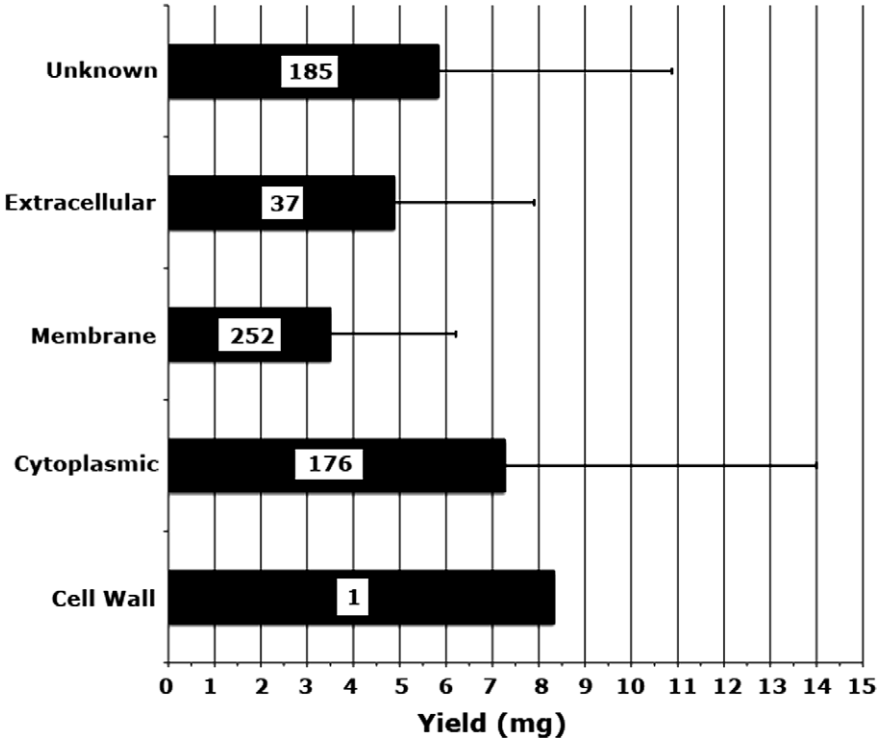

Fig. 4. Relationship of protein yield to predicted subcellular location. Graphical representation of average yield in milligrams of proteins grouped according to their predicted subcellular location as determined by PSORTb analysis [11]. With the exception of the one and only cell wall protein (MAP3434), the data show cytoplasmic proteins had the highest average yield and membrane proteins had the lowest average yield. Yield differences between these two protein categories are statistically significant as determined by the Wilcoxon test. Error bars represent standard deviation of the mean.

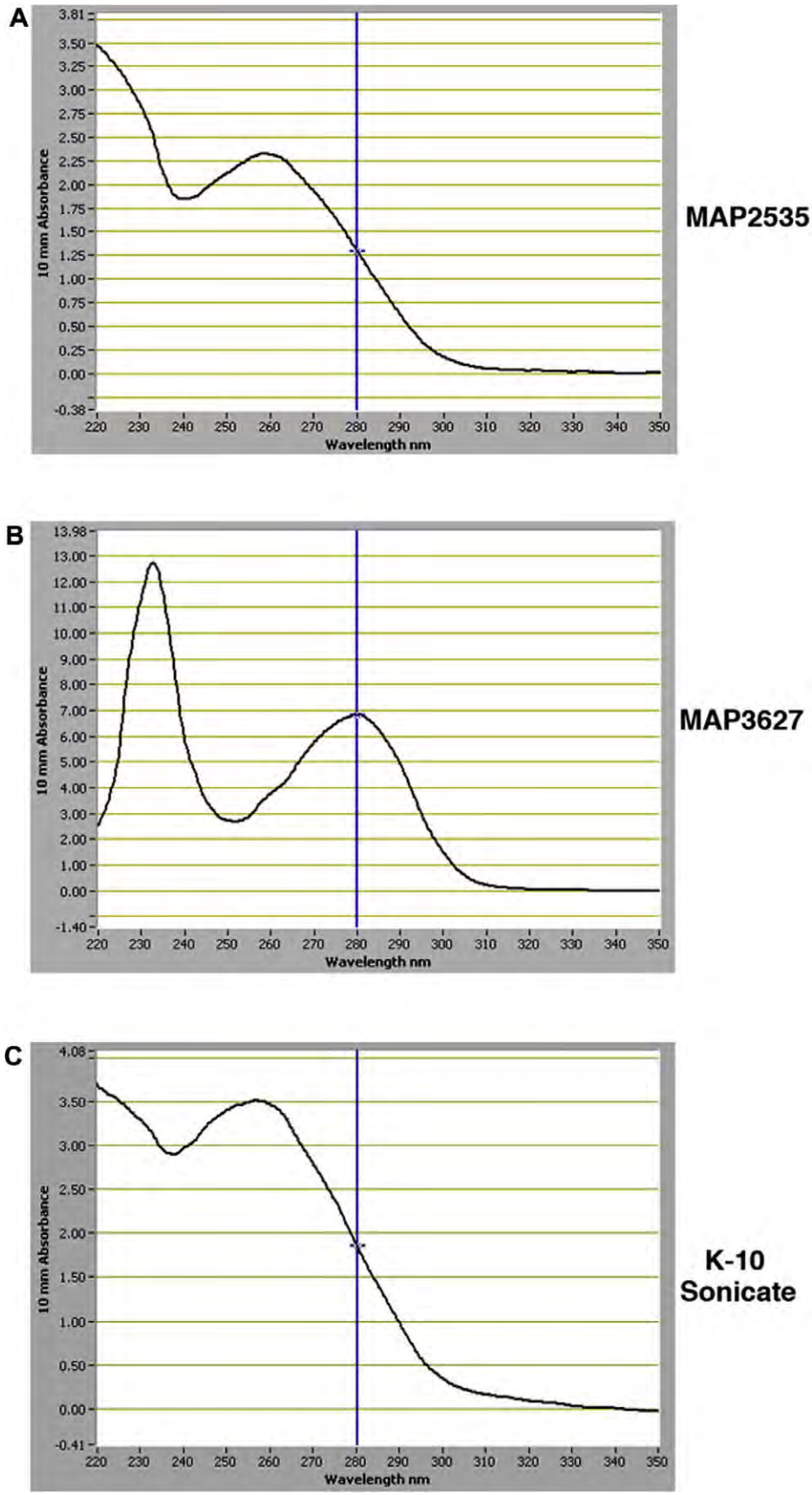

Fig. 5. Absorbance spectra of recombinant proteins. Spectrophotometric analysis shows the different absorbance maximums for recombinant proteins. MAP2535 showed an absorbance maximum at 260 nm (A) whereas MAP3627 showed an absorbance maximum at 280 nm (B). A whole cell extract of *M. avium* subsp. *paratuberculosis*, which contains most of the native proteins showed an absorbance maximum at 260 nm (C). This sonicated protein prep was not affinity purified.

arranged according to cellular location shows wide variability as indicated by error bars in Fig. 4. However, statistical analysis shows the membrane proteins had the smallest yields with an average of less than 4 mg/l of culture. The yield differences between the membrane proteins compared to cytoplasmic proteins and extracellular proteins are statistically significant as determined by the Wilcoxon test ($P < 0.005$). Furthermore, among the coding sequences that did not express in *E. coli* (Supplemental Table 2), 68% are predicted membrane proteins whereas only 12% are predicted cytoplasmic proteins. This percentage may actually be higher as the locations of 10 proteins could not be determined using this algorithm and therefore were classified as unknown.

Protein absorbance spectra

Examination of the absorbance spectrum from 220 to 350 nm revealed three distinct absorbance peak maximums among the recombinant protein set. 632 proteins showed an absorbance peak maximum at 280 nm while 19 proteins displayed a peak maximum at 260 nm. Two proteins (MAP0585 and MAP0790) displayed a peak maximum at 270 nm. Fig. 5A and B shows the two most common spectral peaks observed from purified recombinant proteins in this study. For MAP2535 (Fig. 5A), the concentration is estimated to be 1.30 mg/ml at 280 nm, but the absorbance peak in this example is really at 260 nm giving a concentration of 2.30 mg/ml at that wavelength. Even though the vast majority of recombinant proteins showed a 280 nm absorbance peak, the whole cell extract of *M. avium* subsp. *paratuberculosis* had a peak at 260 nm (Fig. 5C).

This shift from the typical 280 nm peak for pure proteins may indicate contamination with nucleic acid because the A260/280 ratios are very high (range 1.24–2.08) among the 19 proteins (Table 1). The commonly accepted average extinction coefficients for 1 mg/ml nucleic acid solutions at 260 and 280 nm is 20 and 10 respectively [15]. Similarly the extinction coefficient values at 260 and 280 nm at a concentration of 1 mg/ml are 0.57 and 1.00 respectively for proteins [15]. Using these extinction coefficients, pure nucleic acid samples would have an A260/A280 ratio of 2.0, while highly pure protein would be 0.57. Similarity searches of these 19 proteins suggest that one of the proteins may be a DNA-binding protein (MAP3024c), but the remaining 18 do not appear to be (Supplemental Table 1).

When the samples were treated with nucleic acid degrading enzymes, there was minimal change in the A260/280 ratio and little to no shift in the absorbance peak (Table 1). Furthermore, the A260/280 ratios remained constant after treatment with proteinase K even though SDS–PAGE analysis demonstrated the protein was degraded (Table 1 and data not shown). As a control, MAP1174c, a protein with a peak maximum at 280 nm, was exposed to proteinase K treatment. Prior to addition of proteinase K, the A280 nm was 3.94 and after treatment, A280 nm = 0.61. The A260/280 ratio was 0.52 and 0.60, respectively. Ethidium bromide stained gels of proteins with 260 nm peak showed a fluorescent smear in the 100 bp size range indicating presence of nucleic acid, whereas proteins with a 280 nm absorbance peak did not show fluorescence. Collectively, these results suggest binding and co-purification of nucleic acids during the affinity purification process. Why these 19 proteins bind nucleic acid and whether binding is specific or non-specific remains unclear.

Chromogenic proteins and E. coli pellet characteristics

In the course of expression and purification, a few proteins displayed chromogenic characteristics. MAP0211, a UDP-galactopyranose mutase, resulted in yellow *E. coli* pellets and also turned the column resin yellow (Fig. 6A) since this enzyme is a typical yellow flavoprotein [16]. MAP3457 (*O*-acetylhomoserine aminocarboxypropyltransferase) and MAP3898 (glycine oxidase) also turned the resin a bright yellow (data not shown). Glycine oxidase is known to bind the FAD cofactor which is yellow [17] and MAP3457 might also bind a riboflavin derivative [18]. MAP1595 (bacterioferritin subunit) and MAP1469c (cytochrome P450) both turned the resin a red–brown tint of color indicating iron binding and heme content (data not shown and Fig. 6A) [19]. MAP3114 (hypothetical protein) turned the resin a light brown–grey.

Table 1
Proteins with an absorbance peak maximum at 260 nm.

MAP ID	A260 nm (protK)[a]		A260/280 protK[b]		A260 nm DNase[c]		A260/280 DNase[d]		Peak shift
Number	Before	After	Before	After	Before	After	Before	After	From 260 nm[e]
MAP0184c	3.75	1.70	1.87	1.85	3.75	1.55	1.88	1.90	No shift
MAP0324	1.45	0.62	1.81	1.86	1.45	0.75	1.81	1.34	265 nm
MAP0573c	1.48	0.39	1.51	1.74	1.48	1.20	1.50	1.49	No shift
MAP1122	1.10	0.72	1.24	1.57	1.11	1.03	1.24	0.86	275 nm
MAP1250	1.52	0.22	1.38	1.98	1.52	0.94	1.37	0.97	275 nm
MAP1331c	1.68	1.30	1.86	1.87	1.67	1.03	1.88	1.07	265 nm
MAP1699	1.16	0.49	1.95	1.93	1.16	0.43	1.96	1.57	No shift
MAP1902c	1.64	0.73	1.87	1.88	1.64	0.99	1.87	1.30	No shift
MAP1903c	3.51	2.80	1.93	2.10	3.51	2.28	1.93	1.97	No shift
MAP1909	3.43	2.10	1.71	1.85	3.43	3.36	1.71	1.30	265 nm
MAP1918c	1.47	0.34	2.05	1.92	1.45	1.41	2.00	1.71	No shift
MAP2100	3.35	2.61	1.94	1.94	3.35	1.19	1.98	1.27	265 nm
MAP2535	2.85	2.80	1.84	1.91	2.85	1.77	1.86	1.63	No shift
MAP3024c	5.87	5.21	2.08	2.0	5.87	5.82	2.02	1.74	No shift
MAP3217c	1.19	0.61	1.51	1.76	1.20	0.87	1.51	1.13	265 nm
MAP3629c	0.89	0.32	1.84	1.87	0.89	0.66	1.85	1.84	No shift
MAP3664	1.49	0.45	1.47	1.69	1.49	1.22	1.47	1.03	No shift
MAP3906	1.53	0.83	1.72	1.80	1.52	0.97	1.70	1.10	No shift
MAP4186	6.24	5.72	1.93	1.84	6.24	6.11	1.94	1.45	No shift
Sonicate ag[f]	4.50	3.66	1.92	1.93	4.51	4.36	1.92	1.65	No shift

[a] Absorbance at 260 nm of affinity purified protein taken before and after proteinase K incubation for 3 h at 37 °C.
[b] 260/280 absorbance ratio before and after treatment with proteinase K.
[c] Absorbance at 260 nm of affinity purified protein taken before and after DNase/RNase incubation for 3 h at 37 °C.
[d] 260/280 absorbance ratio before and after DNase/RNase incubation for 3 h at 37 °C.
[e] The shift in the absorbance peak maximum from 260 nm after treatment with DNase/RNase.
[f] The whole-cell sonicated antigen of *M. avium* subsp. *paratuberculosis*.

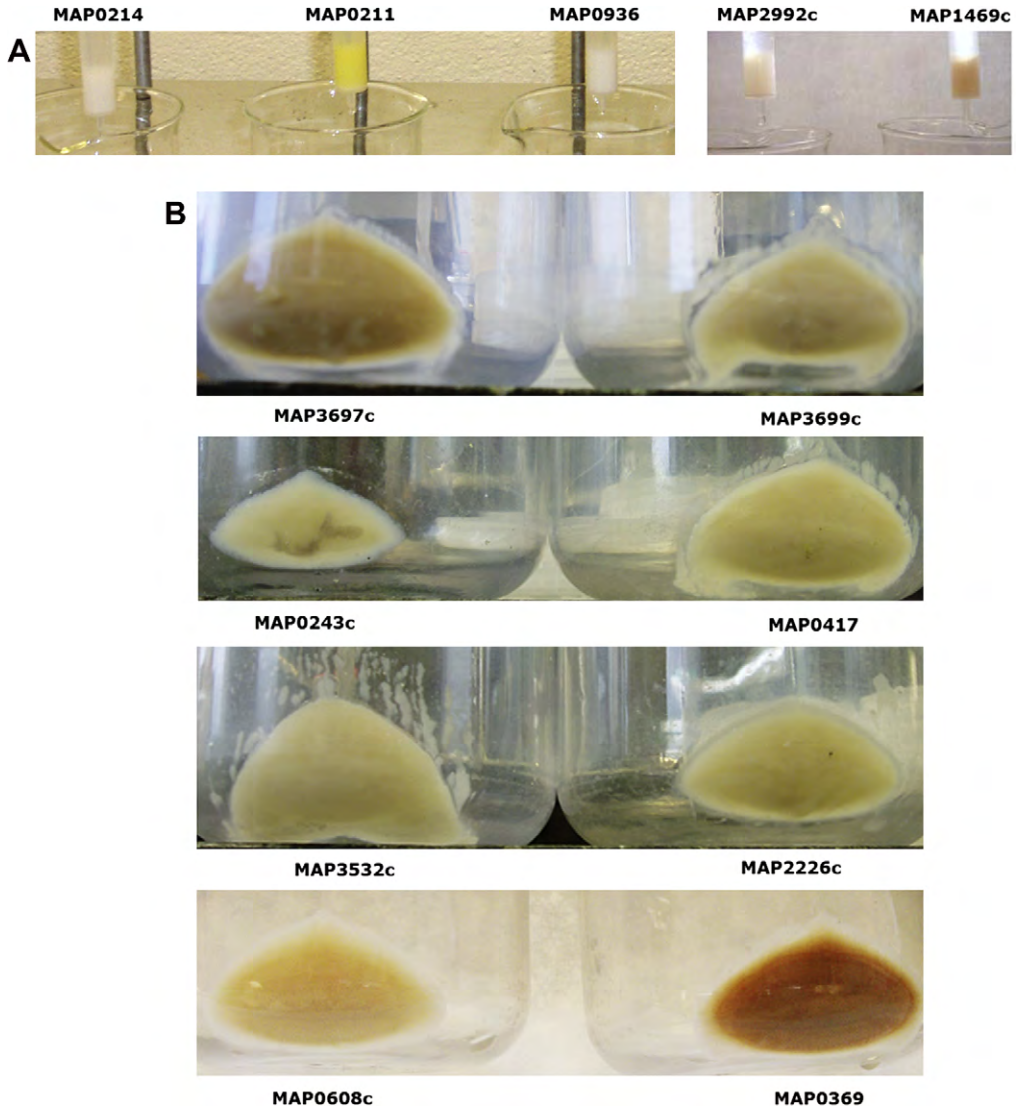

Fig. 6. Phenotypic changes in recombinant *E. coli*. (A) Shown are chromatography columns containing a 5-ml bed of amylose resin. Chromogenic recombinant proteins turn the white column resin different colors. Proteins such as MAP0211 give the resin a yellow color in comparison to the typical white resin seen in columns loaded with *E. coli* extracts containing MAP0214 and MAP0936. Likewise, the resin from purification of MAP1469c appears as a red–brown tint in comparison to MAP2992c. (B) Changes to the IPTG-induced and harvested *E. coli* pellets could be observed in some expression experiments. MAP0417 and MAP3699c expression yielded slimy pellets that quickly slid down to the bottom of the centrifuge bottle after the media was decanted. Pellets from MAP3997c, MAP0243c and MAP2226c were more typical in that they formed "tight" pellets. The harvested pellet from MAP0369 expression resulted in a dark orange color in comparison to MAP0608c.

MAP0094 (hypothetical protein), which is not present in the closely related *M. avium* subsp. *avium*, turned the resin brown. Other proteins, including MAP3532c (ABC type exporter), MAP0417 (type II secretion system protein), MAP3699c (integral membrane protein) (Fig. 6B) and MAP1609c (antigen 85B) (data not shown), when over-expressed in *E. coli* resulted in a slimy pellet texture that was unable to remain tightly associated to the side of a centrifuge bottle. Still other proteins caused the *E. coli* pellets to be very difficult to resuspend. MAP0771 (hypothetical protein), MAP2447c (UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase) and MAP2979 (D-alanyl-D-alanine carboxypeptidase) are three proteins that gave sticky biofilm-like characteristics to the pellet. Some pellets became very stringy, showing long, clinging strands of intact, unbroken cells when resuspended. *E. coli* expressing MAP3056 (lipoprotein) is an example of this characteristic (Fig. 7B). Other proteins that gave *E. coli* this characteristic include MAP1182c (membrane protein), MAP1628 (membrane protein), MAP1680c (secreted protein), MAP1829c (membrane protein), MAP2499 (ABC transporter), MAP2942c (copper sensitivity protein), MAP2979 (D-alanyl-D-alanine carboxypeptidase), MAP3307c (hypothetical protein), MAP3852c (hypothetical protein) and MAP4138c (hypothetical protein). Electron microscopy shows electron-translucent areas within *E. coli* expressing MAP3056 (Fig. 7A). These areas are seen at either pole of the rod-shaped bacteria or just underneath the outer membrane. Collectively, these observations show how the protein alters the phenotype of *E. coli* and may also contribute to the phenotypic characteristics of mycobacteria.

Discussion

Ideally, a protein collection should be comprehensive, representing most of the coding sequences in the genome of an organism. In practice, this is very difficult to achieve because of the number of genes and labor-intensive steps involved in cloning, expression and purification. High throughput expression and purification methods are being developed that will soon make this ap-

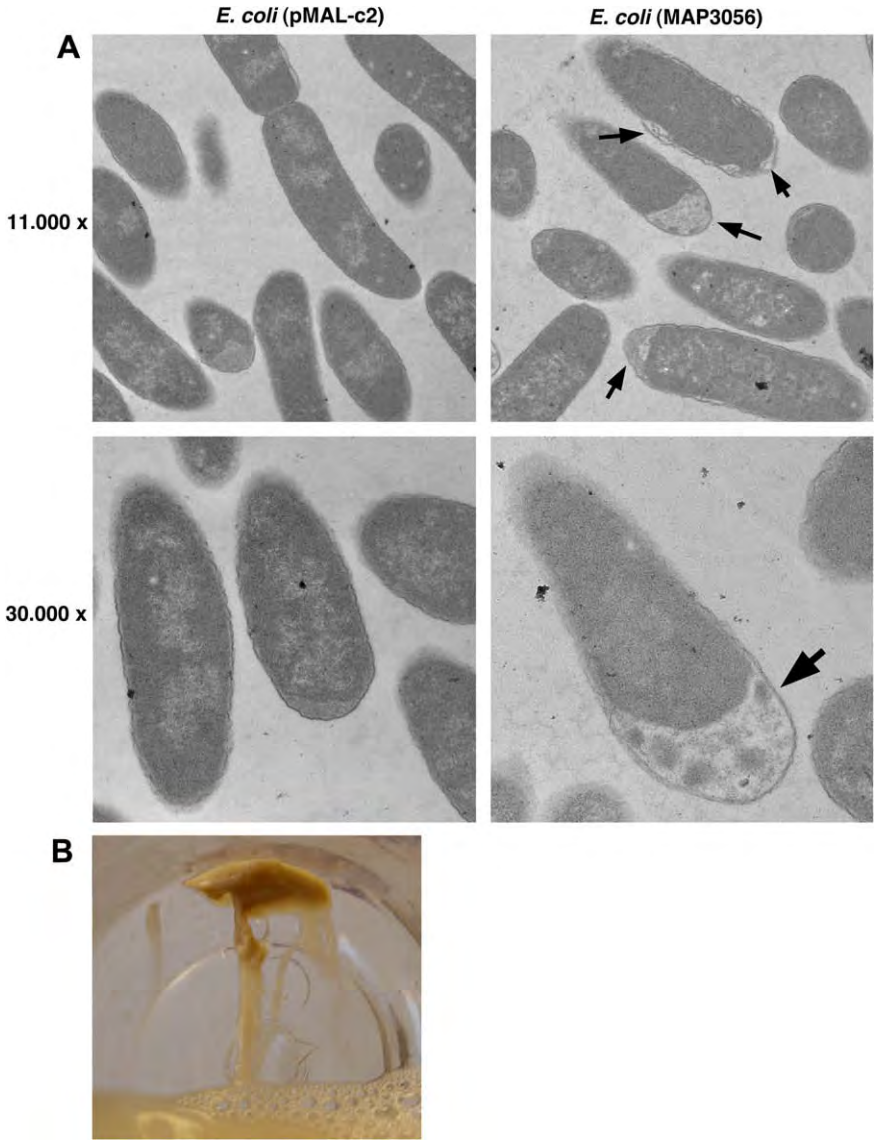

Fig. 7. Observable changes to *E. coli* induced to express MAP3056, a putative lipoprotein. (A) Scanning electron microscopy of *E. coli* cells containing the parent plasmid pMAL-c2 in the left panels and *E. coli* (MAP3056) in the right panels. The magnification is indicated in the left margin and arrows point to electron-translucent areas not observed for *E. coli* (pMAL-c2). (B) Resuspension of an *E. coli* (MAP3056) cell pellet harvested from a fresh LB-broth culture shows the cell paste sticking and forming "long strings" of unbroken cells after induction with IPTG. This image, looking into the top of the centrifuge bottle, was taken prior to any cell lysis.

proach more achievable [20,21]. In this study, well over 600 proteins have been expressed and purified, representing 14.9% of the total *M. avium* subsp. *paratuberculosis* proteome. To our knowledge, only a select few microorganisms have a more extensive collection, including *Pseudomonas aeruginosa* [22], and *Francisella tularensis* [23]. Furthermore, this repository contains the largest number of purified recombinant proteins known for the maltose binding protein expression system. These 651 proteins representing *M. avium* subsp. *paratuberulosis* gene products provides a valuable reagent resource and Supplemental Table 1 can be used as a guide for future investigators initiating their own expression studies. From this information investigators now have an idea of expected yield and can make more informed choices about different expression conditions.

In pilot studies, 10 *M. avium* subsp. *paratuberculosis* 6× his-tagged proteins were produced in our laboratories, a few of which have been described previously [6]. Seven of these had an MBP-fusion counterpart in the current study (MAP0862, MAP0865, MAP0900, MAP1272c, MAP2121c, MAP2128c and MAP3968c). However, all of these his-tagged proteins were highly insoluble in non-denaturing conditions and the yield was poor. These observations made this method of purification untenable for high-throughput production of 100s of recombinant proteins. Insolubility is a common problem with purified recombinant proteins from mycobacteria. Of the 12 *M. leprae* recombinant his-tag proteins, none were soluble regardless of the growth conditions or bacterial strains used [24]. In contrast, MBP promotes solubility of recombinant proteins [25,26]. Nonetheless, the MBP system is not without its limitations. For example, the MBP tag can complicate downstream analysis because it is large (42-kDa) and can elicit an immune response in some animals. Therefore, it is always important to incorporate the MBP tag itself as a control in experimental studies. A distinct disadvantage with the currently available MBP fusion vectors is the requirement for using factor Xa to cleave the MBP fusion partner after expression and affinity fipurification. Factor Xa is a relatively expensive protease and its steric requirements for binding to its cleavage site often result in very slow or incomplete cleavage in our hands. However, new alterna-

tives such as TEV protease may alleviate this limitation [27]. Despite the large size of MBP, this tag does not appear to affect the function of purified proteins as *M. avium* subsp. *paratuberculosis* transcriptional regulators still work in DNA binding assays [28,29].

It is typically thought that membrane proteins are much more difficult to express in high yields when compared to proteins in the cytoplasm. This observation is consistent with the results obtained in this study. Six putative membrane proteins that yielded no protein in *E. coli* displayed a drop in optical density of the culture following induction with IPTG (Supplemental Table 2). This phenomenon was reported previously for the membrane proteins encoded by MAP0176 and MAP0243c [14] and suggests the mycobacterial proteins produced in those expression experiments are toxic to *E. coli*. MAP0243c in particular was difficult to obtain a clone that did not have a frameshift mutation near the start of the open reading frame. It is annotated as an unknown membrane protein, but it clearly has toxic effects on *E. coli*. The MAP0243c sequence is also present in several other species of mycobacteria, including *M. intracellular*, *M. tuberculosis*, *M. kansasii*, *M. marinum*, *M. leprae*, etc.

A distinct set of 19 proteins displayed an absorbance peak maximum at 260 nm. For all protein spectra, there are two characteristic absorbance peaks representing protein constituents. One ranges from 200 to 230 nm and a second peak occurs at 255–285 nm [30]. Peptide bonds show strong absorbance at the lower wavelength range whereas aromatic amino acid side chains strongly absorb at the higher range. It is a common misconception that 280 nm is the only characteristic spectral absorbance peak for proteins. It has been reported that the absorbance of a protein at 280 nm depends on the content of trp, tyr and cystine (disulfide bonds) [15,30]. We have no data on the cystine content for the proteins in this study as cystine content is identical to disulfide bond content; however, there are no significant differences in trp and tyr content between the 260 nm proteins and the 280 nm proteins. To distinguish if the 260 nm peak was due to a characteristic of the protein itself or because of the presence of nucleic acid, a series of experiments were performed. These include the addition of nucleic acid or protein degrading enzymes followed by spectral analysis. Those results showed that the spectrum remained remarkably consistent regardless of treatment, yet degradation of the target biomolecule was confirmed by separation electrophoresis. Most conclusively, ethidium bromide fluorescence was observed in proteinase K treated samples, but not in nuclease treated samples. Therefore, nucleic acids apparently bind to these 19 proteins, either specifically or non-specifically, and are co-purified with the recombinant protein.

Although intense research is being conducted to develop faster and more reliable methods for diagnosis of Johne's disease, there are still significant knowledge gaps concerning the molecular pathogenesis of this organism that might aid in vaccine design strategies. Due to the intracellular location of the pathogen during infection, a safe and effective vaccine is likely to be either a live attenuated or possibly a subunit vaccine. In order to produce an effective subunit vaccine, new candidate protein antigens must be identified. Our laboratory has already used a subset of these proteins to build a protein array for novel antigen discovery [7,31]. The results have provided a unique look into the humoral immune response of *M. avium* subsp. *paratuberculosis* during Johne's disease progression. The current study lays the foundation to identify additional antigens recognized by the host immune response at different stages of disease. By evaluating sera from infected cows at the subclinical and clinical stages of infection, this approach may eventually lead to vaccine protection studies using the identified antigens.

It is clear that powerful tools for research must be created to study the *M. avium* subsp. *paratuberculosis* proteome. With a resource that enables production of all proteins in the proteome, it becomes possible to analyze the activities of proteins in cell growth, maintenance, regulation, survival and pathogenesis. Additionally, this resource can provide reagents for functional proteomic experiments, including structural studies and defining protein–protein interactions. Finally, tools such as this repository build on genomic information by aiding in the functional annotation of the hundreds of hypothetical proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2010.03.019.

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Genomic and transcriptomic studies in *Mycobacterium avium* subspecies *paratuberculosis*

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ABSTRACT

Microarray technology is an important tool in functional genomic research. It has enabled a deeper analysis of genomic diversity among bacteria belonging to the *Mycobacterium avium* complex (MAC). In addition, the expression of thousands of genes can be studied simultaneously in a single experiment. With the complete genome sequence of a bovine isolate of *M. avium* subspecies *paratuberculosis*, and the independent construction of DNA microarrays in our laboratories, transcriptomic studies for this veterinary pathogen are now possible. Furthermore, the bovine genome sequence project is completed and bovine arrays have been developed to examine host responses to infection with *M. avium* subsp. *paratuberculosis*. Collectively, genomic and transcriptomic data has yielded novel insights surrounding the genetic regulation and biology of Johne's disease.

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1. Introduction

Important and foundational research that includes genome sequencing and microarray design has been completed for the veterinary pathogen *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*). Subsequent studies using this foundation have led to a better understanding of the genomic diversity and biology of this bacterium. Much of the focus on *MAP* is warranted as it causes a disease of considerable economic impact to dairy industries worldwide. It is estimated that annual losses to the U.S. dairy industry from Johne's disease exceed $220 million USD (Ott et al., 1999). Specifically, lower lactation performance and early culling from the herd are the main reasons for such losses (Raizman et al., 2009). Johne's disease presents as a granulomatous inflammation of the intestinal tissue and regional lymph nodes due to a massive influx of monocytes and macrophages. This inflammation effectively prevents absorption of nutrients, and therefore cattle in the later stages of disease show significant weight loss and diarrhea. However, the infection is chronic and it takes years to observe clinical signs. Transmission of the organism is via young calves feeding on milk contaminated with *MAP*. The bacterium is also shed in the feces, therefore fecal-oral route is another mode of transmission.

The field of functional genomics has revolutionized our understanding of the process of transcription, as it couples the power of complete genome sequencing with the miniaturization of PCR products or oligonucleotide arrays, allowing the generation of information about the total cell transcriptional responses in defined conditions. In the genus *Mycobacterium* alone, metabolic studies (Kendall et al., 2007; Micklinghoff et al., 2009; Talaat et al., 2007), virulence gene expression (Abomoelak et al., 2009; Agarwal et al., 2007), pseudogene transcription (Williams et al., 2009), host response to infection (Beisiegel et al., 2009), transcriptional regulators and the genes they affect (Fontan et al., 2009; Kendall et al., 2007; Ward et al., 2008) have all been advanced using microarray technology. The explosive growth of publications using microarrays has prompted

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development of the MIAME guidelines (Brazma et al., 2001) to ensure minimal reporting standards for microarray data, and subsequent technological advances in array production allowed for more sophisticated techniques like ChIP-on-chip technologies (chromatin immunoprecipitation followed by DNA microarray) for the genome-wide detection of binding sites for DNA-binding proteins (Sala et al., 2009). Recently, high-density tiling arrays with short oligonucleotide overlaps have already allowed a more detailed study of transcription in *MAP*, *M. leprae*, *Caulobacter crescentus*, *Listeria monocytogenes* and *Bacillus subtilis* (McGrath et al., 2007; Rasmussen et al., 2009; Akama et al., 2009; Toledo-Arana et al., 2009; Wu et al., 2006, 2007). We now know that the microbial transcriptome is much more complicated than previously thought, and includes long antisense RNAs and many more noncoding RNAs than identified previously (Rasmussen et al., 2009; Toledo-Arana et al., 2009). Tiling arrays advance transcriptomics because they are not bound by the annotation errors in genomics. In fact, they can be used to identify transcriptionally active genes missed by annotation as we discovered in our laboratories (unpublished data). The only technology not adopted yet for Johne's disease is ChIP-on-chip experiments. Nonetheless, important insights into Johne's disease have been gained from both the host and pathogen perspectives using microarrays.

2. *MAP* genomic diversity

MAP is a member of the *Mycobacterium avium* complex (MAC). Other members of the MAC group include *M. avium* subspecies *avium* (*MAA*), *M. avium* subspecies *silvaticum* (*MAS*), *M. avium* subsp. *hominsuis* (*MAH*) and a second species *M. intracellulare* (*MI*). Cattle can be infected systemically with *MAA* and *MAH*, but humans are infected preferentially with *MAH*. All members of the MAC are highly genetically similar, although the small distinctions between *MAA* and *MI* are now well established (Boddinghaus et al., 1990; De Smet et al., 1996; Feizabadi et al., 1997; Thorel et al., 2001). The genome sequences available for members in the MAC group are only *MAP* K-10 and *MAH* strain 104. These two genomes clearly demonstrate the close genetic similarity between these subspecies and furthermore, DNA–DNA hybridization studies have long ago shown a strong genetic similarity between *MAP* and *MAA* (Hurley et al., 1989; Saxegaard et al., 1988; Yoshimura and Graham, 1988). More recently, a greater than 98% nucleotide identity was shown to exist when comparing the majority of the *MAP* and *MAH* genomes to each other (Bannantine et al., 2003). Preliminary data suggested that *MAP* and *MAS* may be even more similar than *MAP* and *MAH* (Paustian et al., 2005). However, more recent genotyping data now shows that *MAH* and *MAS* are closer to each other than they are to *MAP* (Turenne et al., 2008). This close genetic relatedness does not explain why members of the MAC group are so phenotypically different.

Genetic diversity among *MAP* isolates has been the predominant theme in published *MAP* genomic studies during 2003 to the present. From repetitive DNA sequences (Amonsin et al., 2004; Bull et al., 2003) to amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) analysis (de Juan et al., 2005; O'Shea et al., 2004), differences on the *MAP* chromosome have been identified and utilized for discriminatory subtyping of isolates. Most of these studies have used the genome sequence of *MAP* to aid in the identification of genetic regions of variability (Amonsin et al., 2004; Overduin et al., 2004; Paustian et al., 2005; Semret et al., 2004). Techniques that both reveal genetic diversity and can be used to discriminate among MAC isolates include short sequence repeat analysis (Amonsin et al., 2004), variable number tandem repeat analysis (Bull et al., 2003; Overduin et al., 2004), PFGE (de Juan et al., 2005), AFLP (O'Shea et al., 2004) and representational difference analysis (RDA) (Dohmann et al., 2003). All were used to distinguish between *MAP* and *MAH*. In one study, optical mapping was used to examine genomic synteny among MAP isolates and showed a great deal of conserved genomic organization (Wu et al., 2009).

Prior to the development of a *MAP* microarray, some regions of the *MAP* genome had been discovered that are not present in *MAH*. Sheridan and coworkers (Sheridan et al., 2003) examined the GS element of *MAP* which was previously found using RDA and is reported absent in *M. avium* subsp. *avium* (Tizard et al., 1998). This 6500-bp region was analyzed in silico using bioinformatics tools, which predicted that coding sequences are involved in GDP-fucose biosynthesis and modification of the oligosaccharide moiety of GPL. Stratmann et al. (2004) also used the RDA technique to find a novel 7-kb ABC-transporter operon located within a 38-kb segment that is flanked by an insertion sequence. Also located on this 38-kb island are several gene clusters thought to be involved in iron uptake. These investigators went further by demonstrating the location and expression of two coding sequences in the ABC transporter operon to support their in silico findings. They found that both MptC and MptD were surface located on the *MAP* bacilli. They concluded that this is the first pathogenicity island discovered in *MAP*. Despite these significant studies, the presence or absence of genes at a whole genome level still remained to be defined.

3. A key tool: the DNA microarray

It was against this background that microarray-based genomic studies were initiated. The goal was to better define genes that were present or missing when comparing genomic DNA from two strains or isolates by competitive hybridization to a microarray. These comparisons might reveal differences that could yield clues to host range or phenotype. For example, it is well known that *MAP* requires exogenous mycobactin J in the culture media for growth whereas *MAA* and *MAH* do not. It has been observed that the *mbtA* gene in the mycobactin biosynthesis operon is truncated in *MAP* compared to *MAH* suggesting that this may be a reason for the mycobactin J requirement (Li et al., 2005).

The first *MAP* microarray consisted of spotted PCR products based on predicted coding sequences in the K-10 genome (Li et al., 2005). This library of PCR products representing *MAP* coding sequences was constructed with primers designed by Primer3 software (Rozen and Skaletsky, 2000) to amplify fragments of 500 bp from each

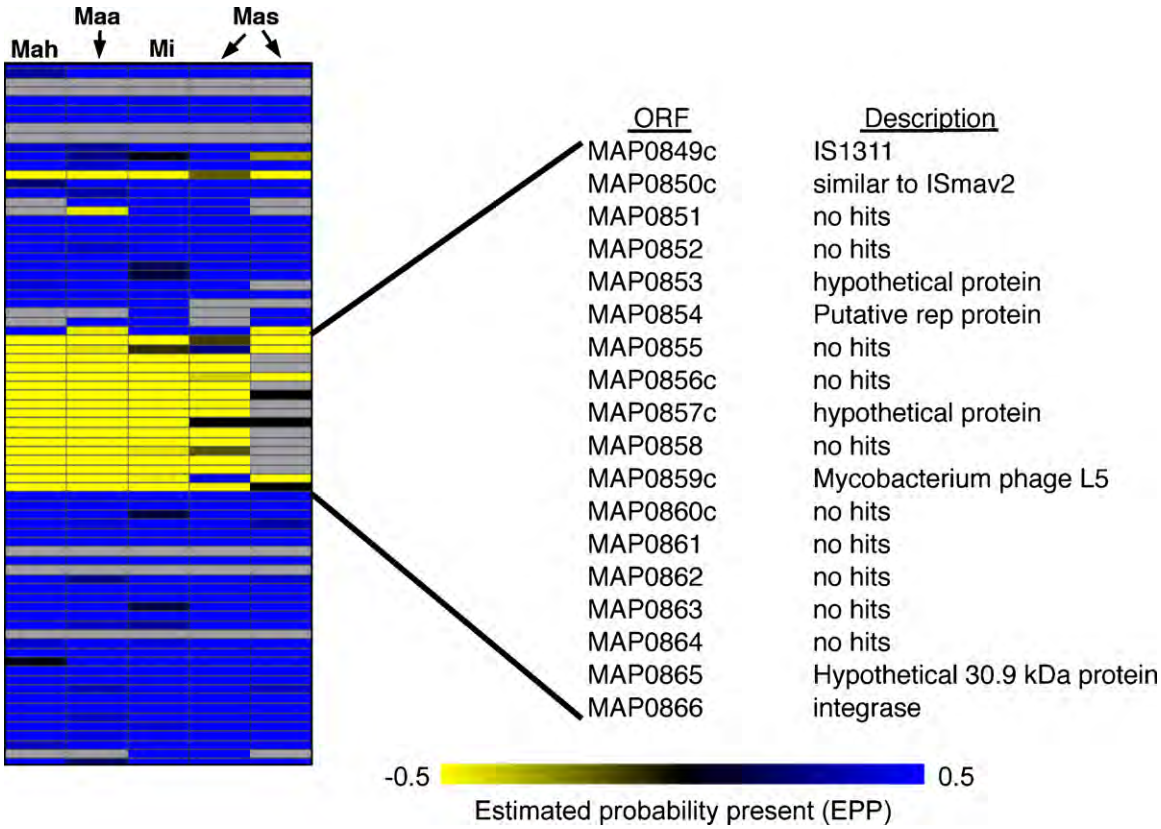

Fig. 1. Comparative genomic hybridization analysis of MAC species. Shown is a clustergram displaying hybridization signals generated from genomic DNA hybridizations of different mycobacterial isolates. Each column in the clustergram represents a single microarray profile from the species indicated at the top. Only the hybridization signals from a region encompassing gene identifiers MAP0821 to MAP0896 are shown. The yellow-to-blue color bar represents low to high log ratios of the hybridization signals generated from each genome relative to that of *MAP* K-10. The genes shown in yellow, indicating a low probability of being present in those genomes, corresponds to LSP4 (Semret et al., 2005), which is an insertion present only in *MAP*. *MAH* = *M. avium* subspecies *hominissuis*, *MAA* = *M. avium* subspecies *avium*, *MI* = *M. intracellulare*, *MAS* = *M. avium* subspecies *silvaticum*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

ORF using purified genomic DNA as template. The array was built with 4110 PCR products representing 95% of the genome. It was used in DNA hybridization experiments to examine genomic diversity among species (Paustian et al., 2005). Using the array of spotted PCR products, some differences in MAC members became evident. Through this approach, 3 defined segments, comprising 24 genes were missing in the *MAP* sheep strain, Telford 9.2 when compared to the bovine strain K-10 (Marsh et al., 2006). One identified region of difference (RD) is shown in Fig. 1. These genomic differences may account for the host specificity observed in sheep strains.

Because only 95% of the genes could be amplified by PCR from this GC-rich bacterium, an oligonucleotide array was designed and built. Every predicted open reading frame in the *MAP* strain K-10 genome is represented on this array. One 70-mer was designed for each gene with a total length of less than 4000 bp, while longer genes were split in half and one 70-mer oligo was designed for each half. This array had the added feature of containing *MAH* sequences that were less than 30% identical to *MAP* genes. Additional details of this microarray design can be found elsewhere (Paustian et al., 2008). Two additional oligo arrays were built using *MAH* strain 104 sequence data (Semret et al., 2004; Wu et al., 2006). One of these arrays contained oligonucleotide probes 70 nucleotides in length (Semret et al., 2004) while the other consisted of tiled oligonucleotides where each gene was represented by 14 pairs of probes (Wu et al., 2006). The *MAH* subspecies is similar enough that these arrays were successfully used to examine genomic differences between *MAP* and other members of the MAC. A second-generation array was constructed that contained both subspecies *paratuberculosis* and *hominissuis*. This microarray contains 60-mer oligonucleotide probes that represent 100% of the annotated genes in both genomes, yet uses only 5744 probes in the final set (Castellanos et al., 2009).

To better understand the host response to infection with *MAP*, a bovine microarray was constructed in Paul Coussens' lab at Michigan State University. The first generation bovine cDNA array used in studies on Johne's disease was a bovine total leukocyte array, termed BOTL-2, that consisted of 721 bovine expressed sequence tags (ESTs) and amplicons representing known genes (Coussens et al., 2002). The known genes included those encoding cytokines IL-1, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α and IFN-γ. This array continued to evolve into the BOTL-3, which had 709 ESTs and 345 amplicons representing known genes (Coussens

et al., 2003) and finally, the BOTL-5, which contained 750 ESTs and 750 known gene amplicons (Murphy et al., 2006; Skovgaard et al., 2006). As described below, unique insights on the bovine immune response were obtained using the BOTL array. The only other host array used in MAP studies is the mouse array for examining response to MAP and MAA infections of murine macrophages. The investigators found a significant decrease in genes that promote inflammatory responses for MAP, but not MAA (Basler et al., 2008).

4. Genomic diversity revisited

All of these *M. avium*-based arrays were initially built to examine genomic diversity and as such they were hybridized with genomic DNA to determine presence or absence of genes. A total of 24 and 18 genomic islands were identified belonging to *MAH* and *MAP*, respectively (Wu et al., 2006) suggesting the mosaic nature of genomes among MAC strains (Alexander et al., 2009). Interestingly, large regions of genomic inversions were found among *MAP* and *MAH* strains (Fig. 2; Wu et al., 2006), another indication of the presence of a common ancestor between members of the MAC group.

With the array constructed, genomic diversity and evolution of the MAC complex organisms began to crystallize into focus. The major genomic differences were identified as insertions or deletions among MAC strains. Large sequence polymorphisms were described and it was suggested that diagnostic sequences might be contained within these regions. A total of 6 genomic insertions specific to *MAP* comprise 125 kb, two of which are putative prophages (Alexander et al., 2009). In contrast, one 10-kb deletion (LSP8) is consistent in all *MAP* strains. Analysis of the LSP regions as insertions or deletions led to the proposal of a biphasic evolution of *MAP* (Alexander et al., 2009). Added to these data was an extensive multilocus sequencing study that suggested *MAH* is the ancestor strain from which *MAP* and *MAA* independently evolved (Turenne et al., 2008). Finally, it appears that *MAP* itself is genetically homogenous with primary differences observed between cattle and sheep strains. Therefore, genome sequencing of a sheep isolate is recommended to complete this knowledge gap. We are currently sequencing an ovine isolate from a Suffolk sheep in the United States. This genome has recently been assembled into a single scaffold and closing remaining gaps will be done quickly. This genome will comprehensively detail all the differences between sheep and cattle isolates of *MAP*.

5. Defining the transcriptome in *M. avium* subsp. *paratuberculosis*

Surprisingly, despite the number of studies using the *MAP* or *MAP–MAH* microarray to identify genomic differences through DNA hybridizations, very few investigators have advanced into transcriptomic experiments using these same arrays. This is disappointing because animal models for Johne's disease need further development (Hines et al., 2007) and transcriptomic studies are expected to reveal which model host might best simulate Johne's disease. Furthermore, because there are immunologically defined stages of Johne's disease, infected asymptomatic, subclinical and clinical, transcriptomic studies might reveal what triggers the progression of the disease into the next stage. Currently, identification of *MAP* gene expression levels within cultured macrophages, such as monocyte-derived macrophages, is a good way to improve understanding of *MAP* pathogenesis. Although the microarray and RT-PCR are the primary methods used in such studies, transcripts of *MAP* infected bovine monocyte-derived macrophages have also been identified by the selective capture of transcribed sequences (SCOTS) method (Zhu et al., 2008). In addition, differential display PCR was used to identify gene expression differences in sheep tissues (Zhong et al., 2009).

There are only a few published studies that use the microarray to examine *MAP* transcriptional responses in defined conditions. These include the response of *MAP* exposed to artificial stress conditions such as low pH, heat and oxidative stress (Wu et al., 2007), exposure to the rhodanine agent D157070 (Bull et al., 2009), iron dependency (Janagama et al., 2009) as well as natural stress conditions (in fecal samples) (Wu et al., 2007). Surprisingly, a large number of genes were activated following exposure to low pH ($n = 597$) indicating the vigorous *MAP* response to acidic conditions encountered during passage through the calf digestive system or inside phagosomes of host macrophages. In addition, *MAP* transcriptomes under heat shock or oxidative stress were very similar to those of *M. tuberculosis* (Wu et al., 2007) suggesting common responsive pathways for these intracellular pathogens.

Moreover, D157070 was shown to kill both actively growing and latent *MAP*. Transcriptome profiles of *MAP* after D157070 treatment for up to 3 days compared to untreated controls resulted in 63 differentially expressed genes (Bull et al., 2009). What was most interesting about this gene set is that 36 were arranged in adjacent clusters or putative operons. One of these clusters (MAP4201 to MAP4211) contained eleven genes, all showing increased transcription in response to D157070 exposure. Furthermore, of the 8 putative operons showing increased transcription, 3 of these may be regulated by the SigL transcription factor (Bull et al., 2009).

Like all living organisms, iron acquisition is critical, but this is especially true for *MAP*. As mentioned above, a phenotypic hallmark that defines *MAP* is the requirement for mycobactin J in the culture media. Without the presence of this siderophore, *MAP* cannot grow. In the presence of iron, the *MAP* transcriptional regulator IdeR binds to a 19 bp consensus promoter sequence called the iron box and represses transcription of genes involved in iron acquisition (Janagama et al., 2009). The MAP oligo array was used to identify genes under the control of IdeR. The *mbtA* was downregulated whereas *bfrA* (iron storage gene) was upregulated in iron-rich laboratory media. In addition, *mbtE* was upregulated in human macrophages, which is a low iron environment. These data demonstrate that *MAP* does regulate these genes in an iron-dependent fashion.

Other data have recently demonstrated that *MAP* increases transcription of mycobactin (*mbt*) synthesis genes (MAP2169c–MAP2178) inside bovine macrophages (Zhu et al., 2008). This iron exochelin is not required by

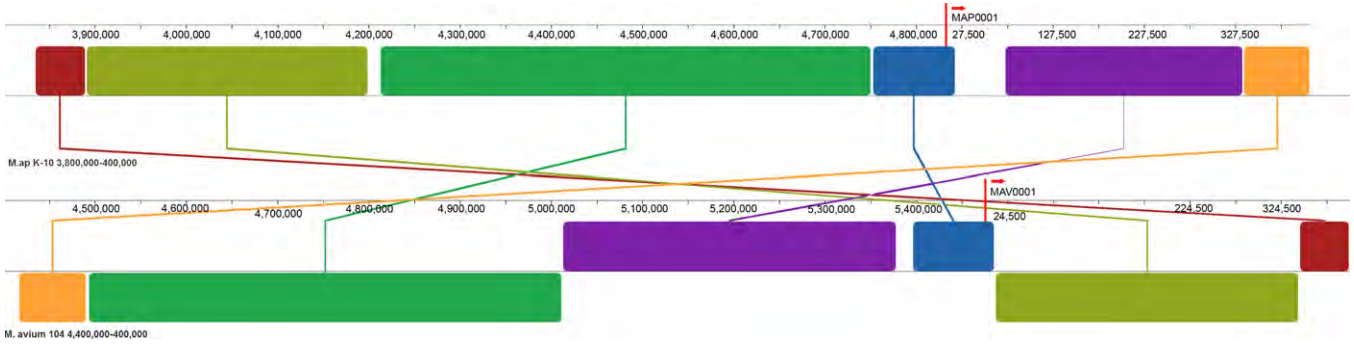

Fig. 2. Extensive genomic rearrangement among MAC species. Genomic inversions between *MAP* K-10 and *MAH* 104 were visualized by aligning the two genome sequences with a multiple genome alignment software, Mauve 2.3 (Darling et al., 2004). Upper panel shows the genome segment of *MAP* K-10, from coordinate 3,800,000, spanning the origin of replication, to coordinate 400,000. This *MAP* K-10 genome sequence was revised based on a previous optical mapping study (Wu et al., 2009), with further re-assignment of genome coordinate according to the new genome synteny. The genome segment of *MAH* 104 (Refseq: NC_008595) between 4,400,000 and 400,000 is shown in the lower panel. Boxes of the same color represent homologous sequence fragments and are connected by lines of the same color. In the lower panel, boxes above the horizontal axis represent *MAH* 104 fragments in the same direction as the counterparts of *MAP* K-10, whereas boxes below the axis represent inverted fragment. The red vertical lines and rightward arrows indicate the location and direction of the first gene in either genome.

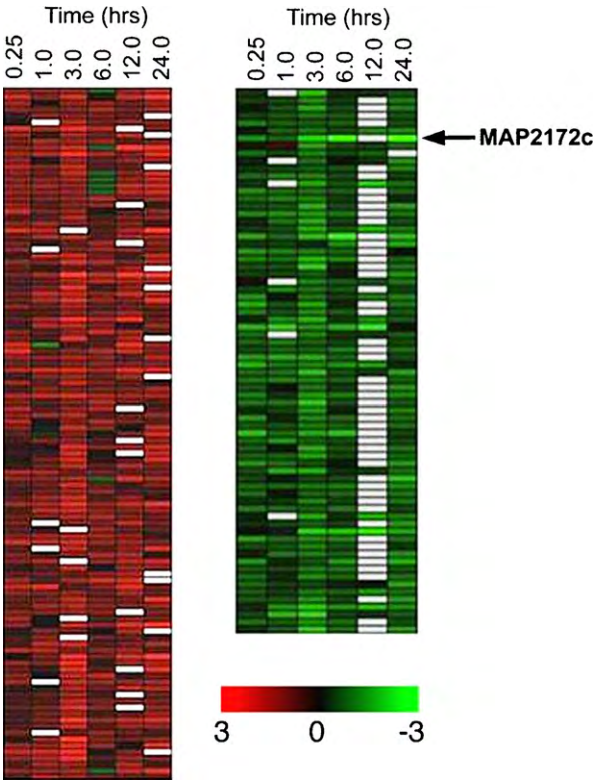

Fig. 3. Differential transcription of *MAP* genes without mycobactin J in the growth media. Shown are two hierarchical clustered plots, one showing 109 genes with increased transcriptional levels (indicated by red color) in response to the absence of mycobactin J and 72 genes with lower transcriptional activity (green color). The time points when samples were taken for RNA extraction are indicated above each plot and indicate the time exposed to media without mycobactin J. MAP2172c, a gene in the middle of the mycobactin biosynthesis operon, is indicated in the right margin. It shows a steady decrease in transcription with time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

other members of the MAC group or other species in the genus. We examined the transcriptional profiles of *MAP* exposed to Middlebrook 7H9 media with and without mycobactin J for 0.5, 1, 3, 6, 12, and 24 h. A total of 109 genes were transcribed at higher levels in the absence of mycobactin J while 72 genes showed reduced transcription (Fig. 3). Although most of the *mbt* genes were not significantly altered in their expression level, MAP2172c clearly showed decreasing levels of transcription as time in media without mycobactin J lengthened (Fig. 3).

Another publication represents the only study to look at the *MAP* transcriptome during interaction with host cells. These investigators examined *MAP* invasion of bovine epithelial cells after the bacteria were inside mammary epithelial cells (MAC-T) or exposed to milk (Patel et al., 2006). This is an important question because transmission of the bacteria is known to occur when calves nurse from an infected dam. These results showed that there was a significant increase in invasion of epithelial cells after MAP was inside MAC-T cells for 24-h and after incubation with milk. Thus the hypothesis is that mammary epithelial cells prime *MAP* for invasion of intestinal epithelial cells, which is thought to be the initial event in Johne's disease. This observation prompted an examination of which genes are upregulated in MAC-T cells. RNA was extracted from *MAP*-infected MAC-T cells at the 24-h time point, converted to cDNA, and hybridized to the *MAP* oligo array. There were 20 genes expressed at a three-fold higher level than the control (Patel et al., 2006). These genes included virulence factors as well as a gene (MAP2751) present uniquely in *MAP*, but has no known function (Bannantine et al., 2004).

6. Host response to *M. avium* subsp. *paratuberculosis* infection

Transcriptomic studies on the host side in response to exposure to *MAP* are farther along in terms of publications. This may be due to the wider availability of arrays through commercial vendors or greater interest in host response versus pathogen response. The sequencing of the bovine genome and other animal hosts has also helped advance studies in this area (Elsik et al., 2009). The transcriptional response to *MAP* infection has been explored for many hosts. These include murine (Basler et al., 2008) and bovine (Murphy et al., 2006) macrophages where *MAA* and *MAP* infections were directly compared. Human macrophages infected with *MAP* strains of diverse genotypes have also been studied (Motiwala et al., 2006). The expression profile of *MAP* infected sheep tissues (Smeed et al., 2009) and bovine illeal tissue (Aho et al., 2003) as well as PBMCs (Coussens et al., 2005, 2004b) has also been performed. Transcriptome analysis on bovine PBMCs showed that *MAP* infected cells are pro-apoptotic; however, very recent data using FACS sorting to capture only infected cells indicates that the opposite may be true (Coussens, unpublished).

The pro-inflammatory defense mechanisms of infected murine macrophages are suppressed during infection with *MAP* but not *MAA* or *MAH* (Basler et al., 2008). An Affymetrix GeneChip® array that contains 22,690 murine transcripts showed pro-inflammatory factors that include lipocalin, TNF-α, IL-1α and IL-1β were expressed at lower levels in MAP-infected macrophages compared to *MAA*- or *MAH*-infected macrophages. However, in bovine monocyte-derived macrophages, TNF-α was not upregulated in either *MAA* or *MAP* infections and IL-6, a pro-inflammatory cytokine was upregulated (Murphy et al., 2006). In fact, there is an overall suppression of macrophage responses by *MAP* as compared to *MAA* (Weiss et al., 2004). These findings suggest that bovine strains of *MAP* generally modulate the host immune response to prevent inflammation whereas *MAA* is more of an inflammatory stimulant. Interestingly, the sheep isolates of *MAP*, regardless of their genotype, differ from the bovine strains in that they induce pro-inflammatory gene expression profiles (Motiwala et al., 2006).

Several studies were completed using bovine peripheral blood mononuclear cells (PBMCs) as the source of the RNA due to the ease of isolation and yield of these cells. Gene expression of bovine PBMCs isolated from cows subclinically or clinical infected has been investigated (Coussens et al., 2002). The PBMCs were incubated with *MAP* for 16-h and gene expression was determined using the BOTL array. They found 16 genes with increased expression and 11

genes with decreased expression when comparing subclinical and clinical cattle PBMCs. Greater expression of IFN-Y and IL-10 and decreased expression of IL-1 receptor were among the genes with different expression levels between the two disease states. Another study examined temporal changes in PBMCs from healthy cattle after exposure to MAP for 2, 4, 8 and 16 h (Coussens et al., 2004a). They saw a decrease in differentially expressed genes as time lengthened, indicating that *MAP* progressively quenched any macrophage response.

Evaluating gene expression locally in the infected tissues has also been studied. This approach is an improvement because gene expression occurring at the site of infection should give a clearer picture of local host response rather than extracting RNA from circulating PBMCs, which is easier to obtain in large quantities. Expression in ileal tissue from MAP infected cows was compared to uninfected control cows (Aho et al., 2003). IL-1α was one gene that was significantly overexpressed and it was concluded that overexpression of this gene may be responsible for many of the clinical signs associated with Johne's disease. Recently, the first study to examine gene expression in the ileum of sheep infected with *MAP* was undertaken (Smeed et al., 2009). The investigators compared asymptomatic sheep with those in the paucibacillary and multibacillary forms of Johne's disease. The cell-mediated immune response that predominates in the paucibacillary form is type 1 $CD4^+$ T cells whereas type 2 $CD4^+$ T cells direct the multibacillary form, which is the late stage of disease. These disease states in sheep were more completely described recently (Gillan et al., 2010). A total of 596 genes represented on the 4824 spot ruminant immuno-inflammatory gene array (Watkins et al., 2008) revealed 36 genes had a fold change of $\geq$1.5 and a $P \leq 0.05$. They not only obtained a list of genes differentially expressed in each disease state, but also discovered a genetic susceptibility in the form of a single nucleotide polymorphism (IGFBP6 G^{3743}), which is not present in asymptomatic sheep (Smeed et al., 2009).

Thus the general trend observed in these host response experiments is that *MAP* is efficient at nullifying the macrophage response and prevents T cell activation. Thus this bacterium is not considered an immune stimulating agent, whereas other members of the MAC group (e.g. *MAH*) are.

7. Conclusions

A lot of work has been completed in this field over the past 2.5–3 years, but much remains to be accomplished. The dominant theme for using microarrays in *MAP* research appears to be defining genetic diversity and examining the host response to *MAP*. Because of this focus, researchers now have an excellent understanding of differences in the genomes of *MAP* isolates and species within the MAC complex. Also, a clearer understanding of how *MAP* affects the host response is beginning to emerge. In general, *MAP* infection results in a strangling of the immune response. But more work needs to be performed on the transcriptome of *MAP* during infection. This area should be a central focus to close the knowledge gap. Another knowledge gap to be close is obtaining the genome sequence of a sheep isolate since genomic and phenotypic differences between bovine and ovine *MAP* isolates are more evident with each new study.

It will be interesting to see whether new technologies (such as deep sequencing) will take over in place of DNA microarrays in both genomics and transcriptomic studies of *MAP*. With the increase of complications associated with Johne's disease in cattle and the potential link to Crohn's disease in humans, we urge for more support from USDA and its newly founded authority (NIFA) for research on Johne's disease. Advances achieved so far in the field of *MAP* genomics and transcriptomics need to be followed by specific gene(s) analyses and discoveries. Such knowledge will be eventually translated to products such vaccines and better control strategies directed towards Johne's disease.

Conflict of interest statement

The authors have no conflicts.

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The *Salmonella enterica* serovar Typhimurium QseB response regulator negatively regulates bacterial motility and swine colonization in the absence of the QseC sensor kinase

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Iron

ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) responds to the catecholamine, norepinephrine by increasing bacterial growth and enhancing motility. In this study, iron with or without the siderophore, ferrioxamine E also enhanced bacterial motility. Iron-enhanced motility was growth-rate dependent, while norepinephrine-enhanced motility was growth-rate independent. The outer membrane catecholate receptors, IroN, FepA and CirA (required for norepinephrine-enhanced growth) were not required for norepinephrine-enhanced motility, nor was ExbD of the energy-transducing TonB-ExbB-ExbD ferri-siderophore uptake system. Examination of the QseBC two-component system revealed that *qseB* and *qseBC* mutants have motility phenotypes similar to wild-type *S.* Typhimurium, while motility of the *qseC* mutant was significantly decreased ($P < 0.01$). Each mutant of the QseBC system, as well as mutants of *qseE* and *pmrA*, responded to norepinephrine with increased motility, suggesting that other genes are involved in norepinephrine-enhanced motility of *S.* Typhimurium. In the swine host, fecal shedding of the *qseBC* mutant was similar to wild-type *S.* Typhimurium, whereas fecal shedding of the *qseC* mutant was significantly decreased ($P < 0.01$). Our data indicate that, in a *qseC* mutant, the QseB response regulator decreases motility and swine colonization; inactivation of the *qseBC* operon restores these bacterial phenotypes, classifying QseB as a negative regulator of bacterial motility and swine colonization.

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1. Introduction

The ability of bacteria to sense, respond and adapt to fluctuations in their environment is crucial for survival, transmission, colonization and pathogenesis. A mechanism that bacteria utilize to sense and respond to environmental signals is phosphotransfer via two-component signal transduction systems [1]. In their simplest form, two-component systems comprise a sensor kinase (typically located in the cytoplasmic membrane) that monitors environmental conditions and a cytoplasmic response regulator that arbitrates the adaptive response, usually involving changes in gene expression [2].

Sperandio et al. [3] have reported that enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 use the two-component system QseBC to respond to epinephrine, norepinephrine and autoinducer-3 (AI-3, from pre-conditioned medium). Clark et al. [4] described the sensor kinase, QseC as a bacterial adrenergic receptor for epinephrine and norepinephrine, and phenotypic responses of EHEC O157:H7 to these hormones include enhanced bacterial motility and virulence gene expression from the locus of enterocyte effacement (LEE) pathogenicity island [3].

In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), two phenotypes have been demonstrated in response to norepinephrine exposure: norepinephrine-enhanced growth in serum-supplemented medium and norepinephrine-enhanced bacterial motility [5–10]. Suppression of bacterial growth in serum-containing medium is due to the presence of transferrin which binds iron, a metal co-factor required by specific bacterial proteins and enzymes [11]. Norepinephrine enhances *Salmonella* growth by functioning as a siderophore in serum-supplemented medium to

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provide iron to the bacterial cell [11]. Similar to EHEC O157:H7, enhanced bacterial motility in response to norepinephrine and preconditioned medium (AI-3) has also been demonstrated for *S.* Typhimurium [10]. Transcriptional analyses using DNA microarrays and real-time RT-PCR identified multiple motility genes that were up-regulated following exposure of *S.* Typhimurium to norepinephrine [10].

In *S.* Typhimurium, the QseBC (PreAB) two-component system was initially described by Merighi et al. as modulating a subset of PmrA-regulated genes including *pmrCAB* and *yibD* [12]. Gene expression of *pmrCAB* and *yibD* is increased in a *qseC* (*preB*) mutant but not in *qseB* (*preA*) or *qseBC* (*preAB*) mutants, indicating a requirement for the response regulator, QseB, for increased gene expression in the absence of the sensor kinase QseC. Recently, genome-wide analysis of the *S.* Typhimurium QseBC (PreAB) regulon demonstrated an increase in expression for a subset of genes within the PhoPQ and PmrAB regulons when *qseB* (*preA*) was expressed from a plasmid in a Δ*qseBC* (*preAB*) mutant compared to a Δ*qseB* (*preA*) strain [13]. Therefore, although QseC functions as a positive regulator of bacterial motility, it serves as a negative regulator of genes within the PhoPQ and PmrAB regulons.

In swine, the *qseC* mutant has decreased competitive fitness in the swine gastrointestinal tract compared to wild-type *S.* Typhimurium [10]. In contrast, a *qseC* (*preB*) mutant of *S.* Typhimurium was not defective in colonization of the mouse spleen and liver compared to mice inoculated with wild-type *S.* Typhimurium [13]. However, competitive fitness for colonization of the mouse spleen and liver was decreased for the *qseBC* (*preAB*) double mutant, and mutation of *qseBC* (*preAB*) caused a two-day delay in mortality compared to mice inoculated with wild-type *S.* Typhimurium [13]. In this study, we examined bacterial motility and swine fecal shedding phenotypes of *qseC* and *qseBC* mutants compared to wild-type *S.* Typhimurium. We demonstrate that only the *qseC* mutant has decreased bacterial motility and colonization fitness and that the presence of the QseB response regulator is required for the diminished phenotypes observed in the *qseC* mutant.

2. Materials and methods

2.1. Bacterial strains and media

The *S.* Typhimurium strains used in this study are listed in Table 1 and were grown in Luria–Bertani (LB) broth or Dulbecco's modified Eagle medium (DMEM; 31053 Invitrogen Life Technologies, Carlsbad, CA) at 37 °C. Antibiotics were used at the concentrations of 100 μg/ml for ampicillin, 50 μg/ml for kanamycin, 30 μg/ml for chloramphenicol and 30 μg/ml for nalidixic acid. Norepinephrine (NE) which is photosensitive, highly oxidized and has a short half-life was used at concentrations of 50 and 100 μM. The concentration of iron chloride ($FeCl_3$) used was 80 μM. Ferrioxamine E (FE) was used at a concentration of 200 ng/ml. All chemicals were purchased from Sigma (St. Louis, MO), unless indicated otherwise.

2.2. S. Typhimurium strain construction by recombineering

PCR Primers for the synthesis of linear DNA fragments to construct *S.* Typhimurium knockout mutants in *fliA* (STM1956), *qseB* (STM3177), *qseC* (STM3178) and *qseE* (STM2564) by recombineering are listed in Table 2. The 5′ end of the primers contain 43–46 bp of nucleotide sequence (bold) that is homologous to the upstream or downstream region of a particular gene. The 3′ end is a universal sequence containing stop codons in all three reading frames for amplification of oBBI 88/89 *cat* or oBBI 92/93 *neo* which contain FRT sites for deletion of the antibiotic resistance gene and were previously described [9,10].

2.3. Swine experiment

Fecal shedding of the *qseC*, *qseBC*, and wild-type *S.* Typhimurium strains in pigs was determined as previously described [9]. Briefly, 15 conventionally raised male and female piglets from *Salmonella* spp. fecal-negative sows were weaned at 12 days (d) of age, shipped

Table 1
Strains.

Strain no.	Strain Background	Genotype	Phenotype	Source
BSX 7	TT22971 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120*/pKD46 *araC bla* oriR101 repA101ts lambda red (*gam* + *bet* + *exo*+)	Ap^R, 30 °C	John Roth via Max Wu
BSX 8	χ4232	Wild-type	Nal^R	Tom Stabel
BSX 28	JF3547 (UK1)	*pagA1*::MudJ *pmrA1*::Cm	Kn^R, Cam^R	John Foster
BSX 31	JF2433 (LT2)	*exbD*::MudJ (*atrD16*)	Kn^R	John Foster [27]
BBS 2	BSX 8 (χ4232)	*exbD*::MudJ (*atrD16*)	Nal^R Kn^R	This Study, BSX 8 X HT BSX 31
BBS 10	BSX 8 (χ4232)	*ygiY*::*cat* (*qseC*)	Nal^R Cam^R	[10]
BBS 108	BSX 8 (χ4232)	Δ*iroN* Δ*fepA* Δ*cirA*	Nal^R	[9]
BBS 120	BSX 7 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120*/pCP20	Ap^R, 30°C	[9]
BBS 174	BSX 7 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120 fliA*::*cat*	Cam^R	This Study
BBS 177	BSX 8 (χ4232)	*fliA*::*cat*	Nal^R Cam^R	This Study, BSX 8 X HT BBS 174
BBS 189	BSX 7 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120 ygiXY*::*neo* (*qseBC*)	Kn^R	This Study
BBS 191	BSX 8 (χ4232)	*ygiXY*::*neo* (*qseBC*)	Nal^R Kn^R	This Study, BSX 8 X HT BBS 189
BBS 192	BSX 8 (χ4232)	Δ*ygiXY* (*qseBC*)	Nal^R	This Study, BBS 191 X HT BBS 120
BBS 234	BSX 7 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120 ygiX*::*neo* (*qseB*)	Kn^R	This Study
BBS 238	BSX 8 (χ4232)	*ygiX*::*neo* (*qseB*)	Nal^R Kn^R	This Study, BSX 8 X HT BBS 234
BBS 242	BSX 8 (χ4232)	Δ*ygiX* (*qseB*)	Nal^R	This Study, BBS 238 X HT BBS 120
BBS 281	BSX 8 (χ4232)	*pmrA1*::Cm	Nal^R Cam^R	This Study, BSX 8 X HT BSX 28
BBS 320	BSX 8 (χ4232)	Δ*fliA*	Nal^R	This Study, BBS 177 X HT BBS 120
BBS 373	BSX 7 (LT2)	*metA22 metE551 trpD2 ilv-452 leu pto* (leaky) *hsdLT6 hsdSA29 hsdB strA120 yfhK*::*neo* (*qseE*)	Kn^R	This Study
BBS 376	BSX 8 (χ4232)	*yfhK*::*neo* (*qseE*)	Nal^R Kn^R	This Study, BSX 8 X HT BBS 373
BBS 380	BSX 8 (χ4232)	Δ*yfhK* (*qseE*)	Nal^R	This Study, BBS 376 X HT BBS 120

Table 2
Primers.

Gene	Oligo	Primer sequences for recombineering (5′ - 3′)
fliA	oBBI 169	**gtgaattcactgtataccgctgaaggtgtaatggataaacactcg**atagctgaatgagtgacgtgc
	oBBI 170	**cttttcgggtgcgatcatgcgcgacctataacttacccagtttgg**catagagcagtgacgtagtcgc
qseB	oBBI 181	**gacggcaacgcgagttaccgcaaggaagaacagatgcgaattttac**gatagctgaatgagtgacgtgc
	oBBI 202	**cgtcaatttcatgcgtcacccagggtgtagccgatgccgtgcacg**catagagcagtgacgtagtcgc
qseBC	oBBI 181	**gacggcaacgcgagttaccgcaaggaagaacagatgcgaattttac**gatagctgaatgagtgacgtgc
	oBBI 97	**ctctgttaccaacttactacggcctcaaatccaccttccgcggc**atagagcagtgacgtagtcgc
qseE	oBBI 204	**gagcgacacgttgaagcgctggtctgttttcccccgttctttacga**tagctgaatgagtgacgtgc
	oBBI 205	**cttcctgcgcgttgtcatccaccagttggatctctccctgcat**agagcagtgacgtagtcgc

to the National Animal Disease Center, Ames, IA and raised in three groups of five in isolation facilities. All pigs were confirmed three times to be fecal-negative for *Salmonella* spp. using bacteriological culture techniques. At 7 weeks of age (day zero), the fifteen pigs ($n = 5$) received an intranasal inoculation of 1 ml PBS containing 1.0×10^9 CFU of BSX 8 (wild-type), BBS 10 (*qseC*) or BBS 192 (*qseBC*). Pig fecal samples were obtained on 0, 1, 2, 3, 5 and 7 days post-inoculation (dpi) for quantitative and qualitative *Salmonella* culture analyses [10]. Statistical analysis of the number of *Salmonella* present in the daily fecal samples (cfu/g) was analyzed by SAS Analyst using the Two Sample t-test for the Means following log normal transformation of the data. Procedures involving animals were lawful and approved by the USDA, ARS, NADC Animal Care and Use Committee.

For quantitative bacteriology, one gram of pig feces was combined with 5 ml PBS, vortexed and 100 μl directly plated to brilliant green agar with sulfadiazine (BGS) containing nalidixic acid (30 μg/ml), ferric ammonium citrate (0.8 g/L) and sodium thiosulfate (6.8 g/L). One hundred microliters of a ten-fold dilution of each fecal sample were also plated, and additional dilutions were performed when necessary. Following 48 hours of incubation at 37 °C, black colonies were enumerated and a single colony from each plate was confirmed to be *Salmonella* by serogroup antiserum agglutination.

Qualitative bacteriology of *Salmonella* was performed as follows: 1 gram fecal samples were inoculated in 10 ml of GN-Hajna (GN) broth and tetrathionate (TET) broth for 24 and 48 hours of growth at 37 °C, respectively. Following incubation, 100 μl of each culture was transferred to 10 ml Rappaport-Vassiliadis medium (RV) and incubated at 37 °C for 18 h. The cultures were streaked on BGS containing nalidixic acid (30 μg/ml), ferric ammonium citrate (0.8 g/L) and sodium thiosulfate (6.8 g/L). Black colonies suspicious for *Salmonella* were confirmed by serogroup antiserum agglutination.

2.4. Motility assay

The motility assay was previously described [10]. Briefly, DMEM 0.3% motility medium without additional supplementation or with supplementation of $FeCl_3$, FE, and/or NE was poured ~24 hours prior to use and allowed to sit overnight in a dark room. The bacterial strains were grown overnight in DMEM medium at 37 °C, 180 rpm. Strains were diluted 1:50 in DMEM and incubated at 37 °C, 180 rpm for ~7 h. Bacteria were pelleted and resuspended in an equal volume of DMEM medium. A 1.25 μl aliquot of bacteria was spotted onto the motility medium, incubated at 37 °C and the bacterial motility zone diameters measured following ~18 h of incubation. Statistical analysis of motility data was analyzed by SAS Analyst (Cary, NC) using the Two Sample *t*-test for the Means prior to converting data to relative motility.

3. Results

3.1. QseBC two component system in swine colonization

Initial characterization of the QseBC (PreAB) system in *S.* Typhimurium revealed that a mutation in the *qseC* (*preB*) gene, but not the *qseBC* (*preAB*) genes, increased the gene expression of *pmrCAB* and *yibD* [12]. However, Merighi et al. demonstrated that a *qseBC* mutant, but not a *qseC* mutant, had reduced competitive fitness compared to wild-type *S.* Typhimurium when simultaneously inoculated into BALB/c mice; furthermore, the *qseBC* mutant had a two-day delay in mortality compared to wild-type *S.* Typhimurium [13]. We have previously demonstrated that a *qseC* mutant has decreased competitive fitness in the swine gastrointestinal tract compared to wild-type *S.* Typhimurium [10]. To investigate the QseBC two-component system further, we constructed a *qseBC* mutant and analyzed the fecal shedding of a *qseC* mutant, *qseBC* mutant and the parental wild-type strain in the swine host. Three groups of pigs ($n = 5$) were intranasally inoculated with 1×10^9 CFU of either the *qseC* mutant, the *qseBC* mutant or wild-type *S.* Typhimurium and monitored for fecal shedding for 7 days pi. No significant difference in fecal shedding of the *qseBC* mutant compared to wild-type *S.* Typhimurium was observed (Fig. 1). In contrast, fecal shedding of the *qseC* mutant was significantly decreased at 2, 3, 5 and 7 dpi compared to wild-type *S.* Typhimurium and at 2, 3 and 5 dpi compared to the *qseBC* mutant ($P < 0.01$). The average swine body temperature (assessed using

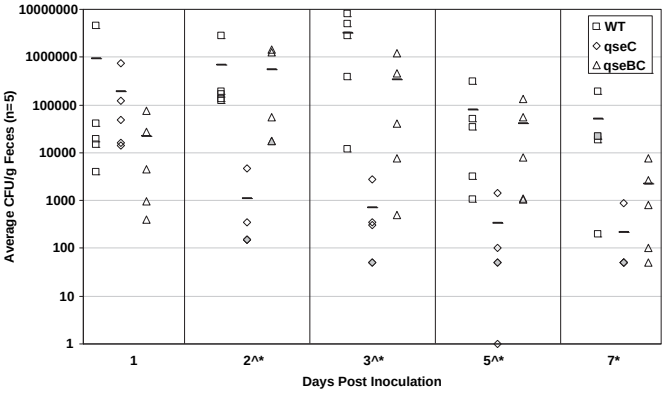

Fig. 1. Decreased swine fecal shedding of the *qseC* mutant compared to the *qseBC* mutant and wild-type *S.* Typhimurium. The horizontal bar for each data set indicates the average daily fecal shedding (CFU/g) for the BSX 8 (WT), BBS 10 (*qseC*) and BBS 192 (*qseBC*) *S.* Typhimurium strains following intranasal inoculation of 7 week old pigs ($n = 5$) with 1×10^9 CFU. Symbols indicate data points from individual pigs and shaded symbols indicate multiple pigs with the same data point. *Significant difference comparing BBS 10 to BSX 8 ($P \leq 0.01$). ^Significant difference comparing BBS 10 to BBS 192 ($P \leq 0.01$).

a rectal thermometer) was significantly increased in swine inoculated with either the wild-type strain (103.7 °F) or the *qseBC* mutant (103.6 °F) compared to the *qseC* mutant (102.5 °F) at 1 dpi ($P < 0.01$). Our results indicate that the *S.* Typhimurium *qseC* mutant but not the *qseBC* mutant has decreased colonization fitness in the swine gastrointestinal tract.

3.2. QseBC two component system in bacterial motility

Another phenotypic difference our previous work has demonstrated is that a *qseC* mutant has decreased bacterial motility compared to wild-type *S.* Typhimurium [10]. Therefore, the QseC sensor kinase enhances both competitive fitness in the swine host and bacterial motility since, in its absence, both phenotypes are reduced. To investigate the role of the QseB response regulator in bacterial motility, we analyzed the motility phenotypes of *qseB*, *qseC*, and *qseBC* mutants of *S.* Typhimurium. In Fig. 2 (white bars), the motility of the *qseB* and *qseBC* mutants was similar to or slightly greater than wild-type *S.* Typhimurium, whereas the motility of the *qseC* mutant was significantly decreased compared to the wild-type strain. This suggests that QseB is a repressor of motility and QseC regulates the repressor activity of QseB: when QseC is absent, decreased motility occurs due to the repressor activity of QseB, and when both QseBC are absent, the repression by QseB is removed and motility is similar to wild-type *S.* Typhimurium.

For wild-type *S.* Typhimurium, addition of the catecholamine, norepinephrine to the medium resulted in enhanced motility (Fig. 2, black bar compared to white bar) [10]. The motility of the *qseB* and *qseBC* mutants was similar to wild-type *S.* Typhimurium in the presence of norepinephrine. Although the motility of the *qseC* mutant is significantly decreased compared to wild-type *S.* Typhimurium (in the presence or absence of norepinephrine), a significant increase in motility is observed for the *qseC* mutant in the presence of norepinephrine (Fig. 2, black bar compared to white bar). Since each of the mutants displayed an increase in motility in the presence of norepinephrine, the exclusive role of the QseBC two-component system in motility of *S.* Typhimurium in response to norepinephrine is questionable.

Given that norepinephrine functions in a siderophore-like manner to provide iron to the bacterial cell [11], the enhanced motility in the presence of norepinephrine could be due to an increase in iron availability. Thus, the motility of wild-type *S.* Typhimurium was assayed on media containing $FeCl_3$ and another siderophore, ferrioxamine E. The saturating concentrations of ferrioxamine E (200 ng/ml) and $FeCl_3$ (80 μM) as well as norepinephrine (100 μM, in the presence of 80 μM $FeCl_3$) were determined to achieve maximum motility (data not shown). In Fig. 3, ferrioxamine E increased the motility of wild-type *S.* Typhimurium similar to $FeCl_3$; the presence of both ferrioxamine E and $FeCl_3$ did not further enhance *S.* Typhimurium motility. At saturating levels of ferrioxamine E and $FeCl_3$, norepinephrine further enhanced the motility of wild-type *S.* Typhimurium (Fig. 4, black bar compared to white bar). To determine if enhanced motility could be due to an increase in bacterial growth rate, the growth rate of each strain was assayed under each growth condition. The presence of $FeCl_3$ (80 μM) and/or ferrioxamine E (200 ng/ml) enhanced the growth of each strain, whereas norepinephrine (100 μM) alone or in the presence of $FeCl_3$ and ferrioxamine E did not increase bacterial growth compared to the growth rate in DMEM alone or DMEM supplemented with $FeCl_3$ and ferrioxamine E, respectively (data not shown). Therefore, the enhanced motility of wild-type *S.* Typhimurium in the presence of $FeCl_3$ and/or ferrioxamine E is likely due to enhanced bacterial growth, whereas norepinephrine-enhanced bacterial motility is independent of bacterial growth rate.

Three iron-regulated outer membrane proteins (IROMPs), IroN, FepA and CirA function as receptors for catecholate-mediated iron uptake by *Salmonella*, and we as well as other investigators have shown the critical role of these IROMPs in norepinephrine-enhanced growth [6,9,14]. We tested whether the IROMPs are required for the observed norepinephrine increase in motility in the presence of $FeCl_3$ and ferrioxamine E: similar to wild-type *S.* Typhimurium, norepinephrine enhanced the motility of a triple IROMP mutant (Δ*iroN* Δ*fepA* Δ*cirA*) by 32% compared to motility on medium containing only $FeCl_3$ and ferrioxamine E (data not shown). Furthermore, a mutation in the *exbD* gene, involved in TonB-dependent energy transduction of iron-siderophore and vitamin B_{12} transport across the bacterial outer membrane [15,16], also did not eliminate norepinephrine-enhanced motility in the presence of $FeCl_3$ and ferrioxamine E (data not shown). These results suggest that contrary to norepinephrine-enhanced growth, the adrenergic-enhanced motility phenotype is independent of the IROMPs and the TonB-ExbB-ExbD complex.

Norepinephrine also significantly enhanced the motility of the *qseC*, *qseB*, *qseBC*, *pmrA*, and *qseE* mutants in the presence of

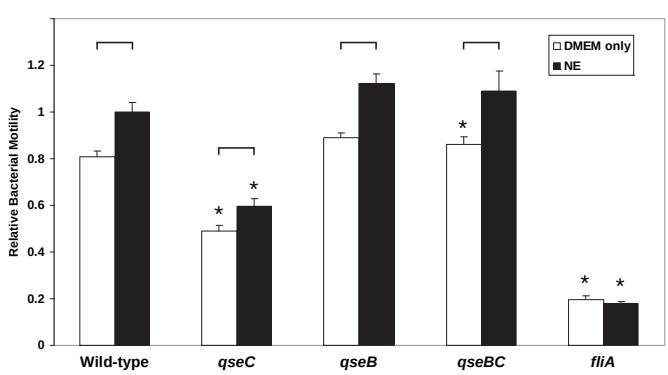

Fig. 2. Norepinephrine enhances bacterial motility of *S.* Typhimurium mutants in the QseBC two-component system. Relative bacterial motility of BSX 8 (Wild-type), BBS 10 (*qseC*), BBS 242 (*qseB*), BBS 192 (*qseBC*) and BBS 320 (*fliA*) *S.* Typhimurium strains following 18 h of incubation on DMEM 0.3% motility agar without supplementation (DMEM only; white bars) or with 50 μM norepinephrine supplementation (NE; black bars). Relative bacterial motility of each strain and culture condition was calculated based on a comparison with wild-type *S.* Typhimurium in the presence of 50 μM norepinephrine. A bracket above the data bars indicates a significant difference between culture conditions ($P \leq 0.01$). An asterisk (*) denotes a significant difference compared to wild-type *S.* Typhimurium under the same condition ($P \leq 0.01$). BBS 320 (*fliA*) represents a motility negative control.

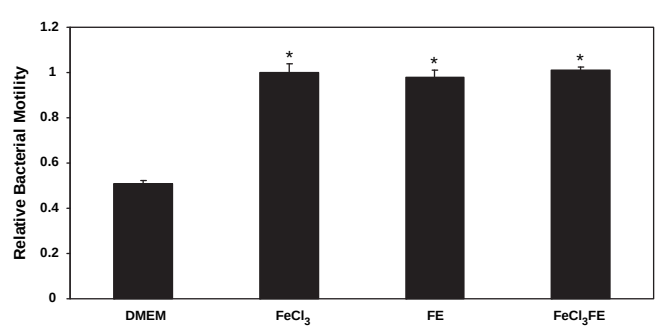

Fig. 3. Iron chloride and ferrioxamine E enhance bacterial motility of wild-type *S.* Typhimurium. Relative bacterial motility of *S.* Typhimurium BSX 8 following 18 hrs of incubation on DMEM 0.3% motility agar without supplementation (DMEM only) or with 80 μM $FeCl_3$ ($FeCl_3$), 200 ng/ml ferrioxamine E (FE) or 80 μM $FeCl_3$, 200 ng/ml FE ($FeCl_3$ FE). Relative bacterial motility of each culture condition was calculated based on a comparison with wild-type *S.* Typhimurium in the presence of 80 μM $FeCl_3$. An asterisk (*) denotes a significant difference compared to DMEM only ($P \leq 0.01$).

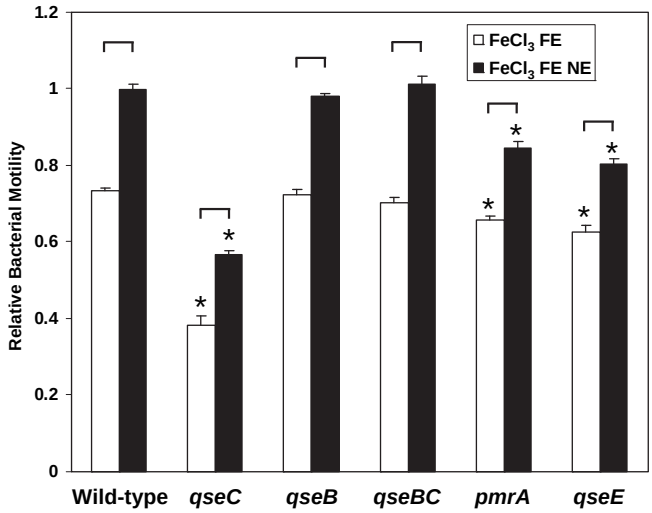

Fig. 4. Norepinephrine enhances bacterial motility of *S.* Typhimurium strains in the presence of saturating concentrations of iron chloride and ferrioxamine E. Relative bacterial motility of BSX 8 (Wild-type), BBS 10 (*qseC*), BBS 242 (*qseB*), BBS 192 (*qseBC*), BBS 281 (*pmrA*) and BBS 380 (*qseE*) *S.* Typhimurium strains following 18 h of incubation on DMEM 0.3% motility agar with 80 μM $FeCl_3$, 200 ng/ml ferrioxamine E ($FeCl_3$ FE; white bars) or 80 μM $FeCl_3$, 200 ng/ml ferrioxamine E, 100 μM norepinephrine ($FeCl_3$ FE NE; black bars). Relative bacterial motility of each strain and culture condition was calculated based on a comparison with wild-type *S.* Typhimurium in the presence of 80 μM $FeCl_3$, 200 ng/ml ferrioxamine E, 100 μM norepinephrine. A Bracket above the data bars indicates a significant difference between culture conditions ($P \leq 0.01$). An asterisk (*) denotes a significant difference compared to wild-type *S.* Typhimurium under the same condition ($P \leq 0.01$).

ferrioxamine E and $FeCl_3$ (Fig. 4, black bars compared to white bars). Previous reports have suggested that the PmrAB and the QseEGF two-component signal transduction systems are possible epinephrine/norepinephrine sensing systems in *S.* Typhimurium [17,18]. Our motility results indicate that mutations in these genes do not prevent *S.* Typhimurium from responding to norepinephrine. However, a significant decrease in motility was detected for the *qseC*, *pmrA*, and *qseE* mutants compared to the parental strain (in the presence or absence of norepinephrine).

4. Discussion

Bacterial motility is a complex phenotype that is modulated by multiple cellular regulators and global regulatory signals [19]. The bacterial motility of the *S.* Typhimurium *qseC* mutant was previously shown by our laboratory and confirmed by Merighi et al. to be decreased compared to the wild-type strain [10,13]. Recently, Merighi et al. demonstrated (and we confirm in this study) that, unlike the *qseC* (*preB*) mutant, *qseB* (*preA*) and *qseBC* (*preAB*) mutants of *S.* Typhimurium do not have defects in motility [13]. Thus, the presence of QseB in the absence of QseC is required for the decrease in bacterial motility. Recently, Kostakioti et al. [20] reported a phosphatase activity for QseC in uropathogenic *E. coli* (UPEC) that is required for QseB dephosphorylation. Similar to our results in *S.* Typhimurium, a *qseC* mutant of UPEC, but not *qseB* or *qseBC* mutants, has decreased bacterial motility and virulence (in a murine cystitis model). The authors demonstrate that phosphorylation of QseB is required for the diminished phenotypes and conclude that the QseC phosphatase activity is critical for regulating QseB activity and subsequent gene expression.

In our motility assays, a significant increase in bacterial motility was observed for *S.* Typhimurium in the presence of norepinephrine. Norepinephrine has been shown to exhibit a siderophore-like activity to supply iron to the bacterial cell [3,11,21]. A number of *S.* Typhimurium genes encoding iron uptake and utilization proteins are required for norepinephrine-enhanced growth in serum supplemented medium [6,9]. In addition, Wang et al. demonstrated that *S.* Typhimurium genes for iron metabolism were induced during swarming, a specialized form of bacterial motility, suggesting that the bacterial cells were increasing iron availability to enhance growth and motility [22]. In our motility assays, supplementation of motility medium with iron or an alternative siderophore, ferrioxamine E also enhanced motility (independent of norepinephrine). Iron-enhanced motility of *S.* Typhimurium in DMEM containing either $FeCl_3$ or ferrioxamine E appears to be due to increased bacterial growth. In contrast, norepinephrine-enhanced bacterial motility was growth rate independent (alone or in the presence of saturating concentrations of $FeCl_3$ and ferrioxamine E). Unfortunately, the question still remains regarding what is responsible for norepinephrine-enhanced motility: increased iron availability or norepinephrine signaling via a bacterial adrenergic receptor (like QseC in *E. coli*)? If the norepinephrine-enhanced motility is due to an increase in iron availability, it is doing so independent of the outer membrane proteins that are required for norepinephrine-enhanced growth since both a triple Δ*iroN* Δ*fepA* Δ*cirA* mutant (catecholate receptors) and an *exbD* mutant (energy-transducing TonB-ExbB-ExbD ferri-siderophore uptake system) exhibit norepinephrine-enhanced bacterial motility in the presence of $FeCl_3$ and ferrioxamine E. TonB-independent, norepinephrine regulation has also been described by Spencer et al. for *S.* Typhimurium gene expression [23].

As for identifying an adrenergic receptor in *Salmonella*, although the motility response of the *qseC* mutant is significantly diminished compared to wild-type *S.* Typhimurium, the *qseC* mutant did respond to norepinephrine, as did the *qseB*, *qseBC*, *qseE* and *pmrA* mutants. We, like Merighi et al. [13], have been unable to show that our QseC-regulated genes are also regulated by norepinephrine. Furthermore, Spencer et al. [23] found no role for QseBC or QseEF in the adrenergic regulation of *S.* Typhimurium genes identified in a transposon mutagenesis screen designed to find genes regulated by neuroendocrine stress hormones. However, Rasko et al. recently demonstrated that transcription of the *S.* Typhimurium *sifA* gene is increased in the presence of norepinephrine and deletion of *qseC* from the *S.* Typhimurium genome eliminated norepinephrine induction of *sifA* [24]. To date, *sifA* is the only *S.* Typhimurium gene that has been demonstrated to be regulated by both norepinephrine and QseC, although neither Merighi et al. [13] nor Karavolos et al. [18] identified *sifA* as a QseBC-regulated gene during their microarray analyses. Currently, the role of the QseBC two-component signal transduction system in norepinephrine-enhanced motility is uncertain, and determining whether *S.* Typhimurium encodes a bacterial adrenergic receptor that modulates motility gene expression will require further investigation.

Previously, we reported up to a 30-fold decrease in competitive fitness for the *qseC* mutant compared to wild-type *S.* Typhimurium during colonization of the swine gastrointestinal tract, as measured by fecal shedding following intranasal inoculation of a 1:1 mixture of the two strains [10]. In this study, we confirmed our previous results, demonstrating that the average fecal shedding of the *qseC* mutant was reduced up to 4000-fold in swine compared to the wild-type strain. Although the *qseC* mutant had significantly decreased colonization fitness in the swine gastrointestinal tract, the fecal shedding of the *qseBC* mutant was not statistically different than wild-type *S.* Typhimurium. Our results in swine are different than the results of Merighi et al. [13] in BALB/c mice who demonstrated a consistent two-day delay in mouse mortality for a *qseBC* (*preAB*) mutant compared to wild-type *S.* Typhimurium. Furthermore, when the *qseBC* (*preAB*) mutant was co-inoculated with wild-type *S.* Typhimurium in mice, these authors reported

a competition infection defect that was not seen when the wild-type strain was co-inoculated with either a *qseB* (*preA*) or *qseC* (*preB*) mutant. The lack of consensus between the in vivo data in swine compared to the mouse highlights potential differences between these host models. Swine are a natural host for *S.* Typhimurium and infection with the pathogen results in enterocolitis, whereas infection of BALB/c mice with *S.* Typhimurium results in systemic disease [25,26]. Our data indicates that a functional QseC sensor kinase is required for optimal gene regulation in the gastrointestinal tract of swine.

In conclusion, this study demonstrated an increase in motility of *S.* Typhimurium in response to iron, norepinephrine, and a combination of norepinephrine and iron. Although iron supplementation of DMEM medium increased the bacterial growth rate, supplementation with norepinephrine did not, indicating that norepinephrine-enhanced bacterial motility is independent of growth rate. Furthermore, norepinephrine-enhanced motility is also independent of the outer membrane proteins that have previously been shown to be required for norepinephrine-enhanced growth (*iroN*, *fepA*, and *cirA*). Mutation of the *qseC* gene (encoding the two-component sensor kinase QseC) decreased motility of *S.* Typhimurium, whereas the *qseB* (response regulator) and *qseBC* (response regulator and sensor kinase) mutants have similar motility phenotypes to wild-type *S.* Typhimurium. Furthermore, the *qseC* mutant has decreased swine gastrointestinal colonization compared to the *qseBC* mutant and wild-type *S.* Typhimurium, an in vivo phenotype that differs from the mouse model.

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Research paper

Methods for transcriptomic analyses of the porcine host immune response: Application to *Salmonella* infection using microarrays

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ABSTRACT

Technological developments in both the collection and analysis of molecular genetic data over the past few years have provided new opportunities for an improved understanding of the global response to pathogen exposure. Such developments are particularly dramatic for scientists studying the pig, where tools to measure the expression of tens of thousands of transcripts, as well as unprecedented data on the porcine genome sequence, have combined to expand our abilities to elucidate the porcine immune system. In this review, we describe these recent developments in the context of our work using primarily microarrays to explore gene expression changes during infection of pigs by *Salmonella*. Thus while the focus is not a comprehensive review of all possible approaches, we provide links and information on both the tools we use as well as alternatives commonly available for transcriptomic data collection and analysis of porcine immune responses. Through this review, we expect readers will gain an appreciation for the necessary steps to plan, conduct, analyze and interpret the data from transcriptomic analyses directly applicable to their research interests.

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1. Introduction

Host-pathogen interactions have been studied at the molecular, cellular, tissue and organismal levels for many years (see, for example, the set of reviews in *Current Opinion in Immunology*, 2007, Vol. 19). At the transcriptomic level, a meta-analysis of microarray data detecting the immune response to many types of pathogens in different human cell types has shown that a core set of ~500 genes are expressed in response to viruses, bacteria (including *Salmonella*) and immune stimulants such as LPS (Jenner and Young, 2005). Because of the ubiquity and ability of *Salmonella* spp. to infect many important vertebrate species, both animal models (Santos et al., 2001; van der Sar et al., 2003) and cell culture systems (see below) have been used to understand *Salmonella* interactions with its host. Early immune responses are thought to be critical in resistance to *Salmonella* (Wick, 2004), and a large number of genes have been implicated in the host response to *Salmonella* (Detweiler et al., 2001; Rodenburg et al., 2007). Many studies have used lipopolysaccaride (LPS) as a model of the transcriptomic response to bacteria (Wells et al., 2003; Bliss et al., 2005; Wurfel et al., 2005; Beck et al., 2006). A direct comparison of the transcriptomic response to LPS versus *Salmonella* showed significant similarity in both the genes responding and the magnitude of the response (Rosenberger et al., 2000).

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The cytokine protein and RNA responses to *Salmonella* have also been studied in the pig; most parameters and genes involved appear to be very similar to those in mouse (Dvorak et al., 2006). Initial interactions of *Salmonella* with the gut tissue have been studied in cell culture (Veldhuizen et al., 2006; Skjolaas et al., 2007), but also in explants of Peyer's patch tissue (Hyland et al., 2006). In the latter study, IL1B and IL8 RNA (but not TNF) were found to increase after exposure to *Salmonella enterica* serovar Choleraesuis (SC) for 2 h. In IPEC-J2 cells, an *in vitro* model of porcine jejunal intestine, *S. enterica* serovar Typhimurium (ST) exposure for 1.5 h increased RNA for TNF, IL8 and CCL20, but induction of these cytokines was not observed in cells exposed to SC (Skjolaas et al., 2007). Using a separate porcine epithelial cell line, exposure to ST for 24 h induced RNA expression for the beta-defensin gene pBD-2 (Veldhuizen et al., 2006).

Immune responses of different types of porcine cells isolated from whole blood have also been studied. The *in vitro* response of pig peripheral blood mononuclear cells (PBMC) to *Salmonella* or mitogen treatment was characterized by increased IL2, IL4, and IFNG RNA, with no effect on IL10 expression, while bacterial F4 fimbrae increased expression of IFNG RNA only (Verfaillie et al., 2001). This group also found cytokine protein levels generally correlated well with RNA expression for the first 24 h of exposure.

Raymond and Wilkie (2004) investigated porcine T cell responses to stimulated dendritic cells (DCs), finding that the T cell response profile depended on how the DC was stimulated and the cytokine milieu during stimulation. They also evaluated monocyte and DC responses to specific pathogen-associated molecular pattern (PAMP) molecules. Treatment with LPS induced expression of TLR4 and T helper 1 (IFNG, IL12p35), T helper 2 (IL13) and regulatory T (IL10) cell response pathways. Cell-specific responses were observed for several of these genes; MHC Class II expression was greater after LPS stimulation in monocytes whereas B7 RNA increased in both cell types (Raymond and Wilkie, 2005). In CD14+ cells isolated from pig spleen, LPS pretreatment was shown to decrease TNF and IL8, but not IL1B. However, gene expression in response to re-application of LPS indicated that pig monocytes undergo a similar LPS tolerance response (Cagiola et al., 2006) to that reported for murine macrophages.

The RNA response of several Toll-like receptor and chemoattractant genes to *S. enterica* serovar Choleraesuis (SC) and *S. Typhimurium* (ST)inoculationof pigs has been reported for a number of tissues (Burkey et al., 2007; Wang et al., 2007, 2008b). Relative to uninfected controls, quantitative real-time PCR (QPCR) analysis showed ST infection greatly increased IL8 expression in MLN and decreased expression of MIF RNA in colon, while in SC infected animals, TLR9 and MIF were decreased in colon and MIF and OPN were decreased in MLN (Burkey et al., 2007). Global transcriptional responses to pathogenic infections in the pig have been reported using microarrays (Afonso et al., 2004; Ledger et al., 2004; Li et al., 2004; Miller and Fox, 2004; Moser et al., 2004; Niewold et al., 2005; Zhao et al., 2006; Uthe et al., 2006; Uthe et al., 2007; Wang et al., 2007, 2008b; Tuggle et al., 2008) and multiple-gene and larger scale QPCR methods (Raymond and Wilkie, 2004; Baltes and Gerlach, 2004; Royaee et al., 2004; Dawson et al., 2005). These studies (reviewed in Tuggle et al., 2007) have begun to identify immune genes involved in the host's response to different pathogen infections. Taken together they have contributed to a better understanding of molecular pathways relating to health and disease in pigs.

This review centers on our approaches to use such transcriptomic data to unravel important pathways controlling the porcine response to *Salmonella*. We will not discuss recent publications on the host transcriptomic response to viruses (Bates et al., 2008; Flori et al., 2008a,b; Durand et al., 2009; Fernandes et al., 2009; Shi et al., 2009; Li et al., 2010; Tomas et al., 2010), to mycobacteria (Galindo et al., 2009), to *Actinobacillus pleuropneumoniae* (Hedegaard et al., 2007; Moser et al., 2008), or to *Toxoplasma gondii* (Okomo-Adhiambo et al., 2006) infections or to non-infectious stimuli or other contrasts (Dvorak et al., 2006; Chowdhury et al., 2007; Nino-Soto et al., 2008a,b; Ponsuksili et al., 2008; Wang et al., 2008a). However, these authors used similar approaches to the broadly applicable methods discussed below. We will also not describe methods to measure miRNAs in tissues of immunological importance, although recent reports have identified miRNAs that are important in the immune response in other species (Pedersen and David, 2008; Bi et al., 2009). A number of reports have described miRNA identification in several porcine tissues focusing on reproduction or muscle development, although single papers have described isolation of swine miRNAs from intestine (Sharbati et al., 2010), or miRNAs potentially interacting with swine influenza virus (He et al., 2009).

2. Steps in producing and exploring transcriptomic data on immune response

2.1. Experimental design and tool choice—what question do you want to ask?

The most important first step in transcriptomic analyses (and in all experiments in fact) is to determine the question to address. In many transcriptome studies, the question is broadly exploratory, along the lines of "what are the genes and pathways that respond to the pathogen of interest in this tissue or cell type?" If so, then tools and processes that capture accurate and sensitive information on the largest numbers of transcripts for the lowest cost are optimal. Because of the lack of available space and complexity of these decisions for each lab, we cannot describe all possibilities but will briefly review the main choices for technologies in this area.

While we focus on the use of microarray technology to collect transcriptomic data in this review, it is by far not the only method, and other technologies such as Differential Display (DD), Suppression Subtractive Hybridization (SSH), and Serial Analysis of Gene Expression (SAGE) have been used to identify differentially expressed (DE) genes during the immune response in pigs (Tuggle et al., 2007 and references therein). These latter technologies, especially DD and SSH, require significant wet lab analyses, as many different combination of primers are required to survey the transcriptome significantly. The popularity of microarrays is due primarily for their breadth of coverage and relative

sensitivity and simplicity over these other methods to generate global RNA profiles. More recent technologies such as RNA Seq (Wang et al., 2009) have not yet been reported in porcine immunogenomics, but a number of groups are developing such data. It is anticipated that RNA-Seq and similar methods based on the new sequencing technologies will replace microarrays in the future, especially for initial screening experiments, due to their anticipated lower cost and broader representation of the transcriptome.

In pigs, there are several options for collecting transcriptomic data and the best approach depends on the level of molecular and bioinformatic expertise available to the lab. If such expertise is minimal, then data collection using a fee-for-service approach is probably most appropriate. One such option is the use of the Affymetrix Porcine GeneChip®, which requires only RNA preparation in one's laboratory; the RNA is then provided to a dedicated service facility which many universities and research institutes have available. Expression data is provided by the Facility ready for statistical analysis as described below. If more "wet-lab" expertise is available, data collection can be less expensive, especially for a large project, through the use of custom oligonucleotide arrays in one's laboratory. cDNA arrays have been replaced by such oligonucleotide arrays, as synthesis costs have dropped significantly and algorithms to minimize cross-hybridization have improved. Thus two major issues with cDNA arrays, the need for highly accurate clone and PCR product tracking during array production, and the concern of cross-hybridization to common domains within multiple cDNAs, are significantly diminished for oligonucleotide arrays, which can also be created for less expense, at least on a per array basis, than purchased arrays. However, in any such cost comparison it is important to consider the labor and materials costs not only on the array production but also the target labeling, hybridization and data acquisition, so that all costs are recognized during the decision-making process. Finally, there are hybrid approaches, where several companies sell arrays for use in individual laboratories or selected universities print arrays for use in labs nationwide, as is done for several of the swine long oligo arrays. In pigs, sets of long (70-mer) oligonucleotides have been designed and validated for transcriptomics research in the past few years (Zhao et al., 2005; Steibel et al., 2009). Most recently, an oligonucleotide array, the Pigoligoarray, with functional annotation for 16,225 of the 18,524 porcine-specific oligonucleotides has been evaluated (Steibel et al., 2009). For the 4 tissues examined, the array was found to be useful for accurate measurement of gene expression on a global scale. In the work described below, we used the Porcine GeneChip®, from Affymetrix, which has 23,937 probe sets with a total of 19,253 annotations currently (see Section 2.3; Couture et al., 2009).

2.2. Statistical analysis of microarray data

There are many excellent reviews of the various approaches to statistical analysis of microarray data (Quackenbush, 2001; Quackenbush, 2002; Roberts, 2008). The MicroArray Quality Control (MAQC) project reviewed major aspects of microarray data analysis in a special issue of Nature Biotechnology (MAQC Consortium, 2006). The following is only a short general synopsis of this topic and focuses on our specific approaches to assess expression response using transcriptomics. We do want to emphasize that any experimental design should incorporate as much biological replication as possible, while eliminating technical replication that had been thought to be important early in the field but is no longer deemed important for microarray analysis. The earliest statistical analysesof microarrays depended on the experimental design of comparing two differently labeled (Cy3, Cy5) samples hybridized to the same array, thus many aspects of the technique that lead to nuisance variation are diminished (Schena et al., 1995). Such work compared levels of Cy3 and Cy5 expression, setting an *ad hoc x*-fold difference in expression as the criteria for declaring a gene as differentially expressed (DE). In many experiments the design was a comparison of test samples to the same control sample (the reference design).

However, an analytical approach based only on a fold change filter is simplistic and an insensitive method to find all differential expression. Experimental designs and data analyses have become statistically more rigorous; details can be found in the reviews listed above. In the work below, we describe our analysis of Affymetrix-based data for which company-provided software is used to produce an estimate of expression for the transcript in question. First, a unique Affymetrix algorithm combines the hybridization signals for a set of probes to estimate the signal for each transcript. The signal for each probeset across the Genechip is normalized. Data normalization is a significant field in its own right, and the type of normalization used depends on the specifics of the microarray technology as well as the kinds of questions to be answered (Quackenbush, 2002). Here we summarize normalization, carried out in the following experiments, as the method to adjust raw individual hybridization signals within a microarray experiment so that results across biological replicates can be combined. Such normalizations often involve an adjustment based on the overall level of hybridization across the microarray, although many refinements including local background measurements and other methods can be applied (Quackenbush, 2002). An ANOVA model is then used to compare responses across time points or treatments. As this method performs many thousands of statistical significance tests at the same time, it is important to correct for such multiple testing by estimating the false discovery rate (FDR) (Storey and Tibshirani, 2003). The FDR q-value provides an estimate of the likelihood that the members of a list of differentially expressed are incorrectly predicted to be DE; commonly used q-values are 0.01–0.1, which indicate that no more than 1–10% of the genes are false discoveries; i.e., the higher the q value the more likely the gene is NOT differentially expressed.

2.3. Bioinformatics analysis of microarray data

2.3.1. Microarray element annotation

Once the genes that differentially respond to infection, treatments, or other variables, have been identified, we can proceed to explore specific biological questions of interest. As shown in Fig. 1, several immediate analyses can

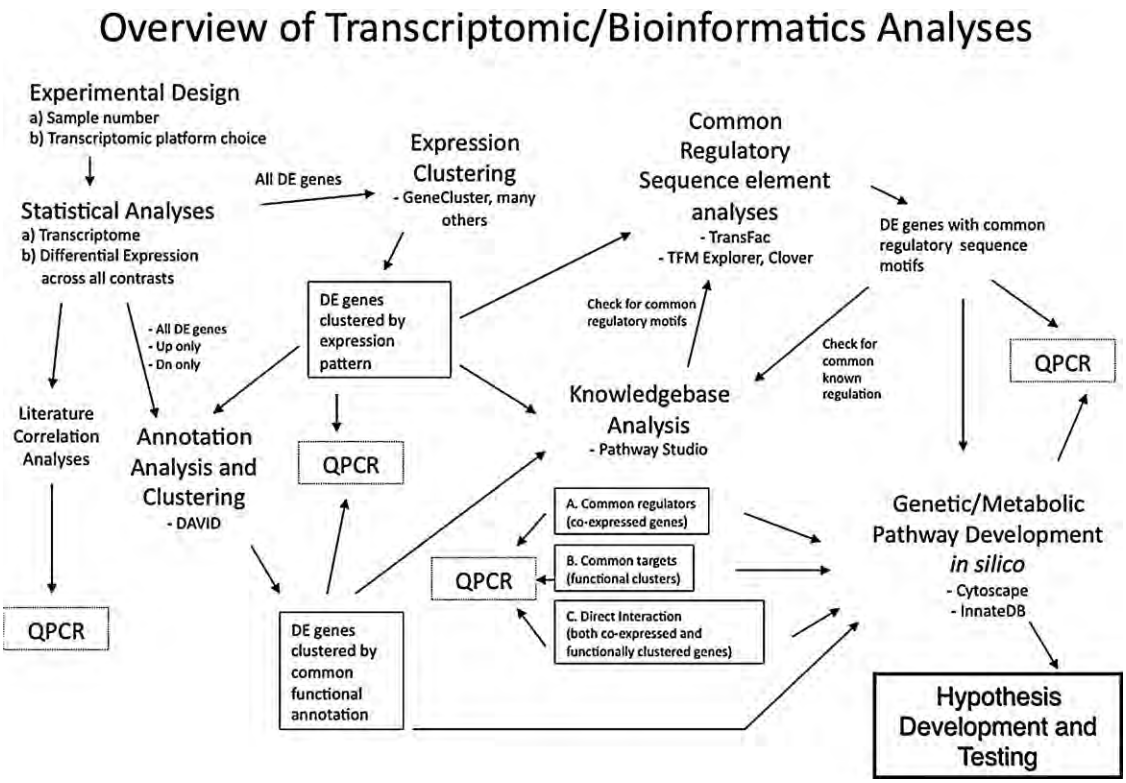

Fig. 1. Overview of transcriptomic/bioinformatics analyses. A schematic view of approaches our collaborative group has developed to analyze porcine transcriptomic data. See text in Sections 2.3.1–2.3.7 for details.

be envisioned. Because all these studies focus on pig gene expression, it is important to remember that most of the available tools rely on the transfer of gene function or annotations to the array elements in the target species (in our case, pig) from closely related and more widely studied species, e.g., human or mouse, through sequence comparison. If it is found that a pig sequence is sufficiently similar to an annotated gene sequence in another species, comparative analysis can be used to predict the identity and function(s) of the porcine transcript based on the annotations associated with the matching sequences in other species. Thus, it is critical to obtain the most up to date and comprehensive annotation of the gene sequences on the microarray.

We have recently assembled all available cDNA sequences to create an Iowa Porcine Assembly (IPA) which is a set of ~140,000 consensus porcine sequences and ~105,000 singletons for all known pig mRNAs (Fig. 2; Couture et al., 2009). We have annotated these sequences using sequence similarity of the porcine consensus sequence to those annotations reported for human, mouse, rat, and bovine sequences in GenBank. These IPA sequences were then used to annotate the Affymetrix Genechip sequence elements; this resulted in excellent annotation coverage (~80%) of the Affymetrix elements. We are now applying our IPA annotation to the Pigoligoarray evaluated by Steibel and colleagues so that information in the future can be more easily integrated across platforms (Couture et al., unpublished data). These annotations will be updated when the draft porcine genome sequence is released, which is expected in late 2010. These annotations are available at www.anexdb.org, where a download file provides up-to-date annotations of the major expression platforms for the pig. In addition, the AnexDB.org website has been organized to assist in transcriptome analyses; we have created data storage, analysis and GEO submission tools (Fig. 2). This includes tools to store the numerical data as well as sample preparation, hybridization protocols and data collection details. Such data can be kept completely private in the database until publication. Most journals now require that all publications reporting microarray data must make available the Minimal Information About a Microarray Experiment (MIAME) information. At AnexDB.org, we provide tools that automate much of this analysis pipeline, including generation of the correctly formatted 'soft' file containing all MIAME information required for NCBI-GEO submission of microarray data.

2.3.2. *Embedding the microarray results into current literature*

A first step for transcriptomic experiments is to compare the list of DE genes with the current literature. This primary step can help integrate the microarray data with known responses at porcine immune response genes. This can be done by individually checking genes for available functional investigations by other groups in PubMed. Using annotation information for the gene symbol of the tran-

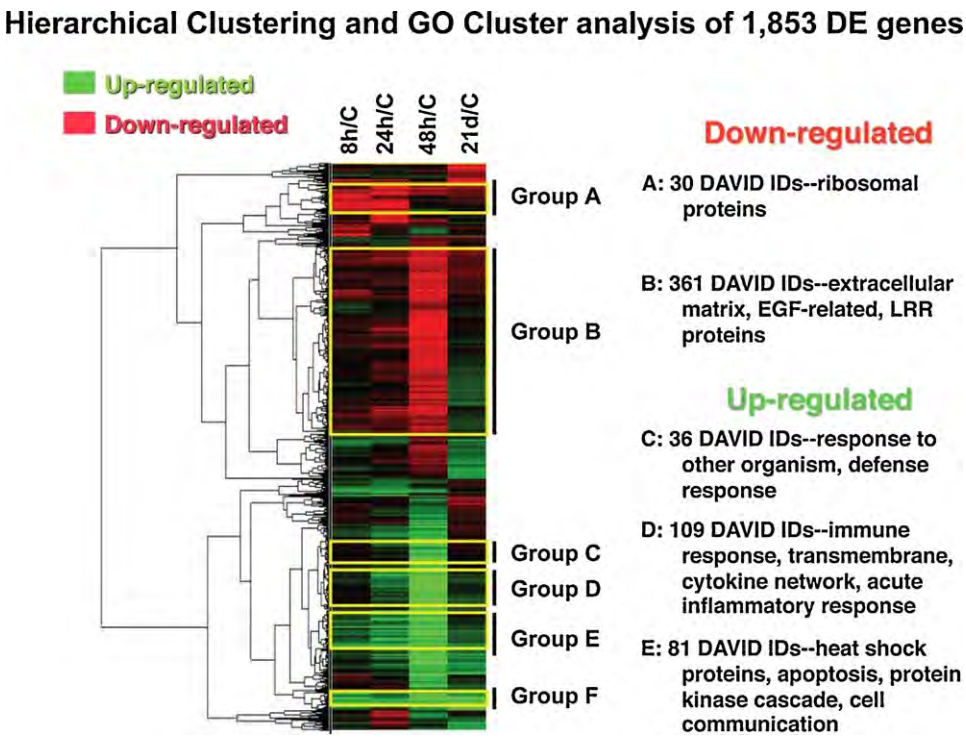

Fig. 2. Schematic diagram of the major parts of the ANEXdb.org website and database for porcine transcriptomic data storage and analysis. See text in Section 2.3.1 for details on the main functions of this bioinformatic resource.

script of interest, a researcher can easily access relevant genome, cDNA and functional information that has been integrated at a number of databases. The most comprehensive of these genome browsers are those available at the NCBI web portal (www.ncbi.nlm.nih.gov) and the EMBL web portal (www.ensembl.org/index.html). Particularly for the porcine genomics community, the latter site has excellent resources for the analysis of the pig genome (see http://www.ensembl.org/Sus_scrofa/Info/Index). Our group has found the Online Mendelian Inheritance in Man (OMIM) database (www.ncbi.nlm.nih.gov/OMIM) and the Online Mendelian Inheritance in Animals (OMIA) (www.ncbi.nlm.nih.gov/omia) to be especially useful as a starting point to learn about specific genes. OMIM contains extensive information on the structure, function, and phenotypes of known mutations in human genes and their counterparts in model organisms. However, the real value of microarray analyses is the power of measuring gene expression of so many genes that the responses of important pathways and networks can be recognized and measured, as described below.

2.3.3. *Function clustering and analysis using Gene Ontology and other gene annotation databases*

Once individual genes in a list of differentially responsive transcripts have been compared to available literature, a new type of exploratory analysis of these genes can be performed. An important question to ask of the data is: are there known functions or other attributes—annotations—for genes in this list that are over-represented compared to a background list of genes? In other words, what are the enriched biological 'signatures' or 'clues' hidden in this list that can help one understand the immune response represented by the list? The most widely used set of annotations are those provided by the Gene Ontology (GO) Consortium, which applies a set of descriptive terms from a defined vocabulary to genes for which some functional data is available. Terms covering three descriptive areas are available: Biological Process, Molecular Function, and Cellular Component.

To determine all annotations for a set of genes, there are a number of software tools that are available. These are proprietary packages (such as Ingenuity or GeneSpring), software available as downloadable local programs (such as GoMiner; http://discover.nci.nih.gov/gominer/index.jsp), as well as free web-based tools, such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) created by NIAID scientists (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003). While DAVID provides a number of different analytical tools, we have primarily used DAVID to annotate lists of genes in whole blood responding to *Salmonella* infection (Huang et al., manuscript in preparation) as well as a number of our other projects (Lkhagvadorj et al., 2009; Lkhagvadorj et al., 2010). In the DAVID on-line tool, one uploads a list of genes to analyze as well as a background gene list. The DAVID tool can calculate the frequency of GO terms associated with all genes in the uploaded list, and calculates those terms that are over-represented relative to the background. Over-representation of other gene annotations, such as KEGG pathways, Protein Information Resource (PIR) information, etc., can also be calculated depending on user specifi-

cations; a recent detailed description of current DAVID functionality is available (Huang da et al., 2009). We have also developed our own specific list of GO terms related to specific immune pathways and functions (a GO-Slim) by using OBO-edit and used it to develop a better understanding of the immune-response specific pathways in gene lists responsive to *Salmonella* infections (Wang et al., 2007, 2008b).

A comment on the selection of the 'background' list of genes is warranted. The default for this list in DAVID is the human genome; this may be inappropriate for any porcine gene list, but especially for those lists created from microarray platforms for which there is either incomplete genome coverage or non-random selection of elements representing transcripts on the array. The modified Fisher's Exact test calculations used by DAVID to determine over-representation—finding a higher frequency of terms in a specified list as compared to the background—depends on the assumption that the background is a set of genes that has a chance to be included in the differentially expressed list. For example, if many genes with a specific term such as "immune response" are present at 10% in a list of genes responding to LPS in macrophage cells, but present at much lower levels in a list of all human genes, this would be returned as an over-represented term. However, it is likely that many of the genes in a human genome background list, contributing to the overall frequencies of GO terms in that list, were not expressed in immune cells and could not be in the DE list. Thus it is more appropriate to use the largest set of genes that could have been in the DE list: the transcriptome for the tissue or cell type under study. Therefore we define our transcriptome, and thus our background list, as all those genes that show at least one hybridization signal above background across our entire set of Affymetrix chips for the tissue of interest. By using GO enrichment approaches such as these, we have shown that annotations for genes up-regulated in mesenteric lymph node from animals infected with SC are enriched in apoptosis, innate immune response and defense response terms, while annotations for down-regulated genes in these tissues are enriched in cell adhesion and calcium ion binding terms (Wang et al., 2008b).

2.3.4. *Expression clustering to find genes with similar transcriptomic response to infection or stimulus*

Depending on the type of experiment, a second valuable global analysis approach is to identify groups of genes that respond similarly to infection or immune stimulation at the RNA expression level. Several methods are available to 'cluster' genes by expression pattern across experimental samples. Such clustering methods (Belacel et al., 2006) can be broadly classified into (a) hierarchical methods or model based methods e.g., probabilistic mixture models (Medvedovic and Sivaganesan, 2002), and (b) model-free methods e.g., spectral clustering (von Luxburg, 2007). In each of these categories, clustering algorithms can generate different types of clusters e.g., non-overlapping clusters of data points or hierarchical organization of clusters. Each class of methods has its own advantages and disadvantages, requiring care in the choice of specific methods in specific settings (Belacel et al., 2006); we have primarily relied on hierarchical clustering, which work well for time course data such as response after infection. Clustering techniques can be particularly powerful for a time course experiment, as not only can expression pattern gene clusters be identified, but inferences can be made as to cause and effect during the immune response. Further, combining expression clustering with GO annotation of specific clusters can be very illustrative. One can find enriched functions that may identify specific pathways activated (or repressed) at specific times, allowing inference of multi-stage gene-gene interactions. For example, a seminal paper in the use of systems biology tools in immunology showed that expression clustering could identify murine regulatory pathways controlling the response to LPS (Gilchrist et al., 2006). This group showed that specific transcription factors (TF) in a cluster of up-regulated genes early in LPS treatment of mouse macrophage cells in culture controlled the expression of other sets of genes that clustered together with a maximal response later than the TF-dominated early cluster.

We have used GeneCluster software to identify sets of genes and their functions responding similarly to infection in mesenteric lymph nodes of SC inoculated pigs (Wang et al., 2008b). As illustrated in Fig. 3 using TreeView software, we detect large clusters of genes down-regulated (Groups A–B) or up-regulated (Groups C–F) at one or more time points during infection. Group A genes, down-regulated for the first 24 h post infection, are over-represented for ribosomal annotations, while Group B genes, only down-regulated by 48 h, are enriched for extracellular matrix proteins (Fig. 3).

2.3.5. *Using promoter sequences of co-expressed genes to find common regulatory motifs*

Genes with a similar response to an immune stimulus are co-expressed, and may be co-regulated; i.e., there may be a common regulatory factor controlling this co-expression response. To look for evidence of a common regulatory factor controlling multiple genes in a list, one approach is to search in the promoter DNA of such genes for over-represented sequence motifs known to mediate TF action. At the time of writing, the porcine genome community was close to completion of a full draft sequence for the pig, but the location of promoters near porcine genes has not yet been established. Due to lack of knowledge of promoter sequences for the pig, we developed perl scripts to obtain the orthologous human promoter sequences for porcine genes shown to be differentially up-regulated due to SC infection. Within these human promoters, we searched for TF motifs using the TransFac database (http://www.gene-regulation.com/pub/databases.html), as well as both TFM Explorer (Defrance and Touzet, 2006) and Clover (Frith et al., 2004) packages to determine over-abundance of motifs in the Group E set of genes (Fig. 3) that were up-regulated by 8–24 h post-inoculation (Wang et al., 2008b). We identified 95% of known NFkB-regulated genes in the Group E gene list, as well as 51 genes that have not previously been shown to be bound by NFkB. Similar results with slightly lower percentages were obtained for a set of genesup-regulated only by 48 hpi. These porcine genes that

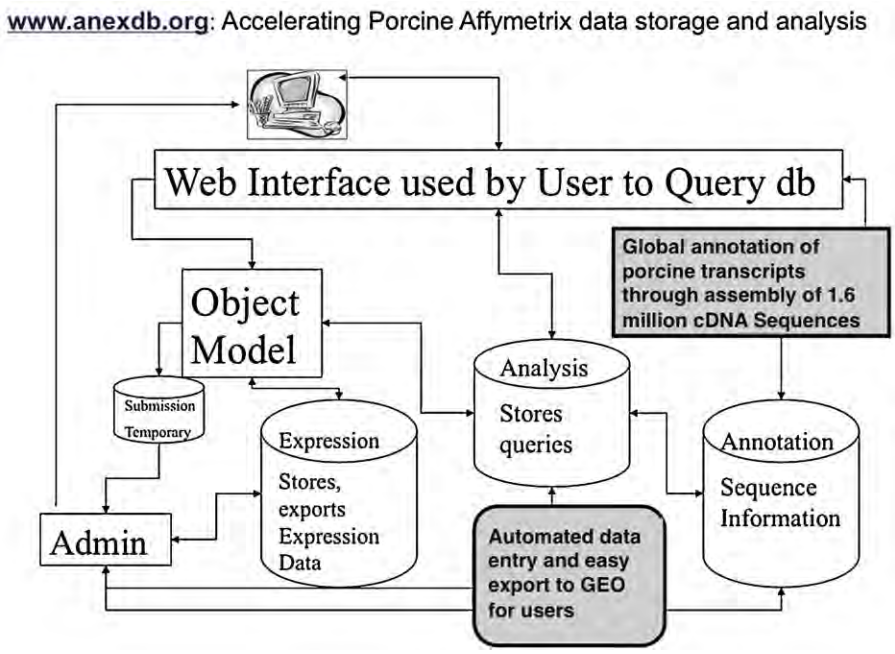

Fig. 3. An example of using hierarchical clustering of gene expression patterns to find co-expressed clusters of genes and the general functions represented by such gene clusters.
Adapted from Wang et al. (2008b).

were co-expressed with many known NFkB target genes (activated early in infection) and with NFkB motifs near their orthologous human promoters are proposed to be previously unrecognized members of an NFkB-dependent regulatory pathway responding to SC infection (Wang et al., 2008b).

2.3.6. Using 'Knowledgebase' text-mining tools to efficiently mine the available literature.

Once you have a list of genes that have some common attribute such as a co-expression pattern or common function, searching the voluminous primary literature to find commonalities among gene list members is of interest but an extremely time-consuming task. We have found value in using a text-mining software tool, such as Pathway Studio (Ariadne Genomics, Inc.), which accelerates the process of such literature searches, and can provide new insight as well. This software accepts a list of genes/proteins and searches a proprietary database that holds published information on relationships between genes, between proteins, as well as gene-protein, small-molecule-gene interactions, etc. It is possible to select a specific type of relationship to search for, such as "direct-regulator of", and specify only those genes/proteins that regulate two or more genes in a list. This would be an example of a search for a "common regulator" of the genes in the list. We recently used Pathway Studio to find the known targets of the NFkB regulatory complex in a list of genes up-regulated by SC early in infection (8–48 h post infection: Wang et al., 2008b). This information was then used to inform further exploratory analyses such as those described above for gene regulatory networks. While we could have found such information by searching many published articles, the software was able to dramatically cut down the time required for such searches, while providing a level of comprehensiveness and a repeatable methodology to the search. A number of other Pathway Studiorelationship filters, such as "common target" (to look for common functions of the gene list), or relationships within the gene list (to look at molecular or regulatory interactions among list members) are available. Furthermore, the software can create publication-quality figures depicting such relationships (Wang et al., 2008b).

2.3.7. Immune network analysis in silico—toward systems understanding of immune response

A higher level analysis that can integrate several of the above datasets is systems biology (Klipp et al., 2005; Bruggeman and Westerhoff, 2007; Gardy et al., 2009). For example, using a network analysis approach, an immunologist could use visualization tools such as those available in Cytoscape (www.cytoscape.org/) or InnateDB (Lynn et al., 2008; www.innatedb.org), to take datasets from many gene expression experiments and, combined with other data such as protein-protein interaction data, use correlations among datasets to draw network diagrams that illustrate the connections between genes (Gardy et al., 2009; Zak and Aderem, 2009). A network diagram, where genes are the nodes and the relationship between any two genes are the edges drawn between those nodes, can help visualize important genes in the immune process; such important genes may have much higher number of connections than on average and are called "hubs" in the network. As well, networks are often drawn such that the edge lengths are related to the correlation values used to create the network; for example, two genes with very similar patterns of expression across many treatments would

Table 1
Number of Affymetrix Porcine GeneChip® probesets showing significant differences in expression in whole blood for contrasts shown.

Criteria	Shed by time	Shed (at t0)	Shed (at t2)	Time (in LS)	Time (in PS)
$q < 0.05$	1442	0	243	171	3379
$q < 0.10$	3308	0	1313	837	4992

t2: 48 h post inoculation LS: low shedder PS: persistent shedder. q value: number of genes with False discovery controlled at 5% or 10%.

have a very short edge length connecting the two nodes. Sets of similarly responding genes across different conditions would cluster in the network and show a high level of connections in a small three-dimensional space. Such approaches can be powerful methods to uncover cryptic networks; the larger and more integrated the datasets, the more powerful the method becomes. Systematic collection of specific immune cell type transcriptomes (Hyatt et al., 2006), and a network analysis of these data and immune response data is being used in human and mouse immunology to move toward a "systems biology" understanding of innate immune responses (Heng et al., 2008; Gardy et al., 2009; Zak and Aderem, 2009). While not used as of yet in livestock transcriptomics, we predict broader use of systems biology approaches in animal species as relevant and useful data accumulate.

2.3.8. *Hypothesis generation and testing*

The analyses (as described above in Sections 2.3.1–2.3.7) can guide hypothesis generation, an important outcome of microarray experiments (Fig. 2). There are many possible paths through the above tools to come to a hypothesis to be tested; it is also possible to develop a hypothesis based on a single such analysis. As discussed above, we found in Wang et al. (2008b) that many genes that were up-regulated in the first 8–24 h post SC infection are known to be involved in the innate inflammatory response, and about ~25% were also previously shown to be regulated by NFkB. We then showed that the majority of the Group E genes do have NFkB regulatory motifs in the promoters of their human orthologs (Wang et al., 2008b). Thus we hypothesize that many of the remaining Group E genes, co-expressed with known NFkB target genes, may in fact be regulated by NFkB; most of these genes were not known to be NFkB targets during infection. We are currently testing a number of these genes for their response to LPS in culture and the dependency of this response on NFkB signaling (Couture et al., data not shown).

3. Use of transcriptomics to find genes associated with quantitative disease resistance traits

In the last few years, RNA profiling has been used to investigate not only the common RNA response to infection across biological replicates, but also the variation in response to LPS treatment among individuals in a population or across inbred strains (Wells et al., 2003; Wurfel et al., 2005; Beck et al., 2006). Different inbred mouse strains showed significant differences in pathway response to LPS challenge (Wells et al., 2003). In an attempt to understand modifiers of human innate response differences to LPS in whole blood *ex vivo*, Wurfel et al. (2005) tested blood from 102 donors, incubating the blood with LPS and measuring levels of 7 cytokines released. They then selected high ($n = 3$) and low ($n = 3$) LPS responders (in terms of cytokines released) and profiled whole blood RNA using a human Affymetrix array. This approach enabled them to identify 80 LPS-responsive genes as well as 36 genes differentially expressed between high and low responders before stimulation.

Recently, we have initiated work to identify genes differentially expressed between pigs with different infection outcomes. We infected 40 individual pigs with ST, and

Table 2
Gene ontology annotation of infection response genes shows distinct differences in persistent shedding and low shedding animals.

Persistent shedders (PS)
Generally: intracellular response genes
↑ Response to biotic stimulus, immune resp.
↑ Proteasome, endopeptidase activity
↑ Protein catabolism
↑ Protein kinase cascade, reg. of NFkB cascade
↑ Programmed cell death, apoptosis
↑ Immunoglobulin domain
↑ Vacuole/lysozyme/lytic vacuole
↑ TOLL receptor signaling, NFkB/IL1R sig.
↑ Multiple sclerosis/diabetes/arthritis
↑ SH2 domain
↑ Pleckstrin
↑ Asthma/lupus-Genetic Assoc
↑ CHOLERA-Genetic Assoc
↓ Signal peptide
↓ Intrinsic to plasma membrane
↓ Signal transducer, receptor activity
↓ Extracellular matrix
↓ Morphogenesis, organ morphogenesis
↓ Cell–cell signaling
↓ Fibrinogen alpha/beta/gamma
↓ Ion channel activity
Low shedders (LS)
Generally: extracellular response genes
↑ Signal peptide, cell communication, receptor
↑ Response to biotic stimulus, immune resp.
↑ Integral to plasma membrane
↑ Extracellular matrix
↑ Cell–cell signaling, structural component
↑ Carbohydrate/heparin binding
↑ Angiogenesis
↑ Tissue/organ development/remodeling
↑ Fibronectin type III
↑ Organ morphogenesis
↓ Cell cycle, M phase
↓ Nucleosome, nucleus/nuclear protein
↓ Nucleic acid binding, reg. of biol. process
↓ Microtubule organizing center
↓ RNA localization, RNA splicing
↓ Response to DNA damage stimulus
↓ Chromosome segregation
↓ Cellular processes—phys., metabolic
↓ Zinc finger, ion binding

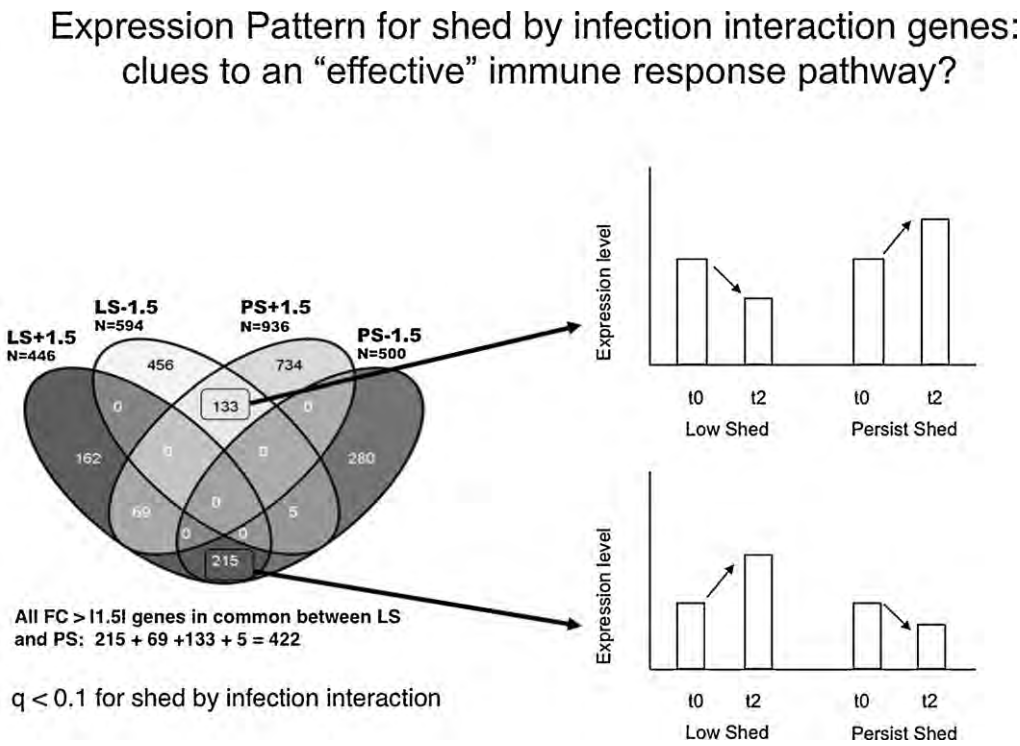

Fig. 4. Genes with significant shed x infection interaction may be clues as to an effective immune response pathway. For example, nearly 50% of genes (215 of 448) showing high up-regulation response to infection in low shedders (LS +1.5) are in common with high down-regulated genes in Persistent shedding animals (PS −1.5). The response of such genes to infection is dependent on the class of animal (LS or PS) in which they are present. The global function of such genes may be useful in understanding variation in immune response to *Salmonella*.

measured numbers of shed bacteria up to 21 days post-inoculation (dpi, Uthe et al., 2009). Four pigs showed shedding only up to 7 dpi, with relatively low numbers of bacteria shed (low shedder phenotype, LS). On the other hand, six pigs shed continuously throughout the test period (Persistent Shedder phenotype, PS). In these 10 animals, we have profiled using Affymetrix technology the peripheral blood RNA before infection (t0) and at the early inflammatory stage (48 hpi, t2) when all animals are shedding bacteria (Huang et al., manuscript in preparation). Interestingly, we find significant numbers of differentially expressed genes in whole blood between LS and PS animals at t2, and many genes also show differential expression during infection in both shedding types (Table 1). Less that 5% of these expression differences are correlated with the numbers of different cell types as measured in complete blood counts (CBCs), indicating that differential expression is not due to changes in cell populations (data not shown). To characterize pathways and functions associated with these phenotypes, we selected genes up-regulated or down-regulated in either Low or Persistent shedders and annotated these genes to identify Gene Ontology functions over- or under-represented in these gene lists (Table 2). This analysis found striking results. First, blood from animals with a PS phenotype has *increased* intracellular-oriented responses and *decreased* extracellular-oriented responses (data not shown). Second, the blood of LS phenotype animals had a nearly opposite response; with *increased* expression of gene annotated in extracellular signaling pathways (Huang et al., manuscript in preparation).

Of special interest is the large number of genes that show significant shed by time interaction, indicating the response to infection (time variable) depends on which phenotypic class is examined (Table 1). To find the genes that show high differences between the phenotype classes, genes with significant shed x time interaction (q-value < 0.1) were sorted by Fold Change (FC) for time (response to infection), separately for LS and for PS animals. Four lists were prepared (FC > +1.5 and FC < −1.5 for each phenotype) and these lists were examined for overlap (Fig. 4). While it is expected that genes with significant shed by time interactions would show different expression between classes, the large numbers of genes with opposite expression patterns (totaling 348 genes; Fig. 4) clearly shows that the immune pathways measured in blood are strikingly different between these two phenotypes. We believe that these genes may indicate pathways controlling a more effective immune response to *Salmonella* infection, as their expression pattern correlates with bacterial load as measured by fecal shedding, and we plan to carefully study these genes for their roles in controlling variation in disease phenotypes during bacterial infection.

4. Summary and future

Clearly the porcine immune response community is just starting to use the tools of trancriptomics and boinformat-

ics to unravel the intricacies of host-pathogen interactions. If we can look to human and mouse studies as a guide, there are tremendous advances in store for researchers using such genomic approaches to study and manipulate porcine immunology and immunogenetics. With the increased use of high-throughput sequencing approaches, researchers will have access to whole-genome datasets with little to no technological limitations in the biological interpretation of the data. The limitations will be only in the imaginations of the scientists to design the optimal experiments to take advantage of these truly extraordinary opportunities for advancing molecular, cellular, and physiological knowledge and to turn such knowledge into understanding and practical application.

Conflict of interest statement

The authors declare there are no conflicts of interest to be disclosed. Funding of several aspects of the work described in this manuscript performed in the authors' laboratories came from the USDA-NRICGP, Iowa State University Center for Integrated Animal Genomics, USDA-Food Safety Consortium, USDA-ARS, and the National Pork Board. None of these study sponsors had a role in writing or submission of this manuscript.

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An outbreak of late-term abortions, premature births, and congenital deformities associated with a *Bovine viral diarrhea virus 1* subtype b that induces thrombocytopenia

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Abstract. *Bovine viral diarrhea virus 1* (BVDV-1) subtype b was isolated from premature Holstein calves from a dairy herd that experienced an outbreak of premature births, late-term abortions, brachygnathism, growth retardation, malformations of the brain and cranium, and rare extracranial skeletal malformations in calves born to first-calf heifers. Experimental inoculation of 3 colostrum-deprived calves aged 2–4 months old with this BVDV isolate resulted in thrombocytopenia, lymphopenia, and leukopenia. Outbreaks of brachygnathism are rarely associated with BVDV, and thrombocytopenia is rarely associated with BVDV-1 strains.

Key words: *Bovine viral diarrhea virus*; brachygnathism, congenital defects; thrombocytopenia.

Infection with *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV-2, respectively; family *Flaviviridae*, genus *Pestivirus*) results in a wide variety of clinical presentations in cattle, including abortion; congenital defects; fetal mummification; persistent infection; diarrhea; sudden death; necrosis of lymphoid tissues and squamous epithelium in the oral cavity, esophagus, rumen, omasum, and skin; crypt epithelial necrosis in the intestinal tract; vasculitis; and immunosuppression.[2,3] A hemorrhagic syndrome associated with thrombocytopenia has previously only been found with BVDV-2 strains.[3] The current study documents the occurrence of an outbreak of congenital deformities, including brachygnathism, late-term abortions, and premature births in Holstein heifers from which a BVDV-1b strain was isolated. Infection of calves with this virus resulted in thrombocytopenia.

In May 2004, first-calf heifers in a dairy with 650 milking cow Holsteins experienced an outbreak of late third-trimester abortions and premature live births with skeletal and neurologic deformities. Over a 4-week period, 19 of 37 heifers in their last month of gestation aborted or gave birth to calves approximately 2–4 weeks early. In the first week of May, 7 of 7 premature stillborn and live calves had notably shortened mandibles and appeared undersized for their age and development. One of these, a live 2-day-old calf (calf A) that had been tube-fed colostrum because it was unable to stand, was submitted to the California Animal Health and Food Safety Laboratory (Tulare, CA). Another live calf (calf B) was euthanized by the referring veterinarian. The euthanized calf and 2 stillborn calves (calves C and D) were necropsied in the field by the referring veterinarian, and their tissues (calf B) or heads (calves C and D) were submitted to the laboratory.

Upon presentation to the laboratory, calf A was in lateral recumbency and unable to obtain a sternal position, and it exhibited ventral strabismus, mild opisthotonus, mandibular brachygnathism, and occasional random paddling. After euthanasia, necropsy revealed that one foreleg was swollen due to marked hemorrhage around a fractured third metacarpal bone; carpal and hock joint fluid were red tinged and of normal consistency. The brain was grossly and histologically unremarkable. Luxol fast blue staining for myelin of medulla and cerebellum was within normal limits. The spinal cord was not examined. Calf B had exhibited neurologic signs, and necropsy by the referring veterinarian revealed ascites and mandibular brachygnathism; the parenchymal tissues were grossly and histologically unremarkable. The brain and spinal cord were not examined. The calvarium of calf C was markedly domed with no bone covering the dorsal central 9 cm of the brain; in addition, the maxilla and mandible were shortened (brachycephaly; Fig. 1), and there was a complete cleft palate. The referring veterinarian reported that there were only short stubs of fleshy tissue where the forelimbs should have been. Calf C had hydranencephaly with red-tinged fluid filling the lateral ventricles and mild cerebellar atrophy. Histologically, the medulla, midbrain, and thin remnants of cerebrum were hypercellular. The cerebral cortex had very thin, poorly organized gray matter and little or no white matter. There was multifocal thinning and mild dysplasia of the granular layer of the cerebellum. Luxol fast blue staining of the medulla and cerebellum revealed decreased myelin throughout. The head of calf D

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Figure 1. Calf C. The maxilla and mandible are shortened (brachycephaly). Marked doming of the head is associated with hydranencephaly.

was slightly domed, and there was mandibular brachygnathism, moderate to marked hydrocephalus, and mild cerebellar atrophy. Histologically, the cerebrum had attenuated, normally organized gray matter, decreased white matter, and increased reserve cells around the distended ventricles. The thalamus, midbrain, and medulla had multiple hypercellular areas. The cerebellum exhibited multifocal thinning and dysplasia of the granular layer. Luxol fast blue staining of medulla and cerebellum revealed multifocal hypomyelination in the cerebellum, particularly in the tips of the folia.

Bovine viral diarrhea virus infection was confirmed in all 4 calves by various methods. Direct BVDV fluorescent antibody testing on frozen tissue sections using a 1:50 dilution of fluorescein-conjugated, porcine-origin, anti-BVDV polyclonal antibody was performed according to the manufacturer's recommendations[a] and was positive on kidney and lung of calf A and kidney of calf B. Immunohistochemistry[1] on formalin-fixed, paraffin-embedded tissue with the use of monoclonal antibody 15c5[b] at a 1:1,000 dilution using the avidin–biotin–immunoperoxidase technique[c] was positive on skin from calves A, C, and D. The real-time reverse transcription polymerase chain reaction (RT-PCR) modified from previously published primers and probes[5] confirmed the presence of BVDV-1 in the kidney and whole blood of calf A, the kidney of calf B, and the brain of calf D. The BVDV real-time RT-PCR was negative on lung and spleen from calf B and brain from calf C.

Sera were submitted from 6 heifers that had affected calves, and BVDV serum neutralization titers ranged from 1:32 to 1:2,048 for BVDV-1 and 1:128 to 1:1,024 for BVDV-2. Of the 6 heifers, 4 had 2-fold higher BVDV-1 than BVDV-2 titers, and one heifer had an 8-fold higher BVDV-1 titer. Only the dam of calf B had a higher BVDV-2 titer (1:128) than BVDV-1 titer (1:32). Calf A received colostrum at birth and had a 1:128 BVDV-1 titer and 1:256 BVDV-2 titer.

The heifers from this dairy had been vaccinated with a single dose of modified live vaccine containing BVDV-1a at 1 week of age. At 5 months of age, heifers were sent to a heifer ranch until 12 months of age when they were moved to a second heifer ranch. At the first heifer ranch, no vaccinations were given. At the second heifer ranch, animals were vaccinated on arrival with a killed vaccine containing cytopathic BVDV-1a and noncytopathic BVDV-1. The home dairy vaccinated lactating cows once a year with a killed BVDV vaccine. The second heifer ranch received animals from 3 other dairies. The heifers returned to the home dairy when they were approximately 6–7 months pregnant and were vaccinated with a killed vaccine containing BVDV-1a and BVDV-2. The pregnant heifers were housed with the dry cows on their return. Approximately 8 weeks before the onset of abortions in the heifers, a number of postpartum cows experienced high fevers (over 40°C) and some had diarrhea. Paired serology on 3 affected cows taken 3 weeks apart revealed a rising BVDV-1 seroconversion in 1 cow, falling seroconversion (acute titer >1:8,196) in a second, and a static titer of 1:2,048 in the third cow. Based on the presumptive diagnosis of BVDV infection, the late lactation cows and dry cows, but not the heifers, were vaccinated with a modified live vaccine containing BVDV-1a and BVDV-2. In July 2003, 9 months earlier, the herd began using a modified live vaccine containing BVDV-1a and BVDV-2 at the postpartum examination, which was the reason they only vaccinated late lactation and dry cows in response to the fever and diarrhea problem.

Tissues from calf A (live born) were submitted to the National Animal Disease Center (Ames, IA) for further characterization. A BVDV was isolated and identified as a BVDV-1b strain based on phylogenetic analysis of the 5′ untranslated region.[7] The BVDV phylogenetic tree in Figure 2 shows the relationship of this strain to other pestivirus strains. Four colostrum-deprived mixed-breed calves, aged 2–4 months, were tested as free of BVDV in buffy coat samples by virus isolation and free of BVDV antibodies in serum. Three of these calves were inoculated with the BVDV-1b isolate, and the fourth served as a nonexposed control. Calves were housed individually in climate-controlled pens. There was no animal to animal contact between pens, but there was shared airflow.

Animals were inoculated via the nasal route with 5 ml of freeze–thaw lysate prepared from infected Madin–Darby bovine kidney cells 5 days after inoculation that contained approximately 1×10^6 tissue culture infectious doses of virus per milliliter. Temperatures were taken daily, and blood samples were collected before inoculation and on days 3, 6, 9, 11, and 13 after inoculation. Lymphocyte and platelet counts were determined using a cytometer per the manufacturer's directions.[d] Two of three inoculated calves and the control had a mild increase of body temperature to a maximum of 0.44°C, and one challenged calf had an increase of 1.22°C (40.2°C for 4 days). Baseline values for white blood cell, lymphocyte, neutrophil, and platelet counts were established on a single blood sample taken from each calf before inoculation.

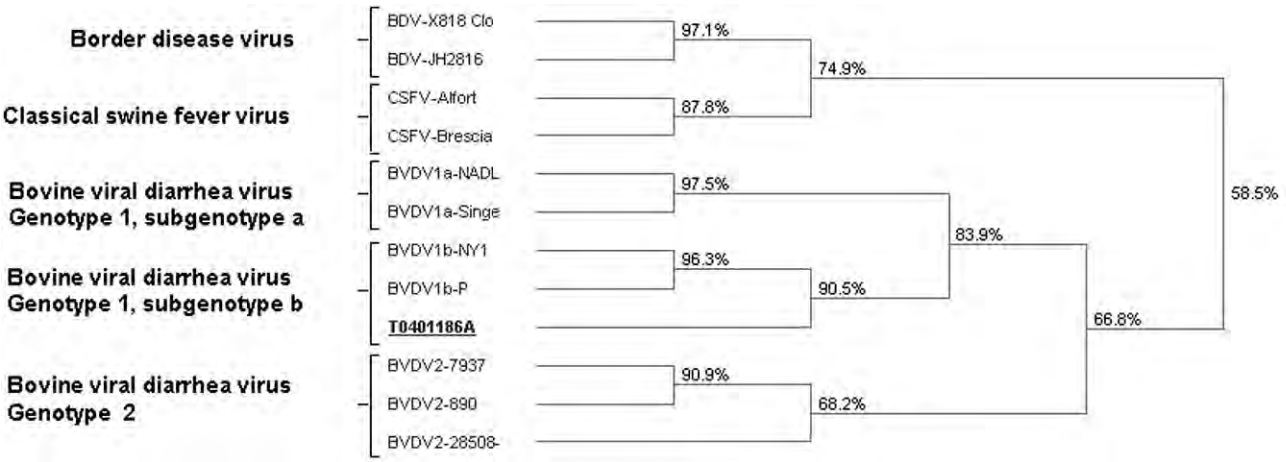

Figure 2. *Bovine viral diarrhea virus* (BVDV) phylogenetic tree demonstrating relationship of virus T0401186A, BVDV genotype 1 subgenotype b, compared to other pestiviruses.

Lymphocyte, total white blood cell, and platelet counts declined in all inoculated calves and rose in the control. Peak declines in lymphocyte counts below baseline occurred on day 3 in 1 calf (33.4% decline) and day 6 in the other 2 calves (20.7 and 44.4%, respectively). All 3 calves had maximal platelet count decreases on day 9 after inoculation. The decreases ranged from 35.5% to 46.8% below baseline with absolute counts of 136,000–199,000 platelets per microliter of blood in BVDV-challenged calves compared with 416,000 for the control calf. On gross necropsy, patchy areas of discolored and collapsed lung were seen in the challenged calves but not the control calf. One challenged calf had petechial hemorrhages in the ileocecal valve and linear hemorrhages in the rectum. Lung tissue from 2 challenged calves was examined histologically. One calf had mild bronchitis and atelectasis and the other had multifocal hemorrhage and areas of alveolar edema and fibrin exudation.

Congenital malformations due to BVDV infection are associated with fetal infection at 75–150 days of gestation and are commonly reported to involve the brain and eye. Eye tissue was not examined in the current study except to note the absence of microphthalmia. Hydrocephalus, hydranencephaly, cerebellar hypoplasia, growth retardation, and mandibular brachygnathism have all been reported with BVDV infection.[2] Mandibular brachygnathism is rarely reported to be associated with BVDV but was found in all 7 calves examined by the referring veterinarian. In the veterinary literature, mandibular brachygnathism has been reported with ocular lesions (cataracts and/or retinal lesions) and cerebellar hypoplasia.[8,9] Natural and experimental reports suggest that BVDV infection between 79 and 90 days of gestation can result in brachygnathism, and this period is also associated with milder cerebellar hypoplasia.[8,9] Exposure to BVDV between 30 and 125 days of gestation can result in persistent infection, which appears to have occurred in the calves in the present study based on the presence of the virus in all 4 calves 6–7 months after the likely onset of fetal infection.

Most BVDV-induced congenital deformities result from infection at 100–175 days of gestation, when the calf is becoming immune competent, and therefore the virus is often no longer present at the time of birth.[2] Failure of limb development has not been reported previously and was found in 1 of the 7 calves that also had multiple craniofacial skeletal and brain deformities. Calves born alive or aborted before and after the visit by the veterinarian were not examined, so the total number of calves with congenital deformities is not known. Exposure to the BVDV-1b isolate at the second heifer ranch could have resulted in a few acutely infected, asymptomatic, shedding heifers returning to the dairy where they exposed the co-housed dry cows to the virus strain, thereby resulting in the fever and diarrhea reported in postpartum cows. These heifers probably gave birth to healthy, congenitally infected, and recovered calves as their exposure was later in gestation. The herd vaccination history of a single yearly killed BVDV vaccine could produce a susceptible population of cows if a heterologous virus strain were introduced. Because the home dairy had begun the use of a modified live BVDV vaccine at postpartum examination approximately 10 months earlier, only late lactation and dry cows had not been vaccinated with modified live virus and were more susceptible to a newly introduced virus strain. The herd owner chose not to pursue testing of calves born to heifers for detection of persistent BVDV infection, so it is unknown what percentage of the live calf crop was infected. The BVDV-1b strain from the outbreak in the current study was associated with premature live births, abortion, multiple congenital defects, persistent infection, postpartum diarrhea, and fevers and, after experimental inoculation, with thrombocytopenia.

The BVDV-2 strains that experimentally cause a hemorrhagic syndrome decrease platelet counts below 100,000 platelets per microliter of blood.[4,10] With the challenge dose used in the experimentally inoculated calves in the present study, the lowest recorded level was 136,000 platelets per microliter of blood. Other BVDV-1 strains have not been previously reported to cause thrombocyto-

penia. Calf A had very extensive hemorrhage associated with a metacarpal fracture, but because a peripheral blood platelet count was not determined, it is uncertain whether BVDV-1b–associated thrombocytopenia may have contributed to the severity of the hemorrhage. Lymphopenia is reported with all BVDV strains regardless of genotype. Thrombocytopenia has only been associated with a minority of BVDV, and those BVDV tend to be strains with increased virulence.[4] Furthermore, although congenital abnormalities have been previously reported with BVDV, the present report documents a relatively large number of animals with multiple fetal malformations and significant impact on lymphocytes and platelets in acute infection making this a potentially good challenge virus to prove vaccine efficacy for fetal protection. More extensive challenge studies using this virus strain have been published and substantiate the limited challenge study results reported in the current study.[6]

Sources and manufacturers

a. American BioResearch Laboratories, Pullman, WA.
b. IDEXX MoAb 15.c.5 (anti-BVDV), IDEXX Laboratories Inc., Westbrook, ME.
c. Vectastain Elite ABC Mouse IgG kit and Vector NovaRED substrate, Vector Laboratories Inc., Burlingame, CA.
d. CDC Technologies Inc., Irvine, CA.

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Characterization of Anti-*Salmonella enterica* Serotype Typhi Antibody Responses in Bacteremic Bangladeshi Patients by an Immunoaffinity Proteomics-Based Technology▽‡

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***Salmonella enterica* serotype Typhi is the cause of typhoid fever and a human-restricted pathogen. Currently available typhoid vaccines provide 50 to 90% protection for 2 to 5 years, and available practical diagnostic assays to identify individuals with typhoid fever lack sensitivity and/or specificity. Identifying immunogenic *S*. Typhi antigens expressed during human infection could lead to improved diagnostic assays and vaccines. Here we describe a platform *i*mmunoaffinity *p*roteomics-based *t*echnology (IPT) that involves the use of columns charged with IgG, IgM, or IgA antibody fractions recovered from humans bacteremic with *S*. Typhi to capture *S*. Typhi proteins that were subsequently identified by mass spectrometry. This screening tool identifies immunogenic proteins recognized by antibodies from infected hosts. Using this technology and the plasma of patients with *S*. Typhi bacteremia in Bangladesh, we identified 57 proteins of *S*. Typhi, including proteins known to be immunogenic (PagC, HlyE, OmpA, and GroEL) and a number of proteins present in the human-restricted serotypes *S*. Typhi and *S*. Paratyphi A but rarely found in broader-host-range *Salmonella* spp. (HlyE, CdtB, PltA, and STY1364). We categorized identified proteins into a number of major groupings, including those involved in energy metabolism, protein synthesis, iron homeostasis, and biosynthetic and metabolic functions and those predicted to localize to the outer membrane. We assessed systemic and mucosal anti-HlyE responses in *S*. Typhi-infected patients and detected anti-HlyE responses at the time of clinical presentation in patients but not in controls. These findings could assist in the development of improved diagnostic assays.**

Salmonella enterica serotype Typhi is a human-restricted pathogen that is the primary cause of enteric fever. It is estimated that *S*. Typhi infects over 20 million individuals and kills approximately 200,000 people globally each year (4). Currently, commercially available typhoid vaccines provide approximately 50 to 75% protection for 2 to 5 years (21), although an anti-typhoid Vi conjugate vaccine demonstrated 90% protection in 2- to 5-year-old children in a large field trial (23). Available and practical diagnostic tests for typhoid fever lack sensitivity and/or specificity (28). Identifying immunogenic *S*. Typhi antigens expressed during human infection could lead to improved diagnostic assays and vaccines.

Infection with *S*. Typhi begins with the ingestion of contaminated water or food. The bacteria invade the gastrointestinal mucosa, translocate to the lymphoid follicles, where they survive and replicate within macrophages, and then disseminate via the bloodstream to the liver, spleen, and intestinal lymph nodes (14). The incubation period is typically 8 to 14 days (22), and symptoms include fever, abdominal pain, anorexia, weakness, potential complications of intestinal perforation, encephalopathy, and gastrointestinal bleeding (14, 34). Clinical studies demonstrate that *S*. Typhi infection stimulates both an intestinal mucosal and systemic humoral and cellular immune response (14, 34). *S*. Typhi is a facultative intracellular pathogen of macrophages, and both cellular and antibody-mediated immune responses are known to play roles in controlling and clearing *S*. Typhi infection (37). Despite this, there are limited

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data on antigen-specific cellular responses during wild-type *S*. Typhi infection in humans. Analyses of cellular immune responses during *S*. Typhi infection have largely used whole-cell preparations or flagellar antigens and have focused predominately on measuring immune responses in recipients of oral live attenuated typhoid vaccines, not in individuals with wild-type disease (24, 25, 40–42, 49).

Antibody responses during wild-type infection have been better studied but have focused largely on a relatively small number of antigens, including O antigen (lipopolysaccharide [LPS]), H antigen (flagellar component), polysaccharide capsular antigen (Vi antigen), heat shock proteins such as GroEL, and outer membrane proteins such as OmpC and -F (13, 34). In addition, gut-derived IgA antibody-secreting cells that recognize LPS, a membrane preparation, or whole-killed *S*. Typhi organisms can be detected in the peripheral blood following natural *S*. Typhi infection or oral typhoid vaccination (16, 43, 50, 54). These cells eventually return home to the gastrointestinal mucosa, where they secrete secretory IgA antibody (36, 43).

A number of immunoaffinity-based techniques that screen protein libraries of pathogens to identify immunogenic antigens have been developed (12, 17, 38), and we have previously reported using one such approach, *in vivo*-induced-antigen technology (IVIAT), to identify immunogenic *S*. Typhi antigens expressed during human infection (12). Another previously described technique, proteomics-based expression library screening (PELS), involves using antibody-charged columns to capture antigens produced by an *Escherichia coli*-based expression system containing an inducible library of a pathogen of interest, with subsequent elution and identification of bound proteins using mass spectrometric analysis (17). Here we describe using a modification of this approach that we have termed immunoaffinity proteomics-based technology (IPT). IPT involves directly screening the pathogen of interest using columns charged with IgG, IgM, or IgA antibody fractions recovered from the blood of infected humans. We applied IPT to *S*. Typhi to gain further insights into immunogenic antigens expressed in patients bacteremic with *S*. Typhi in Bangladesh.

MATERIALS AND METHODS

Bacterial strains and lysate preparation. We obtained wild-type *S*. Typhi CT18 from the Salmonella Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada. To maximize the protein expression profile of *S*. Typhi prior to applying bacterial preparations to antibody-charged columns, we separately grew CT18 to mid-log phase and stationary phase at 37°C, with aeration in two media: Luria broth and low-magnesium (10 μM $MgCl_2$)-containing minimal medium. The latter medium/condition is known to induce the PhoP regulon, involved in the intramacrophage survival of *S*. Typhi; we chose this condition to maximize the expression of antigens expressed *in vivo* during human infection (3). We resuspended cell pellets in Tris-buffered saline (TBS)–2% *n-octyl*-β-D-glucopyranoside (Sigma)–2 mM Mg–2× EDTA-free Roche Complete protease inhibitor (pH 8.0) and lysed cells using 0.1 mM silica beads (MP Biomedical, Solon, OH) and a Mini-Bead-Beater-8 (BioSpec Products, Bartlesville, OK).

Study subject selection, sample collection, and processing. Individuals (3 to 59 years of age) presenting to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), with fever of 3 to 7 days' duration (≥39°C) who were suspected of having enteric fever, who lacked an obvious focus of infection, and for whom we lacked an alternate diagnosis were eligible for enrollment. We collected venous blood (5 ml from children <5 years old and 10 ml from all others) at enrollment (day 0), on day 6, and on day 20. We cultured 3 to 5 ml of day 0 blood by using a BacT/Alert automated system, subculturing positive bottles on MacConkey agar, and identifying colonies using standard biochemical tests and reaction with *Salmonella*-specific antisera (51). Following collection of blood, all patients were initially treated with oral ciprofloxacin or cefixime or injectable ceftriaxone; these were continued for up to 14 days at the discretion of the attending physician. Using this approach, we identified individuals with *S*. Typhi bacteremia (n = 10). We also collected acute- and convalescent-phase control plasma from Bangladeshi patients with documented *Vibrio cholerae* infection (n = 5) and single blood samples from North American volunteers with no history of *Salmonella* infection or vaccination (n = 3).

To analyze mucosal IgA responses, we recovered peripheral blood mononuclear cells (PBMCs) from typhoid (days 0, 6, and 20) and cholera (days 2 and 7) patients. Activated mucosal lymphocytes migrate from intestinal tissue and circulate within peripheral blood before returning to mucosal tissues (15, 43). This migration peaks 1 to 2 weeks after intestinal infection and may be measured by using PBMCs in an antibody-secreting cell (ASC) assay or in supernatants recovered from harvested PBMCs (the "antibody-in-lymphocyte supernatant" [ALS] assay) (36, 43). To recover PBMCs, we diluted heparinized blood in phosphate-buffered saline (PBS; 10 mM [pH 7.2]) and isolated PBMCs by density gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). We resuspended isolated PBMCs to a concentration of 10^7 cells/ml in RPMI 1640 complete medium (Gibco, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (HyClone, Ogden, UT), 100 U of penicillin/ml, 100 μg of streptomycin/ml, 100 mM pyruvate, and 200 mM L-glutamine (Gibco) (43). We incubated cells for 48 h at 37°C with 5% CO_2, collected supernatants containing secreted antibodies, and added a protease inhibitor solution as previously described (43).

This study was approved by the human studies committees of the ICDDR,B and Massachusetts General Hospital.

Plasma preparation for antibody enrichment, coupling of antibodies to HiTrap NHS-activated HP columns, and capture of *S*. Typhi proteins. Equal volumes of acute-phase (day 0) and convalescent-phase (day 20) plasma from four Bangladeshi patients with culture-confirmed *S*. Typhi bacteremia were pooled to create a total volume of 1 ml of acute- and 1 ml of convalescent-phase samples. We diluted each pooled plasma sample 1:1 with PBS, pH 7.4, and recovered IgG fractions using GE Healthcare HiTrap protein G high-performance (HP) columns, IgA fractions using GenWay Seppro human IgA-IgY microbeads, and IgM fractions using GE Healthcare HiTrap protein IgM HP columns, all according to the manufacturers' instructions. We bound recovered antibody fractions to HiTrap *N*-hydroxysuccinimide (NHS)-activated HP columns, applied bacterial lysates prepared as described above, and eluted bound proteins as previously described (17, 20). To assess nonspecific binding, we also eluted lysate proteins bound to columns that had been blocked but to which we had not loaded antibody.

Sample preparation for MS analysis. Following elution from each HiTrap NHS column, protein samples were concentrated using a Vivaspin 5-kDa 2-ml spin filter according to the manufacturer's instructions. To each concentrated sample, we added 50 μl of 8 M urea-2% SDS-150 mM NH_4HCO_3-10 mM dithiothreitol (DTT)-1× lithium dodecyl sulfate (LDS), pH 8.5, and incubated the mixtures at 37°C for 60 min. We next cooled the alkylated samples with iodoacetamide, quenched them with excess DTT, and separated samples on a 10% Bis-Tris NuPAGE MOPS (morpholinepropanesulfonic acid) gel (Invitrogen). We fixed gels in destain (50% methanol and 7.5% acetic acid), rehydrated them, stained them with Simply Blue Safestain (Invitrogen), cut them horizontally into slices, and destained them until transparent. We rinsed gel samples with three alternating washes of 50 mM ammonium bicarbonate and acetonitrile, cooled them, resuspended each gel slice in trypsin (5.5 μg/ml in 50 mM ammonium bicarbonate-10% acetonitrile), and incubated them at 37°C for 24 h for digestion of proteins. We extracted peptides with one rinse of 50 mM ammonium bicarbonate-10% acetonitrile, followed by one rinse of 50% acetonitrile-0.1% formic acid, and prepared samples for mass spectrometry by lyophilization and rehydration in 20 μl 5% acetonitrile-0.2% formic acid.

LC-MS analysis. We loaded eluted and digested samples into 96-well plates for mass spectrometry (MS) analysis on an LTQ-Orbitrap XL (Thermo Fisher Scientific). For each run, we injected 10 μl of each reconstituted sample using a Thermo Scientific MicroAutosampler. We performed reverse-phase chromatographic separation using 3-μm Hypersil Gold C_{18} medium packed into a fused-silica, 75-μm-inner-diameter, 20-cm-long column running at 250 nl/min from a Surveyor MS pump with a flow splitter. A gradient of 5 to 40% acetonitrile in 0.2% formic acid was produced and the over 150 min. The LTQ-Orbitrap was run in a top 8 configuration at a resolution of 60,000 for a full scan, with monoisotopic precursor selection enabled +1 and unassigned charge states rejected. The analysis on the LTQ-Orbitrap instrument was carried out with collision-induced dissociation (CID) fragmentation.

Peptide identification and statistical analysis. We identified peptides using SEQUEST (Thermo Fisher Scientific) through the Bioworks browser, version 3.3.1 SR1. Tandem MS (MS/MS) data were searched using 10-ppm mass accu-

racy for precursor *m/z* and a 0.5-Da window for fragment ions. Fully enzymatic tryptic searches with up to three missed cleavage sites were allowed. Oxidized methionines were searched as a variable modification, and alkylated cysteines were searched as a fixed modification. *Salmonella* databases for CT18 were downloaded from the EMBL-EBI database and supplemented with common contaminants. We employed a reverse database strategy (5) using concatenating reversed protein sequences for each database entry in SEQUEST. We filtered peptides for each charge state to a false discovery rate (FDR) of 1% and then grouped peptides into proteins using Occam's razor logic. We used spectral counting to compare changes in protein abundance in fractions eluted from columns charged with antibody fractions to those in fractions eluted from blocked columns not containing antibody, and we required that proteins be associated with at least 3 spectral counts to be included in our analyses. After normalizing results of duplicate samples, we averaged total spectral counts and used a G-test (45) while controlling for a positive false-discovery rate (47) to test for significant differential protein detection between samples. Protein functional classification was based on J. Craig Venter Institute annotations (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi).

Detection of HlyE-specific antibodies in plasma and mucosal ALS fluid by ELISA. We confirmed the screening results obtained by IPT by using standard enzyme-linked immunosorbent assays (ELISAs), with hemolysin E (HlyE) as a model antigen. HlyE was purified as previously described (55). We measured anti-HlyE IgG and IgA responses using a microtiter plate ELISA format and plasma from culture-confirmed typhoid and cholera patients, as well as North American volunteers. We also measured IgA responses in antibody-in-lymphocyte supernatant (ALS) fractions recovered from circulating mucosal lymphocytes from infected patients (43). To detect antigen-specific responses, we added 100 μl of plasma samples (diluted 1:200 for IgG and IgA) in PBS-Tween 20-0.1% bovine serum albumin (BSA) or 100 μl (1:2 dilution for IgA) of ALS specimens to plates coated with 100 ng of HlyE/well and detected responses using horseradish peroxidase-conjugated human IgA or IgG antibodies (Jackson Laboratories, Bar Harbor, ME) (1:1,000 in PBS-Tween 20-0.1% BSA for IgA and IgG isotypes). We developed and read plates as previously described (43). To compare across plates, we divided readings of unknown samples by readings of an in-house pooled standard, multiplied by 100, and expressed results as ELISA units. We used paired *t* tests to determine if there was a statistically significant difference between results for samples.

RESULTS

Proteins identified by IPT. Using mass spectrometry, we identified 57 *S.* Typhi proteins grown under various conditions whose capture by affinity-purified IgG, IgM, and IgA antibody fractions from acute- and convalescent-phase plasma of patients with *S.* Typhi bacteremia was significantly increased compared to the capture by the column not containing antibody (IgG, column 29; IgA, column 2; and IgM, column 44) (Table 1 and see Table S1 in the supplemental material). These proteins could be grouped into a number of functional categories (Table 2). The most highly represented groups included proteins for energy metabolism and nutrient acquisition, protein and amino acid biosynthesis, and cellular processes, including pathogenesis and virulence, as well as proteins located in and involved in the synthesis of the cell envelope. Among the identified proteins were a number under the control of the PhoP regulon, including PagC, HlyE, and PhoN (6, 8, 9, 27). We also identified heat shock proteins GroEL and DnaK, outer membrane proteins, including OmpA, and other proteins involved in pathogenesis and virulence, including CdtB, PltA, and STY1364.

Comparison of proteins bound by acute- or convalescent-phase antibody fractions, by isotype. When considering proteins bound on the IgM columns, we identified 40 proteins bound by acute- but not convalescent-phase plasma IgM, including GroEL and DnaK, and 4 proteins bound by both acute- and convalescent-phase IgM samples, including HlyE. When considering proteins bound on the IgG columns, we identified 29 proteins in total, including 27 proteins bound by acute-phase samples, 18 proteins bound by convalescent-phase samples, and 16 proteins bound by both acute- and convalescent-phase samples. Proteins bound by the IgG columns included HlyE, PagC, PhoN, CdtB, PltA, OmpX, OmpA, outer membrane adhesin STY0351, GroEL, and bacterioferritin. The only proteins bound by the IgA columns were the flavoprotein WrbA, a protein involved in tryptophan biosynthesis (57), and elongation factor protein TufA.

Confirmation of immune responses to HlyE by standard techniques. To further characterize the immunogenicity of a protein identified in our screening procedure, we measured anti-HlyE responses in plasma and ALS fluid of *S.* Typhi-infected patients and controls. We detected significant increases in anti-HlyE IgG, but not IgA, in the convalescent-phase plasma compared to levels in the acute-phase plasma of patients infected with S. Typhi ($P \leq 0.01$) but not *V. cholerae* (Fig. 1A and B). We also detected significantly higher anti-HlyE IgG titers in the convalescent-phase plasma of *S.* Typhi patients than in North American controls ($P < 0.05$) (Fig. 1A). We detected a significantly elevated anti-HlyE IgA level at the time of clinical presentation using the mucosal ALS assay ($P < 0.05$) in *S.* Typhi patients compared to the level in cholera patients (Fig. 1C).

DISCUSSION

A number of high-throughput immunoaffinity screens have been developed to evaluate host-pathogen interactions during human infection, including IVIAT and PELS (11, 12, 17, 38, 39). Here we describe a further advance of such approaches, IPT, and used this to identify 57 *S.* Typhi proteins recognized by antibodies of various isotypes from individuals bacteremic with *S.* Typhi in Bangladesh. In comparison to other techniques, IPT has the advantage of directly using the pathogen of interest for screening of antibody responses and of being able to characterize these responses by isotype class. Antibody isotype analysis may provide insight into the stage of infection during which antigenic presentation occurs for pathogens like *S.* Typhi, which have both mucosal and systemic phases of infection.

Although an inducible expression library can be used to generate the proteins used in immunoaffinity screening, the generation of this library in an *E. coli*-based system is complicated by difficulties in definitively attributing the captured peptides to the pathogen's proteins or to a homolog in *E. coli* (33). For these reasons, we used *S.* Typhi itself as the source of our protein pool for the IPT modification of the PELS technique. To maximize the number of *S.* Typhi proteins present in the pool used in our analysis, we grew *S.* Typhi under a variety of growth conditions, including those known to induce the PhoP regulon, that mimic conditions encountered in the intracellular environment during human infection (3).

Another important issue for any immunoaffinity screening assay is the optimal negative control. The fact that many *S.* Typhi proteins share homology with corresponding *E. coli* proteins not only complicates attribution of immunoreactive peptides by mass spectrometric analysis but also complicates selection of optimal control plasma for use in immunoaffinity

TABLE 1. *S.* Typhi proteins bound by acute- and convalescent-phase serum antibody fractions from bacteremic patients and identified by mass spectrometric analysis[a]

Protein group and Swiss-Prot ID	Protein name(s)	Gene locus	Protein description	IgG		IgA		IgM	
				A	C	A	C	A	C
Cellular processes, virulence, and pathogenesis									
Q8Z7B8		STY1364	Hypothetical periplasmic protein	X	X				
Q8Z727	HlyE	STY1498	Hemolysin E	X	X			X	X
Q8Z6B2	PagC	STY1878	Outer membrane invasion protein	X	X				
Q8Z6A7	CdtB	STY1886	Cytolethal distending toxin subunit B homolog	X	X				
Q8Z6A4	PltA	STY1890	Putative pertussis-like toxin subunit	X	X				
Cell envelope									
Q8Z939		STY0351	Possible outer membrane adhesion	X	X				
Q8XH17	OmpX	STY0872	Outer membrane protein X	X	X				
Q8Z7S0	OmpA	STY1091	Outer membrane protein A	X	X				
Q8Z5I6	RfbH	STY2300	Putative dehydratase RfbH	X				X	
P67913	HldD	STY4085	ADP-L-glycero-D-manno-heptose-6-epimerase					X	
Amino acid biosynthesis									
Q8Z9A8	DapD	STY0236	2,3,4,5-Tetrahydropyridine-2,6-dicarboxylate	X				X	
Q8Z7N9	WrbA	STY1155	Flavoprotein WrbA	X		X		X	
Q8Z3B6	MetE	STY3594	5-Methyltetrahydropteroyltriglutamate-homocysteine					X	
Q8Z381	IlvC	STY3648	Ketol acid reductoisomerase	X				X	
Q8Z123	ArgI	STY4807	Ornithine carbamoyltransferase	X				X	
Biosynthesis of cofactors, prosthetic groups, and carriers									
P66039	RibH	STY0456	6,7-Dimethyl-8-ribityllumazine synthase					X	
P0A2E2	GlyA	STY2802	Serine hydroxymethyltransferase	X	X			X	X
Central intermediary metabolism									
Q8Z3Q5		STY3330	Possible oxidoreductase	X				X	
Q934J6	PhoN	STY4519	Nonspecific acid phosphatase		X				
P65749	Ppa	STY4773	Inorganic pyrophosphatase	X	X				
Energy metabolism									
Q8Z9F0	AceE	STY0175	Pyruvate dehydrogenase E1 component					X	
Q8Z9E9	AceF	STY0176	Dihydrolipoamide acetyltransferase component		X				
Q8Z9E8	LpdA	STY0177	Dihydrolipoamide dehydrogenase					X	
P66870	SucC	STY0781	Succinyl-CoA ligase					X	
Q8XF12	TrxB	STY0956	Thioredoxin reductase	X				X	X
Q8XGV7	PflB	STY0973	Formate acetyltransferase 1					X	X
Q8Z7F1	Adh	STY1302	Alcohol dehydrogenase					X	
Q8Z747	YdcW	STY1467	Gamma-aminobutyraldehyde dehydrogenase					X	
P0A1P1	GapA	STY1825	Glyceraldehyde 3-phosphate dehydrogenase	X				X	
Q8Z5J3	Gnd	STY2290	6-Phosphogluconate dehydrogenase, decarboxylating	X				X	
Q8Z4T0	TalA	STY2710	Transaldolase A					X	
P64077	Eno	STY3081	Enolase	X	X				
Q8XFG7	Fba	STY3226	Fructose 1,6-bisphosphate aldolase	X	X			X	
P65703	Pgk	STY3227	Phosphoglycerate kinase	X	X			X	
P65693	PrkA	STY3809	6-Phosphofructokinase					X	
Q8Z2D5	AldB	STY4116	Aldehyde dehydrogenase B					X	
Q8Z1T0	Qor	STY4441	Quinone oxidoreductase					X	
Q8XFR3	AspA	STY4685	Aspartate ammonia-lyase					X	
Q8Z0U3	DeoC	STY4918	Deoxyribose-phosphate aldolase	X					
Fatty acid and phospholipid metabolism									
Q8Z7C7	FabI	STY1352	Enoyl-(acyl carrier protein) reductase (NADH)					X	
Protein fate									
Q8Z9R1	DnaK	STY0012	Chaperone protein DnaK					X	
Q8Z934	PepD	STY0361	Aminoacyl-histidine dipeptidase	X				X	
Q7AMH5	ClpB	STY2849	Chaperone protein ClpB					X	

Continued on following page

TABLE 1—*Continued*

Protein group and Swiss-Prot ID	Protein name(s)	Gene locus	Protein description	IgG		IgA		IgM	
				A	C	A	C	A	C
P0A1D4	GroEL	STY4690	60-kDa chaperonin	X	X			X	
Protein synthesis									
P67564	SerS	STY0961	Seryl-tRNA synthetase					X	
Q8XGK9	RpsA	STY0981	30S ribosomal protein S1					X	
Q56112	AsnS	STY1004	Asparaginyl-tRNA synthetase					X	
Q8Z5W1	AspS	STY2109	Aspartyl-tRNA synthetase					X	
P0A1H4	FusA	STY4352	Elongation factor G					X	
Q8Z118	ValS	STY4814	Valyl-tRNA synthetase					X	
P0A1H6	TufA, TufB	STY4353 STY3739	Elongation factor Tu	X	X	X		X	
Biosynthesis of purines, pyrimidines, nucleosides, and nucleotides									
Q8Z4Q3	GuaA	STY2751	GMP synthase					X	
Regulatory function									
Q8Z3E9	SspA	STY3523	Stringent starvation protein A					X	
Transcription									
Q8Z320	RpoB	STY3732	DNA-directed RNA polymerase subunit beta					X	
P0A7Z8	RpoA	STY4383	DNA-directed RNA polymerase subunit alpha					X	
Transport and binding									
Q8Z1Y0	Bfr	STY4355	Bacterioferritin	X	X				
Unclassified									
Q8Z5C3	MetG	STY2384	Methionyl-tRNA synthetase					X	

[a] ID, identifier; A, bound by immunoglobulin in acute-phase samples; C, bound by immunoglobulin in convalescent-phase samples; X, presence; CoA, coenzyme A.

comparisons. We therefore compared the immunoreactivities of *S*. Typhi proteins identified using columns charged with antibody from infected humans to reactivities with blocked columns not charged with antibody. We also compared proteins captured using columns charged with antibody fractions recovered from the same patient at two different phases of infection: the acute and convalescent stages. Using these approaches, we identified a number of *S*. Typhi proteins known to be immunogenic, including HlyE, PagC, and GroEL, supporting the validity of our approach.

TABLE 2. Functional classification of *S*. Typhi proteins captured by acute- and/or convalescent-phase-antibody columns

Functional classification of captured proteins	No. of proteins
Energy metabolism	19
Protein synthesis	7
Amino acid biosynthesis	5
Cellular processes, virulence, and pathogenesis	5
Cell envelope	5
Protein fate	4
Central intermediary metabolism	3
Transcription	2
Biosynthesis of cofactors, prosthetic groups, and carriers	2
Fatty acid and phospholipid metabolism	1
Biosynthesis of purines, pyrimidines, nucleosides, and nucleotides	1
Transport and binding proteins	1
Regulatory functions	1
Unclassified	1

Hemolysin E (HlyE), also referred to as cytolysin A (ClyA) or SheA, is a pore-forming toxin that contributes to the cytotoxicity and invasion of epithelial cells and also affects bacterial growth within human macrophages (6, 7). HlyE is expressed under the control of the PhoP regulon (6) and shares >90% amino acid identity with ClyA in *E. coli* K-12 (30). ClyA has cytotoxic activity for both murine and human macrophages (19). Of the *Salmonella* serovars, the gene for HlyE was initially thought to be uniquely found within the *S.* Typhi and *S.* Paratyphi A genomes (30, 55); however, a recent study by Fuentes et al. suggests that it is found in several other *S. enterica* serovars known to cause systemic infection humans (7). PagC is also expressed under the control of the PhoP regulon (8, 27), and we previously identified PagC in our IVIAT screen of immunoreactive proteins following *S*. Typhi infection, including demonstrating a specific anti-PagC antibody response in the plasma of Bangladeshi patients infected with *S*. Typhi (12). In the current study, we also identified PhoN, a PhoP-regulated phosphatase, as well as heat shock proteins GroEL and DnaK, as immunoreactive. Overall, heat shock proteins are often immunogenic (32, 52), and immunization of mice with purified *S*. Typhi GroEL induces an IgG response and confers 70 to 90% protection against lethal intraperitoneal challenge with *S.* Typhi and *S.* Typhimurium in a murine model (31).

A number of studies have shown that *S.* Typhi outer membrane proteins are immunogenic during human infection (2, 29), and we identified OmpX and OmpA in our assay. OmpA

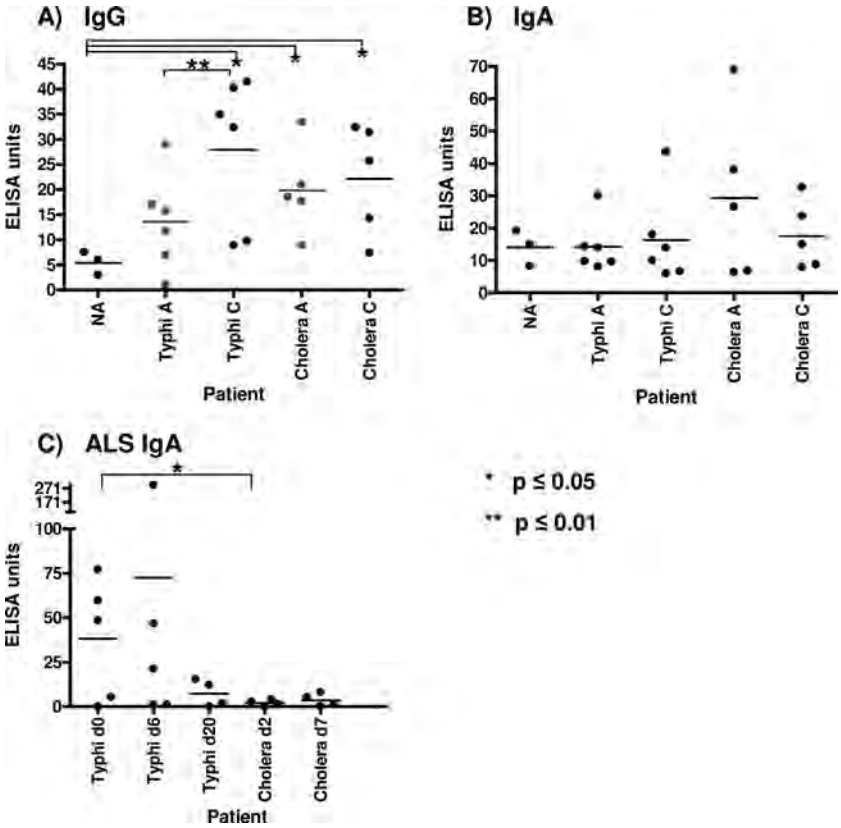

FIG. 1. Characterization of anti-HlyE immune responses. Anti-HlyE IgG (A) and IgA (B) responses in the plasma of North American volunteers (NA) and acute-phase (day 0) and convalescent-phase (day 20) plasma of patients infected with *S.* Typhi or *V. cholerae* (control). (C) Anti-HlyE IgA ALS responses in day 0, day 6, and day 20 samples of patients infected with *S.* Typhi and in day 2 and day 7 samples of patients infected with *V. cholerae* (control).

has previously been shown to be immunogenic in mice following *S*. Typhimurium infection (18, 35, 44, 48). We also identified STY0351, an outer membrane adhesin with an OmpA-like transmembrane domain and with homology to PagC.

We also identified CdtB (STY1886), PltA (STY1891), and STY1364, all of which are present in *S.* Typhi and *S*. Paratyphi A but rarely found in other *Salmonella* serovars (26). CdtB (STY1886) is a homolog of the active subunit of a cytolethal distending toxin found in a number of bacterial pathogens, including *E. coli*, *Shigella dysenteriae*, *Haemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, and *Helicobacter hepaticus* (10). CdtB expression is upregulated intracellularly and induces cell cycle arrest of host cells by causing DNA damage leading to distention of cells and enlargement of nuclei (10). Interestingly, CdtB forms a complex with PltA (STY1890) and PltB (STY1891), pertussis-like toxins that mediate its delivery into host cells (46). STY1364, a putative periplasmic protein, shares homology with PltB (STY1891, 30% identity) and ArtB, a putative ADP-ribosyltransferase toxin of *S*. Typhimurium DT104 (73% identity). All 3 proteins (CdtB, PltA, and STY1364) are upregulated in minimal media, an *in vitro* condition that approximates that of the intracellular macrophage (1).

With regard to the immunoreactivities of antibodies of various isotypes, we found that the IgM fraction collected during the acute stage of illness recognized the largest number of *S.* Typhi proteins, including HlyE, GroEL, and DnaK. Such a result is consistent with early-stage infection and subsequent isotype maturation. The next largest group of *S*. Typhi proteins reacted with either acute- or convalescent-phase IgG antibody, including PagC, HlyE, CdtB, PltA, PhoN, and GroEL. The fact that both acute- and convalescent-phase antibody recognized many of these antigens may reflect the probable 2- to 3-week period between ingestion of infecting *S*. Typhi organisms, the onset of symptoms, and the collection of samples. Consistent with an evolving immune response, we identified several *S*. Typhi proteins that reacted only with convalescent-phase IgG, including the PhoP-regulated protein PhoN. We also identified an IgG response against bacterioferritin, a protein involved in iron homeostasis (53).

To validate the results of the anti-*S*. Typhi responses identified in our screening assay, we assessed anti-HlyE responses by standard ELISA using purified protein and plasma from Bangladeshi patients with documented *S*. Typhi bacteremia and compared them to patients with *V. cholerae* infection or plasma from North American controls. von Rhein et al. have previously shown that anti-HlyE serum antibodies can be detected in *S*. Typhi-infected patients by Western blotting (56). We used an ELISA-based format and plasma from Bangladeshi patients whose samples were not used in the screening IPT assay and confirmed anti-HlyE IgG, but not IgA, responses. We also detected significant increases in anti-HlyE

IgG responses from the acute to the convalescent phase of illness in patients infected with *S*. Typhi but not in the negative-control cholera patients.

To test for a potential mucosal immune response to HlyE, we also evaluated anti-HlyE IgA responses using ALS samples. We recently found that performing an IgA-based ALS assay using an *S*. Typhi crude membrane preparation during acute-stage illness could be used to differentiate patients with typhoid from those with other illnesses (43). We were thus intrigued by our detection of an anti-HlyE IgA response in ALS fluid from patients with typhoid, which was absent in patients with cholera in Bangladesh, a setting where both infections are endemic. The identification of a specific anti-*S*. Typhi serological response at the time of clinical presentation of patients with typhoid fever could be the foundation for an improved diagnostic assay.

In summary, IPT is a rapid screening tool that can be used to identify proteins that elicit a serological immune response in infected hosts. Using this technology, we identified a number of immunogenic proteins of *S*. Typhi, including a subset previously known to be immunogenic (supporting the validity of the approach), a subset under the control of the PhoP regulon involved in intracellular survival, and a subset present in the genomes of human-restricted *S*. Typhi and *S*. Paratyphi A but largely absent from zoonotic and wide-host-range *Salmonella* bacteria. Many of the proteins are components of the outer membrane and involved in energy metabolism, protein synthesis, and iron homeostasis or are biosynthetic and metabolic enzymes; a significant subset are involved in virulence and intracellular survival. Our identification of *S*. Typhi proteins by IPT may facilitate targeted analysis of subsets of *S*. Typhi antigens in cellular immunologic studies. Additionally, our identification of HlyE by IPT, confirmation of differential anti-HlyE reactivities in convalescent- versus acute-phase plasma of patients with *S*. Typhi bacteremia, and detection of a mucosal anti-HlyE immune response present at the time of the clinical presentation of patients with typhoid fever but absent in control patients are of potential diagnostic significance.

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BRIEF REPORT

Identification and molecular cloning of a novel porcine parvovirus

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Abstract A novel porcine parvovirus, PPV4, was identified in the lung lavage of a diseased pig coinfected with porcine circovirus type 2. This virus exhibits limited similarity to its closest relative, bovine parvovirus 2, but resembles viruses of the genus *Bocavirus* (bovine parvovirus, canine minute virus and human bocavirus) that encode an additional ORF3. The ORF3 of PPV4 is predicted to encode a protein of 204 amino acid residues, which is similar in size to the ORF3-encoded proteins of the bocaviruses. Whereas the ORF3-encoded proteins of bocaviruses share significant similarity with each other, the PPV4 ORF3 encoded protein does not exhibit homology with any protein in the GenBank non-redundant database.

Keywords *Parvovirinae* · Porcine parvovirus · Phylogeny

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Parvoviruses are small, non-enveloped icosahedral viruses. These viruses are ubiquitous, and they have been isolated from many animal species. Based on their host range, viruses of the family *Parvoviridae* are divided into two subfamilies: *Parvovirinae*, which includes viruses that infect birds and mammals and *Densovirinae*, which includes viruses that infect insects and other arthropods [10]. The *Parvovirinae* and *Densovirinae* viruses do not share any nucleotide sequence homology. The International Committee on Taxonomy of Viruses has further classified the *Parvovirinae* viruses into five genera: *Dependovirus*, *Bocavirus*, *Erythrovirus*, *Parvovirus* and *Amdovirus*. Recently, a new genus, Hokovirus, was proposed to include a group of newly identified porcine isolates (designated PPV3 in this report) and bovine isolates (designated BPV4 in this report) that clustered with the human PARV4 and PARV5 viruses [14].

The genomes of members of the subfamily *Parvovirinae* are linear single-stranded DNA genomes of about 5 kilobases that contain terminal palindromic sequences. In general, the genome contains two major open reading frames (ORFs), coding for the non-structural protein(s), located at the 5′-end, and the capsid protein(s), located at the 3′-end. An additional ORF3 has been observed among viruses of the genus *Bocavirus* [bovine parvovirus (BPV1), canine minute virus (MVC) and human bocavirus (HBoV)], and ORF3 is located in the middle of the viral genome between ORF1 and ORF2 [2, 10]. Recently, it was reported that ORF3 of MCV is essential for viral DNA replication [20]. Parvoviruses have been proposed to replicate their genomes via a modified rolling hairpin model that involves a hairpin transfer mechanism [3, 5, 8]. In this model, two obligatory dimeric genome replicative intermediates (RF) generated via the terminal palindromic sequences are synthesized during viral DNA replication

[6]. These dimeric RFs contain a sense-strand and a minus-strand, i.e., a double-stranded genome, that are covalently linked (via terminal palindromic self-priming) either at the left end or right end of the linear genome and give rise to head-to-head or tail-to-tail concatameric molecules.

In this work, we cloned a novel porcine parvovirus from a circular or a head-to-tail concatameric template. Since it is the fourth (nearly) complete DNA sequence of a porcine parvovirus described, this virus is designated PPV4. Phylogenetically, PPV4 does not cluster with the three previously identified porcine parvoviruses but exhibits limited homology to bovine parvovirus 2 (BPV2) [1]. Incidentally, a recent publication based on partial nucleotide sequence reported identification of a novel porcine boca-like virus [4] among pigs co-infected with porcine circovirus type 2 (PCV2). The ORF3 of this porcine boca-like virus and PPV4 share low nucleotide identity (45%) and amino acid identity (17.5%), which indicate that this boca-like virus and PPV4 are two distinct parvoviruses.

PPV4 was identified among swine suffering from an acute-onset disease of high mortality in North Carolina during late 2005 [7]. Case reports of this disease indicated that various viral and bacterial pathogens were identified in tissues from swine infected with PCV2. PCR analysis showed that porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus, and several different serovars of porcine enterovirus were isolated from one or more of the affected herds. PCV2 was identified in essentially all pigs from each of the herds. Tissue homogenates consisting of lung, lymph node, spleen, and heart were prepared from fresh frozen tissues of the affected swine. The tissues were triturated with sterile sea sand via mortar and pestle. Serum-free minimum essential medium was used to reconstitute a 10% solution by weight. The solution was clarified at 1,000×*g* for 10 min and filtered through a 0.45-micron filter. Two colostrum-deprived cesarean-derived (CDCD) pigs, pigs 753 and 754, were inoculated oronasally with 2 ml of the 10% tissue homogenates and monitored for clinical disease. Both pigs developed severe respiratory disease and had to be euthanized. Pig 754 was euthanized on day 16 post-infection and pig 753 was euthanized on day 21 post-infection.

To explore whether there were any unknown viruses present in the diseased pigs, a random shotgun sequencing experiment was conducted. RNA from a lung lavage sample of pig 753 was extracted, randomly amplified and cloned into pCR4.TOPO (Invitrogen, Carlsbad, CA, USA) as described previously [23] and sequenced using standard Sanger chemistry [17] on an ABI 3730xl sequencer. The resulting sequence reads were trimmed to remove vector and primer sequences and then assembled using Phred/Phrap [9]; (Phil Green, http://weeds.mgh.harvard.edu/goodman/doc/). Contigs with sequence similarity to known viral sequences were identified using tBLASTx. Three hundred thirty-four clones were sequenced. Alignment of the sequence reads to the GenBank NR database yielded 19 sequences with limited identity to viruses in the family *Parvoviridae*. Nucleotide sequences belonging to other virus families were not detected. The 19 parvovirus-related sequences were assembled by Phred/Phrap into two contigs that were experimentally verified by generating PCR amplicons the length of the contigs. Deduced amino acid sequence homology was analyzed with the translated BLAST search. Contig 1 was 1,815 base pairs (bp) long and had 34% amino acid identity to its top hit, BPV2 capsid protein. Contig 2 was 2,993 bp long and had 39% identity to the non-structural protein of BPV2. Primers at nt 3,681–3,702 and nt 4,206–4,183 (based on c17 of Fig. 1) were then designed to span the gap between the two contigs. PCR was conducted with total cellular DNA from pig 753 that was prepared using a STAT-60 DNA extraction kit purchased from Tel-Text B, Inc. (Friendswood, TX, USA). The PCR reaction was carried out with 0.1 μg of DNA in the presence of 10 mM Tris–HCl (pH 8.3), 0.2 mM each of the four deoxynucleotide triphosphates, 100 pM each of the primers, and 2.5 U of Taq polymerase. The reaction mixture was heated at 94°C for 1 min, amplified for 34 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 5 min 15 s, and then kept at 68°C for 7 min. The resulting contig was 4,851 bp in length.

Based on the DNA sequence obtained from the initial random amplification and the above PCR experiment, diverging primers were designed to obtain additional nucleotide sequences upstream and downstream of the 4,851-nucleotide contig. Nested PCR was conducted with the pig 753 lung lavage DNA. In first-round PCR, primers X (tga acc cat aca ttg tac ca) and Y (gtc tac ttc ttc taa ctg ca) were used (Fig. 1a). In second-round PCR, an aliquot of the first-round products was amplified with the common primer C (ctc cgc ctg ccg aac agc ga), and one of three primers: #1 (ttt cca gat tac atg ggt tc), #2 (tga gac tcc tgg cac aga tg) or #3 (tta cat atg aaa tgg aat gg). In each case, a prominent PCR product of the expected size was obtained. The results demonstrated that a PPV4 molecule in head-to-tail configuration was present in the pig 753 DNA sample. To obtain the unit-length head-to-tail PPV4 sequence, two abutting primers of opposite polarity located at the unique *Kpn*I site of the viral genome were used for PCR. The primers used were PKpnF-gga agt ggt acc ttc tct tac aca tca ac (forward orientation) and PKpnR-gga agt ggt acc tgt ttt cag ttg gtg at (reverse orientation), and the *Kpn*I site is located at nt 3,342–3,348 of the clone 17 (c17) sequence. After amplification, several PCR products were visualized on an agarose gel. One of the products was approximately 6,000 bp long, and the rest were less than 4,000 bp long (Fig. 1b). The 6,000-bp PCR product was excised from the

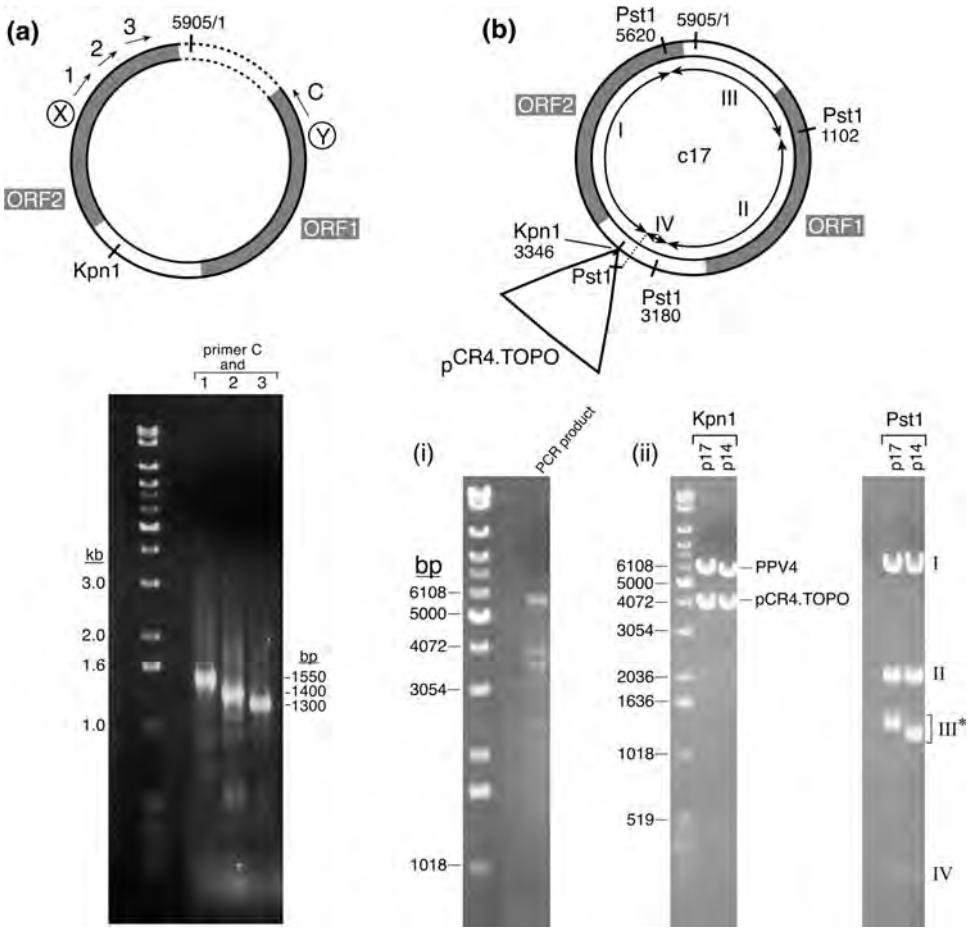

Fig. 1 **a** A schematic diagram denoting the PCR oligonucleotide primers and the strategy to obtain the head-to-tail covalent linkage nucleotide sequence (indicated by dotted lines) from a circular molecule. Nested PCR was performed. The first-round PCR was conducted with primers X and Y (data not shown). *Bottom panel* the second-round nested-PCR was conducted using a common primer C with either primer 1, 2 or 3, as indicated at the top of each lane of the agarose gel. **b** Schematic diagram of plasmid p17, which contains the PPV4 c17 genome inserted into pCR4.TOPO. ORF1, ORF2 and restriction enzyme sites of *Kpn*I and *Pst*I are denoted. *Bottom panel* agarose gel analysis of plasmids p14 and p17. (*i*) PCR products obtained using two divergent primers, PKpnF and PKpnR, at the *Kpn*I site of c17. (*ii*) The restriction enymes (*Kpn*I and *Pst*I) used are indicated at the top of each panel. *Kpn*I digestion releases the entire double-stranded PPV4 genome (5.9 kb) from the cloning vector pCR4.TOPO (4.0 kb). PstI digestion yielded four fragments, labeled *I–IV*, and DNA fragment III, which differs between c14 and c17, is indicated by *asterisk*

gel and cloned into the pCR4.TOPO plasmid. A miniprep plasmid DNA kit (Promega, Madison, WI, USA) was used to isolate nucleic acids from overnight TOP10 (Invitrogen, Carlsbad, CA, USA) bacterial cultures, and DNA electrophoresis was carried out in 1% agarose gel. Two plasmids (p14 and p17) containing the PPV4 DNA clones (c14 and c17), respectively, were obtained. As shown by the *Pst*I restriction enzyme analysis, c14 is slightly smaller than c17. The DNA clones were then sequenced using primer walking. It was confirmed that c14 is 125 nucleotides (nt) shorter than c17 and the deleted sequence is located at nt 127–251. In addition, there are 15 nt differences between these two clones. The GenBank accession numbers for c14 and c17 are GQ387500 and GQ387499, respectively. PCR was also conducted with total cellular DNA purified from the lung lavage of pig 754. The partial PPV4 nucleotide sequence obtained was essentially identical to the parvovirus DNA sequence derived from pig 753. No further experimentation was conducted with the viral sequences from pig 754. To investigate whether PPV4 was present in the field cases of the 2005 outbreak, all of the pigs examined in the original study [7] were tested for PPV4 by PCR. The results showed that only two pigs (n6 and n10) of Farm 1 from North Carolina were positive for PPV4.

Phylogenetic analysis was performed on the PPV4 nucleotide sequences using full-length or nearly full-length genomes from all species of the subfamily *Parvovirinae* as described previously by Lau et al. [14]. Nucleotide and amino acid sequences were aligned with CLUSTAL, and phylogeny was inferred using MEGA4 [21]. The evolutionary history was inferred using the Neighbor-Joining method [16]. The evolutionary distances were computed

using the Maximum Composite Likelihood method [22] and are in units of number of base substitutions per site. The bootstrap consensus tree inferred from 10,000 replicates was taken to represent the evolutionary history of the taxa analyzed [11]. Branches corresponding to partitions reproduced in fewer than 50% of the bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches [11]. Pairwise global comparison of putative amino acid sequences coded for by ORF1, ORF2, and ORF3 were conducted by aligning the sequences for each ORF using the Needleman-Wunsch algorithm [13, 15]. Based on comparative nucleotide sequence, PPV4 did not cluster with any of the known parvoviruses, including the three previously identified porcine isolates (Fig. 2). Instead, PPV4 is most similar to BPV2. Separately, phylogenetic analyses were conducted with the deduced ORF1 or ORF2 amino acid sequences, and essentially identical results were obtained (data not shown).

The c17 PPV4 clone is 5,905 base pairs long. Since the nucleotide sequences of the left and right ends of the linear viral genome have not been determined, the ends of the genome are arbitrarily assigned so that the genome conforms to the general organization of parvoviruses having two major ORFs: ORF1 located at the 5′-end and ORF2 at the 3′-end. PPV4 has an additional ORF3 located between ORF1 and ORF2, which is a feature of the viruses belonging to the genus *Bocavirus*. Analyses were conducted to compare the ORFs of PPV4 with those of other PPVs and BPVs and members of the genus *Bocavirus* at the amino acid level.

1. ORF1: PPV4 ORF1 is 1,794 nt long and capable of coding for a protein of 588 residues. BLAST search showed that it is most closely related to the putative nonstructural protein of BPV2 and the replication proteins of adeno-associated viruses 1 (AAV-1) (45.8% identity) (GenBank accession number FJ872544) of the genus *Dependovirus*. It shares 33.6% amino acid residue identity with BPV2 and less with the other porcine and bovine parvoviruses. PPV4 shares 17.4–20.5% amino acid identity with the bocaviruses, while the bocaviruses share 24.8–33.5% identity among themselves.
2. ORF2: PPV4 ORF2 is 2,184 nt long and capable of coding for a protein of 728 residues. BLAST search showed that it is most closely related to the putative structural protein of BPV2 and the capsid proteins of various parvoviruses. It shares 24.5% amino acid residue identity with BPV2 and less with the other porcine and bovine parvoviruses. PPV4 shares 18.7–20.3% amino acid identity with the bocaviruses, while the bocaviruses share 41.4–45.6% identity among themselves.
3. ORF3: PPV4 ORF3 is 612 nt long and capable of coding for a protein of 204 residues. BLAST search showed that ORF3 did not have significant homology to any protein in the GenBank non-redundant protein database. With respect to coding capacity, it is similar to that of the bocaviruses. The ORF3 of BPV1, HBoV and MCV is capable of coding for a protein of 213, 219 and 188 residues, respectively. The PPV4 ORF3-encoded protein shares only 4.9–11.2% amino acid identity with the bocaviruses, while the bocaviruses share 43.3–47.0% identity among themselves. Whereas the PPV4 ORF3 does not overlap with either major ORF, MVC overlaps with ORF1, HBoV overlaps ORF2 and BPV1 overlaps with both ORF1 and ORF2.

In summary, we report the identification of a novel porcine parvovirus, PPV4. In this study, a 5,905-nt PPV4 sequence was obtained after PCR amplification using two diverging primers from the unique *Kpn*I site of the viral genome. Thus, the templates for the generation of the genomic clones, c14 and c17, were either circular or head-to-tail concatameric molecules in which the left and right ends of the linear genome were covalently linked. Based on the genome organizataion of parvoviruses, it is expected that both the left end and the right end of the linear PPV4 genome are located between nt 1 and 848 (Fig. 1b). However, it is possible that some terminal nucleotides of the linear genome are missing in c17 and c14 during formation of the head-to-tail configuration. The genomes of parvoviruses are single-stranded linear DNA molecules; however, it has been reported that the genomes of AAV-2, a member of the genus *Dependovirus*, can persist in tissues as head-to-tail monomeric and concatameric circles [18, 19]. The presence of head-to-tail concatameric PPV4 genomes in tissues of infected animals suggests that this virus may be able to establish persistence in its host.

Phylogenetic analysis showed that the porcine and bovine parvoviruses exhibit high degrees of diversity. In general, the porcine or bovine isolates do not cluster phylogenetically with their respective viruses from swine or cattle. Among the porcine parvoviruses, PPV1 belongs to the genus *Parvovirus*, PPV3 belongs to the proposed genus Hokovirus, PPV4 and the porcine boca-like virus [4] resemble members of the genus *Bocavirus*, and PPV2 does not cluster with any known isolates. A similar phenomenon has also been reported for the bovine parvoviruses [14]. There are four bovine parvoviruses, but they also do not cluster together phylogenetically (Fig. 2). BPV1 belongs to the genus *Bocavirus*, BPV2 and BPV3 [1, 10] do not cluster with any other known isolates, and BPV4 belongs to the proposed genus Hokovirus. Interestingly, one porcine

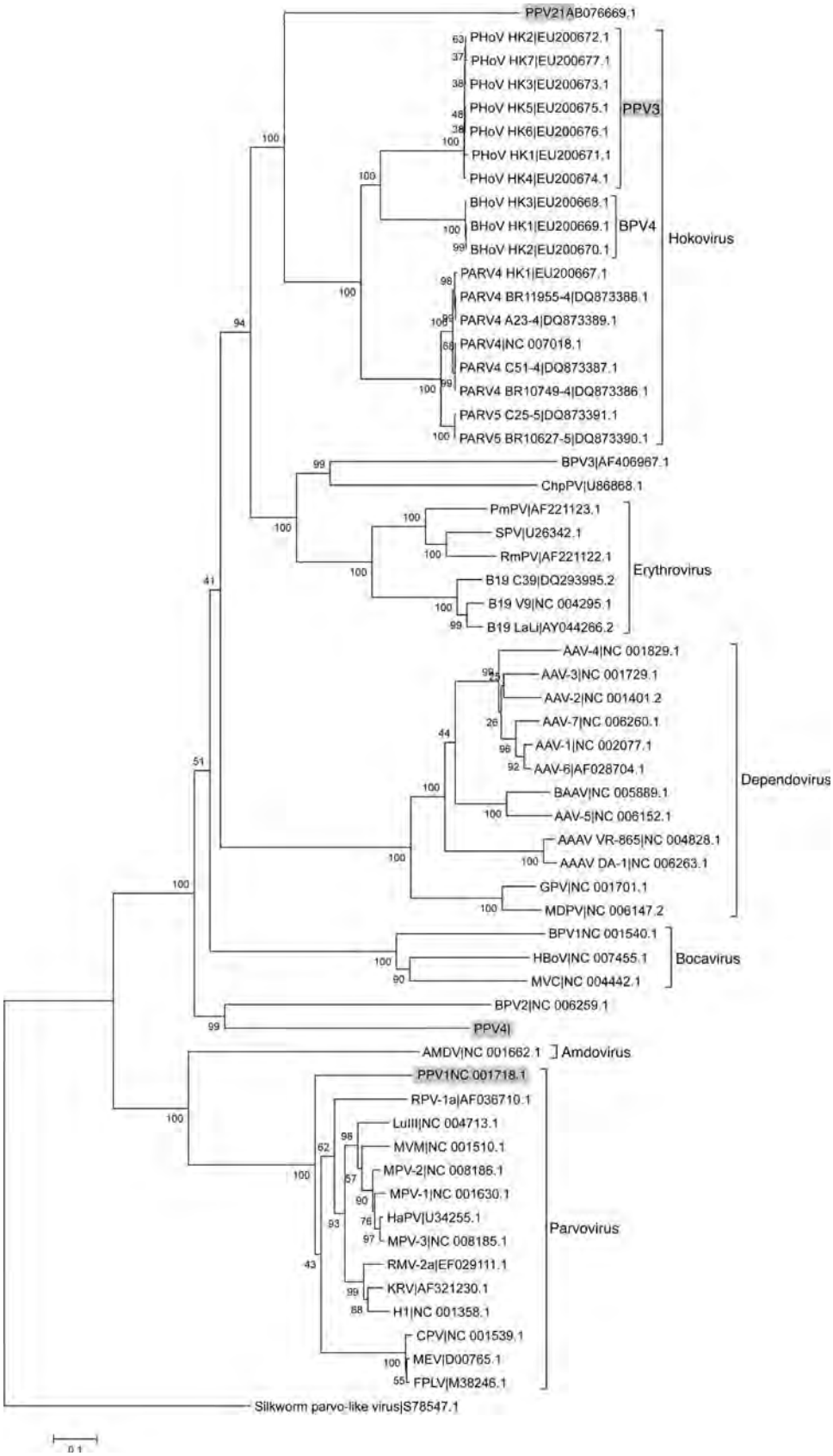

Fig. 2 Evolutionary relationships of PPV4 and other related parvoviruses. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 6,684 positions in the final dataset. Phylogenetic analysis was conducted using MEGA4 [21]. The porcine parvoviruses are denoted in *shaded boxes*

(PPV3), one bovine (BPV4) and two human parvoviruses (PARV4 and PARV5) are closely related and form the proposed genus Hokovirus [14].

Our analysis revealed several distinct characteristics of PPV4. Whereas PPV4 is most closely related to BPV2 phylogenetically, the coding capacity and genome organization of PPV4 are more similar to those of viruses of the genus *Bocavirus*. PPV4 is most closely related to BPV2 with respect to ORF1 and ORF2 phylogenetically; however, BPV2 does not contain a distinct ORF3 situated between the two major ORFs. Similar to the bocoviruses, PPV4 encodes an additional ORF3 located in the middle of the genome, but the deduced protein of PPV4 ORF3 is quite distinct from the ORF3-encoded proteins of the bocaviruses.

The pathogenic potential of porcine parvoviruses is not fully understood. PPV1 is ubiquitous in swine, and it causes reproductive failure in pregnant females [24]. PPV2 has been detected in swine serum but has not been incriminated in any disease [12]. The pathogenic nature of PPV3 and PPV4 remains to be determined. PPV3, a member of the proposed genus Hokovirus, has been found in tissues of healthy and sick pigs [14]. In this study, PPV4 was identified in a co-infection with porcine circovirus type 2 (PCV2) from field cases of severe PCV2-associated disease. PCV2 has been associated with multiple disease syndromes in swine and is the primary agent leading to postweaning multisystemic wasting syndrome (PMWS). It has been reported that other pathogens enhance the severity of PMWS during co-infection with PCV2. From this study, it is not clear whether PPV4 can cause disease on its own or if PPV4 contributed to the disease phenomenon in the CDCD pigs. At any event, the detection of PPV4 among diseased pigs that exhibit PCV2-associated PMWS warrants further investigation into the pathogenic nature of PPV4.

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Understanding the Mechanisms of Immunopathogenesis of Human and Bovine Tuberculosis

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Abstract: Extensive investigations have revealed that zoonotic pathogens in the *Mycobacterium tuberculosis* complex (MTBC) evolved from a common ancestor. Although all the members can cause disease in one or more species of mammals, *Mycobacterium tuberculosis (Mtb)* and *M. bovis (Mbv)* are the major pathogens affecting humans and livestock. The pathogens are genetically distinct, but the mechanisms by which they dysregulate the immune response and cause disease are similar, suggesting a common strategy can be used to control both pathogens. Recent advances have shown NK and NKT cells, γδ T cells and multiple newly identified T cell subsets are involved in the immune response to mycobacteria. A complex network of cytokines regulates their response to mycobacteria during the development and loss of protective immunity. Elucidation of how mycobacteria modulate the network and cause disease will provide the knowledge needed to develop an effective vaccine.

Key words: Cytokine network, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, tuberculosis

Introduction

*Mtb*and *Mbv* are the major pathogens in the MTB complex causing disease in humans and livestock. They have co-evolved with humans during the transition to a pastoral existence and domestication of ruminants as a source of food and fiber approximately 40,000 years ago. Genomic analysis has shown they evolved from a common prototypic ancestor into two clades defined by presence or absence of *M. tuberculosis* deletion 1 (TbD1). The data suggest both clades arose in humans, with the TbD1$^-$ clade 1 co-evolving mainly in humans and the TbD1$^+$ clade 2 co-evolving in humans, ruminants and other species [1]. *Mtb* is the main pathogen in humans in countries with advanced control programs for tuberculosis, with new drug resistant variants increasing health risk to the community at large. However, *Mbv* remains a significant problem where wildlife reservoirs are presentin some of these countries [2-3]. Both *Mtb* and *Mbv* remain a problem in countries with less advanced or no control program. The highest prevalence of infection with *Mtb* and *Mbv* is in caretakers in close contact with animals where infection with both pathogens isendemic [3].Control has been difficult because of wild life reservoirs, the lack of a diagnostic assay that can detect animals during the early or latent stage of infection and also because of the lack of an effective vaccine. Development of a vaccine has been impeded by a lack of a full understanding of the mechanisms regulating the development and persistence of protective immunity. Approximately 90% of humans infected with *Mtb* do not develop clinical disease [4]. It is presumed most of these individuals becomelatently infected and that they have developed a protective immune response that controls but does not eliminate the pathogen.It is not clear whether some of these individuals developed sterile immunity. Limited information is available on whether a comparable proportion of humans infected with *Mbv* also develop protective immunity. Comparison of pathogenicity of *Mtb* with *Mbv* in cattle has shown *Mtb* is less virulent than *Mbv*,indicating cattle may develop a latent infection with *Mtb* similar to what occurs in humans [5]. The comparison also suggests a larger proportion of humans infected with *Mbv* may develop disease rather than a latent infection,because of the greater virulence of *Mbv*. Of more importance, however, is the observation that many humans infected with *Mtb* develop a protective immune response that persists following infection. This indicates that it should be possible to develop a protective vaccine to *Mtb* and the more virulent *Mbv*. To accomplish this objective there is a need to determine the signature of protective immunity in latently infected individuals and identify the trigger(s) used by pathogenic mycobacteria to dysregulate protective immunity. An effective vaccine must elicit an immune response that blocks the pathways used by the pathogens to modulate the immune response and cause disease. The objective of this short commentary is to summarize progress in characterizing the immune response to *Mtb* and

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Mbv and discuss how better use of a bovine model can accelerate development and validation of a vaccine for both pathogens.

Immunopathogenesis

As shown in the summary diagram (Fig. 1), recent studies have revealed the immune response is regulated by a network of cytokines and chemokines secreted by antigen presenting cells (APC) and different lineages of lymphocytes [6-8]. Details on how the network modulates the immune response are still evolving with new insights being gained from study of autoimmunity and the immune response to infectious agents. Cumulative studies show dendritic cells (DC) play a pivotal role in shaping the innate and adaptive immune response [9-12]. Cytokine signaling that occurs following initial uptake of antigens or pathogens activate NK, NKT, and γδ T cells [13-16], cells that comprise the innate immune system. These cell subsets are the initial source of newly identified proinflammatory cytokines IL-17 and IL-22, granulysin, and IFN-γ, the primary cytokine that primes DC for initiation of adaptive immunity [17-21]. Studies of the immunopathogenesis of tuberculosisindicate initial infection is under immune control, mediated in part by the innate immune system. Phagocytosis of pathogenic mycobacteriaby immature DC (iDC) leads to differentiation and maturation of DC (mDC) that subsequently activate innate immune cells. Activated NK and NKT cells develop the capacity to kill infected DC and Mϕ as well as cell free mycobacteria through secretion of granulysin [13-14,20,22-26]. Activated γδ T cells are also a source of IFN-γ, IL-17, and granulysin and may play a role in priming DC for antigen presentation [27-29]. Cytokine signaling by primed DC activates naïve T cells to differentiate into CD4 and CD8 subsets producing IFN-γ, essential for maintenance of immune protection against mycobacteria, and subsets secreting either or both IL-17 and IL-22 [4,30-33]. One or more of the subsets are also the source of granulysin. IL-12 and IL-23 differentially secreted by mDC modulate differentiation of naïve T cells towards IFN-γ producing T cell subsets or subsets producing IL-17 and/or IL-22 [4,9,32]. In conjunction with the innate response, the early adaptive response controls infection. In spite of these mechanisms of protection, however, immune control is lost. Efforts to control infection by vaccination in mouse models have been unsuccessful [4,31]. Vaccination has increased survival but protective immunity is still lost. Studies in mice and humans suggest loss of protection is attributable to development of regulatory T cells (Tr). Support for this possibility derives from experiments in a mouse model that showed Tr cells interfere with clearance and effecter activity toward mycobacteria [34]. Studies in humans haverevealed the presence of Tr cells in peripheral blood of patients with active TB [35-38]. The frequency of antigen specific Tr cells was observed to behigher at sites of lesions and in pleural effusions from patients with active disease, suggesting Tr cells mediate their effect by interfering with effecter T cell activity at the site of lesions [36]. These studies indicate *Mtb* dysregulates protective immunity by modulating the immune response to favor development of Tr cells that interfere with rather than suppress effecter activity. This could account for the presence of a mixed population of effecter and regulatory T cells in patients with progressive disease.

Although the mechanisms used by mycobacteria to dysregulate protective immunity have not been identified, recent findings provide insights that may lead to identification of the mechanisms. Comparison of the frequency of subsets of CD4 T cells secreting IFN-γ, IL-17, and/or IL-22 has shown persistence of protective immunity is associated with a higher frequency of the subsets in healthy latently infected subjects than in patients with active disease[39]. A

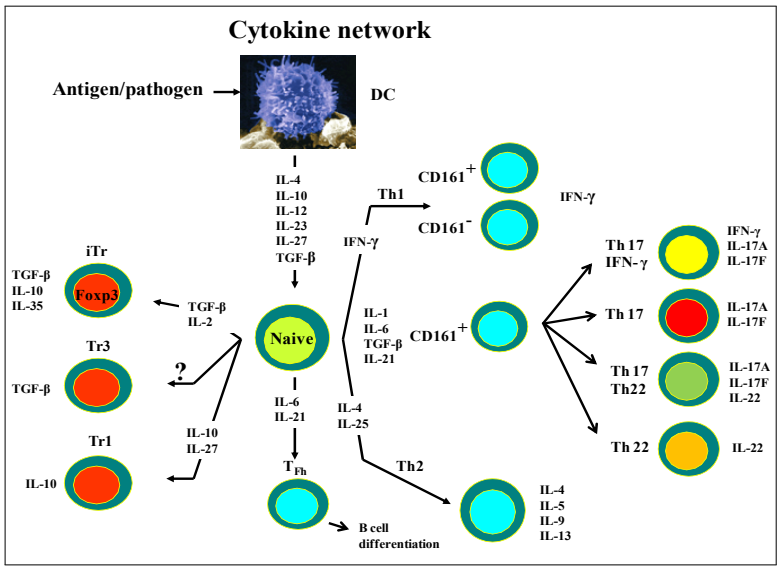

Fig. 1. Schematic showing the main pathways of naïve CD4 T cell differentiation modulated by the cytokine network. The diagram is based on studies in humans and mice [6-8;46-48]. Interaction with innate immune cells is not shown. Signaling mediated through pathogen-derived molecules can induce differentiation of immature dendritic cells (DC) into DC producing combinations of cytokines (IL-4, IL-10, IL-12, IL-23, IL-27) that determine the direction of T cell differentiation. Combinations of cytokines produced by activated cells modulate differentiation of T cells to effector cells producing the cytokines indicated. Additional studies are needed to characterize Th1 subsets defined by expression of CD161. Additional studies are also needed to determine the number of Th17 and Th22 subsets exist in ruminants.

closer examination of the cytokine profile of patients with latent infection and active disease has shown a potential relation between IFN-ɣ and IL-24 that distinguishes patients with clinical progressive disease from healthy latently infected subjects [40-41]. Patients with progressive TB exhibited low levels of IL-24 and IFN-ɣ compared to levels in latently infected subjects following in vitro stimulation of PBMC with ESAT-6. Of particular interest, the importance of IL-24 in expression of IFN-ɣ was demonstrated by addition of exogenous IL-24to stimulated cultures of PBMC from patients with progressive TB. Addition of IL-24 to the cultures increased expression of IFN-ɣ. In contrast, addition of antibody to IL-24 to ESAT-6 stimulated cultures of PBMC from latently infected subjects decreased expression of IFN-ɣ. The studies showed that the enhancing effect of exogenous IL-24 on expression of IFN-ɣ in PBMC from clinical patients was associated with increased expression of IL-12, IL-23, and IL-27 and decreased expression of Foxp3. The findings are consistent with *Mtb* (*Mbv*) targeting IFN-ɣ/IL-12 and IL-17/IL-22/IL-23 pathways to dysregulate immune function. Further studies should provide an understanding of the mechanisms used by *Mtb* (*Mbv*) to modulate immune function.

Bovine model of tuberculosis

The mouse model has been favored for the study of tuberculosis because of the advantages of size, maintenance costs, availability of strains of mice with variation in resistance, and gene knock out strains to dissect the immune response to *Mtb*. The model has clearly advanced our knowledge considerably on understanding immunopathogenesis [4,31]. However, a bovine model may be more appropriate for extending recent findings that have emerged from comparative studies focused on analysis of the mechanisms ofimmune protection that control infection in latently infected humans. Previous proposals to develop a model have only considered use of *Mbv* in the model [5,42-43]. However, a model that includes both pathogens should prove more useful. Advantage can be taken of the differences in pathogenicity of *Mtb* and *Mbv* that have persisted throughout the evolution of these pathogens during the transition of humans from a hunter-gatherer mode of existence to a pastoral existence and domestication of livestock [1]. Both pathogens can infect multiple species but they differ in their capacity to cause disease. It can be inferred that the difference is associated with differences in the capacity of the immune system of different species to develop a persistent protective immune response. This is reflected in the apparent host range of *Mtb* and *Mbv*. *Mbv* has the broadest host range, causing disease in multiple species. Humans are the primary host of *Mtb*. Though less well documented, a latent state of infection also occurs with *Mbv* [5]. This suggests the mechanisms used by *Mtb* and *Mbv* to dysregulate protective immunity are similar with the difference being that *Mbv* is more virulent and capable of triggering dysregulation at a higher frequency than *Mtb*.Analysis of how the bovine immune system responds to the pathogens should reveal the mechanism by which pathogenic mycobacteria dysregulate protective immunity. It has been recognized for some time that *Mtb* is less virulent in cattle than in humans. The basis for this difference is attributable to the capacity of the bovine immune system to develop what appears to be sterile immunity. A recent comparison of infection and expression of disease with *Mbv*, *Mtb*, and *M. kansasii* (*Mkan*) in cattle showed infection with *Mbv* leads to colonization and pathology whereas infection with *Mtb* leads to transient colonization and no pathology and infection with *Kan* leads to no colonization or pathology [44]. Both cellular and humoral immunity were elicited by each bacterium. The IFN-ɣ response was similar and persisted for *Mbv* and *Mtb* while the response to *Mkan*was less pronounced and waned during the study. The antibody response persisted in animals infected with *Mbv* and waned in animals infected with *Mtb*.

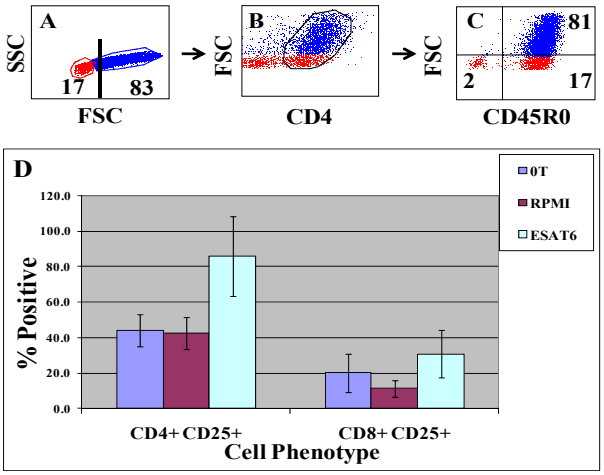

Fig. 2. Flow cytometric analysis of the immune response to ESAT-6 in calves experimentally infected with Mbv (N = 5). PBMC were isolated and analyzed at T0 and following culture with ESAT-6 for 6 days. Cells were labeled with anti-CD4 (or –CD8), -CD25, and -CD45R0 to distinguish naïve from memory T cells (CD45R0) and CD25 to identify cells proliferating in response to antigenic stimulation. Electronic gates were placed on small resting (red) and large proliferating (blue) cells (side and forward light scatter) to color code activated proliferating cells (Fig. 2A). Additional gates were placed on CD4 and CD8 to electronically isolate each cell population for analysis (Fig. 2B). The activated proliferating CD4 and CD8 memory T cell populations were identified for analysis, using FSC vs CD45R0 (Fig. 2 C). The histogram shows the percent of activated CD4 and CD8 memory T cells present at the initiation of culture (T0) and after 6 days of culture in RPMI culture medium alone or with ESAT-6. Both CD4 and CD8 memory T cells proliferated in response to antigenic stimulation (Fig. 2D).

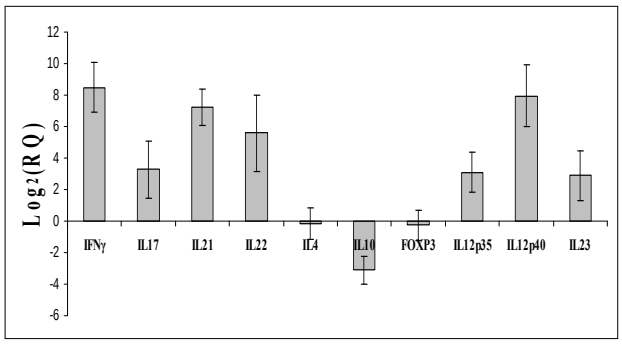

Fig. 3. Cytokine profile obtained from PBMC from calves experimentally infected with Mbv (n = 5) stimulated with ESAT6 for 6 days measured by real time RT-PCR. The results represent the relative quantification (RQ) of cytokines in PBMC stimulated with ESAT-6 compared to PBMC cultured in medium alone [49]. The data are presented as the means ± the SD in log2scale. Expression of IL-12p35, IL12p40 and IL-23 (p19) indicates macrophages and some dendritic cells were present in the antigen stimulated cultures and expressed the indicated cytokine genes. The expression of IFN-γIL-17, IL-22, and IL-21 shows stimulation of PBMC resulted in the proliferation of multiple T cell subsets expressing the respective cytokine genes. Expression of IL-4, IL-10, FOXP3 were low indicating cell subsets expressing these genes were low or absent. It remains to be determined how many subsets of T cells secrete combinations of the cytokine in ruminants.

This clear difference in the immune response to *Mbv* and *Mtb* shows the bovine model offers an unique opportunity to extend the recent studies in humans that showedan apparent correlation between expression of IFN-ɣ and IL-24 and immune status of latently infected subjects and patients with active disease. Recent exploratory studies with calves experimentally infected with *Mbv* show infection elicits a similar immune response. Flow cytometric analysis of the response to ESAT-6 has shown a strong proliferative CD4 and CD8 memory T cell response develops in PBMC from calves experimentally infected with *Mbv* (Fig. 2). Examination of the cytokine profile of stimulated cells by quantitative RT-PCR studies hasshown experimental infection with *Mbv*leads to up-regulation in expression of genes in cattle that are also modulated in latently infectedsubjects and patients with active disease. It can be inferred from the expression of IFN-ɣ, IL-17, IL-21, and IL-22 that the responding population of cells is comprised of multiple subsets of T cells expressing different combinations of cytokines (Fig. 3). The exploratory study shows that the molecular tools have been developed and are available to pursue comparative analysis of the immune response in the bovine model comparinganimals with progressive disease with animals that have brought infection under immune control. The comparative model can also be used to extend studies on the efficacy of experimental vaccines [45]. The findings should reveal how the immune response is dysregulated and provide valuable insights needed for development of an effective vaccine.

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Yeast cell-wall products containing β-glucan plus ascorbic acid affect neonatal *Bos taurus* calf leukocytes and growth after a transport stressor[1]

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ABSTRACT: The objectives were to ascertain whether a yeast cell-wall derivative that was 1.8% β-glucan in combination with ascorbyl-2-polyphosphate could improve innate immunity and mediate transportation stress in neonatal calves, and to compare the 1.8% β-glucan yeast cell-wall derivative with a more purified yeast cell-wall derivative (70% β-glucan). Treatments were 1) an unsupplemented control (CNT); 2) 113 g of a 1.8% (approximately 2%) β-glucan derivative of yeast cell walls plus 250 mg of L-ascorbic acid phosphate (BG2); or 3) 150 mg of a purified β-glucan fraction from yeast cell walls (approximately 70% β-glucan) plus 250 mg/feeding of L-ascorbic acid phosphate (BG70). Calves (n = 39) were transported for 4 h, placed in outdoor hutches, and randomly assigned to treatments. Treatments (mixed with a milk replacer) were individually fed twice daily for 28 d. Calves were offered calf starter, free choice, throughout the study. Weekly starter intake and BW were measured, and fecal samples were collected for *Salmonella* Typhimurium and *Escherichia coli* O157:H7 PCR analysis. Blood was collected immediately before transport (d 0) and on d 3, 7, 10, 14, 21, and 28 after transport. Starter intake and DMI were less ($P < 0.05$) at d 28 for the BG2 and BG70 treatments compared with the CNT treatment. Hematocrit percentages increased ($P = 0.002$) throughout the experiment. White blood cell counts (treatment × time interaction, $P = 0.066$) were less for the calves supplemented with BG70 than for those supplemented with BG2 ($P = 0.01$) or for CNT calves ($P = 0.04$) on d 28. Granulocyte counts changed ($P = 0.04$) throughout the experiment. A trend ($P = 0.077$) for a treatment × time interaction was detected for peripheral blood mononuclear cell counts (PBMC). Counts of PBMC were greater ($P = 0.006$) for the BG2 treatment compared with the CNT treatment on d 3. Calves given the BG70 supplement had fewer PBMC than those given the BG2 supplement on d 21 ($P = 0.03$) and 28 ($P = 0.05$). Fibrinogen concentrations were affected only by time ($P = 0.002$). Time effects were detected for phagocytosis ($P = 0.005$), oxidative burst ($P < 0.001$), expression of cluster of differentiation 18 ($P = 0.001$), and increased cluster of differentiation 18 ($P = 0.006$). Phagocytosis was less ($P = 0.05$) for calves in the BG70 group than for those in the CNT group. Percentage of calves positive for *E. coli* O157:H7 was greatest ($P \leq 0.05$) for those in the BG2 group on d 7 compared with those in the other treatments. The BG2 and BG70 supplements both increased feed intake, but only the BG2 supplement increased *E. coli* shedding on d 7, and the BG2 and BG70 supplements varied in modulating immune functions, indicating differences in yeast extract function.

Key words: cattle, β-glucan, immunity, transportation, vitamin C, yeast cell wall

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INTRODUCTION

Transporting dairy cattle multiple times during their lives has become a routine practice because of farm specialization for different ages of growing calves and heifers, and because sometimes environmental protection issues can limit the number of animals that can be kept on the property. The effect that early transport may have on neonatal calves has not been well established. However, age at transport (<1 to >3 wk)

[1]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

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is negatively correlated with morbidity and mortality (Knowles, 1995; Eicher, 2001).

Dietary supplements are possible immune modulators that may assist calves during immunity development and frequent managerial stressors. Two such supplements are yeast extracts (specifically β-glucan components) and vitamin C (ascorbic acid). Beta-glucan has been shown to modulate innate immune function in several species (Pedroso, 1994; Rodriguez et al., 2003; Lowry et al., 2005). Ascorbic acid also modulates innate immunity and health measures. Neonatal dairy calves fed a diet containing ascorbic acid had less nasal and ocular discharge, which indicated better mucosal health (Eicher-Pruiett et al., 1992).

Fewer studies have examined the effects of β-glucans and ascorbic acid when administered together. Eicher et al. (2006) reported a synergistic relationship with a yeast extract with β-glucans and ascorbic acid on pig growth and feed efficiency. Modulation of fish innate immune responses occurred when β-glucan was fed with ascorbic acid (Verlhac et al., 1998).

The objectives of this study were to ascertain if a yeast cell-wall derivative that was 1.8% β-glucan in combination with ascorbyl-2-polyphosphate could improve innate immunity and mediate transportation stress in neonatal calves, and to compare the 1.8% β-glucan yeast cell-wall derivative with a more purified β-glucan (70% β-glucan) yeast cell-wall derivative.

MATERIALS AND METHODS

Animal care and use was approved by Purdue Animal Care and Use Committee.

Animals and Treatments

Calves from the Purdue Dairy Teaching and Research Center were used for the study (20 bulls and 19 heifers). Calves were transported once on Monday, when they were at least 3 d of age and less than 10 d of age (mean age = 6 d). Variables were measured 28 d after the transport. All transport occurred during moderate fall and spring climates (4.4 to 27.8°C and 50 to 73% humidity; n = 16 in fall, and n = 23 in spring). At approximately 24 d of age (±5 d), calves were dehorned. No vaccinations were given during the study.

The 39 calves were blocked by birth date and assigned randomly to 1 of 3 treatments (n = 13 calves/treatment) in a randomized complete block design. Treatments were 1) a positive control of a 1-time subcutaneous electrolyte treatment (Plasma-lyte A, Baxter Health, Deerfield, IL; containing 140 mEq/L of Na^+, 5 mEq/L of K^+, 3 mEq/L of Mg^{2+}, 98 mEq/L of Cl^-, 23 mEq/L of gluconate, and 27 mEq/L of acetate; **CNT**); 2) a yeast cell-wall derivative containing 1.8% (rounded as 2%) β-glucan (EnergyPlus, Natural Chem Group LLC, Houston, TX) plus ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ; **BG2**); or 3) β-glucan extracted from yeast cell walls (approximately 70% β-glucan; BetaRight, BioThera, Eagan, MN) plus ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ; **BG70**). Supplement amounts were 113 g/feeding of 1.8% β-glucan and 250 mg/feeding of ascorbyl-2-polyphosphate, 150 mg/feeding of 70% β-glucan (equivalent to the β-glucan component of BG2) and 250 mg/feeding of ascorbyl-2-polyphosphate, or subcutaneous electrolytes without glucose. Supplements were delivered twice daily throughout the study and electrolytes were given 1 time only. Ascorbyl-2-polyphosphate alone was not tested as a treatment in this experiment because in preliminary work (our unpublished results), yeast extract and ascorbyl-2-polyphosphate acted synergistically and the objective of this experiment was to ascertain the effects of the β-glucan component of yeast extract in the combination of supplements.

Approximately 2.5 h after the morning feeding, the calves were weighed, blood was collected by jugular venipuncture, a fecal sample was collected, and calves were loaded by walking up a ramp onto a 4.88-m aluminum trailer. Transport consisted of a 4-h journey (0800 to 1200 h). The trailer was bedded with approximately 10 cm of straw, and space was restricted to allow space (approximately 1 m^2/calf) for each calf to lie down. On return to the dairy, CNT calves were given the subcutaneous electrolytes (37°C, given equally over each shoulder), and all calves were placed in individual outdoor polyethylene hutches (2.18 × 0.97 m) with wire fencing (approximately 1.5 × 0.97 m). Supplements were fed in milk replacer after the transport, beginning with the first evening feeding. Calves were weighed weekly at approximately 2.5 h after the morning feeding. Jugular blood samples were collected in 8.5-mL acid citrate dextrose vacuum tubes (trisodium citrate = 22.0 g/L, citric acid = 8 g/L, and dextrose = 24.5 g/L) before transport on d 0 and after transport on d 3, 7, 10, 13, 21, and 28. Samples were stored on ice until processed in the laboratory.

Calves were fed 4 L of pooled colostrum within 12 h after birth and were fed twice daily for the first 2 d of life. From d 3 throughout the remainder of the experiment, calves were fed 4.45 kg/d of a 20% protein and 20% fat (all milk protein) milk replacer. Milk replacer was reconstituted at 12.5% milk solids using a wire whisk and was divided into 2 equal feedings per day throughout the 28-d experiment. At each feeding (0600 and 1530 h), supplement treatments were added to batches of bucket-fed reconstituted milk replacer by treatment. Grain-based starter (formulated to provide 21% CP) was provided free choice.

Jugular blood was collected into 10-mL heparinized tubes from calves after the first 2 feedings of colostrum, between 24 and 48 h after birth. Plasma was removed after centrifugation of blood at 1,800 × *g* at 4°C for 20 min for confirmation of adequate passive transfer of im-

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munoglobulins. All calves were determined to have adequate immunoglobulin absorption by plasma protein concentration determination (passive transfer; >5.5 g of protein/dL of plasma; McVicker et al., 2002).

Sample Analysis

Differential cell counts of total white blood cells, peripheral blood mononuclear cells (**PBMC**), and granulocytes, and hematocrit percentages and fibrinogen concentrations were measured using a QBC VetAuto-Read Hematology Analyzer (IDEXX, Westbrook, MA). Phagocytosis and oxidative burst of neutrophils were determined using a modification of the whole blood assay of Böhmer et al. (1992). Whole blood (450 μL) was incubated at 37°C for 1 h before adding 50 μL of dihydrorhodamine (29 m*M*; Calbiochem, San Diego, CA) to each tube. After a 10-min incubation, 50 μL (time 0) was removed and placed into 1 mL of lysing buffer (Sigma, St. Louis, MO) for 1 min. Isotonicity was restored by adding 1 mL of 1× Hanks' Balanced Salt Solution (**HBSS**; Invitrogen Corporation, Carlsbad, CA). Fifty microliters of Pansorbin (Calbiochem), which had been incubated with propidium iodide (100 mg/mL) for 30 min and washed twice, was added to the 450 μL of blood stained with dihydrorhodamine. After incubation at 37°C for 10 min, a 50-μL sample (time 10) was removed and lysed as with the time-0 sample. All samples were washed twice and resuspended in HBSS. The time-0 cells served as the control cells for each sample. Percentages of cells expressing cell surface cluster of differentiation (**CD**)18 (adhesion molecule) were measured using flow cytometry. Whole blood (500 μL) was incubated at 23°C for 30 min using fluorescein isothiocyanate-labeled CD18 (Dako, Carpinteria, CA). After lysing and resuspending in HBSS, the percentage of cells fluorescing and the mean fluorescence were determined for 5,000 cells, using a Coulter Elite flow cytometer (Beckman Coulter Inc., Hialeah, FL) with a 488-nm air-cooled argon laser for excitation and a 525-band pass for fluorescein isothiocyanate labels and a 575-band pass for PE labels. Percentage of cells expressing CD18 above the control cells and percentage of cells exhibiting increased fluorescence of CD18 above a second gate were calculated.

Microbial Analysis

Fecal samples were kept on ice for transport to the laboratory and frozen immediately at −80°C. The samples were shipped to the National Animal Disease Center, USDA-ARS, Ames, IA, for analysis of *Salmonella* Typhimurium and *Escherichia coli* O157:H7.

Enrichment of Salmonella Typhimurium and E. coli, and Extraction of the PCR Template. Feces were weighed and 1:10 (wt/vol) of gram-negative broth:tripicase soy broth was added to a 50-mL conical centrifuge tube, vortexed, and incubated (6 to 18 h at 37°C with shaking). All centrifugations were at 25°C. Tubes were centrifuged (1,000 × *g* for 2 min), and then 50 μL of the clarified liquid was transferred to a screw-capped tube containing 950 μL of gram-negative broth:tripicase soy broth and centrifuged (2 min at 14,000 × *g*), and the supernatant was discarded. To the remaining pellet, 200 μL of InstaGene matrix (Catalog No. 732-6030, Bio-Rad Laboratories, Hercules, CA) was added, the tube was vortexed for 10 s and incubated (boiling water bath for 8 to 10 min), vortexed for 10 s, and centrifuged at 14,000 × *g* for 3 min. One-hundred microliters of supernatant was transferred to a fresh tube (avoiding the pellet) and frozen immediately at −80°C until used in PCR analysis. One microliter of supernatant was used per 10 μL of PCR reaction volume.

Detection of Salmonella Typhimurium Phagetype DT104 and E. coli O157. The presence of *Salmonella* Typhimurium and *E. coli* O157:H7 serotypes in the enrichments was detected by multiplex real-time PCR using primers and probes (Carlson et al., 1999; Sharma and Carlson, 2000). However, the *E. coli* O157:H7 primers and probe were replaced with a newer set developed recently (Sharma, 2002). Conditions for PCR were 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.2 m*M* deoxynucleotide 5′-triphosphate, 450 n*M* of each sipB-sipC (DT104) primer, 300 n*M* of *E. coli* primer, 100 n*M* of each fluorogenic (TaqMan) probe, and 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Cycling conditions included a 10-min, 94°C activation step followed by 40 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 45 s. On completion of the 40 cycles, the data were analyzed using Applied Biosystems Sequence Detection System software. Positive reactions were determined by examining the $R_n/\Delta R_n$ (where R is the cycle threshold) and the increase in fluorescence of individual samples.

Statistical Analysis

Data were analyzed as a randomized complete block design with repeated measures over time using a mixed model (PROC MIXED, SAS Inst. Inc., Cary, NC) with a compound symmetry covariance structure specified (Littell et al., 1996). Model statements included terms for the fixed effects of treatment and time and treatment × time interactions, including the baseline pretransport data as time 1. Block (birth date) and sex were tested as random variables. Sex was removed from the model because it was not significant. Significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$. However, when weak trends ($P \leq 0.15$) for interactions were found, pair-wise comparisons were separated using the Fisher protected LSD in SAS. Chi-squared analysis in SAS was used to evaluate the presence of microbial populations, with α set at 0.05.

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Table 1. Means (±SE) of BW and feed intake (as fed) of calves either provided a 1-time subcutaneous electrolyte injection (control; CNT) immediately after a 2-h transport or fed supplements containing 2 or 70% β-glucan extracts from yeast cell walls (as fed) plus 250 mg/feeding of ascorbyl-2-polyphosphate (BG2 or BG70, respectively) for 28 d after transport

Item[1]	*P*-value[2]	Before transport, d 0	Day after transport			
			7	14	21	28
BW, kg						
CNT	Time = 0.001	44.5 ± 1.4	45.9 ± 1.3	47.8 ± 1.3	51.4 ± 1.6	55.4 ± 1.34
BG2		43.9 ± 1.4	46.0 ± 1.3	47.4 ± 1.4	50.9 ± 1.6	55.2 ± 1.34
BG70		42.6 ± 1.6	45.0 ± 1.5	46.5 ± 1.6	49.2 ± 1.8	54.1 ± 1.34
ADG, g						
CNT	Time = 0.001	—	259 ± 79	297 ± 79	492 ± 79	492 ± 67
BG2		—	285 ± 73	203 ± 73	431 ± 78	506 ± 64
BG70		—	357 ± 82	166 ± 86	376 ± 90	531 ± 75
Calf starter intake, g/d						
CNT	Treatment × time = 0.007	—	131 ± 101	325 ± 101	476 ± 101	1,032 ± 88[a]
BG2	Time = 0.001	—	85 ± 101	133 ± 101	501 ± 101	653 ± 88[b]
BG70		—	69 ± 117	159 ± 117	524 ± 117	620 ± 101[b]
DMI,[3] g/d						
CNT	Treatment × time = 0.007	—	650 ± 103	843 ± 103	995 ± 103	1,552 ± 103[a]
BG2	Time = 0.001	—	614 ± 95	703 ± 95	1,057 ± 95	1,161 ± 82[b]
BG70		—	593 ± 108	697 ± 108	1,029 ± 108	1,036 ± 92[b]
G:F						
CNT	Time = 0.08	—	0.40 ± 0.12	0.37 ± 0.12	0.51 ± 0.12	0.35 ± 0.10
BG2		—	0.57 ± 0.12	0.32 ± 0.12	0.44 ± 0.12	0.46 ± 0.09
BG70		—	0.60 ± 0.13	0.26 ± 0.13	0.34 ± 0.13	0.64 ± 0.11

[a,b]Means within day without common superscripts differ ($P < 0.05$). Superscripts are provided when an effect was detected.

[1]Treatments: CNT = electrolyte injection (Plasma-lyte A, Baxter Health, Deerfield, IL); BG2 = yeast cell-wall derivative containing 1.8% (approximately 2%) β-glucan (EnergyPlus, Natural Chem Group LLC, Houston, TX) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ); BG70 = β-glucan (approximately 70%) extracted from yeast cell walls (BetaRight, BioThera, Eagan, MN) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products).

[2]*P*-value of significant effects or trends for main effects and interactions.

[3]DMI included milk-replacer solids and starter.

RESULTS

Growth Performance

Although BW were not different ($P > 0.10$) among treatments, intake of dry feed only on d 28 (1,032, 653, and 620 g/d for CNT, BG2, and BG70, respectively) and DMI (dry feed plus milk-replacer DM; 1,552, 1,161, and 1,036 g/d for CNT, BG2, and BG70, respectively) only on d 28 were less ($P < 0.01$) for BG2 and BG70 than for CNT (Table 1). Feed efficiency was not different among treatments ($P > 0.10$), but a trend ($P = 0.08$) for a time effect was detected such that G:F was least on d 14 and greater on d 21 and 28.

Hematology

Hematocrit and leukocyte counts are summarized in Table 2. Hematocrit percentages tended to be greater ($P = 0.09$) for the BG2 treatment than for the BG70 and CNT treatments. Hematocrit percentages increased ($P = 0.002$) throughout the 28-d experiment. White blood cell counts (treatment × time interaction, $P = 0.066$) were less for the calves given BG70 compared with those given BG2 ($P = 0.01$) and for CNT calves ($P = 0.04$) on d 28. Granulocyte counts changed ($P = 0.04$) throughout the experiment, with no differences among treatments detected ($P > 0.10$). A trend ($P = 0.077$) for a treatment × time interaction was detected for PBMC counts. Peripheral blood mononuclear cell counts were greater ($P = 0.006$) for calves treated with BG2 than for CNT calves on d 3. Calves given the BG70 supplement had fewer PBMC than calves given the BG2 supplement on d 21 ($P = 0.03$) and on d 28 ($P = 0.05$). Ratios of granulocytes to PBMC were not different ($P > 0.10$) among treatments.

Fibrinogen

Plasma fibrinogen concentration (Figure 1) was greater for calves before transport (time effect, $P = 0.002$). Fibrinogen concentrations decreased throughout the study until d 21 and 28. No differences among treatments were detected ($P > 0.10$).

Leukocyte Function

Differences among treatments in leukocyte function (Figure 2) were evident only for phagocytosis. Calves in the BG70 group had fewer ($P = 0.02$) cells positive for phagocytosis compared with calves in the CNT group and tended ($P = 0.08$) to have fewer cells positive for phagocytosis than calves in the BG2 group. Time was a significant factor for phagocytosis ($P = 0.005$), oxida-

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Table 2. Means (±SE) of hematocrit and leukocyte counts of calves either provided a 1-time subcutaneous electrolyte injection (control; CNT) immediately after a 2-h transport or fed supplements containing 2 or 70% β-glucan extracts from yeast cell walls (as fed) plus 250 mg/feeding of ascorbyl-2-polyphosphate (BG2 or BG70, respectively) for 28 d after transport

Variable and treatment[1]	*P*-value[2]	Before transport, d 0	Day after transport					
			3	7	10	14	21	28
HCT, %								
CNT	Treatment = 0.09	29.4 ± 1.9	30.3 ± 2.0	29.7 ± 1.9	34.0 ± 1.9	31.7 ± 1.7	31.6 ± 1.4	34.0 ± 1.3
BG2	Time = 0.002	31.8 ± 1.8	31.9 ± 1.9	34.3 ± 1.8	33.0 ± 1.8	32.8 ± 1.6	34.4 ± 1.4	37.9 ± 1.4
BG70	Treatment × time = 0.15	28.9 ± 2.1	27.2 ± 2.2	27.6 ± 2.1	27.6 ± 2.1	29.4 ± 1.9	30.1 ± 1.7	32.6 ± 1.5
WBC, $\times 10^6$								
CNT	Treatment × time = 0.066	11.5 ± 1.0	10.1 ± 1.7	10.7 ± 1.3	11.0 ± 1.1	11.2 ± 0.9	10.5 ± 1.0	12.5 ± 1.1[a]
BG2		11.1 ± 1.0	12.4 ± 1.5	12.9 ± 1.4	10.4 ± 1.0	10.1 ± 0.8	12.2 ± 1.0	14.4 ± 1.2[a]
BG70		13.3 ± 1.1	12.6 ± 1.6	13.1 ± 1.3	10.1 ± 1.2	10.8 ± 1.0	9.1 ± 1.2	10.2 ± 1.2[b]
GRN, $\times 10^6$								
CNT	Time = 0.04	4.3 ± 0.8	4.3 ± 1.3	3.9 ± 1.0	3.8 ± 0.7	4.3 ± 0.6	3.9 ± 0.7	5.2 ± 0.9
BG2		4.2 ± 0.7	4.4 ± 1.3	5.1 ± 1.0	3.1 ± 0.7	3.4 ± 0.6	4.5 ± 0.7	5.2 ± 1.0
BG70		5.3 ± 0.9	5.0 ± 1.5	6.0 ± 1.2	3.6 ± 0.8	3.9 ± 0.6	2.6 ± 0.8	3.4 ± 1.0
PBMC, $\times 10^6$								
CNT	Treatment × time = 0.077	7.2 ± 0.7	5.8 ± 0.6[b]	6.9 ± 0.6	7.3 ± 0.6	7.0 ± 0.5	7.0 ± 0.6[ab]	7.3 ± 0.6[ab]
BG2		7.1 ± 0.7	8.2 ± 0.6[a]	7.9 ± 0.5	7.3 ± 0.5	6.9 ± 0.5	7.5 ± 0.6[a]	8.3 ± 0.5[a]
BG70		7.8 ± 0.8	7.1 ± 0.5[ab]	6.5 ± 0.6	5.6 ± 0.6	6.7 ± 0.6	5.4 ± 0.7[b]	6.4 ± 0.6[b]
GRN:PBMC								
CNT		0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.3
BG2		0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.3
BG70		0.9 ± 1.2	0.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.8 ± 0.3

[a,b]Within a day, means of a variable without common superscripts differ ($P \leq 0.05$).

[1]Variables: HCT = hematocrit; WBC = white blood cell counts; GRN = granulocytes; PBMC = peripheral blood mononuclear cells. Treatments: CNT = electrolyte injection (Plasma-lyte A, Baxter Health, Deerfield, IL); BG2 = yeast cell-wall derivative containing 1.8% β-glucan (EnergyPlus, Natural Chem Group LLC, Houston, TX) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ); BG70 = β-glucan (approximately 70%) extracted from yeast cell walls (BetaRight, BioThera, Eagan, MN) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products).

[2]*P*-value of significant effects or trends for main effects and interactions.

tive burst ($P = 0.001$), CD18 expression ($P = 0.001$), and increased fluorescent CD18 expression ($P = 0.006$). All measures of leukocyte responses increased over the 28-d experiment.

Microbial Analysis

The frequency of *Salmonella* Typhimurium DT104 detection in weekly fecal samples was not different ($P > 0.10$) among treatments (data not shown). The percentage of calves positive for *E. coli* O157:H7 was greater ($P = 0.04$) for BG2-supplemented calves than for BG70-supplemented and CNT calves on d 7 (Figure 3). There was a time effect ($P = 0.05$) of calves positive for *E. coli* O157:H7 for all treatments.

DISCUSSION

Body weight loss occurred for all treatments after transport. Although not typical, studies have shown that calves may have decreased BW gain in the second or third week of life (Eicher et al., 1994; Todd et al., 2000). This was evident in both the BG2 and BG70 treatments on d 14. Although animals from both the BG2 and BG70 treatments gained more BW in the fourth week than in the previous week, feed intake did not increase as dramatically as in the CNT treatment. The total BW of the calves by d 28 was also similar to that of the CNT calves. This observation implies that transport was not as great a stressor for which dietary supplements might be beneficial, as seen with a weaning stressor in other species. Neonatal pigs supplemented with β-glucan and ascorbic acid had improved BW gain at weaning (Eicher et al., 2006) compared with control pigs and pigs supplemented with ascorbic acid only. However, nutrient supplementation can have unexpected results when paired with stressors such as transportation. For example, feeding vitamin C alone after transport resulted in depressed keyhole limpet antibody titers of calves (Tyler and Cummins, 2003).

Relatively large morbidity and mortality rates during the weeks after transport have been observed previously (Trunkfield and Broom, 1990; Knowles, 1995). Immunological responses to a transport stressor are not evident until d 7 after the transport of neonates (Tyler and Cummins, 2003), implying that the effects of stress are delayed in young calves compared with mature cattle. Additionally, Knowles et al. (1997) noted that young transported calves do not have the same sharp increases in heart rate, plasma cortisol, and glucose that are typical of older transported animals (Eicher and Burton, 2005), which may explain some

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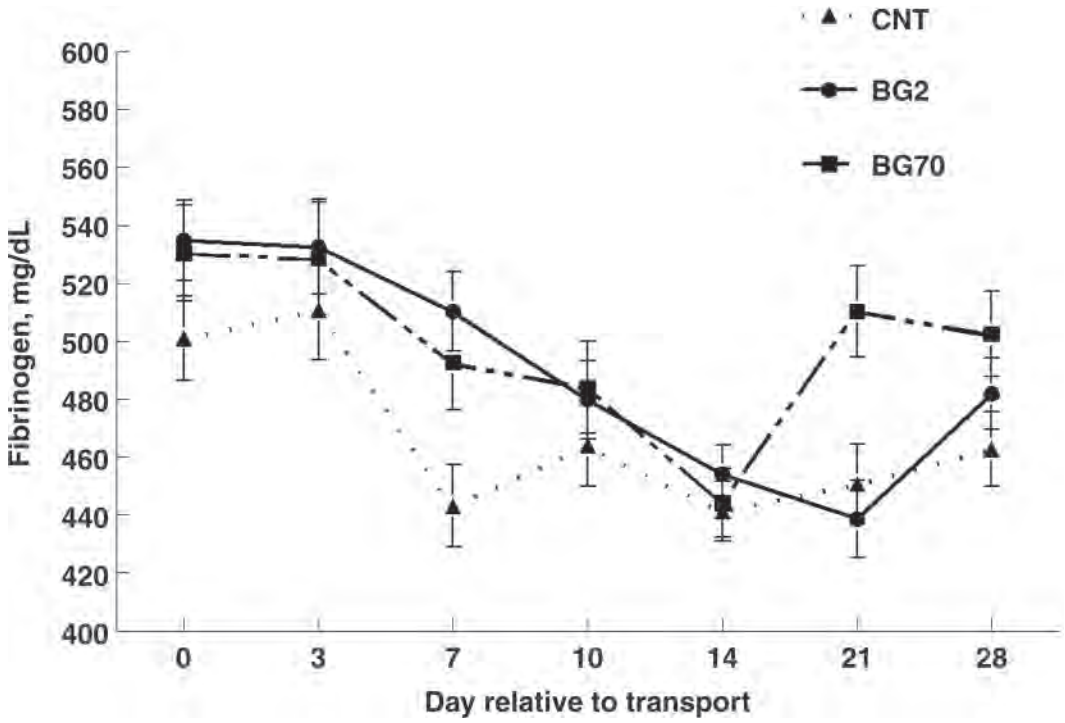

Figure 1. Plasma fibrinogen concentrations of calves given a control (CNT), 2% β-glucan plus ascorbyl-2-polyphosphate (BG2), or 70% β-glucan plus ascorbyl-2-polyphosphate (BG70) treatment. Time effect ($P = 0.002$). Data are least squares means ± SE. CNT = 1-time subcutaneous electrolyte injection (Plasma-lyte A, Baxter Health, Deerfield, IL); BG2 = 113 g/feeding of yeast cell-wall derivative containing 1.8% β-glucan (approximately 2%; EnergyPlus, Natural Chem Group LLC, Houston, TX) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ); BG70 = β-glucan (approximately 70%) extracted from yeast cell walls (BetaRight, BioThera, Eagan, MN) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products).

of the responses in the current study. However, in the present study, PBMC counts were greater for calves supplemented with BG2 and tended to be greater for calves supplemented with BG70 than for CNT calves at d 3. In contrast to the d 3 response, white blood cell counts were less for calves supplemented with the more purified β-glucan and ascorbic acid (BG70) at 28 d, and differences between the β-glucan treatments were evident in PBMC counts at d 28. This observation implies that dietary treatment takes approximately 3 wk to alter blood cell counts, but with the transport stressor, early supplementation may be beneficial by stabilizing immune responses. Additionally, we observed an overall effect of BG70, but not BG2, to suppress the phagocytosis by leukocytes to near CNT values. This implies some differential leukocyte activity that could be attributed to something other than the β-glucan factor, such as mannan and starch concentration differences in the supplements or other factors resulting from yeast growth conditions and extraction processes.

Fibrinogen is an acute phase protein that is a known indicator of stress of transported cattle (Arthington et al., 2003). Neonatal calves have increased fibrinogen concentrations after birth (Thornton et al., 1972). Calves in this study ranged from 3 to 10 d of age at transport, during which fibrinogen concentrations decrease rapidly, as reported by others (Thornton et al., 1972). Therefore, detection of treatment effects may have been obscured by the increased concentrations after birth. Fibrinogen concentrations of calves in all treatments decreased throughout the experiment to near adult concentrations.

Published data on cattle transport are derived from older, weaned calves (Steinhardt and Thielscher, 2000; Odore et al., 2004; Ishizaki et al., 2005), different from those used in the current study. However, studies of neonatal calves and transport stress have demonstrated the importance of metabolic differences in neonatal vs. older calves (Todd et al., 2000). Metabolic changes have been reported to affect the shedding of commensal and pathogenic bacteria (Bach et al., 2004). Preconditioning of mature cattle for long transports (15 h) was shown to alleviate the *E. coli* O157:H7 detected in feces (Bach et al., 2004). Our approach was to postcondition, rather than precondition, neonatal calves that were consuming primarily liquid feed. However, we noted that β-glucan increased the number of days that feces were positive for naturally occurring *E. coli* O157:H7. This observation could have 2 opposite implications. The consequences are not acceptable if we assume that feces that are positive for a pathogen are undesirable and indicative of infection and that they have potential for the spread of disease or carcass contamination at slaughter. However, if we postulate that detecting *E. coli* in the feces could indicate its clearance from the intestine and not gut colonizing, then more days of detection could

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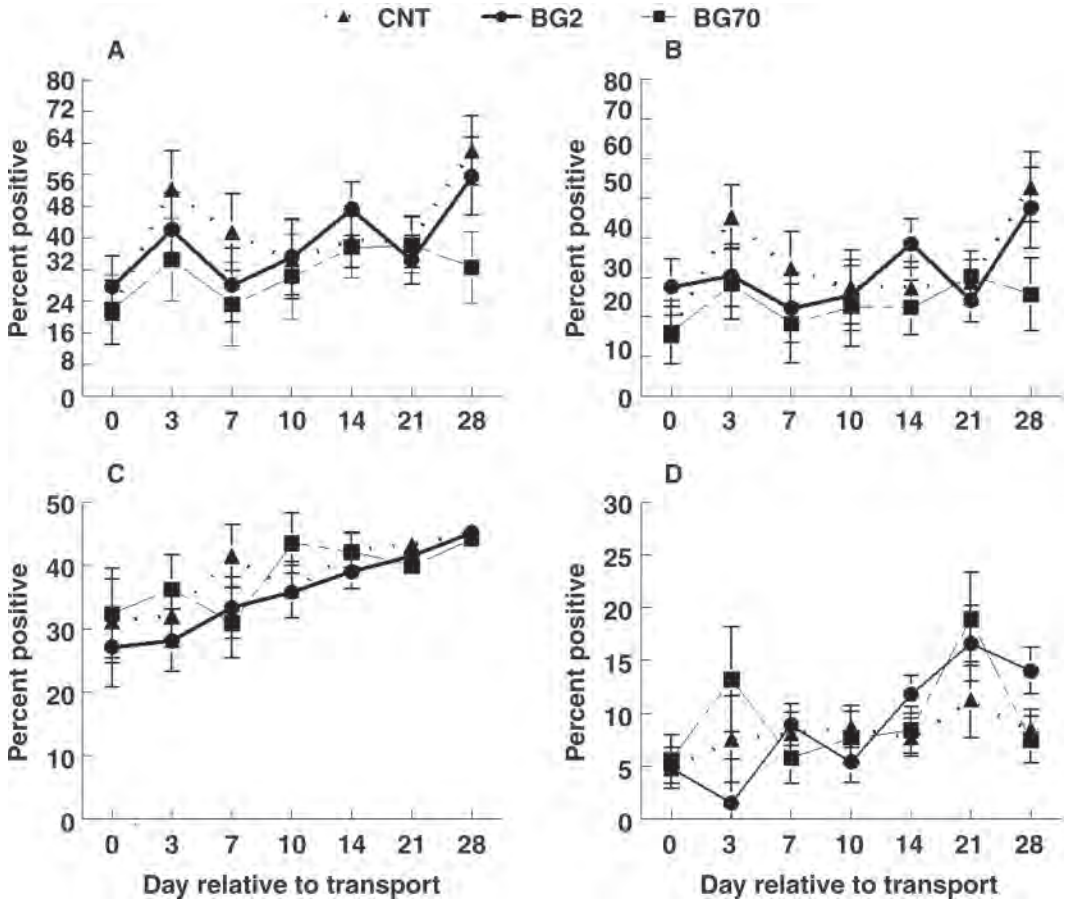

Figure 2. Percentage of cells with fluorescence from phagocytosis of unopsonized *Staphylococcus aureus* (panel A) and oxidative burst (panel B). Phagocytosis was greater ($P = 0.05$) for cells from control calves (CNT) compared with cells from calves given 70% β-glucan (BG70), and a time effect was detected ($P = 0.005$). Oxidative burst showed a trend ($P = 0.10$) for CNT calves to have more cells with fluorescence from oxidative burst compared with calves in the BG70 treatment. Percentage of cells fluorescing with fluorescein isothiocyanate-labeled cluster of differentiation (CD)18 antibody (panel C) increased over the study ($P = 0.001$). Percentage of cells that exhibited increased CD18 fluorescence (panel D) also increased over the study, peaking on d 21 ($P = 0.006$). Data are least squares means ± SE. CNT = 1-time subcutaneous electrolyte injection (Plasma-lyte A, Baxter Health, Deerfield, IL); BG2 = 113 g/feeding of yeast cell-wall derivative containing 1.8% β-glucan (approximately 2%; EnergyPlus, Natural Chem Group LLC, Houston, TX) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ); BG70 = β-glucan (approximately 70%) extracted from yeast cell walls (BetaRight, BioThera, Eagan, MN) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products).

be considered a positive indicator. Because cattle lack a Shiga toxin receptor, most *E.* coli O157:H7-infected cattle remain healthy (Pruimboom-Brees et al., 2000). However, the pathogen can live and replicate within the intestinal tract, creating a potential human health hazard when it is shed in the feces.

Therefore, we are possibly observing 2 or 3 mechanisms of supplementation with β-glucan plus ascorbic acid. First, we saw an effect on feed intake by d 28. From these data, we cannot determine if it is a physical mechanism, such as a slowing of intestinal motility, or if it is a physiological modulation of metabolism. Second, in both supplements we saw an effect of β-glucan to tend to increase the number of days that calves were positive for *E. coli* O157:H7 or the percentage of calves that were positive earlier than CNT calves. It is not known how the β-glucan interacts with a liquid diet, as opposed to a diet consisting primarily of dry feed. However, that may be an advantage of providing the β-glucan during the first 3 wk for increasing pathogen shedding rather than colonization. Third, we saw an attenuation of leukocyte function that occurred with the more purified β-glucan supplemented at lesser concentrations. There was a divergence in the response of the 2 supplements for many of the immune measures at 21 and 28 d. Therefore, there is a need to elucidate whether this is a form or concentration effect (i.e., whether the β-glucan alone, delivered at the same concentration as the mannan and β-glucan (EnergyPlus), could show the same response). Additionally, it is important to investigate whether the BG2 immune response is more protective against pathogens. Challenge studies are necessary to determine if an activated or quiescent immune system before challenge is beneficial. These data from the current study imply that these yeast cell-wall extracts plus ascorbic acid supplements may be beneficial to enhance dry feed consumption and may be beneficial to alleviate transport stress in calves, but the benefits of the 2 supplements need to be determined because they may have immunologically opposite effects.

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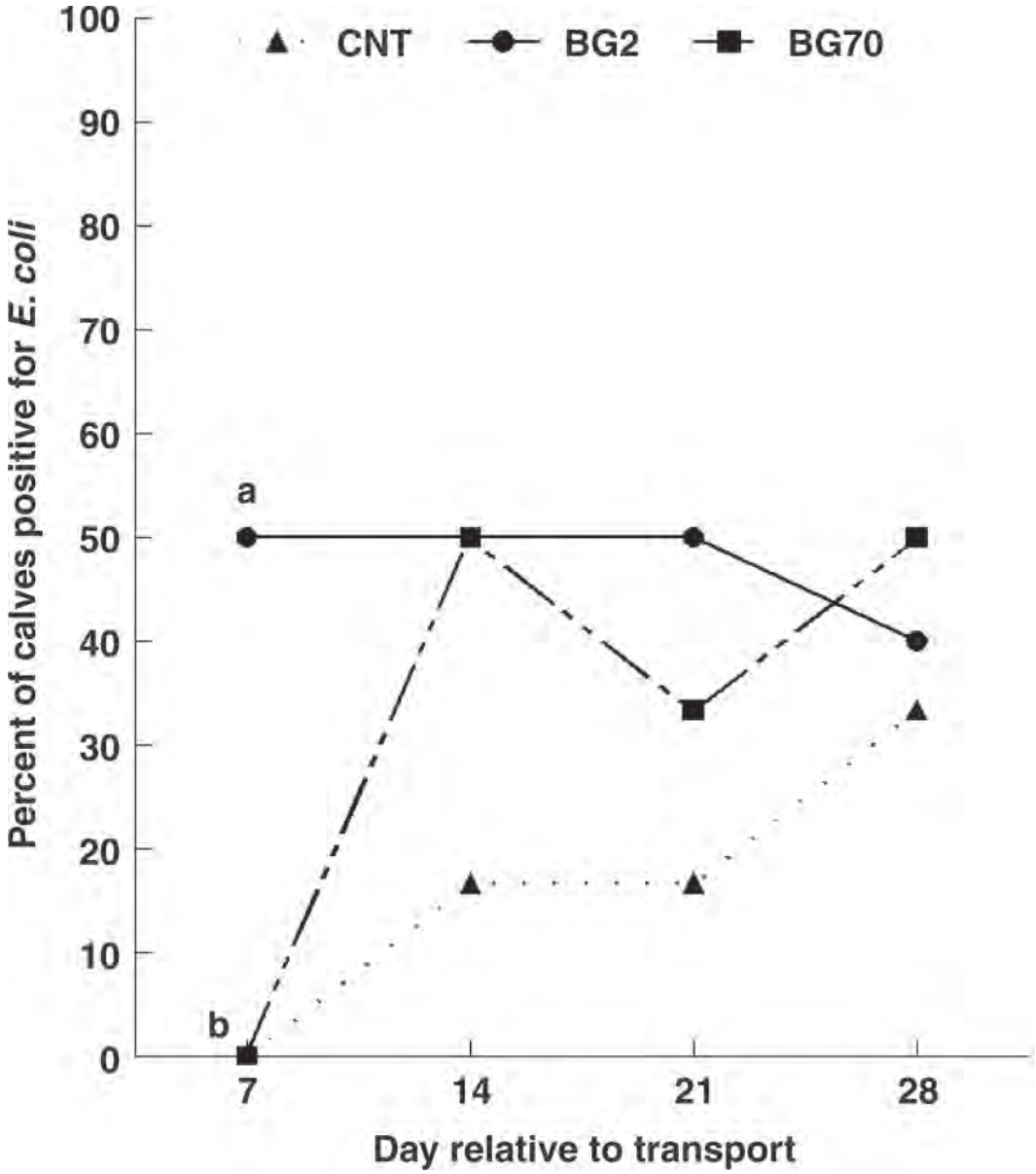

Figure 3. Percentage of days calves were positive for *Escherichia coli* O157:H7 for each treatment by week. [a,b]Means within a day without a common letter differ ($P \leq 0.05$). Data are least squares means ± SE. CNT = 1-time subcutaneous electrolyte injection (Plasma-lyte A, Baxter Health, Deerfield, IL); BG2 = 113 g/feeding of yeast cell-wall derivative containing 1.8% β-glucan (approximately 2%; EnergyPlus, Natural Chem Group LLC, Houston, TX) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products, Parsipanny, NJ); BG70 = β-glucan (approximately 70%) extracted from yeast cell walls (BetaRight, BioThera, Eagan, MN) plus 250 mg/feeding of ascorbyl-2-polyphosphate (Stay-C, DSM Nutritional Products).

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Vaccine efficacy of porcine reproductive and respiratory syndrome virus chimeras

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ABSTRACT

The vaccine efficacy of six PRRSV Type 2 infectious clones, including five chimeras and a strain-specific deletion mutant, were examined using a respiratory challenge model in growing swine. The chimeras were constructed from different combinations of a licensed modified live vaccine (Ingelvac® PRRS MLV) and a virulent field isolate (*wt* MN184) which differ by 14.3% on a nucleotide basis, while the deletion mutant tested had a broad deletion in the nsp2 region of strain MN184. The appearance of antibodies and virus characterization revealed regions of the genome that could influence PRRSV replication *in vivo*. Swine growth, clinical signs and lung lesions were also monitored. Average daily weight gain was negatively and directly impacted by some vaccines, and after challenge, vaccination with different constructs led to variable weight gain. We determined that 3 of the tested chimeras, including two previously published chimeras [1] and one in which strain MN184 ORF5-6 was placed on the background of Ingelvac® PRRS MLV were able to prevent lung consolidation to a similar extent as traditionally prepared cell-passaged attenuated vaccines. The study suggested that only specific chimeras can attenuate clinical signs in swine and that attenuation cannot be directly linked to primary virus replication. Additionally, the strain MN184 deletion mutant was not found to have been sufficiently attenuated nor efficacious against heterologous challenge with strain JA-142.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) emerged to cause clinical problems in animals in the early 1990s on separate continents [2,3]. Since then, the virus has spread to most swine producing regions of the world. PRRSV has been found to vary as much as 40% in nucleotide sequence and has been separated into two genotypes, European (Type 1) and North American (Type 2), based upon their original isolation location and date. As a result of this overwhelming diversity, the swine immune response is often not cross protective. PRRSV also induces a poor immune response in most animals. This incomplete protection appears to be due to several factors including the nature of the virus, the genetics of swine host and the complication of co-infection with other pathogens [4,5]. One traditional method of vaccine preparation, culturing the virus over several *in vitro* cell passages in order to attenuate clinical symptoms, has resulted in several available products for use in the field [6,7]. However, this can lead to incomplete protection against heterologous PRRSV strains and an uncertainty in selection of the appropriate vaccine for routine use [5]. A newer approach has been to evaluate infectious cDNA clones of PRRSV, which can represent chimeras or site-specific changes that may potentially increase the immune response, and may also have specifically engineered deletions and/or insertions to provide markers for vaccine identification [1,8–16].

Limited reports using this new approach have suggested that specific PRRSV chimeras can provide direct attenuation of clinical signs in either a respiratory model or a reproductive failure model [1,10]. Our previous studies have demonstrated that two reciprocal chimeras (rMLVORF1/MN184 and rMN184ORF1/MLV) of Type 2 PRRSV strains, Ingelvac® PRRS MLV and *wild-type* (*wt*) isolate MN184, could attenuate clinical signs of young swine after heterologous challenge with PRRSV strain SDSU73 in a respiratory challenge model [1]. Therefore, it was hypothesized that additional chimeras created from the same parent strains, but with different

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regions of the genome exchanged, would be more efficacious in the face of virulent challenge than commercially available products and perhaps provide an alternate method of PRRSV attenuation.

To extend these original studies, we examined the two original [1] as well as three additional chimeras, plus a deletion mutant recombinant of *wt* strain MN184, under different challenge conditions. The new genomic areas of study were chosen to consider the contributions of key regions of a virulent strain in inducing protection against subsequent heterotypic virus exposure. PRRSV ORF5-6 code for the major viral attachment domain, ORF7 encodes the nucleocapsid protein that surrounds the genome and interacts with the structural proteins in the virion, and the 3′UTR is critical to successful transcription of subgenomic RNAs and replication [17,18]. The additional chimeras were synthesized using an Ingelvac® PRRS MLV backbone. In addition, the replicase region known as nonstructural protein 2 (nsp2) has been shown to be immunogenic, contains hypervariable segments, encodes a protease responsible for replicase cleavage and harbors B-cell epitopes [12,19–25]. Thus, in order to examine a possible role for nsp2 in protection, recombinant strain MN184 was modified by removing 618 bases of the nsp2 coding region. The six engineered viruses were used as vaccines in parallel with two conventionally attenuated PRRSV strains, Ingelvac® PRRS MLV and newly prepared MN184 (MN184-P102). The vaccinated animals were then challenged with *wt* Type 2 strain JA-142. The appearance of antibodies and virus characterization were followed over the course of the study. The results of these assays revealed regions of the genome that influence PRRSV replication *in vivo*. Swine growth, clinical signs and lung lesions were also monitored. Average daily weight gain was negatively and directly impacted by some vaccines, and after challenge, vaccination with different constructs led to variable weight gain. We also found that only the original chimeras and the one in which strain MN184 ORF5-6 was placed on the background of Ingelvac® PRRS MLV were able to prevent lung consolidation after strain JA142 challenge to a similar extent as the cell-passaged attenuated vaccines. The outcomes of this study suggested that only specific chimeras can attenuate clinical signs in swine and that attenuation cannot be directly linked to primary virus replication. Additionally, a large deletion in the nsp2 region of strain MN184 was not sufficient to reduce the pathogenicity of that strain, or serve as an adequate vaccine against heterologous challenge with strain JA-142.

2. Materials and methods

2.1. Cells and viruses

MA-104 cells (ATCC CRL2621) or MARC-145 cells, both African Green monkey kidney cell lines which support the growth of PRRSV, were cultured in minimum essential medium (EMEM, SAFC Biosciences M56416) with 10% fetal bovine serum (Invitrogen) at 37 °C, 5% CO_2. PRRSV vaccine Ingelvac® PRRS MLV and *wt* isolate MN184 were previously described [1,26]. Two recombinant viruses, rMLV and rMN184, and two chimeric viruses, rMLVORF1/MN184 and rMN184ORF1/MLV, were rescued from cDNA clones (GenBank EF484031–EF484034) described previously [1]. Other recombinant viruses were generated as described below. MN184-P102 was prepared by successive passages of MN184C (GenBank EF488739) on MARC-145 cells at Boehringer Ingelheim Vetmedica, Incorporated. PRRSV strain JA-142 (AY424271) was used as a heterologous challenge virus for the swine studies and has also been characterized previously [27,28].

2.2. Construction of PRRSV cDNA clones

Different sections of pMLV were replaced with comparable sections of pMN184 using specific restriction enzyme sites (Fig. 1) or the primers listed in Table 1. The correct nucleotides of the exchanged regions of every clone were confirmed by DNA sequencing. The nucleotide and amino acid changes as a result of the cloning are listed in Table 2.

pMLV/MN184ORF5-6 (GenBank Accession FJ629369) possessed nucleotides (nt) 1–13,650 and 14,823–15,452 of pMLV; nt 13,651–14,822 were exchanged for pMN184 13,257–14,429 by PciI and SmaI restriction digest of subclone IV of each full-length plasmid (Fig. 1). pMLV/MN184ORF7-3′UTR (GenBank Accession FJ629370) consisted of nt 1–14,822 of pMLV; nt 14,823–15,452 were replaced by pMN184 14,430–15,060 by SmaI digestion of subclone IV of each full-length plasmid. In both constructs, the new subclone IV replaced its counterpart in pMLV. The specific regions targeted span nucleotides 13,789–14,391 (ORF5), 14,376–14,900 (ORF6) and 14,890–15,452 (ORF7 and 3′UTR) of the parental virus, Ingelvac® PRRS MLV.

pMLV/MN184-3′UTR (GenBank Accession FJ629371) was obtained in the following manner. One PCR product was ampli-

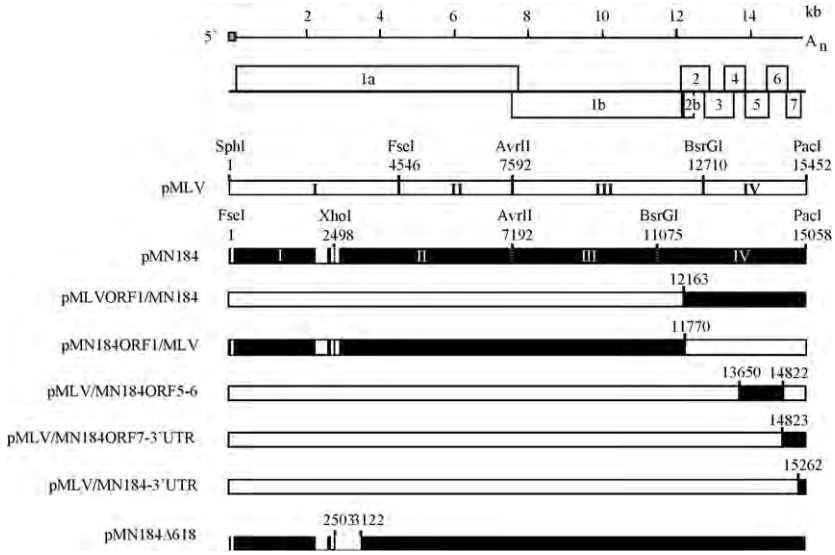

Fig. 1. Genome schematic of PRRSV, the two infectious clones initially derived (pMLV and pMN184 [1]) and chimeric and deletion mutants prepared from these clones for the present study.

Table 1
Primers used in preparation of recombinant PRRSV infectious clones. Forward primers indicated by a slash (/) following the name and reverse primers by a slash before the name. Primers were positioned based on genomic sequences pMLV (MLV; EF484033) or pMN184 (MN184; EF484031) (most primers anneal to both sequences).

Primer	Nucleotide Position	Sequence
Synthesis of PRRSV recombinants		
MLV-ORF6-F/	MLV 14,192–14,217	5′-GCTACGCGTGTACCAGATATACCAAC
/MLV-ORF7-R	MLV 15,249–15,277	5′-CAAGAATGCCAGCTCATCATGCTGAGGGT
184-3′UTR-F/	MN184 14,856–14,884	5′-ACCCTCAGCATGATGAGCTGGCATTCTTG
/184-3′UTR-R	MN184 15,011–15,099	5′-GTCTTTAATTAACTAG$(T)_{30}$AATTTCGGC
184-3122-F/	MN184 2494–2503/3122–3136	5′-AAGCTCGAGCTGTGGGTTTGTGATG
/184-4083-R	MN184 4065–4091	5′-AAAACCGGTCGCACAGGTCGACAAGTG

Table 2
Nucleotide and amino acid changes to recombinant viral parent due to clone construction.

Construct	Region	NT Changes	AA Changes
pMLV/MN184ORF5-6	GP4	11	0
	GP5	81	31
	M	37	6
pMLV/rMN184ORF7-3′UTR	M	5	2
	N	26	6
	3′UTR	9	–
pMLV/MN1843′UTR	3′UTR	9	–
pMN184Δ618	nsp2	Δ618	Δ206

fied using pMLV and primer pair MLV-ORF6-F/MLV-ORF7-R and another product representing the 3′UTR of MN184 was amplified from pMN184 using 184-3′UTR-F/184-3′UTR-R. Overlapping PCR was then completed with both PCR products and MLV-ORF6-F/184-3′UTR-R. The PCR product was then digested with SmaI and PacI and cloned into subclone IV of pMLV, which was then used to replace part IV in pMLV. The final pMLV/MN1843′UTR construct possessed nucleotides nt 1–15,261 of MLV with only the 3′UTR (15,262–15,452) replaced with 14,869–15,058 of pMN184.

As reported, *wt* strain MN184 has a tripartite deletion totaling 393 bases in the nsp2 region when compared to other sequenced viruses [20]. It was of interest to assess additional nucleotide deletions in regards to MN184 strain virulence reduction and the capacity of the mutated virus to protect against strain JA-142 challenge. One PCR product was amplified using pMN184 and primer pair 184-3122-F/184-4083-R, which was then digested with XhoI and AgeI, cloned into subclone II of pMN184 and then into a full-length viral plasmid. As a result, a 618 nucleotide segment of nonstructural protein 2 (nt 2504–3121) was removed from pMN184 to produce the final pMN184Δ618 construct (14, 440 bp; GenBank Accession FJ629372).

2.3. Rescue of viruses

The cDNA clones were linearized with PacI and then transcribed *in vitro* (mMessage Machine Kit, Applied Biosystems). RNA transcripts (2.5 μg) of each clone were subsequently transfected into confluent MA-104 cells using DMRIE-C (Invitrogen), as described previously [1]. The transfection supernatants were collected when cytopathic effect (CPE) was approximately 80% and cell debris was then removed by centrifugation at 4000 × *g*. Recombinant viruses were passaged on MA-104 cells a total of 4 times to yield viral stocks sufficient in volume and titer to allow for vaccination studies. The rescued viruses were named rMLV/MN184ORF5-6, rMLV/MN184ORF7-3′UTR, rMLV/MN184-3′UTR and rMN184Δ618. Total RNA was extracted from an aliquot of passage 4 supernatant of each rescued virus and analyzed by RT-PCR followed by 3′ end sequencing of approximately 4000 bases.

2.4. Swine study

The study utilized 100, healthy 3-week old, commercial crossbred piglets from a PRRSV seronegative herd to examine PRRSV vaccine efficacy in a respiratory challenge model. Animals were housed in a conventional setting at Veterinary Resources Inc. in Ames, Iowa and were under the supervision of a veterinarian. Throughout the duration of the study, all animals received food and water *ad libitum*. All laboratory personnel and animal caretakers involved with the study were blinded to the treatments given to the respective groups.

The study consisted of 10 groups, including 6 infectious clones (groups 1–6), *wt* MN184 at passage 102 (MN184-P102; group 7), Ingelvac® PRRS MLV (group 8), heterologous challenge virus *wt* JA-142 (group 9), and a strict control group (group 10) (Table 3). Animals were required to test negative for PRRSV antibody by HerdChek® PRRS ELISA 2XR and then randomly assigned by weight into each treatment group prior to vaccination (IDEXX Laboratories Inc., Westbrook, ME). Viral titers were determined by $TCID_{50}$/ml (Table 3) [29,30]. All viruses were diluted with minimum essential medium (MEM; Sigma, St. Louis, MO) containing 2% fetal bovine serum (FBS) (Sigma, St. Louis, MO) in order to deliver 4.79 logs of virus in 2 ml, intramuscularly, to each animal. The challenge control (group 9) and strict control (group 10) groups received only dilution medium. For testing purposes, 10–15 ml of blood was collected from each animal on Days 0, 3, 7, 14, 21, 28, 31, and 35. Serum was separated from the clotted blood and stored for a maximum of 24 h at 4 °C for testing. Aliquots were then frozen at −70 °C.

Table 3
Treatment list, titer information and whole genome percent nucleotide identity to the challenge virus.

Group #	Treatment	Original titer (Log_{10} $TCID_{50}$/ml)	Percent nucleotide identity to JA-142
1	rMN184ORF1/MLV	5.02	84.2
2	rMLVORF1/MN184	5.70	90.2
3	rMLV/MN184ORF5-6	5.63	90.7
4	rMLV/MN184ORF7-3′UTR	5.50	90.9
5	rMN184Δ618	5.35	90.9
6	rMLV/MN184-3′UTR	4.96	80.4
7	MN184-P102	4.49	83.1
8	Ingelvac® PRRS MLV	4.09	91.0
9	N/A		
10	N/A		

The challenge model used in this study is consistent with the model used in the original characterization of the chimeras [1]. This model calls for virulent challenge three weeks after vaccination (Day 21) and necropsy at five weeks post-vaccination (Day 35). Challenge timing was chosen since the vaccine component of the chimeras, Ingelvac® PRRS MLV, has proven to be efficacious three weeks post-vaccination even though the vaccine virus may still be causing some viremia [31]. Therefore, it was expected that the chimeras would exhibit similar characteristics. Necropsy at two weeks post challenge allowed for maximal detection of lung lesions that correlate with *in vitro* testing procedures [32]. The animals in groups 1–9 were challenged intranasally with 3.8 logs in 2 ml (1 ml per nostril) of virulent JA-142 strain of PRRSV at cell passage 4. At necropsy all animals were humanely euthanized and assessed for gross lung lesions.

2.5. Clinical evaluation

General observations of each animal in the study were taken from Day 0 through Day 19 and anything abnormal was noted. From Day 20 through Day 35, clinical observations were noted and anything considered abnormal was recorded. Individual observations consisting of behavior, respiration, and cough were also recorded based on a numerical index from 1 to 4 that reflected the severity of the diseased state for each category. For instance, a normal animal received a score of 3 (3×1 each for behavior, respiration and cough), an animal exhibiting maximum clinical signs received a 9 (3×3), and a deceased animal received a cumulative score of 12 (3×4). In addition, animals were weighed 3 days before vaccination (Day −3), at challenge (Day 21), and at necropsy (Day 35) for average daily weight gain testing. The lungs of all animals in the study were evaluated at necropsy for percent consolidation due to PRRSV infection. Lungs were scored for each individual lobe, as well as an overall level of gross lung pathology using a standard scoring system [33]. The observation score equaled the sum of all the individual lobe scores.

2.6. Serology

Serum samples were analyzed for PRRSV antibody using the IDEXX HerdCheck® PRRS ELISA 2XR. The tests were performed as described by the manufacturer's instructions. Samples were considered positive for PRRSV antibodies if the sample-to-positive (S/P) ratio was at least 0.4.

2.7. Viremia detection by virus isolation and quantitative RT-PCR

To qualitatively determine viremia, virus isolation was performed on all serum samples from all collection days. Each animal was tested by inoculating 100 μl of serum individually onto 3-day-old MA-104 cells in a 48-well tissue culture plate which was then evaluated 8 days later for signs of cytopathic effect. The percent of positive animals at each bleed date was then recorded. To attain a relative quantity of viral RNA present, quantitative RT-PCR (qRT-PCR) was also performed on all serum samples. The QIAamp® Virus BioRobot® MDx Kit was used in conjunction with the BioRobot Universal System from Qiagen (Qiagen Inc., Valencia, CA) to extract the viral RNA from the serum per manufacturers recommendations. To detect US PRRSV nucleic acid, the North American Tetracore qRT-PCR kit (Tetracore, Inc., Rockville, MD) was used as described previously [29].

2.8. Nucleotide sequence analysis

Viral RNA was extracted from serum samples from each animal at day 21. To obtain a consensus nucleotide sequence of the structural genes at this time point, RNA extracts were pooled for each group and submitted for nucleotide sequence determination (oligonucleotide primers available on request). Sequences were analyzed using Geneious Pro Version 4.7.5 (Biomatters Limited). Approximately 4000 bases were sequenced at the 3′end of the viral genome. In the case of the nsp2 deletion mutant, rMN184Δ618, a 500 base section spanning the deletion site was examined.

2.9. Statistics/biometrics

All data were imported into SAS version 9.1 for management and preliminary analysis. Data listings and summary statistics by treatment group including mean, median, standard deviation, standard error, range, 95 percent confidence limits, coefficient of variation, and frequency distributions were generated for all variables where appropriate. All parameters were compared among groups 1–9 and pair wise between groups 1–9. Group 10 (strict controls) was not included in the analyses other than summary statistics. In compliance with the methods recommended by the United States Department of Agriculture Animal Plant Health Inspection Agency, only two-sided results were reported and all comparisons were at $\alpha = 0.05$. All data were transferred to Prism 4 (Graphpad Software, Inc.) for additional statistical analyses and optimal formatting prior to publication. Virus isolation data used Fisher's exact test to determine the number of animals positive/negative per group ratio. Weights and average daily weight gain (ADWG) were tested by two-way analysis of variance (ANOVA). Lung scores for each group were analyzed by one-way ANOVA and Bonferroni's Multiple Comparison Test (95% confidence interval).

3. Results

3.1. Recovery of viruses

Two chimeric viruses, rMLVORF1/MN184 and pMN184ORF1/MLV, were previously generated [1]. Four other recombinant PRRSV full-length cDNA clones, pMLV/MN184ORF5-6, pMLV/rMN184ORF7-3′UTR, pMLV/MN184-3′UTR and pMN184Δ618 were constructed in a similar manner (Fig. 1). To verify whether these four additional cDNA clones were infectious, linearized pMLV/MN184ORF5-6, pMLV/rMN184ORF7-3′UTR, pMLV/MN184-3′UTR and rMN184Δ618 were transcribed *in vitro* and the synthetic RNAs were subsequently transfected into MA-104 cells. Day 3 post-transfection, all four transfections resulted in the appearance of CPE, indicating that the genetic exchange between the two different strains and the 618 base deletion in the nsp2 region of MN184 did not have a severe effect on the *in vitro* growth properties of the recombinants. Sequence analyses of around 4000 bases at the 3′-end of the genome confirmed that these four viruses were recovered from the respective recombinant PRRSV with no or a few scattered changes (data not shown). All chimeras were passaged 4 times on MA-104 cells in parallel with parental rMLV and rMN184 as well as Ingelvac® PRRS MLV vaccine and *wt* MN184. At each passage, onset of CPE in rMLV/MN184ORF5-6 and rMLV/MN184-3′UTR infected MA-104 cells was similar to those infected with rMLV, but appeared 1 day later for rMLV/MN184ORF7-3′UTR infected cells. CPE for rMN184Δ618 infected cells was similar to rMN184 at all four passages. Passage 4 viruses were titered and used to infect 10 animals/group in the vaccination study (Table 3).

3.2. HerdChek ELISA

After vaccination, all animals in Groups 1, 2, 3, 5, 7, and 8 tested positive by IDEXX PRRS ELISA prior to strain JA-142 virulent challenge (Day 21). Group 6 (rMN184Δ618) had 9 positive animals

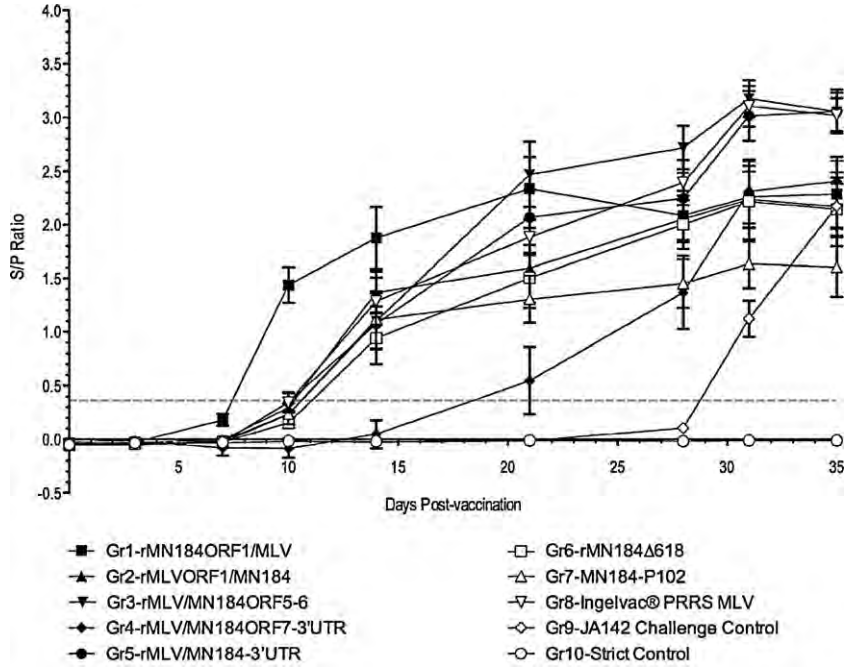

Fig. 2. Mean PRRS ELISA 2XR S/P ratios. The dashed line at 0.4 S/P ratio designates threshold value above which titers are considered positive for anti-PRRSV antibodies.

prior to challenge while Group 4 (rMLV/MN184ORF7-3′UTR) had only 4 positive animals and the appearance of the antibodies in this latter group was delayed (Fig. 2 and data not shown). Antibodies appeared in Group 1 animals 3 days prior to all other vaccination groups. In addition, the S/P ratios suggested that all of the animals in groups 1–9 seroconverted to either their respective vaccine or the challenge material by Day 31 of the study. The Strict Control (group 10) had no positive tests throughout the duration of the study. Fig. 2 indicates the day when the individual groups became positive for PRRSV specific antibodies and the trend for all treatment groups.

3.3. Virus Isolation

Virus isolation analysis confirmed that at least 3 of the 10 animals in each treatment group were viremic by Day 3 in groups 1, 2, 3, 5, 7, and 8 (Fig. 3). Only (10%) of the animals in Group 4 were positive on Days 3 and 14, the only positive results obtained for rMN184ORF7-3′UTR prior to challenge. No viremia was detected for Group 6 (rMN184Δ618) animals until Days 14 and 21, which then showed positive results for only 10% and 20% of the animals, respectively. The results suggested that each engineered recombinant virus was capable of some level of viral replication in the swine host, although it is evident that rMN184ORF7-3′UTR (Group 4) and rMN184Δ618 (Group 6) were less successful at replicating inside the animal host as compared to the other groups, as measured by virus isolation on MARC-145 cells. We had detected antibodies to Group 6 virus (Fig. 2) with similar kinetics to all other treatment groups (except Group 4) that might be indicative of replication of this virus in the absence of overt CPE due to infection of MA-104 cells. PRRSV vaccine strains MN184-P102 and Ingelvac® PRRS MLV confirmed viral replication within the host, although replication of Ingelvac® PRRS MLV from swine serum samples on cultured cells was more apparent than replication of MN184-P102 at all time points. Viremia continued after virulent heterologous PRRSV challenge in all groups except the strict control.

In order to assess the ability of the immune response to reduce the replication of the JA-142 challenge virus after vaccination with each of the candidate viruses, we compared the levels of viremia for all treatment groups to that of the Challenge Control Group on Day 35, when presumably most virus remaining in the animals would

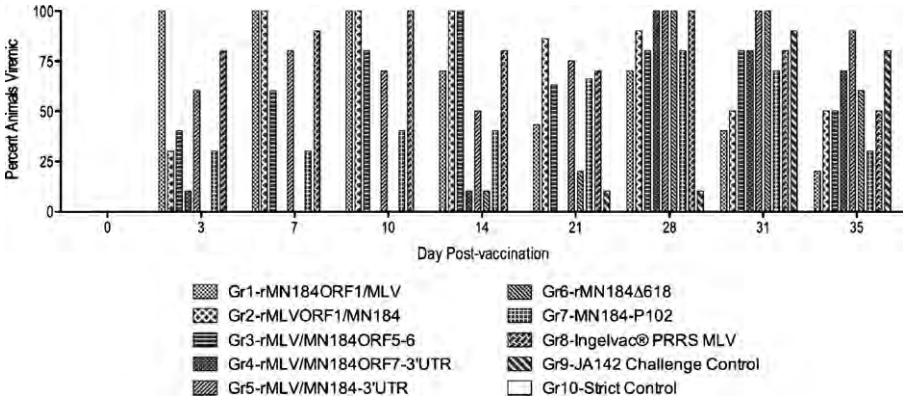

Fig. 3. Viral load, determined by virus isolation on MA-104 cells, in swine serum at all time points after intramuscular inoculation with 4.79 logs of each virus. The challenge control and strict control groups received only dilution medium. On Day 21, all animals except the strict control group were intranasally challenged with 3.8 logs of strain JA-142. Results for each animal of each group were collated and the percent positive per group was then determined.

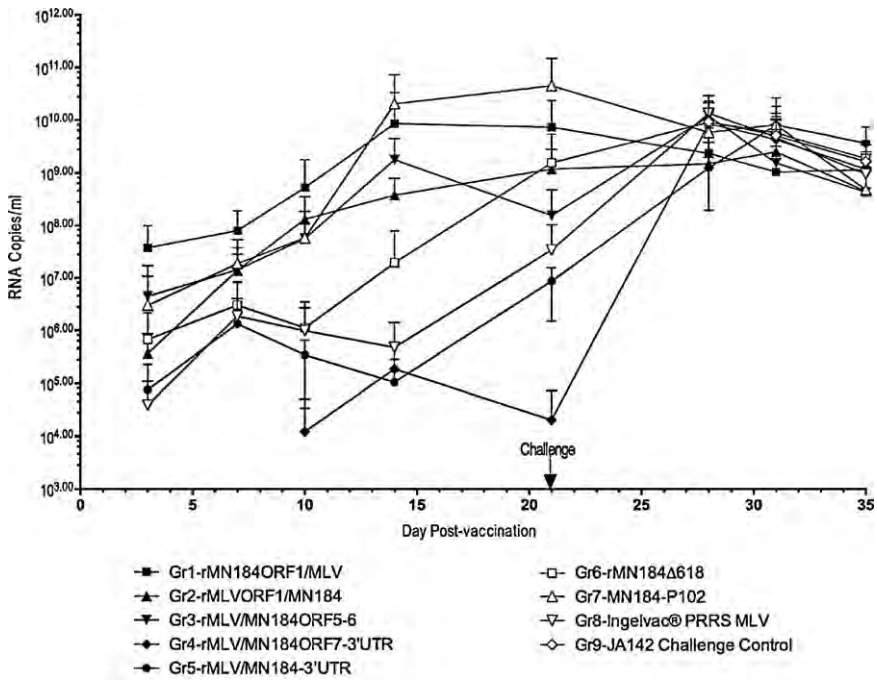

Fig. 4. Viral load was determined at all time points by qRT-PCR and plotted as viral RNA copies/ml serum. Results were plotted as the mean and error bars signify standard deviation. No viral RNA was detected in group 10 animals.

be the challenge PRRSV strain. Groups 1–3 (chimeric viruses) and Groups 7–8 (traditionally prepared vaccines) had a statistically significant ($p \leq 0.0001$) lower percentage of viremia as compared to the Challenge Control (Group 9) at this time point (Fig. 3).

3.4. Real Time RT-PCR

PRRSV RNA was detected in all pigs of treatment Groups 1–8 prior to challenge except for Group 4 (rMLV/MN184ORF7-3′UTR), for which only 3 of the 10 animals were positive for viral RNA by the day of challenge (Fig. 4). On Day 10, the various treatment viruses could be separated into two discrete categories. Those that had high levels of circulating viral RNA ($>10^7$) include Groups 1–3 (chimeric viruses) and Group 7 (MN184-P102) while those that had less amounts of viral RNA ($<10^6$) included Groups 4–6 and 8. Viral RNA detected in Group 6 animals suggested that this virus (rMN184Δ618) initially replicated at a lower rate, but eventually achieved RNA levels at Day 21 approximately equal to the viruses initially showing a higher level of circulating viral RNA. Group 4 animals revealed only 3 of 10 animals with circulating PRRSV RNA until after challenge, suggesting the virus does not replicate well in swine with a nucleocapsid gene and 3′UTR different from the rest of the pMLV genome. The remaining two viruses, inoculated into animal Groups 5 (rMLV/MN184-3′UTR) and 8 (Ingelvac® PRRS MLV), never reached above 10^7 RNA copies/ml until after challenge.

On Day 28, after virus challenge with heterologous strain JA-142, PRRSV RNA was found in all animals, providing evidence of successful challenge conditions. No treatment groups were statistically different for viral load as compared to the Challenge Control, suggesting little or no effect on JA-142 replication in swine by prior vaccination, when analyzed by qRT-PCR (Fig. 4). These results are considerably different from those obtained with virus isolation (Fig. 3).

3.5. Nucleotide sequence analysis

RT-PCR followed by nucleic acid sequencing of the products was completed on pooled and extracted serum samples from day 21. Approximately 1000 bases of ORF1b, the entire structural protein region and most of the 3′UTR were examined to ensure the animals remained infected with the respective test virus. In all cases, no discrepancy between the consensus nucleotide sequence with the input viral genome was found (data not shown). Furthermore, all viruses showed very little nucleotide variation after 21 days in 10 different animals. This indicated that all of the viruses, including the chimeras, were not undergoing demonstrable nucleotide change in the regions examined during the course of the experiment.

3.6. Average daily weight gain

To assess the gross clinical effects of PRRSV vaccination and challenge on swine, all animals were weighed at each time point. From this data, average daily weight gain (ADWG) was derived for Days −3 to 21 (before challenge) and Days 21–35 (after challenge)(Fig. 5). Prior to virulent challenge, Group 10 (Strict Control) had a statistically significant ($p \leq 0.01$) higher ADWG than only rMN184ORF1/MLV (Group 1), signifying that only Group 1 vaccination significantly reduced animal growth during the period before challenge (identified as A in Fig. 5). After challenge, Groups 1 (rMN184ORF1/MLV), 5 (rMLV/MN184-3′UTR), 6 (rMN184Δ618), and 9 (JA-142 Challenge Control) showed significantly reduced ADWG ($p \leq 0.01$) compared to control animals. This reduced ADWG may be due to insufficient protection of animals by prior vaccination in Groups 1, 5 and 6. To monitor the ability of the various viruses to protect against reduced weight gain after JA-142 challenge, ADWG was compared to Group 9 animals (Challenge Control)(identified as B in Fig. 5). In this comparison, rMLVORF1/MN184 (Group 2), rMLV/MN184ORF5-6 (Group 3), rMN184ORF7-3′UTR (Group 4), MN184-P102 (Group 7), and Ingelvac® PRRS MLV (Group 8) showed a statistically significant ($p \leq 0.05$) higher ADWG than the JA-142 Challenge Control group.

3.7. Clinical observations

Very few animals exhibited clinical signs after primary infection with the test viruses or with controls, with only one out of

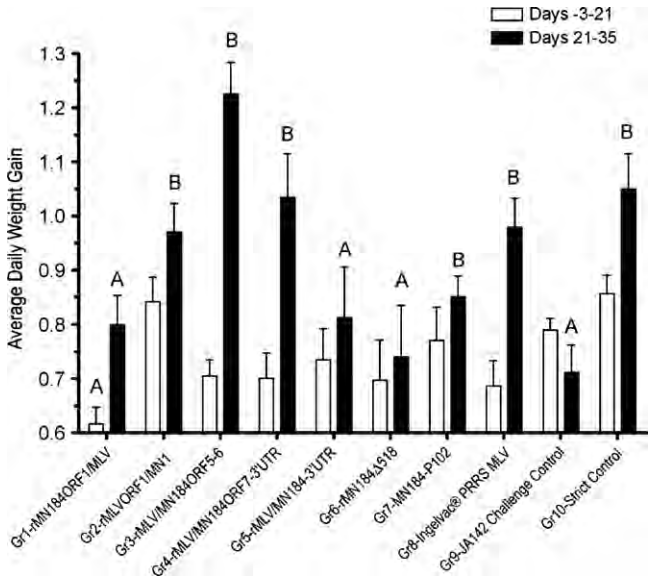

Fig. 5. Growth effects of chimeric and parental viruses on swine. All experimental pigs were weighed at Days −3, 21 and end of study. The average daily weight gain (ADWG) from 10 pigs in each group was calculated at period of −3 to 21 and 21–35 dpi. The mean was plotted and the standard error of the mean (SEM) represented as error bars. Statistically significant (≤0.01) lower average daily weight gain than the Strict Control group for the relevant time period was specified by the letter A. Statistically significant (≤0.01) higher average daily weight gain than Challenge Control group for Days 21–35 is represented by the letter B.

ten animals in each of Groups 5–7 showing mild discomfort (data not shown). This suggests that all treatments, although replicating variably in the host, did not mimic overt PRRS disease typically seen in the field. Only one animal in Group 6 experienced sustained mild lethargy and/or an intermittent cough after challenge (data not shown). In all, the mild clinical signs were to be expected and were a typical response to PRRSV infection in high health herds. The symptoms were not severe enough to have an effect on the outcome of the study, as attending veterinarians determined that no medication was necessary for resolution of clinical signs for all animals enrolled in the study.

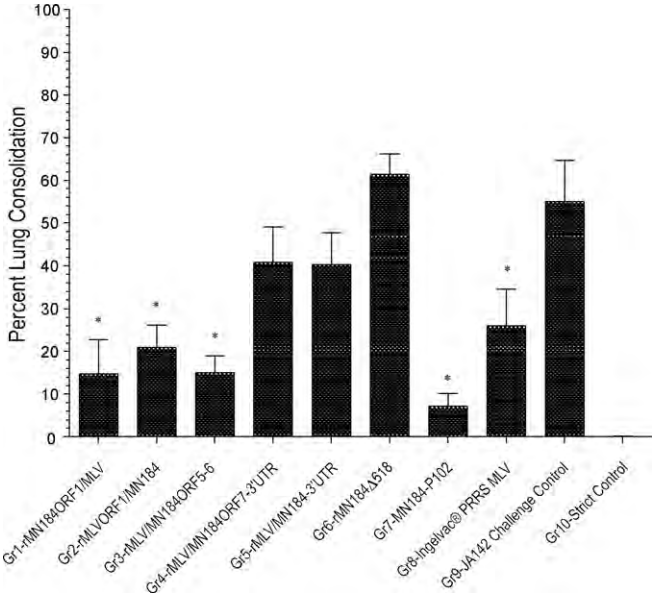

Fig. 6. Average lung scores were recorded at 35 dpi. The results were plotted as mean values of gross lung lesions from 10 pigs in each group, and the SEM values from different pigs designated by error bars. An asterisk indicates the average lung score of the group is lower than the challenge control group (*; $p < 0.01$ to $p < 0.05$).

3.8. Lung pathology

Upon completion of the study (Day 35), all animals were necropsied and assessed for lung pathology (Fig. 6). When compared to the Challenge Control (group 9), five treatment groups exhibited a statistically significant reduction in gross lung lesions. Those groups were: rMN184ORF1/MLV (Group 1; $P < 0.001$), rMLVORF1/MN184 (Group 2; $P < 0.01$), rMLV/MN184ORF5-6 (Group 3; $P < 0.001$), the recently developed vaccine MN184-P102 (Group 7; $P < 0.001$) and, to a lesser degree, Ingelvac® PRRS MLV (Group 8; $P < 0.05$). Three groups, rMN184ORF7-3′UTR (Group 4), rMLV/MN184-3′UTR (Group 5), and rMN184Δ618 (Group 6), did not appear to have sufficient protection against the development of pulmonary lesions in the strain JA-142 respiratory challenge model as the average lung scores of these three groups were not significantly different (>0.05) than the average score of the Challenge Control Group, which had over 50% of the lung displaying lesions. The Strict Control (group 10) had no lung lesions, thus indicating a valid challenge and successful bio-containment.

4. Discussion

In this report, a respiratory challenge model was used to examine the vaccine efficacy of five chimeras and a deletion mutant engineered from PRRSV Type 2 strain viral clones that differed by 14.3% on a nucleotide basis. Two chimeras, rMN184ORF1/MLV (Group 1) and rMLVORF1/MN184 (Group 2) had been previously shown to successfully protect swine against challenge with heterologous PRRSV strain SDSU73 [1]. The percent nucleotide identities between the Group 1 and 2 chimeras and SDSU73, over the available SDSU73 ORF2-7 sequence (EF442775), were 92.9% and 89.7%, respectively. For this study, the nucleotide identities based on complete genome comparisons to strain JA-142 ranged from 80.4 to 91.0% (Table 3), and yet two of the four most efficacious vaccines were of lower identity. One conclusion to draw from these comparisons is that percent similarity is not an accurate measure for determining which vaccine formula will provide the best protection from challenge, as has been shown previously for ORF5 only [34]. Rather, PRRSV protection after vaccination seems to be directed towards specific gene regions that influence genome replication kinetics and/or viral interaction with the swine host. Since both ORF1 reciprocal chimeras protected against strain SDSU73 and now strain JA-142, and both replicated well in swine, we firmly established that genome components from both viral nonstructural and structural regions can influence the ability to protect against heterologous challenge [1]. The present work also suggests that simple exchange of just the ORF5-6 region of strain MN184 can protect against challenge with strain JA-142, possibly increased over the traditionally prepared Ingelvac® PRRS MLV vaccine. This specific data reveals similar findings as those completed using a reproductive challenge model and infectious clones of two other PRRSV strains, attenuated vaccine Prime Pac PRRS® and virulent NVSL #97-7895 [10]. The rMN184 nsp2 deletion mutant (Group 6) also provided interesting results. As in previous study findings, where full-length rMN184 did not protect against challenge with strain SDSU73 [1], rMN184Δ618 did not protect against challenge with strain JA-142. The challenge viruses were different between those two studies, so additional parallel experiments must be completed to substantiate this preliminary finding. However, the data suggested that deletion of much of the nsp2 hypervariable region did not improve protection from heterologous PRRSV challenge. All of the data confirmed that PRRSV attenuation is complex, and may

involve interactions between individual viral component and/or host factors.

Novel findings concerning viral fitness *in vivo* were also presented. Virus isolation, which requires another round of MA-104 cell infection and growth, revealed that rMLV/MN184ORF7-3′UTR and rMN184Δ618 both replicated at a slower rate than most other viruses before challenge (Fig. 3). rMLV/MN184-3′UTR replicated quite well *in vitro*. However, when samples were directly assessed for the level of serum vRNA by qRT-PCR, rMLV/MN184-3′UTR along with rMLV/MN184ORF7-3′UTR may have replicated very poorly in swine, suggesting a PRRSV strain does not easily tolerate a nucleocapsid gene or protein and/or a 3′UTR different from the rest of the genome. rMN184Δ618 showed evidence of adequate replication *in vivo* when monitored by qRT-PCR, different from what was detected by the virus isolation technique. The implications of this finding are that viral fitness must be directly examined in the host, that replication of chimeric viruses in the host animal are not predictable and, therefore, one must assess several parameters when evaluating viruses for pharmaceutical use. We have also shown that some chimeric viruses can be readily utilized as vaccines, although traditionally prepared vaccines can perform as well or better against specific virulent strains.

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Review

In vivo growth of porcine reproductive and respiratory syndrome virus engineered nsp2 deletion mutants

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ABSTRACT

Prior studies on PRRSV strain VR-2332 non-structural protein 2 (nsp2) had shown that as much as 403 amino acids could be removed from the hypervariable region without losing virus viability in vitro. We utilized selected nsp2 deletion mutants to examine in vivo growth. Young swine (4 pigs/group; 5 control swine) were inoculated intramuscularly with one of 4 nsp2 deletion mutants (rΔ727–813, rΔ543–726, rΔ324–523, rΔ324–726) or full-length recombinant virus (rVR-2332). Serum samples were collected on various days post-inoculation and analyzed by HerdChek* ELISA, PRRSV real time RT-PCR, gamma interferon (IFN-γ) ELISA, and nucleotide sequence analysis of the entire nsp2 coding region. Tracheobronchial lymph node weight compared to body weight was recorded for each animal and used as a clinical measurement of viral pathogenesis. Results showed that all deletion mutants grew less robustly than full-length recombinant virus, yet all but the large deletion virus (rΔ324–726) recovered to parental viral RNA levels by study end. Swine receiving the rΔ727–813 mutants had a significant decrease in lymph node enlargement compared to rVR-2332. While swine infection with rVR-2332 caused a rapid rise in serum IFN-γ levels, the IFN-γ protein produced by infection with 3 of the 4 deletion mutant viruses was significantly reduced, perhaps due to differences in viral growth kinetics. The rΔ543–726 nsp2 mutant virus, although growth impaired, mimicked rVR-2332 in inducing a host serum IFN-γ response but exhibited a 2-week delay. Targeted sequencing showed that all deletions were stable in the region coding for nsp2 after one swine passage. The data suggested that the selected nsp2 deletion mutants were growth attenuated in swine, altered the induction of serum IFN-γ, an innate cytokine of unknown function in PRRSV clearance, and pointed to a domain that may influence tracheobronchial lymph node size.

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1. Introduction

One of the most economically important diseases of swine worldwide is caused by porcine reproductive and respiratory syndrome virus (PRRSV) (Neumann et al., 2005). This virus, belonging to the *Nidovirales* order, *Arteriviridae* family of positive-sense single-stranded RNA viruses, can cause reproductive failure in pregnant sows, respiratory disease in growing swine and asymptomatic infection in boars. PRRSV is also subject to high frequency viral recombination, which has led to rapid evolution and the existence of a multitude of strains belonging to one of two genotypes, Type 1 (European-like) and Type 2 (North American-like). Growing evidence suggests the swine immune response is often ineffective against heterologous PRRSV strains primarily because of the nature of the virus itself (Faaberg et al., 2006; Lopez and Osorio, 2004; Plagemann, 2004a,b).

With the advent of reverse genetics for several strains of PRRSV, portions of the genome can be directly assessed for disease function. One such area is the region of the ORF1 replicase protein known as non-structural protein 2 (nsp2) (Snijder et al., 1995b; Tian et al., 2007; van Hemert and Snijder, 2008), the most variable section of the genome (Han et al., 2006). Based on considerable research on equine arteritis virus (EAV), the nsp2 protein is processed from the ORF1 protein by the action of the papain-like protease in nsp1β and the self-encoded PL2 cysteine protease, and acts as a co-factor for the nsp4 serine protease (3CL) in processing downstream viral cleavages (den Boon et al., 1995; Snijder et al., 1994, 1995a,b; van Hemert and Snijder, 2008; Wassenaar et al., 1997). In addition, EAV nsp2 induces double membrane vesicles, probably through its predicted transmembrane spanning regions, and serves as an anchor for the virus replication complex (Pedersen et al., 1999; Snijder et al., 2001). Nsp2 of both genotypes of PRRSV is considerably larger than that of EAV and contains, in addition to the signature motifs described above, an extended N-terminal hypervariable region (HV1) and a middle hypervariable region (HV2). HV2 can differ in length by up to almost 150 amino acids. The two hypervariable regions of recombinant Type 2 strain VR-2332 (rVR-2332) were shown to contain amino acid segments not essential for replication in cell culture (Han et al., 2007) and recently, the cysteine protease domain (PL2) of strain VR-2332 was shown to possess both *cis* and *trans* cleavage activities and to cleave an expressed nsp2–3 construct at or near a downstream the predicted conserved G|G|G tripeptide (nsp2 amino acid residues 1196–1198) (Han et al., 2009; Ziebuhr et al., 2000).

The PL2 protease may also interact directly with cellular proteins. Shown through bioinformatics to be related to the mammalian ovarian tumor domain (OTU) containing protease family, PRRSV PL2 enzyme is thought to be a new member of this class of novel cysteine proteases. The sequence conservation among over 100 OTU members centers on the motifs containing the putative catalytic Cys and His residues, which arterivirus PL2 proteases possess (Makarova et al., 2000). PRRSV nsp2, when expressed individually in transfected 293T cells, decreases the level of ubiquitin and interferon-stimulated gene 15 (ISG15) conjugates (Frias-Staheli et al., 2007), indicating that the PL2 protease may deubiquitinate ubiquitinylated proteins. Protein ubiquitination has been shown to play a critical role in innate and adaptive cellular immune system induction by regulating several aspects of antiviral immunity such as NF-kB signaling, toll-like receptor/interleukin I signaling, MHC class I and II antigen presentation, and induction of type I IFN by the cellular viral sensor retinoic acid-induced gene I (RIGI) (Liu et al., 2005). The OTU domain of EAV decreases the activation of an NF-kB-responsive promoter after TNFα treatment, which may indicate that the NF-κB signaling pathway could be compromised by the presence of the arterivirus PL2 protease (Frias-Staheli et al., 2007; Hayden et al., 2006; Lenschow et al., 2007).

Nsp2 has also been shown to possess B-cell epitopes in both genotypes of viruses (de Lima et al., 2006; Oleksiewicz et al., 2001) and to induce B-cell responses (Chen et al., 2010; de Lima et al., 2006; Fang et al., 2004; Johnson et al., 2007; Mulupuri et al., 2008; Yan et al., 2007). Recently, using a type I PRRSV infectious clone, six identified immunodominant nsp2 B-cell epitopes [ES2–ES7; Oleksiewicz et al., 2001] were deleted. Three of these small domain deletions (ΔES3, ΔES4 and ΔES7) allowed the recovery of viable virus. The deletion mutants showed different levels of peak viremia in swine, with ΔES3 showing increased viral load over the other two mutants as well as parental rescued virus. However, the B-cell responses were similar when measured by IDEXX HerdChek, and neutralizing antibody responses were varied among all swine. Thus, removal of these B-cell epitopes did not appear to have an effect on the antibody responses. Only IL-1β and TNFα expression levels were different, showing a much lower level of expression after cells were stimulated with the ΔES3 mutant than with the ΔES4 and ΔES7 deletion viruses, or with the parent virus. Importantly, no lung lesion data was given (Chen et al., 2010).

In this report, we used four nsp2 deletion mutant viruses derived from the infectious clone of Type 2 PRRSV strain VR-2332. Three of these deletion viruses (rΔ727–813, rΔ543–726, rΔ324–523) were shown to be roughly similar in their in vitro growth kinetics, in terms of plaque forming units/ml, compared to parent virus (rVR2332). The virus with the largest nsp2 deletion (rΔ324–726) grew more slowly and produced an atypical cytopathic effect. Each mutant produced a smaller plaque size than rVR2332, and the large deletion mutant (rΔ324–726) produced no discernable plaques (Han et al., 2007).

Strain VR-2332, when administered to healthy pigs under defined hygienic experimental conditions, often produces an extremely mild clinical disease, as was noted in this study. Here, infection of growing swine with rVR-2332 and four engineered nsp2 deletion viruses were used to assess their impact on viral replication and to examine potential changes in the host response. Serum samples taken at various times were examined for anti-PRRSV antibody, the level of virus or viral RNA detected, and the stability of the deletions. We found that anti-PRRSV antibody levels were quickly induced by all but the mutant virus with the largest nsp2 deletion (rΔ324–726), that all inoculated viruses would replicate animals but at different rates, and that the engineered nsp2 deletions were stably maintained. For examination of the innate immune response, we chose to survey serum IFN-γ, as a previous

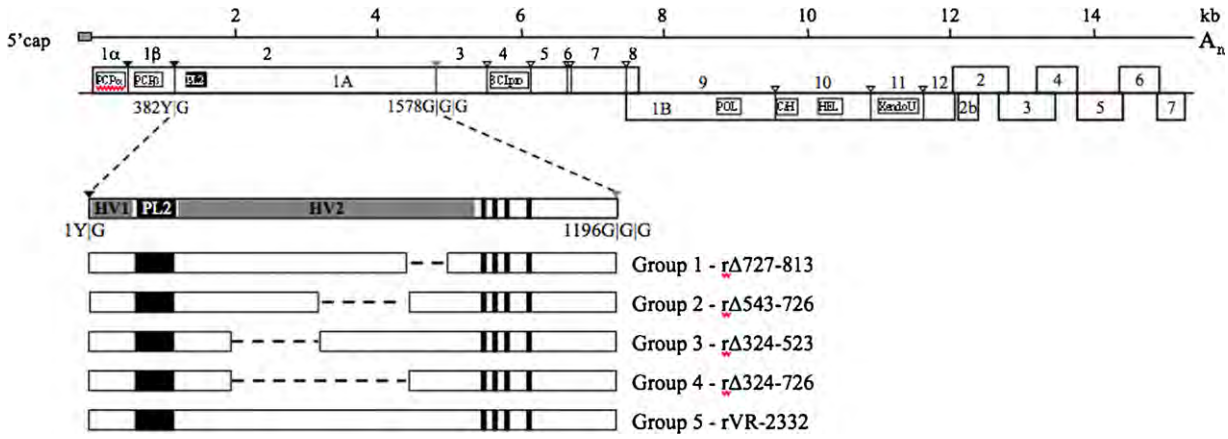

Fig. 1. Genome and PRRSV deletion mutant schematic. Illustration of treatment groups 1–5 used for deletion mutant growth analyses and pathogenesis assessment. Downward arrows indicate predicted cleavage sites processed by PRRSV encoded PCPα and PCPβ (papain-like cysteine protease α and β; ▼) chymotrypsin-like cysteine protease (PL2; ▼) and serine/3C-like protease (3CLpro; ▽). Other signature motifs are also indicated: POL, RNA dependent RNA polymerase; C/H, cysteine/histidine rich; HEL, helicase; XendoU, *Xenopus laevis* homolog poly(U)-specific endoribonuclease. The short black bars (⌘) indicate predicted nsp2 transmembrane regions. Hypervariable regions of nsp2 (HV1 and HV2), defined previously, are shown in solid grey (Han et al., 2006).

study had shown the level of this cytokine rose soon after infection with Type 2 PRRSV strains SDSU73 and JA142, peaked after 10 days and remained in the serum for approximately 3 weeks (Wesley et al., 2006). We found that similar high serum IFN-γ levels were induced by recombinant PRRSV strain VR-2332, but that the nsp2 deletion mutants differed from the parent virus in that all but one mutant did not elicit IFN-γ, and the mutant that did elicit this innate cytokine (rΔ543–726) showed a marked delay in doing so. Lastly, clinical severity was completed through lymph node weight to body weight measurements, as the ratio had been shown to be a reliable indicator of lymph node enlargement (Mengeling et al., 2003). In this study, only the smallest deletion mutant virus (rΔ727–813) resulted in decreased lymph node hyperplasia.

Previous reports of lymphoid hyperplasia and preferential proliferation of undiversified B cells during PRRSV infection have suggested a unique interaction between the virus and the host immune system (Butler et al., 2007; Lemke et al., 2004), and the identification of B-cell and T-cell epitopes in the nsp2 region bear this out (Chen et al., 2010; Fang et al., 2004). In the present study, results suggested that the change in viral growth kinetics of the engineered nsp2 mutant viruses of strain VR-2332 may be related to the quality of the host response, as was indicated previously with Type 2 strain JA142 (Greiner et al., 2000). Only the smallest nsp2 deletion resulted in a virus (rΔ727–813) that significantly reduced both the IFN-γ levels in serum and the degree of lymphadenopathy observed at necropsy, which corresponds to a deletion of only 4 predicted B-cell epitopes. Removal of B-cell epitopes located elsewhere in nsp2 reduced IFN-γ levels, but did not alleviate lymph node enlargement. Thus, no direct correlation between the numbers of nsp2 predicted B-cell epitopes and the level of lymph node involvement. However, removal of individual sections of nsp2 hypervariable region 2 was shown to have a differential effect on the viral kinetics and clinical disease phenotype.

2. Materials and methods

2.1. Cells and viruses

MARC-145 cells were cultured in minimum essential medium (EMEM, SAFC 56416C) with 10% fetal bovine serum at 37 °C, 5% CO_2. The recombinant viral strain VR-2332 (rVR-2332) and four VR-2332 nsp2 deletion mutants (rΔ727–813, rΔ543–726, rΔ324–523, rΔ324–726) have been described previously (Fig. 1) (Han et al., 2007). The engineered mutants correspond to deletion sizes of 87 amino acids (AA), 184AA, 200AA and 403AA, respectively. Recombinant viruses were sequentially passaged 3–6 times in MARC-145 cells until the individual titers were greater than 1×10^5 $TCID_{50}$/ml. The nucleotide sequences of the amplified recombinant viruses confirmed that no further changes in the nsp2 region had occurred (Han et al., 2007).

2.2. In vivo infection

The in vivo studies were performed at the National Animal Disease Center under its Animal Care and Use Committee approval. Twenty-five healthy pigs at 4 weeks of age were obtained from a herd free of PRRSV and randomly assigned to 6 treatment groups, 4 pigs each to groups 1–5, and 5 pigs to group 6. Groups 1–6 received the following treatments: Group 1 – nsp2Δ727–813, Group 2 – rΔ543–726, Group 3 – rΔ324–523, Group 4 – rΔ324–726, Group 5 – rVR-2332 and Group 6 – sham inoculation (negative control; NC). On day 0, all animals in groups 1–5 were inoculated intramuscularly with the respective virus (1×10^4 $TCID_{50}$/pig) or MEM for NC pigs. Swine were observed daily for general health status. Serum samples were collected from all animals on 0, 2, 4, 7, 9, 11, 14, 21, 28 and 35 days post-inoculation (dpi). On 35 dpi, all animals were weighed and then tracheobronchial lymph nodes were excised and weighed. For each pig, the ratio between the lymph node weight and body weight were calculated (LN/BW ratio). The mean LN/BW ratio for each group was recorded for each group and used as a clinical measurement of viral pathogenesis (Mengeling et al., 2003).

2.3. Serology

Serum samples were examined for serological response with the PRRS 2XR enzyme-linked immunosorbent assay, which utilizes recombinant PRRSV antigens (ELISA; IDEXX Laboratories). A sample was considered positive for antibodies to PRRSV if the sample-to-positive (S/P) ratio was equal to or greater than 0.4. At day 35, convalescent serum neutralization was monitored by immunofluorescence (IFA) (Yoon et al., 1994).

2.4. Serum interferon γ ELISA

Levels of IFN-γ in serum samples at all time points were determined by an ELISA according to manufacturer's recommendations (BioSource, #KSC4022). The assay measures IFN-γ protein

Table 1
Primers used in analyses of viruses. Forward primers indicated by a slash (/) following the name and reverse primers have a slash before the name.

Primer	VR-2332 Nt	Sequence
5′-1050/	1050–1075	5′-TCGCCATGCTAACCAATTTGGCTATC-3′
5′-1264/	1264–1283	5′-GTTGAGCCTAACACGTCGCC-3′
/3′-1711	1711–1736	5′-GACATCCCAGGGGTCACAGTGACAGT-3′
5′-2107/	2107–2125	5′-GACCTGTACCTCCGTGGTG-3′
5′-2167/	2167–2188	5′-CGCCCGCCACGCGTAATCGACA-3′
5′-2392/	2392–2413	5′-CTAACCGCCGTGCTCTCCAAGT-3′
5′-2811/	2811–2832	5′-CCCACCTGAGCCGGCAACACCT-3′
5′-3330/	3330–3355	5′-GCGCGAGGCATGTGATGCGACTAAGC-3′
/3′-3348	3348–3373	5′-GCGTAGCAGGGTCATCAAGCTTAGTC-3′
5′-3666/	3666–3691	5′-CTCCGAGGATAAACCGGTAGATGACC-3′
/3′-3846	3846–3870	5′-CCGGGATCCTTGGTCAAAGAGCCTTTCAGCTTTT-3′
/3′-4257	4257–4287	5′-CCGGGATCCGCCCAGTAACCTGCCAAGAATG-3′
/3′-4300	4617–4635	5′-CTGGGCGACCACAGTCCTA-3′
/3′-5300	5613–5632	5′-GAGTTCTGAAAGCACCCTCA-3′

(0–500 pg/ml) against a standard curve based on recombinant swine interferon gamma. The ELISA plates were read at 450 nm on a SPECTRAmax 190 Microplate Spectrophotometer (Molecular Devices Corporation). Serum levels of IFN-γ were interpolated from a standard curve generated by SOFTmax PRO Version 3.1 software (Molecular Devices Corporation). IFN-γ data were analyzed using a mixed linear model for repeated measures. Linear combinations of the least squares means estimates for serum IFN-γ were used in a priori contrasts after testing for either a significant ($P < 0.05$) effect of PRRSV challenge strain (5 rVR-2332 constructs or sham-inoculated controls), or interaction effect between study day and PRRSV challenge strain. Comparisons were made between groups challenged with each PRRSV isolate and non-challenged controls at each time-point using a 5% level of significance ($P < 0.05$) to assess statistical differences.

2.5. Viremia detection by virus isolation and quantitative Real-Time RT-PCR

To initially determine if the animals were viremic, virus isolation was performed with serum samples collected on all days and lavage fluids at the time of necropsy. Briefly, swine samples (50 μl serum, 200 μl lavage fluid) were inoculated into MARC-145 cells separately and the percent found positive for cytopathic effect (CPE) in each group was calculated (data not shown). Positive samples were then titered on fresh MARC-145 cells. The log value of titer/ml was recorded and graphed for each positive sample. Since all PRRSV constructs or isolates might not replicate in the same manner on MARC-145 cells, Real-Time RT-PCR was performed on identical extracted RNA to further assess PRRSV replication. An in-house standardized quantitative Real-Time RT-PCR, targeting the conserved genomic region corresponding to Ingelvac® PRRS MLV nucleotides 14,947–15,051, was used to detect, in duplicate, the PRRSV RNA copy number in 8 μl of viral RNA purified from serum.

2.6. RT-PCR and nucleotide sequencing

In order to confirm deletion size and ensure accidental contamination with full-length virus did not occur, viral RNA from sera containing virus from 2 animals per group was extracted and subjected to differential analysis using One-Step RT-PCR (Qiagen). In order to detect the virus used to inoculate the animals, the following primer pairs were utilized with 4 μl viral RNA from 2 pigs/group: Group 1 – 5′-2811/3′-3846 (808 bp product); Group 2 – 5′-2392/3′-3846 (936 bp product); Group 3 – 5′-2167/3′-3348 (608 bp product); Group 4 – Primer pair: 5′-2167/3′-4257 (904 bp product); Group 5 – 5′-1050/3′-1711 (674 bp product); NC group – 5′-1050/3′-1711 (no product expected). To detect inadvertent parental virus contamination in Groups 1–4, the following primer pairs were utilized: Group 1 – 5′-3666/3′-4257 (592 bp product); Group 2 – 5′-3330/3′-3846 (517 bp product); Group 3–5′-2811/3′-3348 (538 bp product); Group 4 – Primer pair: 5′-3330/3′-4257 (955 bp product); Group 5 – 5′-2811/3′-3348 (538 bp product); NC group – 5′-2811/3′-3348 (no product expected). Nested PCR was completed on samples from all four pigs from Group 4 at all time points. First round RT-PCR was completed with the primers 5′-2107/3′-4300 (1320 bp product). Second round PCR used 5 μl of the first round product diluted 1:100 and standard conditions using the primer pair 5′-2167/3′-3846 (492 bp product). The primers used for these analyses are listed in Table 1. The individual RT-PCR products were cloned into pGEM-T, amplified in DH5α and then one to two plasmids of each were purified and submitted for sequence analysis to confirm retention of the nucleotide sequence deletion (data not shown). To determine the nucleotide sequence variation after 35 days of infection, the entire nsp2 gene was amplified from two swine in each group. The swine chosen were not necessarily the same swine sampled for deletion verification and preliminary sequence analysis. The PCR products, at 35 day postinfection, were of low abundance and could not be sequenced directly. The products were cloned and amplified, as described above, and then two nsp2 containing plasmids each were submitted for sequence analysis. In this way, a concise indication of clonal variation was obtained.

2.7. Software utilized

Graphpad Prism version 4.0c was used to display HerdChek ELISA and Real-Time RT-PCR results, where the mean and standard error of the mean (SEM) were chosen for representation of individual sample variability within a group. Geneious Pro 3.8.5 (Biomatters Ltd.) was used to examine nucleotide and protein sequences. Alignments were then transferred to Showalign, an EMBOSS (European Molecular Biology Open Software Suite) program embedded in the eBioX 1.5.1 graphical interface (The Linnaeus Centre for Bioinformatics, open source), for suitable display using EBLOSUM90 substitution (Henikoff and Henikoff, 1992).

3. Results

3.1. Immunogenicity and replication of chimeric and parental viruses in pigs

Groups of 4–5 pigs were inoculated with different recombinant viruses (depicted in Fig. 1) or used as a negative control group. Results of anti-PRRSV antibody levels determined by HerdChek* ELISA are illustrated in Fig. 2. All animals were seronegative to PRRSV until 9 days postinfection. By day 14, anti-nucleocapsid antibodies were discerned in Groups 1 (rΔ727–813; 87AA), 2 (rΔ543–726; 184AA) and 5 (rVR-2332), as normally seen with PRRSV field isolates. Group 3 (rΔ324–523; 200AA) swine did not seroconvert until day 21 and one out of four pigs in Group 4 (rΔ324–726; 403AA) seroconverted at day 21 and another at day 35. The two remaining animals in Group 4 did not seroconvert in the course of this study, although one displayed an S/P ratio of 0.387, near the baseline set by the manufacturer for seroconversion (0.400). The data suggested that 87AA or 184AA deletions in the nsp2 hypervariable region 2 carboxyl terminus had no detrimental effect on seroconversion of swine when compared to the parental recombinant clone, but that a 200AA deletion near the N-terminus of the hypervariable region and a 403 AA deletion, which included the majority of the region, retarded the appearance of anti-nucleocapsid antibodies. There were no statistical differences detected in the S/P ratios induced by the recombinant viruses on days 28 and 35, except for the large deletion virus.

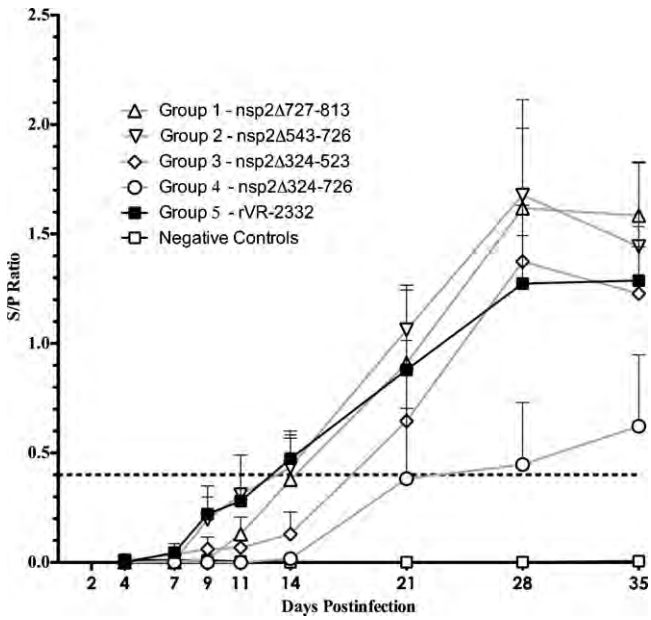

Fig. 2. HerdChek* ELISA, detecting antibodies elicited against PRRSV nucleocapsid protein, were determined for all serum samples and then plotted as the mean and standard error of the mean for each group of pigs at all time points. Serum samples ≥0.4 (indicated by dotted line) were considered positive, as per manufacturer's guidelines (IDEXX).

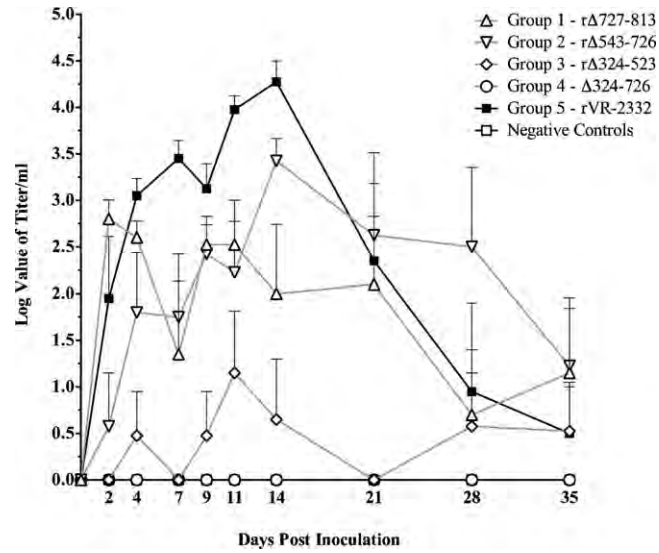

Fig. 3. Virus titer results ($TCID_{50}$/ml) on all virus isolation positive serum samples. Virus isolation was performed with all serum samples and lavage fluids. Positive samples were then titered on fresh MARC-145 cells.

Lastly, no neutralization was detected in serum from any animal (data not shown).

The percentage of serum and lavage samples positive by virus isolation was determined and then titered to assay the ability of the recombinant viruses to replicate in swine (Fig. 3). A delay in viral growth compared to rVR-2332 (native nsp2) was detected for all but Group 1 challenged swine. Group 2 virus growth in swine was only slightly delayed. Virus was isolated only sporadically from swine in Group 3, and never for Group 4 swine. Also evident from this analysis is that although the detectable virus titers from serum had declined by day 35, most infected swine still showed variable amounts of virus in the lung lavage fluid at necropsy, as is typical for PRRSV infection (Table 2).

3.2. Real-Time RT-PCR detection of PRRSV RNA

The fact that some Group 4 swine seroconverted yet did not show evidence of PRRSV replication suggested that virus isolation might not be ideally sensitive. To further evaluate the levels of viral replication, serum samples at all days postinfection for all treatment groups were analyzed by quantitative Real-Time RT-PCR (Fig. 4). Until day 14, there were three discrete levels of viral RNA detected. Swine infected with rVR-2332 had viral RNA amounts typical of PRRSV infection with wt strain VR-2332 (10^4–10^6 RNA copies/ml). Groups 1 (rΔ727–813), 2 (rΔ543–726) and Group 3 (rΔ324–543) swine exhibited viral RNA at 10–100-fold lower

Table 2
Day 35 lung lavage virus isolation and PRRSV Real-Time RT-PCR results.

Group	Virus isolation[a]	Mean	SEM	Copies PRRSV RNA	Mean	SEM
Negative control	0	0	0	0	0	0
	0			0		
	0			0		
	0			0		
	0			0		
1	2.9	3.2	0.6	18,170.0	40,914.1	22,903.6
	1.9			1396.3		
	4.6			105,791.8		
	3.3			38,298.2		
2	0	1.6	1.1	110.4	53,049.7	52,873.5
	1.8			418.4		
	0			0		
	4.5			211,670.1		
3	1.8	1.5	0.5	1388.7	4638.5	2473.1
	0			135.1		
	1.8			11,014.8		
	2.3			6015.5		
4	0	0	0	0	94.2	94.2
	0			376.7		
	0			0		
	0			0		
5	0	1.7	0.7	3386.3	7779.3	5880.9
	1.8			1485.9		
	3.3			25,350.0		
	1.8			895.0		

[a] Log value of titer/ml.

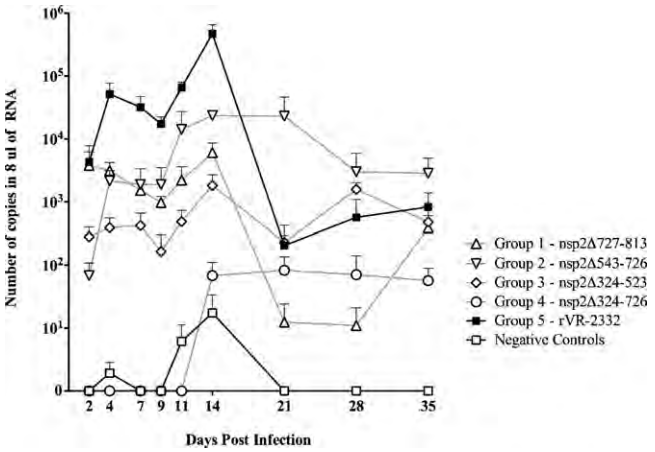

Fig. 4. Real-Time RT-PCR results, performed in duplicate, for all serum samples. Quantitative RT-PCR was used to detect, in duplicate, the PRRSV RNA copy number in 8 μl of viral RNA purified from serum.

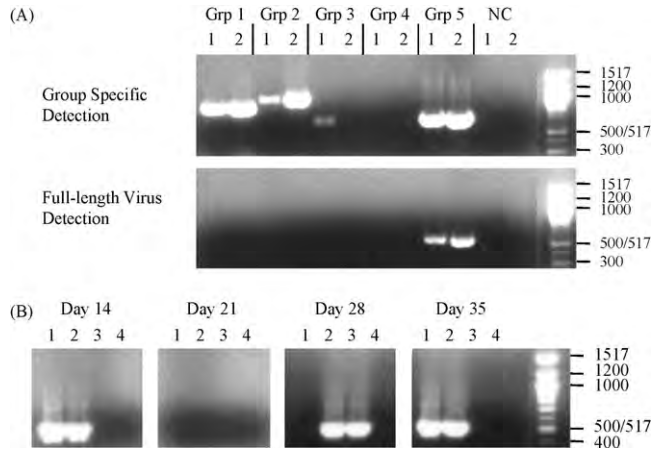

Fig. 5. (A) RT-PCR detection of PRRSV construct used to infect 2 pigs/group (using days 4 and 7 viral RNA from serum samples; day 7 shown) in Group 1 – 808 bp product, Group 2 – 936 bp product, Group 3 – 608 bp product, Group 4 – 904 bp product, Group 5 – 674 bp product, and NC Group – no product expected. RT-PCR to confirm absense of rVR-2332 virus contamination in Group 1 – 592 bp product, Group 2 – 517 bp product, Group 3 – 538 bp product, and Group 4 – 955 bp product. Included were identical RT-PCR conditions for two pigs from Group 5 – 538 bp product (positive control) and NC group – no product expected. (B) Nested PCR was completed on Group 4 at time points that viral RNA was detected by Real-Time RT-PCR (492 bp product).

levels. Lastly, Group 4 (rΔ324–726) showed viral RNA above background only after 14 days of in vivo infection. NC swine showed only background levels of RNA. Also, after day 14, viral detection in all treatment groups was diminished and varied from 10^1 to $10^{4.5}$ copies in 8 μl of purified viral RNA.

3.3. Verification of input virus

At days 4 and 7, viral RNA from 2 pigs/group was tested by RT-PCR with relevant primer pairs to ensure that the virus detected in the different groups corresponded with the input virus and that no aberrant contamination with parental strain rVR-2332 was seen. As shown in Fig. 5A, Groups 1–3 viruses were confirmed to have successfully replicated in swine although at lower levels in Group 3 (barely detectable in pig 2), and there was no evidence of full-length virus growth in any of the deletion virus treatment groups. Nested RT-PCR was used to verify Group 4 mutant virus (Fig. 5B), where the correct size band was variably seen in 3 of the 4 pigs in serum samples from days 14, 28 and 35.

3.4. PRRSV nsp2 protein sequence stability

In order to assess the stability of the nsp2 gene deletion during replication in swine, viral RNA from all pigs/group were extracted from serum taken at day 35. All deletion virus swine sera at this time were first examined for maintenance of nucleotide sequence immediately surrounding the respective engineered deletion site, and the NC swine sera were similarly analyzed using primers encompassing all of the nsp2 deletion regions. All animals retained the correct nsp2 nucleotide sequence (data not shown). After successful RT-PCR amplification of the entire nsp2 gene from 2 pigs/group, 2 DNA products/pig were cloned and 2 of the individual clones were used for nucleic acid sequencing. Silent and mostly single nucleotide changes were scattered throughout the nsp2 gene but it was uncertain as to whether these were due to viral growth in the animal or acquired during amplification and cloning. However, the amounts of nucleotide variation shown in the deletion mutants and rVR-2332 after 35 days of viral growth compared to the starting viruses were similar, suggesting that the mutated nsp2 regions were as stable as the native gene. No universal nucleotide changes from rVR-2332 were seen. Most importantly, all engineered deletions were stable, possessing no additional insertions or deletions in nsp2 (Fig. S1). Some predicted amino acid changes were detected in one or both clones of each mutant after alignment with the inoculating virus sequence. Only four amino acid changes were seen in all sequenced products of the individual mutants [Group 1 – H653Q; Group 3 – G648E and R710W; Groups 2–5 – K1125E (using full-length nsp2 residue numbering)], but all showed 99.2–99.6% amino acid similarity to the respective parent nsp2 sequence and the significance of these changes could not be determined (Fig. S1).

3.5. Serum IFN-γ levels induced by PRRSV infection

In order to establish whether the previous observation that two Type 2 strains induced highly levels of IFN-γ (Wesley et al., 2006) was also seen for strain VR-2332 infection, and to investigate the effect of nsp2 deletions on the IFN-γ levels, all serum samples were assayed. One pig from group 3 (rΔ324–523) had an extraordinarily high IFN-γ level at 7 days (552 pg/ml), which was reproducible. This data point was 4.5 times greater than the next highest serum value detected at any point in the study in any pig (data not shown). When the data value for that pig on that day was removed from the analysis, the group mean ($n = 4$) for that day was 0 (versus 138 pg/ml). The daily standard deviations with this pig excluded from the data set ranged from 0 to 26. The standard deviation for serum IFN-γ levels in all other pigs on day 7 was 5.0, suggesting the value for this one pig was an extreme statistical outlier. Therefore, an analysis of the overall data set was run with the value for this pig on that study day omitted. The effect of removing this data point reduced the treatment group standard error for the least squares mean estimate on day 7 from 21.2 to 7.9. A pattern of IFN-γ response to PRRSV strain VR-2332 emerged when this pig at day 7 was removed from consideration (Fig. 6). The rVR-2332 virus induced a significant biphasic increase in serum IFN-γ levels. The initial increase was observed at day 2 post-inoculation followed by increases peaking on day 11 post-inoculation. These increases were statistically significant on post-inoculation days 2, 11 and 14 and began to decline by day 21. Overall, based on the nsp2 deletion mutant strain of VR-2332 given to pigs, there was a significant effect on elevation of serum IFN-γ levels ($P = 0.0005$). Two groups of pigs receiving different virus constructs (rVR-2332 and rΔ543–726) had significant increases in serum IFN-γ levels above zero over the course of the study. There was a low background level of detectable serum IFN-γ in the non-inoculated control pigs, but rVR-2332 and rΔ543–726 groups were

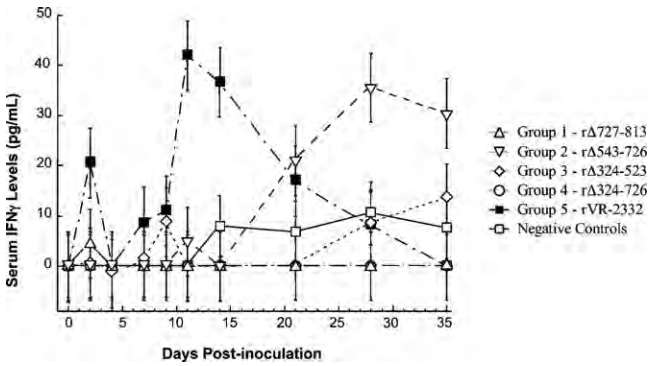

Fig. 6. Interferon γ protein ELISA for all serum samples were analyzed using a mixed linear model for repeated measures.

Table 3
One-way analysis of variants (ANOVA) data obtained from lymph node (LNW) and body weight (BW) comparison on day 35.

Group	*N*	Mean LNW/BW (SD[a])	*P*-value[b]
Negative control	5	0.0738 (0.0207)	
Group 1	3	0.1130 (0.0095)	>0.05
Group 2	4	0.3228 (0.0570)	<0.001
Group 3	4	0.2018 (0.0641)	<0.01
Group 4	4	0.0985 (0.0241)	>0.05
Group 5	4	0.2300 (0.0659)	<0.01

[a] Standard deviation.
[b] In comparison with negative control group.

still different from the sham-inoculated control group ($P = 0.0007$ and 0.04, respectively). Swine in groups 1, 3 and 4 did not produce any IFN-γ above background controls when inoculated with the respective PRRSV deletion mutant.

3.6. Clinical assessment

In order to assess possible changes in virus pathogenicity due to deletion of nsp2 hyvariable region 2 amino acids, all animals were weighed (body weight, BW) at necropsy and then the tracheobronchial lymph nodes were harvested and weighed (lymph node weight, LNW). The resulting LNW/BW fraction, when compared to that of control animals, is a measure of lymph node enlargement (Mengeling et al., 2003). As shown in Table 3, one-way analysis of variance revealed that infection with two deletion viruses, Group 1 and Group 4, did not differ significantly from the negative control animals (>0.05), and showed a significant difference from the rVR-2332 group (<0.05). Since rΔ324–726 (Group 4) did not replicate well in swine, the significance of the lower LNW/BW ratio could not be appropriately evaluated. The other swine groups showed typical lymph node enlargement, a diagnostic indicator of infection with PRRSV. Thus, only one nsp2 deletion mutant of strain VR-2332 did not definitively cause lymph node adenopathy, rΔ727–813.

4. Discussion

Previous work by several investigators had shown that nsp2 deletions in both PRRSV genotypes are naturally seen in the field (Fang et al., 2004, 2007; Gao et al., 2004; Han et al., 2007; Li et al., 2007; Ropp et al., 2004; Zhou et al., 2008). Furthermore, bioinformatic analyses have suggested that nsp2 contains several putative B-cell epitopes (de Lima et al., 2006; Fang et al., 2004), and research has suggested that this protein may be highly immunogenic (Johnson et al., 2007; Mulupuri et al., 2008). Investigators have also shown that insertion of foreign genes such as green fluorescent protein into the nsp2 deletion region has resulted in viable virus, although foreign gene instability has been noted (Fang et al., 2006; Han et al., 2007; Yoo et al., 2004). In our own prior studies, we had determined the essential regions of nsp2 for cell culture passage of PRRSV Type 2 strain VR-2332 (Han et al., 2007). However, no swine disease phenotype has been associated with regions of PRRSV nsp2, although porcine high fever disease of China was originally attributed to a deletion in nsp2 (Li et al., 2007; Zhou et al., 2009, 2008).

In this investigation, we chose four of the previously engineered nsp2 deletion mutants for analysis of viral growth and corresponding disease characteristics in swine. Three deletion mutants possessed in vitro growth phenotypes very close to the parent virus, but the mutant with the largest deletion, rΔ324–726, had been previously found to replicate poorly in cell culture. The targeted section for deletion was located within nsp2 hypervariable region 2 and contained 24 predicted antigenic epitopes (Kolaskar and Tongaonkar, 1990). The recombinant viruses chosen had as few as four (rΔ727–813) and as many as 20 (rΔ324–726) of these epitopes removed. However, other undescribed nsp2 interactions with the immune system, such as the ability to deubiquitinate cell proteins, may have also been compromised as a result of defined nsp2 deletion. We sought to understand the differential effects nsp2 HVR2 deletion had on virus in vivo growth kinetics and potential correlations with the antibody responses, number and position of putative B-cell epitopes, the innate immune response protein INF-γ and particular clinical disease manifestations over the 35 day experiment.

We found that all of the mutant viruses replicated in vivo, and all but the virus with the large deletion (rΔ324–726) induced a strong IDEXX anti-nucleocapsid antibody response. The response mirrors the viral growth in swine as measured by virus isolation, with rVR-2332 reaching the highest titers and gradually decaying over time. The three smaller deletion mutants (87, 184 or 200 AA removed) grew approximately 10–100-fold less vigorously, but were nonetheless able to induce antibodies to the nucleocapsid protein rapidly. A notable delay in antibody response was seen in three of four animals infected with the mutant harboring the largest nsp2 deletion, but was the first indicator that this virus had indeed replicated in swine. We also examined PRRSV RNA copies detected by Real-Time RT-PCR, and noted that this measurement approximated the virus isolation results, but was more sensitive to viral nucleic acid and was able to discern a small and stable amount of the large deletion mutant viral RNA at later times in infection. The shape of most curves in both assays suggests a cyclical nature for PRRSV growth. When the lung lavage fluids were examined by the same assays, it appeared that the viruses infecting Group 1 (rΔ727–813, 87 AA deletion) and 2 (rΔ543–726, 184 AA deletion) animals were able to persist in the lungs as well as native VR-2332 nsp2. We also sought to determine if the viral mutants were genetically stable during replication in swine, and found that although there were periodic silent nucleotide changes and some amino acid changes, no further editing of the engineered deletions in nsp2 was detected, suggesting the virus adapts readily to mutation in this hypervariable part of the replicase protein, as has been amply noted in the field (Fang et al., 2004, 2007; Gao et al., 2004; Greiner et al., 2000; Han et al., 2007; Li et al., 2007; Ropp et al., 2004; Zhou et al., 2008). Sequence analysis of the entire genomes of these interesting viral mutants after growth in swine, which are underway, will have to be completed in order to examine changes that may have occurred outside of the nsp2 region.

IFN-γ, an important cytokine produced during the early stages of infection by macrophages, natural killer (NK) cells, T cells and alternative cells types is a mediator for cellular immunity. Previous work reported that PRRSV Type 2 strains JA-142 and SDSU-73 induced release of IFN-γ into the serum of infected animals whereas two other viruses, porcine coronavirus and swine influenza virus, did

not induce comparable IFN-γ serum levels (Wesley et al., 2006). In order to examine whether this effect was seen after infection with PRRSV strain VR-2332 and to test if deletion of parts of the nsp2 HVR 2 would alter the response, the serum samples from the present study were assayed in the same manner, but with additional time points included. Again, we noted the paradoxically high levels of IFN-γ in serum of swine that had been infected with full-length recombinant virus rVR-2332, but it also appeared to be biphasic in nature. However, only one deletion mutant, rΔ543–726, was able to generate a similar response in swine, although it was delayed by approximately 2 weeks. The other mutants showed no discernable rise serum IFN-γ above the control group. When compared to the virus load diagrams, the peak of IFN-γ appeared to precede the peak of viral titer or RNA load detected, which may have revealed a unique feature of infection caused by this persistent virus. The role of this interesting cytokine in the serum of PRRSV infected animals is thought to be critical but is still poorly understood. In addition, the effect of virus growth kinetics and the amount of circulating virus on inducing serum INF-γ must be further studied.

Although no obvious clinical signs were noted in this study, we sought to examine whether deletion of separate polypeptide sequences of nsp2 would have an effect on any disease parameter. LNW/BW ratios revealed that only one mutant, rΔ727–813, caused a discernable decrease in lymph node enlargement over that seen with rVR-2332. Thus, the smallest deletion with only 87AA acids removed from nsp2 was able to induce diminished clinical disease whereas the larger upstream 187AA and 200AA deletions had no moderating effect. Bioinformatic analysis of the 87AA region did not yield any noticeable protein motifs, although the high proline content (10%) suggests a highly structured protein region. Finally, when comparing the putative antigenic regions discussed above, the regions containing 10 such motifs did not reduce the lymphadenopathy, while a region with only 4 domains significantly decreased this measurable sign of pathogenicity. Whether the reduction of the predicted B-cell epitopes results in an attenuation/exacerbation of other clinical signs must be examined using alternative PRRSV strains. However, the viral mutants described in this report will be extremely useful in the search for other modulators of PRRSV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.07.024.

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Research paper

Differential expression of cytokine transcripts in neonatal and adult ovine alveolar macrophages in response to respiratory syncytial virus or toll-like receptor ligation

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ABSTRACT

Alveolar macrophages (AMϕs) secrete regulatory molecules that are believed to be critical in maintaining normal lung homeostasis. However, in response to activating signals, AMϕs have been shown to become highly phagocytic cells capable of secreting significant levels of pro-inflammatory cytokines. There is evidence to suggest that susceptibility of Mϕ subpopulations to viral infection, and their subsequent cytokine/chemokine response, is dependent on age of the host. In the present study, we compared bovine respiratory syncytial virus (BRSV) replication and induction of cytokine responses in neonatal ovine AMϕs to those cells isolated from adult animals. While neonatal AMϕs could be infected with BRSV, viral replication was limited as previously shown for AMϕs from mature animals. Interestingly, following BRSV infection, peak mRNA levels of IL-1β and IL-8 in neonatal AMϕ were several fold higher than levels induced in adult AMϕs. In addition, peak mRNA expression for the cytokines examined occurred at earlier time points in neonatal AMϕs compared to adult AMϕs. However, the data indicated that viral replication was not required for the induction of specific cytokines in either neonatal or adult AMϕs. TLR3 and TLR4 agonists induced significantly higher levels of cytokine transcripts than BRSV in both neonatal and adult AMϕs. It was recently proposed that immaturity of the neonatal immune system extends from production of pro-inflammatory cytokines to regulation of such responses. Differential regulation of cytokines in neonatal AMϕs compared to adult AMϕs in response to RSV could be a contributory factor to more severe clinical episodes seen in neonates.

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1. Introduction

Infants and young children are commonly infected with respiratory syncytial virus (RSV), which causes severe lower respiratory tract infections manifesting itself as acute bronchiolitis and pneumonia (Durbin and Durbin, 2004; Welliver, 1983). Bovine respiratory syncytial virus (BRSV) is a major etiological agent of lower respiratory tract disease in calves (Van der Poel et al., 1994). RSV tropism for the lower respiratory tract is supported by active replication within lung epithelial cells, inducing production of mediators that are chemotactic to cells of the immune system. Infiltration of antigen presenting cells, lymphocytes and mononuclear cells is detected within lung tissue after RSV infection (Kurlandsky et al., 1988; Tripp et al., 1999). Within the alveolar space, alveolar macrophages (AMϕs) play a

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crucial role in clearance of respiratory pathogens as well as the production of pro-inflammatory and immunomodulatory mediators upon stimulation by microorganisms (Wilmott et al., 2000). The presence of AMϕs in the lower respiratory tract enables them to come in contact with RSV. In vivo data from adult humans confirm the presence of RSV within AMϕs of transplant patients (Panuska et al., 1992). AMϕs infected in vitro with RSV has been documented in humans (Panuska et al., 1990), mice (Stadnyk et al., 1997), guinea pigs (Dakhama et al., 1998), and calves (Liu et al., 1999). Production of pro-inflammatory mediators including IL-6, IL-8, TNFα, and RANTES (Becker et al., 1991; Franke-Ullmann et al., 1995; Kaan and Hegele, 2003; Miller et al., 2004), and immunomodulatory IL-10 (Panuska et al., 1995) have been described in RSV-infected AMϕs. Recently, it was discovered that human AMϕs can produce IL-4 after phorbol-12-myristate-13-acetate and calcium ionophore stimulation (Pouliot et al., 2005). The immunoregulatory mediators that are produced by AMϕs make them a critical cell type with a key role in host defense and the early innate immune response to RSV.

Neonatal infants have an ineffective immune response to RSV, as evidenced by the prevalence of recurrent infections during childhood (Henderson et al., 1979). Disease manifestations in response to RSV infection vary greatly among individual children. Children with severe RSV infections were shown to have predominant type 2 cytokine response leading to progression of the disease (Bendelja et al., 2000; Roman et al., 1997). Older children and adults generally exhibit mild symptoms. Similar age-dependency in disease severity is seen in RSV infections in cattle and sheep. In a previous study, we utilized an in vivo infection model to show that AMϕs isolated from BRSV-infected neonatal lambs express increased levels of IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12p40 mRNA transcripts compared to control animals (Fach et al., 2007), similar to the aforementioned findings reported in other host species. The susceptibility of Mϕs to viral infection, and their subsequent response to infection, is likely dependent on age of the host and differentiation stage of these myeloid lineage cells. To our knowledge, there have been no direct comparisons of neonatal and adult AMϕ responses to RSV infection. In the present study, we first examined whether neonatal and adult ovine AMϕs differed in permissiveness to viral infection. Next, we analyzed cytokine transcriptional profiles induced in BRSV-infected AMϕ isolated from neonate or adult sheep. Finally, BRSV-induced cytokine responses were compared to those induced by UV-inactivated BRSV or TLR agonists (synthetic dsRNA polyriboinosinic:polyribocytidylic acid (poly (I:C); TLR3) or lipopolysaccharide (LPS; TLR4)).

2. Materials and methods

2.1. Viral inoculum

Bovine RSV strain 375 was propagated on adherent bovine turbinate cells. By 7 days of incubation or when over 90% of viral induced cytopathic effect was observed, flasks were frozen at −80 °C. Within 2 days, the flasks were thawed and all media pooled. After thorough mixing and sterile filtration, viral inoculum was aliquoted and stored at −80 °C. Using a standard plaque assay, the tissue culture infectious dosage ($TCID_{50}$), for the virus was determined to be $10^{4.9}$ $TCID_{50}$/ml (3.97×10^4 plaque forming units (pfu)/ml). The viral inoculum was negative for bovine viral diarrhea virus by PCR (not shown). For in vitro studies, BRSV strain 375 was inactivated by UV irradiation using a Stratagene UV Stratalinker 1800 (3000 μW/cm^2; Stratagene, La Jolla, CA) on the auto-crosslink mode. Twenty-two milliliters of BRSV 375 in a sterile 150 mm × 15 mm petri dish was irradiated 10 times, and the dish was gently swirled each time in between treatments. Inactivation of UV-BRSV was confirmed by testing inoculum on fetal bovine turbinate cells.

2.2. Isolation of alveolar macrophages (AMϕs)

Healthy 2–4-day-old lambs ($n = 8$) and 2–9-year-old adult sheep ($n = 7$) of mixed Rambouillet and Polypay breeds were used for the following experiments. At the time of necropsy, the lungs were aseptically removed, lavaged with sterile PBS and the collected cells were pipetted into sterile 50 ml conical tubes. Lavaged cells were centrifuged at 805 × *g* for 15 min at 4 °C. The supernatants were aspirated off and cells resuspended in 5 ml of supplemented medium (SM; RPMI 1640 with 2% lamb serum, 2 mM L-glutamine, 25 mM HEPES and 1% antibiotic–antimycotic solution (all from Gibco, Carlsbad, CA). Cells were transferred to a large (150 mm × 15 mm) sterile petri dish containing 30 ml of SM. After 2 h incubation at 5% CO_2 and 37 °C, any non-adherent cells and the media were aspirated off and adherent AMϕs washed in warm, sterile PBS. The cells were released from the bottom by the addition of PBS and gentle scraping with a sterile rubber policeman. The AMϕs in PBS were pipetted into 50 ml conical tubes and centrifuged at 453 × *g* for 5 min at 4 °C. The supernatants were aspirated off and AMϕs were resuspended in 1–2 ml of AMϕ medium then enumerated on a hemacytometer.

2.3. In vitro stimulation of AMϕs

Sterile, ninety-six well round bottom plates were used for the in vitro assays. AMϕs were adjusted to 1×10^6 cells in 200 μL of SM. The cells were exposed to *Escherichia coli* LPS O55:B5 (1 μg/ml; Sigma–Aldrich, St. Louis, MO), poly (I:C) (1 μg/ml; Amersham Biosciences, Piscataway, NJ), BRSV 375 at a multiplicity of infection (MOI) of 0.5, 1.0, or 2.0, or UV-inactivated BRSV 375 (UV-BRSV) at a MOI of 1.0. After 90 min of incubation, BRSV treated wells were washed by aspiration of the supernatant and cells rinsed with warm RPMI and 200 μL of fresh SM was replenished. Supernatants were collected and 300 μL of RLT buffer, from the RNeasy Mini RNA Isolation Kit (Qiagen, Valencia, CA), were added to cells and collected at 2, 6, 18 and 24 h.

2.4. Immunohistochemistry

Alveolar macrophages (1×10^6 cells) were incubated in chamber slides (Nalge Nunc International, Naperville, IL) with 2 ml SM and BRSV at a MOI of 1. After 90 min

incubation at 5% CO_2 and 37 °C, any remaining extracellular virus and medium was aspirated off and the cells washed in warm RPMI. Two milliliters of fresh SM was added to each chamber and slides were incubated at 5% CO_2 and 37 °C for a total of 24 h. The medium was aspirated off and the slides were washed with PBS and fixed for 10 min with 100% methanol at room temperature. The slides were dried and rehydrated in 1× Tris buffer and blocked with normal goat serum (Kirkegaard Perry Labs, Gaithersburg, MD) for 30 min at room temperature. The slides were washed in 1× Tris buffer and incubated with polyclonal anti-BRSV antibody conjugated to FITC (50 μL; VMRD Inc., Pullman, WA) at 37 °C for 30 min. To visualize nuclei, slides were washed with 1× Tris buffer and incubated with 4′,6-diamidino-2-phenylindole, dilactate (DAPI, 300 nM; Molecular Probes) for 5 min at room temperature. The slides were washed with 1× Tris buffer and cover slips mounted with a 50–50 glycerin and PBS solution. Alveolar macrophages were examined using a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Inc., Exton, PA). Images were prepared using Adobe Photoshop version CS and InDesign version 3.

2.5. RSV plaque assay

A previously published plaque assay (McKimm-Breschkin, 2004) was utilized in the present study, with the minor modification that primary ovine fetal turbinate epithelial (OFTu) cells were used as the cell type. This was based on results of preliminary viral plaque experiments comparing OFTu cells, bovine turbinate epithelial cells, and human A549 cells. It was determined, that for BRSV, OFTu cells gave the best plaque visualization. Confluent cells were inoculated with BRSV or supernatants from BRSV-infected AMϕs and incubated for 1 h at 37 °C. The inoculum was removed and 3 ml of overlay (DMEM/F12 + 0.3% agarose) was added to each petri dish. Samples were incubated up to 6 days at 37 °C, 5% CO_2. Subsequently, at selected time points postinoculation, cells were fixed overnight with 1% paraformaldehyde in PBS. Following fixation, agarose overlay was decanted, plates rinsed with dH_2O, and air-dried. Cells were stained with 0.5% neutral red for 2 h at RT, washed, and allowed to air dry.

2.6. Total RNA extraction and cDNA synthesis

Enriched AMϕs were pelleted in sterile 1.5 ml eppendorf tubes. Cells were resuspended in 350 μL of RLT buffer from the RNeasy Mini RNA Isolation Kit (Qiagen, Valencia, CA) and stored at −80 °C. Total RNA was isolated using the RNeasy Mini RNA Isolation Kit (Qiagen) according to manufacturer's instructions. Contaminating genomic DNA was removed during RNA isolation using an on-column RNase-Free DNase I digestion set (Qiagen) according to manufacturer's instructions. Total RNA was eluted by the addition of 40 μL of DNase–RNase free water. 500 ng to 1 μg of total RNA from each sample was reverse transcribed using oligo$(dT)_{12-18}$ primers (Invitrogen, Carlsbad, CA).

2.7. Cytokine and BRSV NS2 mRNA real time PCR assays

Primers were designed specifically for SYBR Green quantification using the Primer Select program (DNASTAR Inc., Madison, WI) with suggested parameters for SYBR Green chemistry that included product size no larger than 100–150 base pairs and a 50 °C annealing temperature. Ovine ribosomal protein S15 was chosen as the endoge-

Table 1
Primer sequences for ovine target and endogenous control genes.

Primer	Sequence (5′–3′)	Length (bp)	Accession no.
IL-1β F IL-1β R	ATGGGTGTTCTGCATGAG AAGGCCACAGGAATCTTG	63	X54796
IL-4 F IL-4 R	GGACTTGACAGGAATCTC CTCAGCGTACTTGTACTC	80	Z11897
IL-6 F IL-6 R	GAGTTGCAGAGCAGTATC GGCTGGAGTGGTTATTAG	75	X62501
IL-8 F IL-8 R	AAGCTGGCTGTTGCTCTC GGCATCGAAGTTCTGTACTC	94	X78306
IL-10 F IL-10 R	GATGCCACAGGCTGAGAACC GCGAGTTCACGTGCTCCTTG	53	U11421
IL-12p40 F IL-12p40 R	AAGTCACATGCCACAAGG CACTCCAGAATGAGCTGTAG	72	AF004024
TNFα F TNFα R	CTCATCTACTCGCAGGTCCTC ACTGCAATGCGGCTGATGG	86	X56756
RiboS15 F RiboS15 R	TACAACGGCAAGACCTTCAACCAG GGGCCGGCCATGCTTTACG	105	[a]
BRSV NS2 F NS2 R	ACCACTGCTCAGAGATTG AATGTGGCCTGTCGTTCATCG	155	NC_001989

F = forward; R = reverse.

[a] Ovine ribosomal protein S15 mRNA sequence provided by Dr. Sean Limesand, Dept. of Pediatrics, University of Colorado Health Sciences Center, Perinatal Research Center, Aurora, CO.

nous control for the genes of interest, which are listed in Table 1. We have used S15 as our endogenous control in several previous publications (Fach et al., 2007; Kawashima et al., 2006; Meyerholz et al., 2006; Sow et al., 2009) and it was previously selected after comparisons to β-actin and GAPDH. In addition, primers were included for a macrophage cell surface antigen, CD14, and cytokeratin 18. Cytokeratins are a subfamily of intermediate filament proteins expressed in the intracytoplasmic cytoskeleton of epithelia. Cytokeratin 18 was included to control for epithelial cell contamination in the lavage cell preparation. Cytokine mRNA transcripts were quantified as previously described (Fach et al., 2007). Briefly, Oligo(dT) cDNA was diluted 1:10 in DNase–RNase free water and 2 μL used for quantification. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used according to manufacturer's instructions. An Applied Biosystems 7300 Real Time PCR Systems machine was used with the same amplification conditions for all genes of interest: 10 min at 95 °C, 15 s at 95 °C, 40 cycles of 1 min at 50 °C, with a dissociation step of 15 s at 95 °C, 30 s at 50 °C, 15 s at 95 °C. All reactions were performed in duplicate. Final relative quantification was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) where the amount of target gene is normalized to an endogenous control (ovine ribosomal protein S15) and expressed relative to control cells (Fach et al., 2007). Primers were validated on the Applied Biosystems 7300 machine by using serial dilutions of total RNA with ovine ribosomal protein S15 and target gene primers, whose values were plotted as the log input amount versus ΔC_T values (target gene C_T—endogenous control C_T) for relative efficiency. Only primers with a slope of less than 0.1 were used, due to similar amplification efficiencies as the endogenous control. Specificity of PCR products was confirmed by sequencing of amplicons.

In sterile eppendorf tubes, 20 μL of total RNA from BRSV 375 inoculum and BRSV oligo(dT) cDNA were treated with RNase I_f (1 U/μL; New England Biolabs, Ipswich, MA) or untreated and all four samples were incubated for 30 min at 37 °C. The digestion of single stranded RNA was stopped by heating the tubes at 70 °C for 20 min. Removal of the RNase enzyme was performed by Microcon column exclusion (100 U columns; Millipore, Billerica, MA) according to manufacturer's instructions. Samples were quantified using BRSV NS2 primers and SYBR Green chemistry as previously described (Fach et al., 2007), with the modification that subsequent time points were expressed relative to the 2 h postinfection time point. No amplification of genomic BRSV NS2 was observed (not shown).

2.8. Statistical analysis

Data were expressed as a fold change relative to control cells or, in the case of BRSV NS2 gene, relative to the 2 h time point. We analyzed the cytokine data for each treatment and gene with the outcome variable ($2^{-\Delta\Delta C_T}$) log transformed. Samples were compared using a one-way ANOVA (Prism, GraphPad, La Jolla, CA). Statistical significance was considered to be $P < 0.05$.

3. Results

3.1. In vitro immunofluorescence detection of BRSV-infected AMϕs

To compare the permissiveness of neonatal and adult AMϕs to BRSV infection, cells were exposed to virus at a MOI of 2 for 90 min. After 24 h of incubation, slides were fixed and subsequently stained using a FITC-conjugated anti-BRSV polyclonal antibody. A subpopulation of neonatal AMϕs expressed BRSV antigen as shown for two individual animals (Fig. 1A and B). BRSV staining was evident in the cytoplasm of infected AMϕs. Alveolar macrophages not exposed to BRSV were included as negative controls as shown for one representative sample (Fig. 1C). Similarly, a subpopulation of BRSV-infected adult AMϕs expressed BRSV antigen as seen in neonatal lamb AMϕs (not shown). Overall, AMϕs from both neonatal lambs and adult sheep are permissive to BRSV infection in vitro. However, additional experiments were utilized to determine whether neonatal AMϕs differed from adult AMϕs in supporting active viral replication after in vitro BRSV infection.

3.2. Validation of BRSV NS2 real-time PCR using primary cells

Upon initiation of replication, the RNA dependent RNA polymerase transcribes the RSV negative sense genome in the 3′ to 5′ direction. Large amounts of the nonstructural proteins NS1 and NS2 mRNA are produced during viral replication, as a result of the genome's transcriptional polarity, where the genes on the 3′ end are transcribed in greater amounts than 5′ genes. Primers were designed to BRSV NS2 mRNA and a real-time PCR assay was used as a measure of BRSV replication in adherent fetal ovine turbinate cells. Data shown for each time point were normalized using an endogenous control and expressed as a fold increase relative to the 2 h time point (Fig. 2A). As can be seen, results of the real-time PCR assay indicated that NS2 mRNA increased in fetal turbinate cells over time. Results of the NS2 real-time PCR assay correlate well with results obtained using a viral plaque assay (Fig. 2B). Earliest viral plaques were visible between days 3 and 4, but were most clearly defined by day 6 of incubation. The data indicate that the NS2 real-time PCR assay can be used as a measure of viral replication.

3.3. Neonatal AMϕs support limited viral replication in vitro

To detect in vitro viral replication within AMϕs, primers were designed to the NS2 mRNA of BRSV. Adult and neonatal AMϕs were exposed to BRSV in vitro for 90 min and the supernatant was removed. The cells were washed in warm RPMI to remove any remaining extracellular virions and fresh medium was added. The supernatants and cells were harvested after 2, 6, 18 and 24 h of incubation. Data from NS2 real-time PCR assay showed that neonatal AMϕs incubated with BRSV exhibited little viral replication and this did not vary significantly with the different MOI

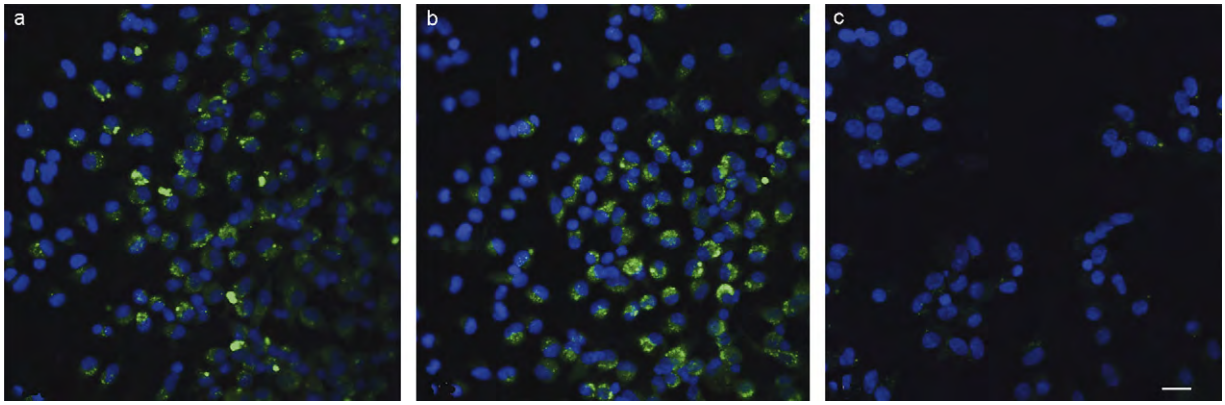

Fig. 1. Bovine respiratory syncytial virus (BRSV) detection in neonatal AMϕs. Neonatal AMϕs were incubated in vitro with BRSV for 90 min. Cells were washed with warm RPMI and replenished with fresh complete medium. After 24 h of incubation, cells were washed and fixed. Cells were stained with FITC-labeled polyclonal anti-BRSV antibody and DAPI for nuclear visualization. Immunofluorescence observed in BRSV-infected AMϕs from two representative individual animals is shown (A and B). A representative micrograph from mock-infected AMϕs is shown in (C). Scale bars: 5 μM.

examined in the present study (Fig. 2C). If the NS2 C_T values for BRSV were expressed relative to the C_T values for UV-inactivated virus there was a significant difference ($P < .05$), but the fold change did not increase between 2 and 24 h (not shown). Adult AMϕs exposed to BRSV produced similar amounts of NS2 mRNA when compared to the neonatal AMϕs exposed to BRSV (Fig. 2D). In either case, NS2 transcription in BRSV-infected AMϕs was significantly lower at 6 and 24 h than observed for BRSV-infected epithelial cells (Fig. 2A compared to Fig. 2C or D). The lack of evidence for

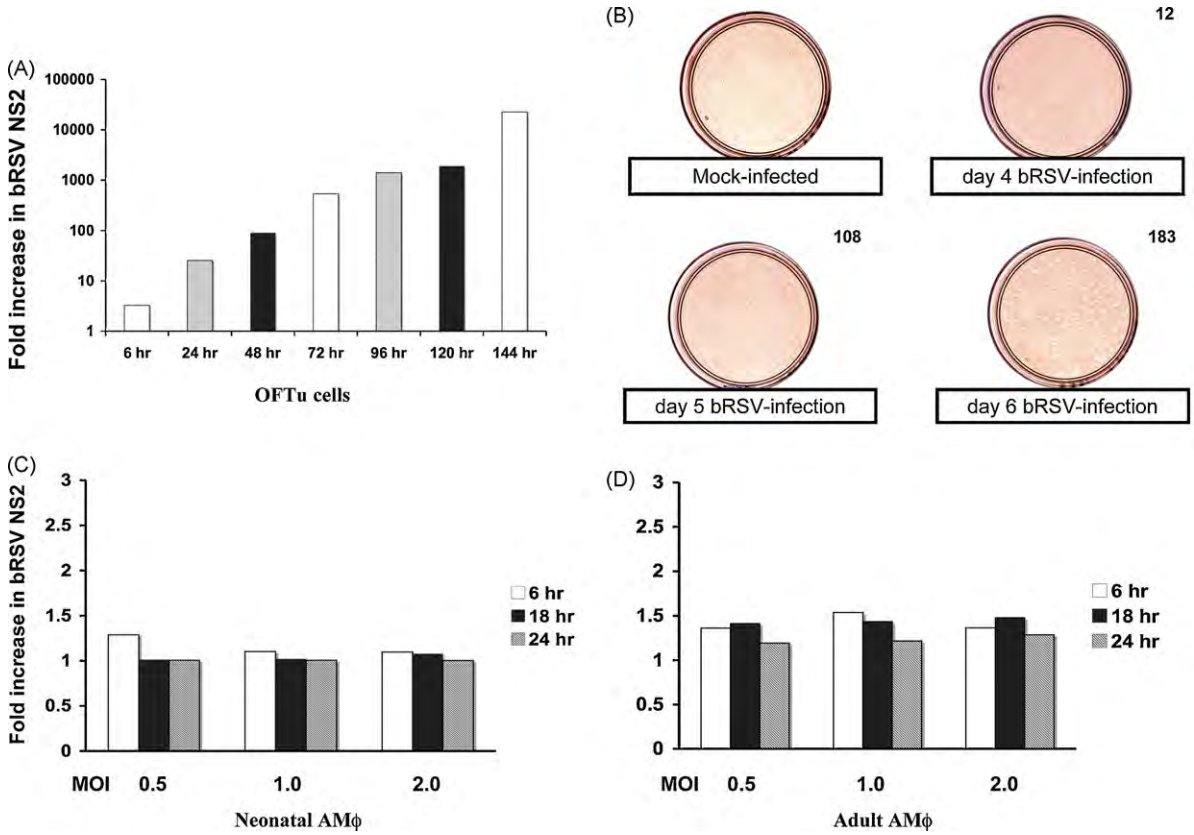

Fig. 2. Bovine respiratory syncytial virus (BRSV) replication in vitro. (A) Fetal ovine turbinate (OFTu) cells were exposed to BRSV and samples harvested at the time points shown. BRSV NS2 mRNA induction was quantified by real-time PCR and data expressed relative to 2 h time point. (B) Viral plaque assay using OFTu cells exposed to BRSV. On days 4–6 postinfection, cells were fixed, stained with 0.5% neutral red, washed and air-dried. Numbers in the upper right corner are the average number of plaques per plate for duplicate samples. A mock-infected sample is presented for comparison. (C) Neonatal ($n = 8$) or (D) adult ($n = 7$) AMϕs were exposed to BRSV at the indicated multiplicity of infection (MOI 0.5, 1.0 or 2.0) for 6, 18, or 24 h. NS2 mRNA induction was quantified using a real-time PCR assay and data expressed relative to 2 h time point.

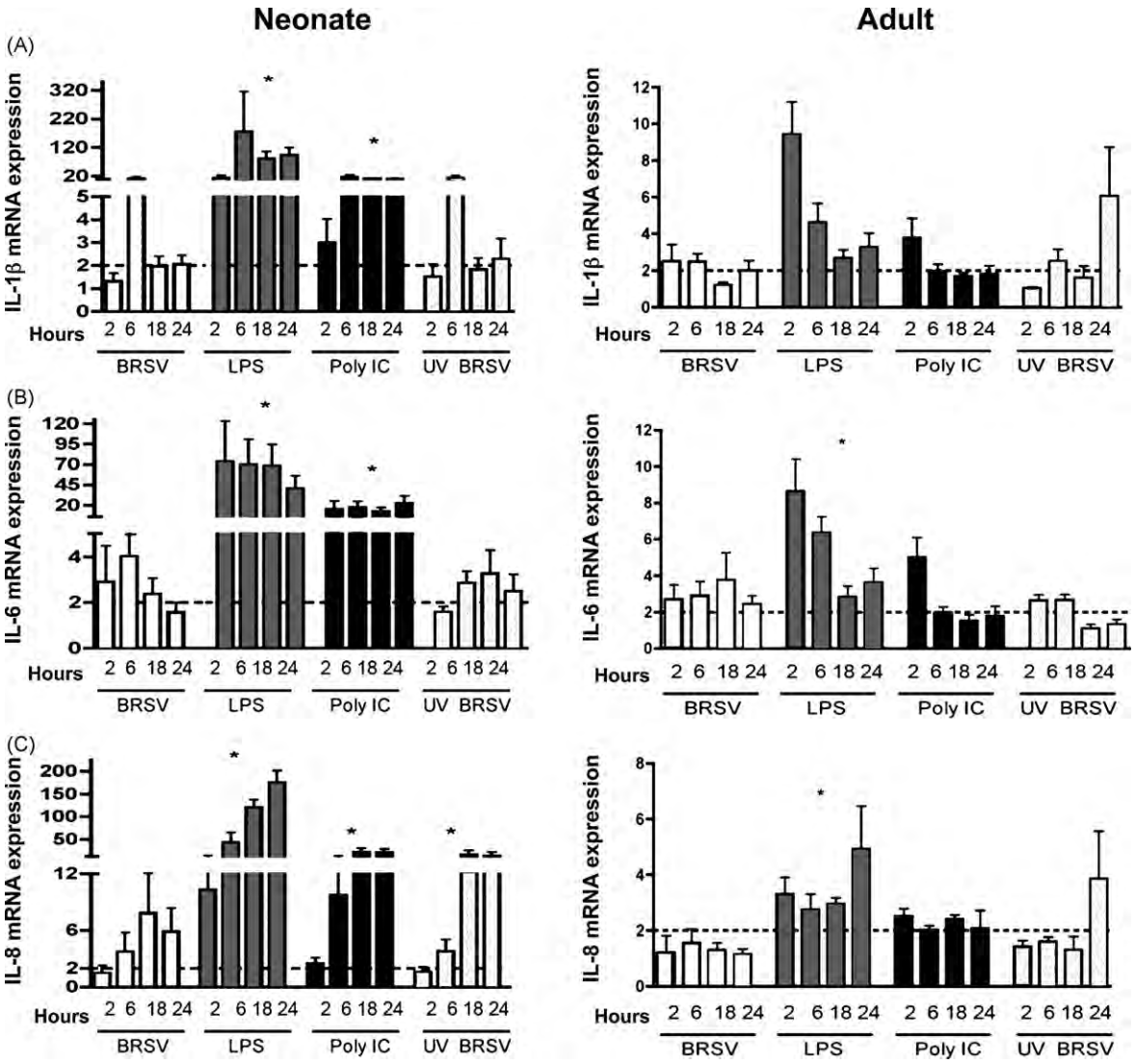

Fig. 3. IL-1β, IL-6, and IL-8 mRNA transcript expression in AMϕs in response to bovine respiratory syncytial virus (BRSV) or TLR ligation. Shown in each panel are data for cells treated with BRSV (MOI = 2), LPS (1 μg/ml), poly (I:C) (1 μg/ml), or UV-inactivated BRSV (MOI = 1) for 2, 6, 18, or 24 h. Total RNA was isolated and cytokine induction was quantified by real-time PCR. Data for IL-1β (A), IL-6 (B), or IL-8 (C) are presented as means ± standard errors of the means relative to non-stimulated controls. Results for neonatal AMϕs ($n = 8$) are shown in the left column, whereas results for adult AMϕs ($n = 7$) are shown in the right column. The dotted line in each panel denotes a 2-fold induction. ANOVA was used to compare TLR agonists or UV-inactivated BRSV to live BRSV-stimulated cells within each panel for each cytokine (*$P < 0.05$).

substantial viral replication in neonatal or adult AMϕs was confirmed by viral plaque assay using 24 h supernatants from RSV-infected AMϕ cultures added to OFTu cells; there were relatively few plaques observed following the 6-day incubation period (not shown). Taken together, our results indicate that neonatal AMϕ do not support BRSV replication to a greater degree than adult AMϕ as evidenced by data from NS2 real-time PCR and viral plaque assays.

3.4. BRSV induces higher levels of vitro pro-inflammatory cytokine transcripts in neonatal AMϕs compared to mRNA levels induced in adult AMϕs

Alveolar Mϕs from neonatal lambs or adult sheep were infected with BRSV and induction of cytokine gene expression at various time points postinfection was determined using real-time PCR assays. To account for the potential for epithelial cell contamination in lavage cell preparations, we included primers for CD14 (macrophage marker) and cytokeratin 18 (epithelial cell marker). AMϕ cell preparations analyzed by RT-PCR did not contain a cytokeratin 18 amplicon, but an epithelial cell control was cytokeratin 18 positive (not shown). Thus, we did not find evidence for significant contribution of epithelial cells to adherent BAL cell-derived mRNA. The data shown herein (Figs. 3–5) demonstrate that BRSV induced a quantitatively different cytokine mRNA response in neonates than in adults. Of note, IL-1β, IL-6, and IL-8 mRNA transcripts were increased 4–20-fold upon viral infection of neonatal AMϕs (Fig. 3A–C). However, these responses in neonatal

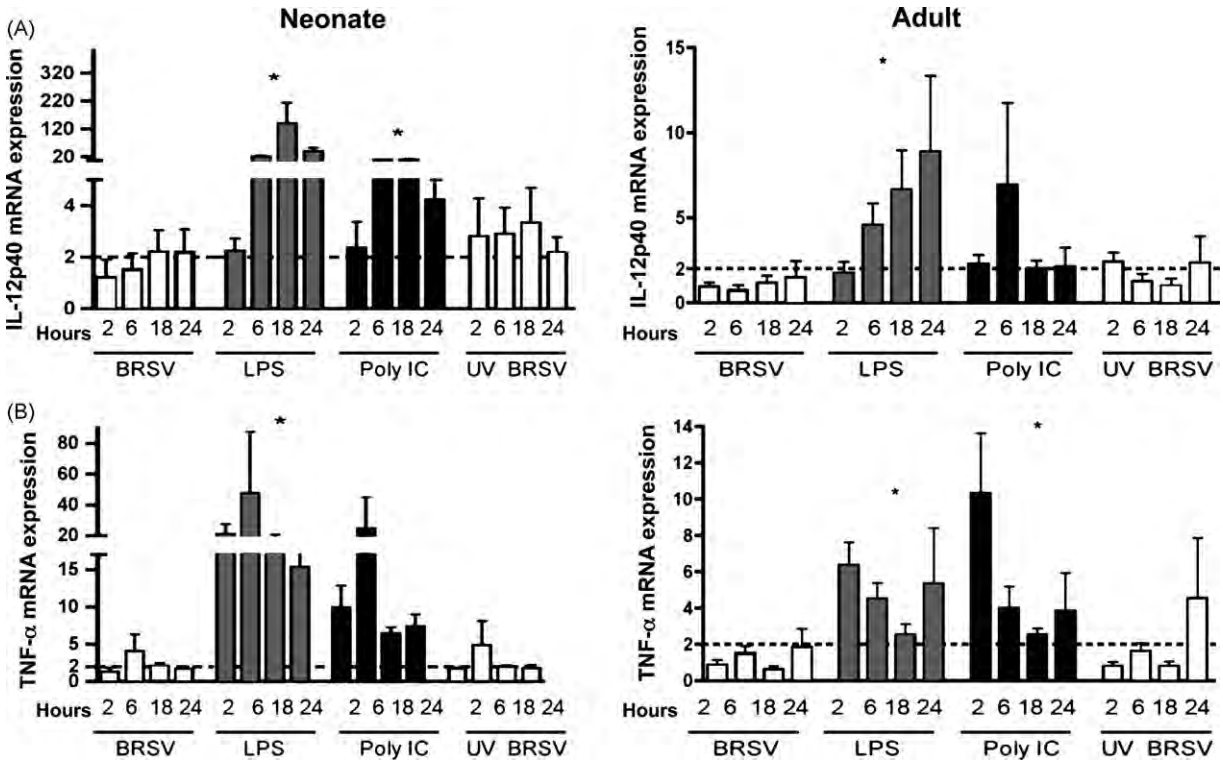

Fig. 4. IL-12p40 and TNF-α mRNA transcripts expression in AMϕs in response to bovine respiratory syncytial virus (BRSV) or TLR ligation. Shown in each panel are data for cells treated with BRSV (MOI = 2), LPS (1 μg/ml), poly (I:C) (1 μg/ml), or UV-inactivated BRSV (MOI = 1) for 2, 6, 18, or 24 h. Total RNA was isolated and cytokine induction was quantified by real-time PCR. Data for IL-12p40 (A) or TNF-α (B) are presented as means ± standard errors of the means relative to non-stimulated controls. Results for neonatal AMϕs ($n = 8$) are shown in the left column, whereas results for adult AMϕs ($n = 7$) are shown in the right column. The dotted line in each panel denotes a 2-fold induction. ANOVA was used to compare TLR agonists or UV-inactivated BRSV to live BRSV-stimulated cells within each panel for each cytokine (*$P < 0.05$).

AM peaked early and were not sustained, similar to the previous report of Matsuda et al. (1996) showing short-term expression of IL-6 or TNF-α in neonatal cord blood monocyte-derived macrophages. By comparison, a 1–4-fold increase in transcripts for IL-1β, IL-6 and IL-8 were observed in adult AMϕs. In general, pro-inflammatory IL-12p40 and TNFα gene transcription increased 2–4-fold after BRSV infection of neonatal and adult AMϕs (Fig. 4A and B). For the time points examined, transcription of IL-4 and IL-10 peaked at 6 h postinfection in neonates, but peaked at later time points postinfection in adults (Fig. 5A and B). Interestingly, AMϕs exposed to either live BRSV or UV-BRSV expressed similar levels of mRNA transcripts of IL-1β, IL-4, IL-6, IL-10, IL-12p40, and TNFα. By comparison, UV-BRSV actually induced significantly more IL-8 gene transcription than live BRSV in neonatal AMϕs. Thus, it is likely that phagocytosis of small numbers of RSV virions would be sufficient to trigger an induction of cytokines/chemokines in neonatal AMϕs.

3.5. TLR agonists induced higher levels of cytokine transcripts in neonatal ovine AMϕs compared to mRNA levels induced in AMϕs from adult animals

LPS is a known inducer of TLR4 signaling and poly I:C (synthetic double stranded RNA) has been shown to signal through TLR3. Data from in previous studies have suggested that RSV may induce cytokine production via signaling pathways dependent on TLR3 (Rudd et al., 2005) or TLR4 signaling (Kurt-Jones et al., 2000). Therefore, we were interested to compare responses from AMϕs stimulated with LPS or poly I:C to responses induced by BRSV. Induction of cytokine gene expression at various time points postinfection was determined using real-time PCR assays. In the present experiments, TLR3 or TLR4 agonists induced significantly higher levels of IL-1β, IL-6, IL-8, IL-10 and IL-12p40 gene transcription in neonatal and adult AMϕs in comparison to the levels of transcription induced by BRSV infection (Figs. 3–5). Moreover, the cytokine induction to TLR agonists was sustained in neonatal AMϕs, whereas the induced cytokine response to BRSV was generally not. In addition, as described for BRSV above, TLR-stimulation of AMϕs isolated from neonates generally exhibited levels of mRNA transcription that exceeded levels seen in adult AMϕs. Of note, levels of peak induction varied based on the specific cytokine examined, the agonist used for stimulation, and age of host from which AMϕs were isolated. Taken together, our results indicate that cytokine/chemokine mRNA expression induced in neonatal AMϕs via TLR3 or TLR4 ligation or BRSV infection was, in general, substantially higher than mRNA transcript levels induced in adult AMϕs.

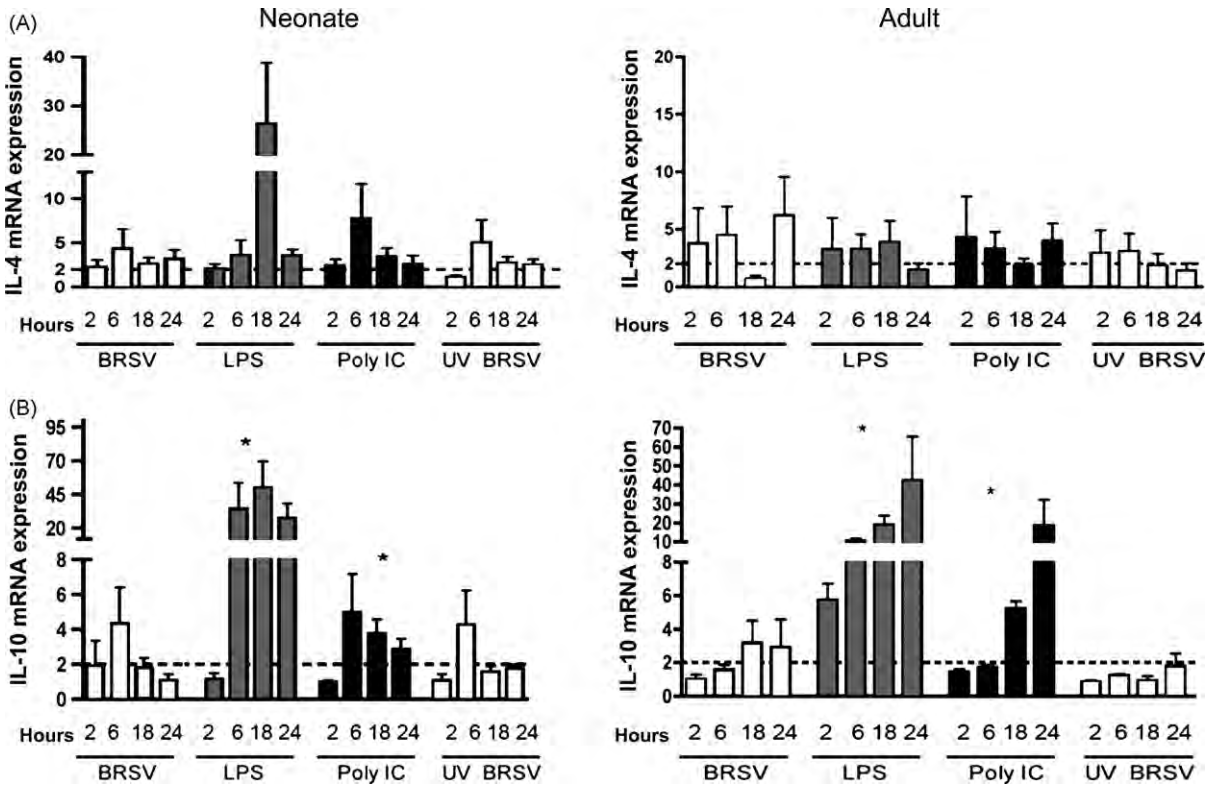

Fig. 5. IL-4 and IL-10 mRNA transcripts expression in AMϕs in response to bovine respiratory syncytial virus (BRSV) or TLR ligation. Shown in each panel are data for cells treated with BRSV (MOI = 2), LPS (1 μg/ml), poly (I:C) (1 μg/ml), or UV-inactivated BRSV (MOI = 1) for 2, 6, 18, or 24 h. Total RNA was isolated and cytokine induction was quantified by real-time PCR. Data for IL-4 (A) or IL-10 (B) are presented as means ± standard errors of the means relative to non-stimulated controls. Results for neonatal AMϕs ($n = 8$) are shown in the left column, whereas results for adult AMϕs ($n = 7$) are shown in the right column. The dotted line in each panel denotes a two-fold induction. ANOVA was used to compare TLR agonists or UV-inactivated BRSV to live BRSV-stimulated cells within each panel for each cytokine (*$P < 0.05$).

4. Discussion

The present study is the first to directly compare BRSV infection or TLR ligation and subsequent induction of cytokine responses in neonatal AMϕ to cells isolated from adult animals. Neonatal and adult AMϕs were permissive to BRSV, however, the virus was demonstrated to minimally replicate within these cells. In general, adult AMϕs produced lower levels of cytokine mRNA transcripts after in vitro BRSV infection compared to cytokine transcripts seen in the neonatal AMϕs, especially IL-1β and IL-8 at the peak of their respective responses. Furthermore, peak cytokine mRNA expression occurred at earlier time points in neonatal AMϕs compared to peak cytokine mRNA expression in adult AMϕs. Finally, TLR3 and TLR4 agonists induced quantitatively higher levels of pro-inflammatory and immunomodulatory cytokine responses than BRSV in both neonatal and adult AMϕs.

Prior investigators have shown that adult AMϕs can be infected with RSV, but viral replication is generally abortive. Given that neonates exhibit greater susceptibility to RSV infection, a key parameter to evaluate would be permissiveness of neonatal AMϕs to RSV infection compared to cells from adults. To investigate viral infection of AMϕs, in vitro assays utilizing neonatal lamb AMϕs inoculated with BRSV were established for direct comparison to responses induced in adult AMϕs. In vitro studies were necessary since immunocompetent adults are generally not susceptible to RSV. Detection of macrophage infection was determined by immunofluorscence microscopy. The immunofluorescence data shows that different subsets of neonatal AMϕs are permissive to RSV infection. To date, it remains unknown why some AMϕs are not infected with the virus, but one study suggests that the maturation state of AMϕs may influence susceptibility to RSV, with immature AMϕs more permissive to RSV infection (Dakhama et al., 1998). Nonetheless, we observed that minimal replication of BRSV occurred in both neonatal lamb and adult sheep AMϕs infected with the virus in vitro, in agreement with previous studies of adult AMϕs from other species (Franke-Ullmann et al., 1995; Stadnyk et al., 1997). We have demonstrated this by two different approaches; utilizing real-time PCR detection of viral NS2 mRNA or a viral plaque assay using supernatants from AMϕ cultures. Further, we have shown that live virus is generally not required for cytokine mRNA induction. Therefore, differences in cytokine transcriptional profiles induced by RSV in neonatal AMϕs compared to adult AMϕs are not a direct result of differences in viral replication.

Previous studies have reported production of pro-inflammatory cytokines and chemokines from AMϕs infected in vitro with RSV. Human, mouse, calf, or guinea

pig AMϕs have been utilized for these in vitro assays. Production of IL-1β, IL-6, IL-8, and TNFα mRNA and protein is seen in AMϕs after infection with RSV (Becker et al., 1991; Franke-Ullmann et al., 1995; Kaan and Hegele, 2003; Panuska et al., 1995). However, AMϕs utilized in previous studies were isolated from different aged subjects, some using adult human, or adult or juvenile laboratory animals for their studies (Becker et al., 1991; Kaan and Hegele, 2003; Miller et al., 2004; Stadnyk et al., 1997). We are aware of studies in which investigators compared neonatal and adult macrophage responses to RSV. However, in those studies, the authors utilized neonatal cord blood-derived and adult peripheral blood-derived Mϕs (Krishnan et al., 2003; Matsuda et al., 1996; Midulla et al., 1989). Midulla et al. (1989) did examine alveolar macrophages in their study, but only isolated these cells from adult subjects. Our study, for the first time, compares neonate and adult AMϕs in parallel to examine the RSV modulation of cytokine mRNA transcripts. Both neonatal and adult AMϕs produced at least a 2-fold increase in gene expression of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40 and TNFα in response to BRSV. However, IL-1β and IL-8 at the peak of their respective responses were found to be several fold higher in neonatal AMϕs than in adult cells. In agreement with our findings, it has been shown that neonatal mice have stronger pro-inflammatory cytokine responses to viral infection than adult mice (Zhao et al., 2008). Moreover, it is interesting to note that IL-1β and IL-8 were found to be the major inflammatory mediators in bronchoalveolar lavage fluid of children with chronic respiratory diseases (Babu et al., 2004). IL-1β has numerous pro-inflammatory effects, including induction of chemokines in bronchial epithelial cells and airway smooth muscle (Edwards et al., 2005; Oltmanns et al., 2003; Wuyts et al., 2003). In RSV illnesses in infants, there is a marked increase in neutrophil numbers which is associated with increased levels of IL-8 (Noah et al., 2002).

Discrepancies exist in the literature as to whether live, replicating virus is needed for cytokine induction. In the present study, results indicate that in vitro BRSV replication is not necessary for induction of cytokine gene expression in neonatal or adult AMϕs. Our results are in agreement with previous studies that reported IL-6, IL-8, and TNFα expression is induced by live RSV and UV- or heat-inactivated RSV exposed human or murine primary AMϕs (Becker et al., 1991; Stadnyk et al., 1997). However, these observations are in contrast to one report where investigators using a murine AMϕ cell line showed that live RSV is required for optimal chemokine induction (Miller et al., 2004). This discrepancy between the observations in the study of Miller et al. (2004) and results from other studies, including our own, may plausibly be explained as a difference due to the use of an AMϕ cell line versus the use of primary AMϕs.

In the present study we quantitatively compared BRSV modulated cytokine gene transcription in AMϕs to that induced by TLR3 and TLR4 agonists. Recently, one study has reported RSV induces chemokine production of IL-8 and RANTES via TLR3 signaling in vitro using transfected TLR3 HEK 293 cells (Rudd et al., 2005). RSV was also shown to up regulate expression of TLR3 on epithelial cell lines. TLR3 engagement initiates cytoplasmic recruitment of TRIF, leading to downstream activation and nuclear translocation of NF-κB family members and transcription of pro-inflammatory genes (O'Neill, 2006). Other studies have investigated the interaction of RSV and TLR4. Engagement of TLR4 by affinity purified RSV A2 F proteins has been shown in vitro to stimulate production of IL-6 by human monocytes and peritoneal macrophages, though it is unknown whether this occurs specifically on AMϕs since they were not examined in these studies (Kurt-Jones et al., 2000). TLR4 stimulation leads to activation of either MyD88 dependent or independent pathways, subsequent activation of NF-κB family members, and ultimately the transcription of pro-inflammatory mediators (O'Neill, 2006). While studies have demonstrated that RSV activates NF-κB via TLR signaling in epithelial cells, cytokine responses of lung APCs to RSV and TLR agonists have not been compared in parallel. Since RSV may potentially interact with TLR3 and TLR4, it was reasonable to compare the cytokine response elicited by BRSV infection compared to TLR3 or TLR4 stimulation of AMϕs. The data in the present study demonstrate that although BRSV induced an upregulation in the cytokine/chemokine response in AMϕs, modest mRNA transcription was generally observed when compared to that induced via TLR ligation. More importantly, TLR signaling induced higher levels of cytokine transcripts in neonatal AMϕs compared to the induction in adult AMϕs. This data fits well with previous published results showing that neonatal mice have higher inflammatory responses to LPS and poly (I:C) than do adult mice (Zhao et al., 2008). They recently proposed that immaturity of the neonatal immune system extends from production of pro-inflammatory cytokines to regulation of such responses. Based on these observations, it will be of interest to delineate mechanisms involved in cytokine transcriptional regulation in neonatal AMϕs compared to adult AMϕs.

Host cellular recognition of pathogenic microorganisms activates signaling modules that are critically involved in regulating cytokine and chemokine induction within the lung microenvironment. Cytokine and chemokine crosstalk in the lung between epithelial cells, professional antigen presenting cells, and other immune cells is important in the outcome and severity of RSV pathogenesis. We have shown a differential induction of the cytokine transcriptional response to RSV infection in neonatal AMϕs compared to AMϕs isolated from adults. Macrophages may be a predominant cell population contributing to severe immunopathology seen in respiratory viral infections. Differential pro-inflammatory cytokine regulation in neonatal myeloid lineage cells compared to analogous cells in adults could contribute to variances in responses to RSV infection.

Acknowledgments

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Rapid and Reliable Single Nucleotide Polymorphism-Based Differentiation of *Brucella* Live Vaccine Strains from Field Strains[▽]

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The reliable differentiation of live *Brucella* vaccine strains from field isolates is an important element in brucellosis control programs. We describe the design, validation, and implementation of a novel single nucleotide polymorphism (SNP)-based typing platform that offers a rapid, reliable, and robust tool to achieve this with improved diagnostic accuracy compared to existing molecular tests. Furthermore, the assays described are designed such that they supplement, and can be run as an intrinsic part of, a previously described assay identifying *Brucella* isolates to the species level (K. K. Gopaul, C. J. Smith, M. S. Koylass, and A. M. Whatmore, BMC Microbiol. 8:86), giving a comprehensive molecular typing platform.

Brucellosis is a zoonotic disease caused by a number of species of the genus *Brucella* (25), which has global implications both economically and socially (8). The most important species in terms of both animal and human disease are *Brucella melitensis* (sheep and goats are the natural hosts) and *Brucella abortus* (cattle are the natural host). While treatment is available for cases of human brucellosis, in other animals, disease control is mediated through culling of infected individuals ("test and slaughter") and/or vaccination of healthy livestock (8). Currently, the three vaccine strains recommended by the World Organization for Animal Health (Office International des Epizooties [OIE]) for brucellosis control are *Brucella abortus* strains S19 and RB51 and *B. melitensis* Rev1 (14, 18). All three vaccines, when administered correctly, can protect livestock from brucellosis but can still cause abortions when administered at the wrong time (4, 10, 21, 22). Furthermore, while the vaccines are considered sufficiently attenuated for animal use, they may still be pathogenic to humans. There are documented cases showing the pathogenic nature of strains Rev1 and S19 in humans (3). In the case of strain RB51, it has been noted that accidental inoculation via open wounds or needle-stick injuries have led to localized infections, but there is no evidence of accidental exposure to this strain leading to brucellosis (2, 6). Both Rev1 and RB51 are also resistant to drugs that may be used in frontline chemotherapy for the treatment of brucellosis in humans. Thus, for the effective monitoring of both brucellosis control programs and human disease it is important to have reliable tests to differentiate vaccine and field strains.

Existing molecular assays. Previously molecular assays to distinguish each of the three live vaccine strains have been described. These target a 702-bp deletion within the *eryCD* locus for *B. abortus* S19 (19), an insertion sequence, IS*711*, in the *wboA* gene of *B. abortus* RB51 (23), and a single nucleotide polymorphism (SNP) within *rpsL* in *B. melitensis* Rev1 (7). However, currently the commonly used assays for these strains consist of time-consuming and labor-intensive individual conventional PCR or PCR-restriction fragment length polymorphism (RFLP) reactions. Furthermore, the marker used for S19 identification is reportedly absent in older variants of this strain, such as NCTC 8038 (19), and vaccines that are derived from these variants may still be in use in some parts of the world (1, 17).

We have previously described a multiple-outcome real-time PCR assay that can define *Brucella* isolates to the species level based on SNPs identified by multilocus sequencing studies (15, 24). In brief, these are competition assays using two differently labeled minor groove binding (MGB) probes targeting either the "species-specific" or "nonspecific" sequence. Depending on the presence or absence of the target SNP, one probe is cleaved in preference to the other, generating an increase in dye fluorescence from the cleaved oligonucleotide (15). To extend the scope of this assay as a genotyping tool, we have sought to incorporate targets that define the three *Brucella* vaccine strains, using data compiled from recently determined genome sequences for S19 (9), RB51 (B. Bricker et al., unpublished data), and Rev1 (http://www.broadinstitute.org/annotation/genome/brucella_group/MultiHome.html and unpublished data).

Target identification and validation. As the derivation of vaccine strain *B. abortus* RB51 from its parent strain 2308 is well described (20), we chose to use only a single SNP, $A_{667} \rightarrow C_{667}$ in BAB1_0649, for assay development. This SNP was identified by comparison of the RB51 draft genome with the sequences of *B. abortus* strain 2308, *B. abortus* strain 9-941, *B. melitensis* strain 16 M, *B. ovis* ATCC 25840, and *B. suis* strain 1330. In order to confirm the specificity of this target for RB51, we sequenced the loci in 68 strains of *B. abortus* corresponding to members of all 28 multilocus sequence analysis types identified in this species to date (unpublished data) and

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TABLE 1. Targets, primers, and probes for the various vaccine MGB assays with associated working concentrations[a]

Vaccine	Target (position)	Gene description	Working concn (nM) of:	
			Probe	Primer
B. abortus S19	BruAB1_0060 (290)[b]	Transcription regulator (*lysR* family)	VAC: 5′-CCGATAGG**A**G AACCG-3′ (150) NON: 5′-CCGATAGG**C**G AACCG-3′ (100)	F: 5′-ACGCTCTGGATCG CATATTCATC-3′ (900) R: 5′-GCACAGATCGGCG AACTG-3′ (100)
B. abortus S19	BruAB1_1114 (450)	Chaperone (*clpX*)	VAC: 5′-CTGACG**C**AAG CCG-3′ (250) NON: 5′-CTGACG**G**AAG CCG-3′ (50)	F: 5′- CACATCTTCGCCG ACATAGC-3′ (700) R: 5′-CATCGATGTGCCG TTCATCATG-3′ (100)
B. abortus RB51	BAB1_0649 (667)[b]	Glutathione *S*- transferase	VAC: 5′-CTCCTCTTCC**G** TATTGG-3′ (250) NON: 5′-CTCCTCTTCC**T** TATTGG-3′ (50)	F: 5′-TGCTGGTTGGCAA GGAAGAG-3′ (100) R: 5′- CGATCAACGCGC CTTCTG-3′ (700)
B. melitensis Rev 1	BMEI0752 (271)[b]	Ribosomal protein S12 (*rpsL*)	VAC: 5′-ACACCC**A**GCA AGTC-3′ (250) NON: 5′-ACACCC**G**GCA AGTC-3′ (50)	F: 5′-TGACACCCTGGGT ATCGAGAA-3′ (700) R: 5′-GTGGCGGTCGTGT GAAG-3′ (100)
B. melitensis Rev 1	BMEI0208 (933)[b]	Gamma-glutamyl phosphate reductase (*proA*)	VAC: 5′-CGCAGG**T**TTT GCC-3′ (50) NON: 5′-CGCAGG**C**TTT GCC-3′ (250)	F: 5′-CGCGTCGAGATAT TCGGTTGA-3′ (900) R: 5′-GGTGCTGGCGCTC TATCC-3′ (100)

[a] The position of the SNP within each target is shown in parentheses. The target SNP is shown underlined and bold within the vaccine (VAC) and nonspecific (NON) probes.
[b] Probes based on the reverse complement of the target SNP region.

26 other *Brucella* strains representing members of all other species and biovars (data not shown).

The origins of both *B. melitensis* Rev1 and *B. abortus* S19 are somewhat less clear, with no comparison to parental strains possible (11, 16). Thus, for additional confidence in the ability of the assay to distinguish vaccine and wild-type strains, two SNPs were selected for each of these assays. For *B. abortus* S19, SNPs $G_{290}\rightarrow T_{290}$ in BruAB1_0060 and $G_{450}\rightarrow C_{450}$ in BruAB1_1114 were chosen based on comparative genome analysis (9). These changes correspond to SNPs on line 9 and line 75, respectively, of Table S2 in the Crasta et al. paper describing the comparison of S19 with other *B. abortus* genomes (9). In order to confirm the specificity of these two targets for S19, we sequenced the region including these markers from the same panel of strains described above to confirm the specificity of the RB51 SNP target.

For Rev 1, we selected the SNP $C_{271}\rightarrow T_{271}$ in BMEI0752 (*rpsL*), which forms the basis of a currently accepted PCR-RFLP assay (7). In addition, based on a whole-genome sequence comparison between *B. melitensis* 16 M and Rev1 (A. R. Wattam, personal communication) and subsequent confirmation from sequencing 95 *Brucella* isolates, we included an additional marker, $G_{933}\rightarrow A_{933}$, in BMEI0208.

Assay development and validation. The locations of primers, probes, and putative gene functions are shown in Table 1. We employed a "checkerboard" approach to optimize concentrations of primers and probes for each assay. Reaction volumes and cycling parameters were as previously published for our multiple-outcome species-defining assays (15) such that these additional assays can simply be included as additional wells in this test. Briefly, assays were set up in a reaction mixture volume of 12.5 μl made up of 6.25 μl TaqMan genotyping mix (Applied Biosystems, Warrington, United Kingdom) with the working concentrations of primers and probes listed in Table 1. For sensitivity determination, genomic DNA extracted by a standard phenol-chloroform method was quantified in triplicate by spectrophotometry (Smartspec Plus; Bio-Rad, Hemel Hempstead, United Kingdom) and diluted to the required concentration in DNA/RNA-free sterile water (Ambion). Reactions were run on an Agilent MX3005p platform (Agilent, La Jolla, CA) using cycling conditions of one hold at 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Analysis was undertaken using the MxPro software provided with the MX3005p.

Sensitivity for each of the assays was established by testing titrations of target DNA. All vaccine MGB assays were shown to have a minimum discriminatory sensitivity of around 100 fg, a result equating to around 30 cells and in line with previous findings (15).

To confirm the specificity of the newly devised vaccine strain identification assays, two large panels of test strains were assembled. The panels consisted of a mix of purified genomic DNA and crude cell extracts (obtained by inactivating material either by immersion in 66% [vol/vol] methanol or by heat inactivation). For the Rev1 MGB assays the specificity of both markers was examined using a panel of 277 isolates consisting predominantly of a worldwide collection of *B. melitensis* field isolates (n = 180) and including an additional 34 Rev1 isolates. For the S19 and RB51 MGB assays, we examined a panel of 498 *Brucella* isolates representing all *Brucella* species and biovars but consisting predominantly of a worldwide collection of *B. abortus* field isolates (n = 245). Furthermore, given previous evidence of the inability of existing conventional assays to identify all S19 isolates (17), we tested all 498 isolates with both the S19 and RB51 conventional PCR tests accepted by the OIE (18).

The results of this validation exercise are shown in Fig. 1. In brief, the endpoint fluorescence readings for vaccine-specific and nonspecific probes for each sample were plotted with the

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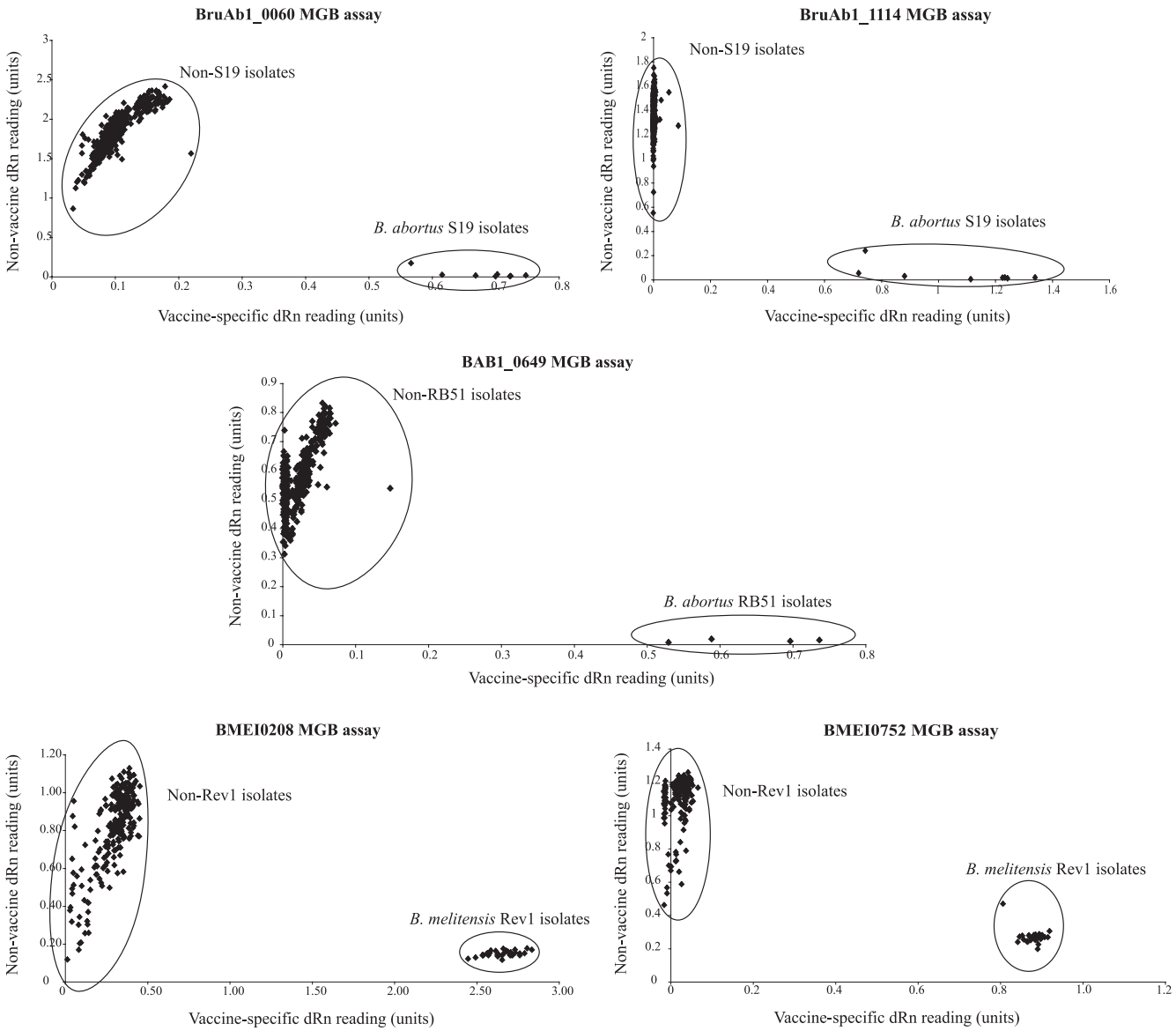

FIG. 1. Allele discrimination plots generated by the individual MGB assays examining either 498 (in the case of strains S19 and RB51) or 277 (in the case of strain Rev1) isolates. In all cases, two distinct populations can be seen: vaccine (closest to the *x* axis) and nonvaccine (closest to the *y* axis).

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former on the *x* axis and the latter on the *y* axis. In all assays the discrimination of vaccine and nonvaccine isolates was clear-cut, with well-defined vaccine and nonvaccine populations. SNP-based typing and the conventional PCR test (18, 23) identified the same four isolates as RB51. However, there was discord between the conventional S19 PCR and MGB assays. The conventional Δ*eryCD* PCR identified only five strains as being S19, while the SNP discrimination assay identified an additional three isolates as S19. These isolates were NCTC 8038, a United Kingdom field isolate from 1964 (64/42), and a Chinese strain (55005) supplied to the VLA in the 1980s. NCTC 8038 was deposited in 1949, before the United States Department of Agriculture (USDA) decided to replace the diverse vaccine stocks circulating at the time with an erythritol-sensitive isolate in 1956 (12). The provenance of the remaining strains is less clear, but the phenotype is consistent with S19, as they have previously been biotyped as CO_2-independent *B. abortus* biovar 1 (L. Perrett, personal communication). To prove the strains were indeed derivatives of S19, we amplified and sequenced gene fragments corresponding to BAbS19_I10690, BAbS19_I10750, BAbS19_I12470, BAbS19_I14270, and BAbS19_I14530, all of which we knew from pilot studies harbored additional SNPs unique to S19 (data not shown). In all five gene fragments from NCTC 8308, 64/42, and 55005, these additional SNPs, consistent with S19, were present, providing strong evidence that they represent S19 derivatives.

Conclusions. The SNP-based assays we have described here can be seamlessly combined with our *Brucella* species identification assays, giving a comprehensive genotyping tool that can rap-

idly, simply, and unambiguously characterize any *Brucella* isolate to the species level and concurrently determine whether it represents a vaccine isolate. The tool works well from crude bacterial extracts, avoiding the need for extensive bacterial processing. In terms of assay performance, we have shown that the BAB1_0649 assay for RB51 provides the same level of discrimination as the existing IS*711 wboA* PCR. The BruAB1_0060 and BruAB1_1114 assays for S19 proved more discriminatory than the existing Δ*eryCD* PCR, which is also the target used in some multiplex conventional PCR tests (5, 13), detecting S19 strains that lack this deletion. This will be particularly useful, as some countries may have generated their own S19 vaccines from stocks apparently originating before the occurrence of the *eryCD* deletion (1, 17). We have also demonstrated that BMEI0208 is a valid target in the differentiation of Rev1, giving the same discrimination as the OIE-accepted BMEI0752 marker. Thus, the assays described here give improved discriminatory capacity relative to conventional PCR tests and provide a unified platform, avoiding the need for multiple different conventional PCR and PCR-RFLP formats. As with all such assays, although we have validated them extensively, further studies with even more extensive collections of field isolates worldwide will undoubtedly be valuable in confirming the specificity of the markers selected for vaccine strains.

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Biological markers of neonatal calf performance: The relationship of insulin-like growth factor-I, zinc, and copper to poor neonatal growth[1]

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ABSTRACT: Raising a heifer calf to reproductive age represents an enormous cost to the producer. Poor neonatal growth exacerbates the costs incurred for rearing, and use of blood variables that may be associated with poorly growing calves may offer predictive value for growth and performance. Thus, the principal objective of the present study was to describe changes in serum IGF-I, zinc, and copper from birth to 90 d in Holstein calves, while accounting for sex and twin status, in poorly growing calves and calves growing well. A second objective was to test the hypothesis that an association exists between these serum variables and morphometric indicators of growth. Measurements of BW, length, and height were recorded at birth and at 30, 60, and 90 d of age. Jugular blood (12 mL) was collected from each calf on d 1 to determine serum total protein, serum IgG, packed cell volume, serum zinc, serum copper, serum IGF-I, and CD18 genotype for bovine leukocyte adhesion deficiency; serum zinc, serum copper, and serum IGF-I (predictor variables) were also determined for each calf on d 2 through 10 and on d 30, 60, and 90. Stepwise multiple regression and logistic regression analyses were used to examine the relationships between the predictor variables and the dependent variables (BW, height, and length at d 30, 60, and 90 of life). Birth weight, sex, serum IGF-I (at all ages), serum copper, and the serum copper-to-zinc ratio were associated, to varying degrees, with the dependent growth variables. Birth weight was consistently the dominant predictor. In conclusion, these results suggest that lighter birth weight, reduced serum IGF-I, and inflammation may be important causes of poor growth in neonatal Holstein dairy calves.

Key words: calf, copper, growth, inflammation, insulin-like growth factor, zinc

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INTRODUCTION

Identifying biomarkers indicative of malnourishment or inflammation that predict poor growth and enable early intervention would reduce overall costs associated with raising dairy replacement heifers. Malnutrition in children and calves is associated with stunted growth (Fawzi et al., 1997; Hoffman, 1997; Mourits et al., 1997), greater morbidity and mortality from pneumonia and diarrhea (Sweeny and Divers, 1990; Yoon et al., 1997), and decreased protein synthesis and breakdown (Manary et al., 1997); malnutrition delays puberty, increases the time from birth to calving, and results in increased costs of raising heifers (Goodger et al., 1989).

Insulin-like growth factor-I is associated with increased somatic growth (Zapf and Froesch, 1986; Yakar et al., 2002), enhanced T-lymphocyte activity (Gelato, 1993; Dorshkind and Horseman, 2001), and stimulation of mammary protein synthesis (Hadsell et al., 1996).

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Cattle on a high plane of nutrition, compared with those on a low plane, have greater serum IGF-I concentrations (Bishop et al., 1989; Radcliff et al., 2004; Brown et al., 2005), although most studies have evaluated calves in laboratory rather than field settings (Baumrucker and Blum, 1994; Baumrucker et al., 1994a,b; Skaar et al., 1994; Daniels et al., 2008). Morbidity and inflammation are negatively associated with calf growth (Waltner-Toews et al., 1986; Paré et al., 1993; Erb, 1994; Jamaludin et al., 1996; Virtala et al., 1996). Serum zinc and copper are biomarkers of inflammation (Graham et al., 1994), and serum immunoglobulin concentration, sex, and twin status may differentially affect the growth of calves (el Bushra et al., 1989; Arrayet et al., 2002), with bulls growing faster than heifers and with compensatory growth in twins allowing similar overall growth compared with singletons. The objectives of this study were to describe changes in blood variables and growth from birth to d 90 in Holstein calves and to test the null hypothesis that there were no associations between serum IGF-I, zinc, and copper and growth, while accounting for sex and twin status.

MATERIALS AND METHODS

All animals were cared for as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

Animals

Holstein bull (n = 211) and heifer (n = 209) calves between 12 and 36 h of age were brought into the calf facility (Foster Farms, Modesto, CA) after being fed approximately 4 L of colostrum at the dairy of origin. All calves were provided by Foster Farms Dairy. All cattle were enrolled during a 6-wk period in March and April. The trial was completed in July. All calves were sequentially allocated into a treatment group (n = 105/group), and a color-coded identification tag was placed in the left ear of each calf, as described previously (Arrayet et al., 2002). Each calf was injected intramuscularly with vitamins A and D (500,000 IU, Boehringer Ingelheim Animal Health, St. Joseph, MO, and 75,000 IU, Butler Co., Dublin, OH, respectively). Calves were housed in individual hutches for the duration of the trial and fed 1.89 L of milk twice daily until they were weaned at 90 d of age. At wk 1 and 4, calves were vaccinated for infectious bovine rhinotracheitis and parainfluenza type 3 with an intranasal vaccine (Nasalgen, Schering-Plough, Omaha, NE). Bull calves were implanted at d 30 with 36 mg of zeranol (Ralgro, Pitman-Moore Inc., Kansas City, KS) and were surgically castrated at d 60. Calves were dehorned, and heifers had permanent identification tags placed in both ears at 4 wk of age. Calves were revaccinated for infectious bovine rhinotracheitis and parainfluenza type 3; vaccinated for bovine virus diarrhea, bovine respiratory syncytial virus, leptospirosis, and clostridia (Cattlemaster 4 + L5, Pfizer Animal Health, Exton, PA; Ultrabac 8, Pfizer Animal Health); and given vitamins A and D (500,000 and 75,000 IU, respectively) at wk 10 of age.

Diets

Calves were fed approximately 3.8 L/d of whole pasteurized waste milk containing 1 of 4 zinc supplements: 40 mg of zinc/kg of DM (that is, no additional zinc; control group) or 100 mg of zinc/kg of DM [control milk with 60 mg of additional zinc added as zinc sulfate (A. L. Gilbert Company, Oakdale, CA), zinc methionine (Zinpro, Eden Prairie, MN), or zinc lysine (Zinpro)]; the details of the milk diet have been published previously (Arrayet et al., 2002). Calves were offered a grain concentrate free choice from birth. The basal concentrate diet contained 40 mg of zinc/kg of DM. Parallel to the zinc supplementation in the milk, the concentrate diets were likewise either 40 or 100 mg of zinc/kg of DM with the same zinc forms as noted above. The diet formulation has also been published previously (Arrayet et al., 2002).

Data Collection

On arrival at the calf ranch, approximately 12 to 36 h after birth and colostrum ingestion, calves were weighed and morphometric measures were determined. Calf BW, length, and height were repeated at 30, 60, and 90 d of age. More detailed morphometric measures from these animals were also obtained and were published in Arrayet et al. (2002). Jugular blood [collected by venipuncture (10 mL), allowed to clot, centrifuged at 800 × *g* for 15 min at 25°C, and aliquoted into 2-mL microfuge tubes and frozen at −20°C; another 2 mL was collected in EDTA] was collected from each calf on d 1 to determine serum total protein, IgG, packed cell volume, serum zinc, serum copper, serum IGF-I, and CD18 genotype [bovine leukocyte adhesion deficiency (**BLAD**)]. Jugular blood [collected by venipuncture (10 mL), allowed to clot, centrifuged at 800 × *g* for 15 min at 25°C, and aliquoted into 2-mL microfuge tubes and frozen at −20°C] was also collected from each calf on d 2 through 10 and on d 30, 60, and 90 for the determination of serum zinc, serum copper, and serum IGF-I.

Blood Variables

The CD18 genotype (homozygous normal or heterozygous for the D128G allele that causes BLAD when present in a homozygous-affected calf) was identified as described previously (Arrayet et al., 2002). Plasma immunoreactive IGF-I was measured in a nonequilibrium assay after removal of IGFBP by acid ethanol cryoprecipitation as described previously (Breier et al., 1991; Chow et al., 1994) using iodinated human recombinant IGF-I (Collaborative Research Inc., Bedford, MA; Hodgkinson et al., 1989). The polyclonal antiserum (UB3-189, provided by L. Underwood and J. J.

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Van Wyk; University of North Carolina, Chapel Hill) was obtained through the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary program (Bethesda, MD). The samples were run in 24 assays, with the same reference control used in each assay. Intraassay variation was 4.5% and interassay variation for controls was 6%. Serum zinc and copper values were measured using published techniques (Graham et al., 1994).

Statistical Analyses

With the data collected, the following variables were examined in the model: twin status; sex; BLAD status; 4 (average over the first 10 d and d 30, 60, and 90) serum zinc, copper, and IGF-I measurements; 4 (same days as the serum zinc and copper measurements) copper-to-zinc ratios; packed cell volume on d 1; serum IgG on d 1; serum total protein on d 1; and the morphometric measurements described above (BW, height, and length on d 0, 30, 60, and 90). The statistical program BMDP 2D (Statistical Solutions, Saugus, MA) was used to examine the descriptive information regarding each variable. Variables with large skewness and kurtosis factors were transformed using either the log or square root function, and the transformation yielding the smallest skewness and kurtosis factors was selected. The following transformations were made: log (d 30 copper-to-zinc ratio), log (d 60 copper-to-zinc ratio), log (serum IGF-I averaged over the first 10 d), log (d 30 serum IGF-I), and square root (d 60 serum IGF-I). No other variables were transformed.

For each statistical run (for each dependent variable), the sample was broken into quintiles, and the bottom 40% (representing poorly growing calves) were compared with the top 40% (representing calves growing well). The statistical programs BMDP 2R and BMDP PR (Statistical Solutions) were used to perform stepwise addition multiple regression and logistic regression analyses, respectively, on the data set. Nine statistical runs were made with each program, using each morphometric variable (BW, length, and height) at d 30, 60, or 90 as the dependent variable. The other nonmorphometric variables, as well as BW at birth, were used as the independent variables.

RESULTS

Of the 211 Holstein bull calves, 35 were twins. A much smaller percentage of the heifer calves were twins (17 twins out of 209 heifer calves evaluated). There was no effect of zinc supplementation on any measures of growth in the current trial (Arrayet et al., 2002). Descriptive data for the calves at different ages stratified by sex and twin status for serum zinc, copper, and the copper-to-zinc ratio are presented in Table 1. Bull calves tended to have reduced serum zinc ($P < 0.06$) and greater serum copper ($P < 0.08$) than heifer calves. This sex effect was lost when the data were expressed as the ratio of copper to zinc. Singletons had significantly less serum zinc ($P < 0.001$), greater serum copper ($P < 0.001$), and a greater copper-to-zinc ratio ($P < 0.001$) than twin calves. Although serum zinc decreased with increasing age, the opposite was observed for serum copper and the copper-to-zinc ratio ($P < 0.001$). Serum IGF-I concentrations (Table 2) were greater in singletons than twins ($P < 0.001$) and in bull calves than heifers ($P < 0.001$), and they increased with increasing age ($P < 0.001$), with heifer singletons exhibiting the greatest increase over time.

The results from the stepwise addition multiple regression analyses for BW, body length, and height are presented in Tables 3, 4, and 5, respectively. These results demonstrate a strong relationship between birth weight and later BW, length, and height in the Holstein calves. For example, in the case of d 30 BW, birth weight accounted for 56% of the variation. The magnitude of the birth weight contribution to the variation diminished with increasing age, but of the independent variables measured, birth weight represented the greatest contribution. Sex and twin status also contributed to the variation observed for BW, length, and height, but those independent variables became less predictive as the animals aged. By d 90 of age, in addition to birth weight, the serum concentration of IGF-I at d 90 showed a distinct relationship to BW, length, and height.

The coefficients for the logistic regression models for BW, length, and height are presented in Tables 6, 7, and 8, respectively. Results from logistic regression models demonstrate, similar to the stepwise addition multiple regression models, that birth weight was the greatest predictor of whether a calf would be in the bottom 40% for BW, overall length, or height. This was true for all 3 ages evaluated (d 30, 60, and 90 of age). Additional associated coefficients for these dependent growth variables were sex, serum IGF-I (at all ages), and the serum copper-to-zinc ratio. For body length, serum copper and heterozygous BLAD status were also associated with poorly growing calves. Table 9 presents the values used to determine the adjusted-risk odds ratio. Values of a hypothetical poorly growing calf were calculated from the calves in the bottom 40% for growth. Similarly, averages of the values from all the calves in the top 40% for growth were used as the reference values. Adjusted-risk odds ratios for the reference group, compared with the poorly growing group, showed greater birth weight, greater serum IGF-I concentrations, and less serum copper than those of the poorly growing group. The reference group also consisted of more male calves than female calves.

DISCUSSION

Birth weight, serum IGF-I, serum copper, and the serum copper-to-zinc ratio were associated with risk of poor growth. Greater birth weight and serum IGF-I concentrations were contraindicative of the poor

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Table 1. Mean ± SEM concentrations of serum zinc, serum copper, and the copper-to-zinc ratio from birth to 90 d of age in Holstein dairy calves, stratified by sex and twin status

Calf and age	Zinc, mg/kg	Copper, mg/kg	Copper:zinc	n
Bull calves				
Singleton				
1 to 10 d[1]	1.12 ± 0.02	0.55 ± 0.01	0.57 ± 0.02	176
30 d	1.18 ± 0.02	0.64 ± 0.01	0.57 ± 0.02	170
60 d	1.04 ± 0.01	0.61 ± 0.01	0.60 ± 0.01	170
90 d	1.01 ± 0.02	0.72 ± 0.01	0.76 ± 0.02	165
Twin				
1 to 10 d[1]	1.30 ± 0.06	0.49 ± 0.01	0.42 ± 0.02	35
30 d	1.33 ± 0.06	0.58 ± 0.02	0.46 ± 0.02	34
60 d	1.07 ± 0.04	0.60 ± 0.02	0.59 ± 0.04	33
90 d	1.06 ± 0.05	0.69 ± 0.03	0.72 ± 0.02	34
Heifer calves				
Singleton				
1 to 10 d[1]	1.15 ± 0.02	0.60 ± 0.01	0.61 ± 0.02	192
30 d	1.24 ± 0.02	0.69 ± 0.01	0.58 ± 0.01	186
60 d	1.08 ± 0.02	0.64 ± 0.01	0.62 ± 0.01	185
90 d	1.03 ± 0.02	0.80 ± 0.01	0.83 ± 0.02	175
Twin				
1 to 10 d[1]	1.36 ± 0.08	0.52 ± 0.02	0.46 ± 0.05	17
30 d	1.30 ± 0.09	0.57 ± 0.04	0.50 ± 0.08	16
60 d	1.19 ± 0.06	0.56 ± 0.02	0.48 ± 0.03	16
90 d	1.08 ± 0.07	0.65 ± 0.04	0.67 ± 0.07	16

[1]Average of first 10 d.

growth rate of a calf, whereas greater serum copper concentration and serum copper-to-zinc ratio were associated with an increased risk of a calf growing poorly. Birth weight was the principal predictor variable associated with the dependent growth variables of BW, body length, and height. The strength of association is most likely attributable to greater genetic potential, with larger animals have larger offspring; however, birth weight also accounts for growth differences attributable to sex and twin status (Arrayet et al., 2002).

Reasons for an association between serum IGF-I and growth have been described previously in mice and rats (Zapf and Froesch, 1986; Hadsell et al., 1996) as well as livestock (Bell et al., 1998; Lammers et al., 1999; Radcliff et al., 2004; Brown et al., 2005). This association is most likely due to an enhanced stimulation of long bone growth, increased nutrient availability (Lowe, 1991), and muscle tissue accretion (Fiorotto et al., 2003) by IGF-I. Further, IGF-I enhances immune function under periods of stress; stress raises immunosuppressive glucocorticoids, and under this condition, IGF-I maintains lymphocytic activity (Dorshkind and Horseman, 2001). A reduction in IGF-I is therefore correlated with diminished immune function while under stress. Malnutrition is a documented stressor that raises glucocorticoids (Dorshkind and Horseman, 2001). The nutrient deprivation also results in depressed IGF-I concentrations, although there is a shift in the remaining IGF-I utilization to support the immune system (Davis, 1998). Thus, periods of stress or malnutrition reduce IGF-I, and the remaining IGF-I is redirected away from stimulating growth.

Inflammation or the nonspecific immune response-induced changes in serum zinc and copper have been described over the past several decades. Under the influence of IL-1, IL-6, tumor necrosis factor, and other

Table 2. Mean ± SEM concentrations of serum IGF-I from birth to 90 d of age in Holstein dairy calves, stratified by sex and twin status

Calf and age	IGF, ng/mL	n
Bull calves		
Singleton		
1 to 10 d[1]	18.38 ± 0.90	176
30 d	12.58 ± 0.60	157
60 d	29.68 ± 1.03	168
90 d	38.70 ± 1.40	165
Twin		
1 to 10 d[1]	12.36 ± 0.88	35
30 d	10.27 ± 0.70	29
60 d	23.83 ± 1.60	33
90 d	34.45 ± 2.45	34
Heifer calves		
Singleton		
1 to 10 d[1]	17.52 ± 0.65	192
30 d	10.93 ± 0.39	177
60 d	24.22 ± 0.78	184
90 d	30.43 ± 0.95	179
Twin		
1 to 10 d[1]	14.22 ± 0.87	17
30 d	11.10 ± 1.49	16
60 d	18.37 ± 2.27	16
90 d	26.45 ± 3.56	17

[1]Average of first 10 d.

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Table 3. Summary of the stepwise multiple regression analyses evaluating the relationships between BW and significant ($P < 0.05$) independent variables recorded at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and variable	Coefficient (SE)	Contribution to adjusted R^2
30 d (359)		
Constant	11.3044	
Birth weight, kg	0.6966 (0.041)	0.560
Sex[1]	−3.0861 (0.443)	0.043
Log_{10} d 30 IGF-I, ng/mL	4.1722 (0.991)	0.023
Log_{10} first 10 d average IGF-I, ng/mL	3.4115 (1.168)	0.011
Serum total protein, g/dL	0.8659 (0.327)	0.007
Twin status[2]	−1.5963 (0.707)	0.004
60 d (347)		
Constant	21.0040	
Birth weight, kg	0.9959 (0.059)	0.425
Square root of d 60 IGF-I, ng/mL	1.3469 (0.305)	0.080
Sex	−4.0453 (0.737)	0.039
Log_{10} d 30 IGF-I, ng/mL	6.5086 (1.594)	0.017
d 60 serum zinc, mg/kg	8.7963 (1.877)	0.013
First 10 d average serum copper, mg/kg	−15.4849 (3.409)	0.010
Log_{10} d 30 serum copper:zinc	12.4281 (2.867)	0.022
Serum total protein, g/dL	1.1781 (0.520)	0.005
90 d (326)		
Constant	51.7819	
d 90 IGF-I, ng/mL	0.2276 (0.042)	0.216
Birth weight, kg	1.0473 (0.099)	0.188
d 90 serum copper:zinc	−11.0274 (1.938)	0.051
Square root of d 60 IGF-I, ng/mL	1.4782 (0.511)	0.022
Log_{10} d 30 IGF-I, ng/mL	7.9304 (2.777)	0.012
Sex	−3.2001 (1.223)	0.009

[1]Bull calf = 0; heifer calf = 1.
[2]Singleton = 0; twin = 1.

Table 4. Summary of the stepwise multiple regression analyses evaluating the relationships between body length and significant ($P < 0.05$) independent variables recorded at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and variable	Coefficient (SE)	Contribution to adjusted R^2
30 d (359)		
Constant	59.6362	
Birth weight, kg	0.3400 (0.027)	0.371
Sex[1]	−0.8681 (0.318)	0.009
Log_{10} first 10 d average serum IGF-I, ng/mL	2.0307 (0.856)	0.008
60 d (347)		
Constant	67.3553	
Birth weight, kg	0.3086 (0.029)	0.276
Square root of d 60 IGF-I, ng/mL	0.4409 (0.149)	0.033
Sex	−1.0661 (0.356)	0.017
Log_{10} d 30 IGF-I, ng/mL	1.8404 (0.808)	0.008
90 d (326)		
Constant	81.3444	
Birth weight, kg	0.3331 (0.039)	0.138
d 90 IGF-I, ng/mL	0.0779 (0.015)	0.100
Log_{10} first 10 d average IGF-I, ng/mL	−5.7258 (1.261)	0.040
Square root of d 60 IGF-I, ng/mL	0.4423 (0.200)	0.018
First 10 d average serum zinc, mg/kg	−2.7009 (0.767)	0.013
First 10 d average serum copper, mg/kg	−7.4169 (2.371)	0.014
d 30 serum copper, mg/kg	5.9685 (1.764)	0.012
Log_{10} d 30 IGF-I, ng/mL	3.1414 (1.107)	0.011
Log_{10} d 60 serum copper:zinc	−6.0487 (2.172)	0.014

[1]Bull calf = 0; heifer calf = 1.

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Table 5. Summary of the stepwise multiple regression analyses evaluating the relationships between height and significant ($P < 0.05$) independent variables recorded at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and variable	Coefficient (SE)	Contribution to adjusted R^2
30 d (359)		
Constant	67.7717	
Birth weight, kg	0.3115 (0.027)	0.404
Twin status[1]	−1.6570 (0.482)	0.010
Sex[2]	−1.0018 (0.299)	0.017
60 d (347)		
Constant	70.9505	
Birth weight, kg	0.2882 (0.024)	0.304
Square root of d 60 IGF-I, ng/mL	0.3387 (0.121)	0.014
First 10 d average serum copper, mg/kg	−3.1183 (1.312)	0.010
Log_{10} first 10 d average IGF-I, ng/mL	1.7749 (0.787)	0.008
90 d (326)		
Constant	79.8708	
Birth weight, kg	0.2562 (0.028)	0.196
d 90 IGF-I, ng/mL	0.0435 (0.011)	0.057
First 10 d average serum copper, mg/kg	−3.9559 (1.541)	0.013
Square root of d 60 IGF-I, ng/mL	0.3013 (0.143)	0.008

[1]Singleton = 0; twin = 1.
[2]Bull calf = 0; heifer calf = 1.

inflammatory mediators, serum copper is increased as ceruloplasmin is exported from the liver, causing serum copper concentrations to increase. Similarly, zinc is sequestered or excreted, although the mechanism for this has not been well described (Keen and Graham, 1989; Graham et al., 1995; Polberger et al., 1996). The negative association of greater serum copper concentrations and greater copper-to-zinc ratios with growth can most likely be attributed to inflammation (Graham et al., 1994). Serum zinc and copper are direct biomarkers of inflammation (Graham et al., 1994), with decreased serum zinc and increased serum copper present during episodes of inflammation. The poorly growing calves had almost 175% greater serum copper concentrations in comparison with the reference group of calves growing well. Likewise, the copper-to-zinc ratio was increased in the poorly growing calves. Beyond reduced feed intake when a calf is ill, inflammation utilizes energy stores in

Table 6. Coefficients of logistic regression models for predicting the odds of a calf growing poorly (bottom 40%) in terms of BW, using independent variables at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and independent variable	Coefficient[1] (SE)	OR[2]
30 d (290)		3,009.2
Constant	16.3100 (2.120)	
Birth weight, kg	−0.3404 (0.042)	
Sex[3]	1.6010 (0.354)	
Log_{10} d 30 IGF-I, ng/mL	−2.3080 (0.880)	
60 d (286)		1,041.8
Constant	14.9200 (2.140)	
Birth weight, kg	−0.3096 (0.041)	
Sex	1.7170 (0.350)	
Square root of d 60 IGF-I, ng/mL	−0.5110 (0.157)	
90 d (264)		106.9
Constant	13.5000 (1.970)	
Birth weight, kg	−0.2587 (0.039)	
d 90 IGF-I, ng/mL	−0.0587 (0.015)	
d 90 serum copper:zinc[4]	2.2310 (0.700)	
Log_{10} d 30 IGF-I, ng/mL	−2.1200 (0.802)	

[1]The selection criteria required that coefficients entering the model be significant at $P < 0.05$.
[2]Estimated odds ratio (OR) comparing a calf having average values of the selected predictors of the group of poorly growing calves with a calf having average values of the predictors of the group of calves growing well (top 40%); comparison calves were assumed to have the same sex.
[3]Bull calf = 0; heifer calf = 1.
[4]Serum copper and serum zinc measured in mg/kg.

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Table 7. Coefficients of logistic regression models for predicting the odds of a calf growing poorly (bottom 40%) in terms of body length, using independent variables at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and variable	Coefficient[1] (SE)	OR[2]
30 d (312)		62.1
Constant	11.4500 (1.350)	
Birth weight, kg	−0.2639 (0.031)	
60 d (267)		55.7
Constant	10.7800 (1.700)	
Birth weight, kg	−0.2145 (0.033)	
Sex[3]	1.1870 (0.314)	
Log_{10} d 30 IGF-I, ng/mL	−2.3630 (0.741)	
90 d (272)		13.1
Constant	−0.2207 (1.830)	
Birth weight, kg	−0.1813 (0.031)	
d 90 serum IGF-I, ng/mL	−0.0438 (0.011)	
Log_{10} first 10 d average IGF-I, ng/mL	4.2760 (0.950)	
Bovine leukocyte adhesion deficiency[4]	−1.2280 (0.472)	
First 10 d average serum copper, mg/kg	5.9560 (1.700)	
Log_{10} d 30 serum copper:zinc	−3.7380 (1.290)	

[1]The selection criteria required that coefficients entering the model be significant at $P < 0.05$.

[2]Estimated odds ratio (OR) comparing a calf having average values of the selected predictors of the group of poorly growing calves with a calf having average values of the predictors of the group of calves growing well (top 40%); comparison calves were assumed to have the same sex and bovine leukocyte adhesion deficiency status.

[3]Bull calf = 0; heifer calf = 1.

[4]CD18 homozygous negative = 0; CD18 heterozygous positive = 1.

the calf that could otherwise be used for growth, compounding the lack of nutrient intake.

The present data indicate that by avoiding exposure to those factors that would cause decreased serum IGF-I and increased inflammation, the risk of a calf growing poorly is decreased. These data also indicate that BW at birth is a very strong predictor variable of growth (multiple regression R^2 values ranged from 37 to 56%, 27 to 43%, and 14 to 22% at d 30, 60, and 90 of age, respectively). Future research efforts should be directed toward devising methods (nutritional, managerial, and pharmacological) to increase serum IGF-I and decrease inflammatory events in neonatal calves. Breeders and producers should also consider selecting for animals with heavier birth weights, but with body conformations that allow for calving ease. Through these tech-

Table 8. Coefficients of logistic regression models for predicting the odds of a calf growing poorly (bottom 40%) in terms of body height, using independent variables at 30, 60, and 90 d of age in northern California Holstein dairy calves

Calf age (n) and variable	Coefficient[1] (SE)	OR[2]
30 d (319)		639.3
Constant	13.6400 (1.660)	
Birth weight, kg	−0.3301 (0.039)	
Sex[3]	1.1030 (0.308)	
60 d (300)		29.7
Constant	10.4500 (1.560)	
Birth weight, kg	−0.2018 (0.029)	
Log_{10} d 30 IGF-I, ng/mL	−2.0320 (0.691)	
Sex	0.7830 (0.280)	
90 d (267)		9.5
Constant	10.2600 (1.520)	
Birth weight, kg	−0.1682 (0.028)	
d 90 IGF-I, ng/mL	−0.0320 (0.010)	
Log_{10} d 30 IGF-I, ng/mL	−1.9090 (0.759)	

[1]The selection criteria required that coefficients entering the model be significant at $P < 0.05$.

[2]Estimated odds ratio (OR) comparing a calf having average values of the selected predictors of the group of poorly growing calves with a calf having average values of the predictors of the group of calves growing well (top 40%); comparison calves were assumed to have the same sex.

[3]Bull calf = 0; heifer calf = 1.

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Table 9. Blood variable values, birth weight, and calf sex for a poorly growing Holstein dairy calf and the reference group used to calculate the logistic regression adjusted-risk odds ratio

Variable	Poorly growing calf[1]	Reference group[2]
Birth weight, kg	38.5	46.8
Sex[3]	1	0.3
First 10 d average IGF-I, ng/mL	12.50	14.33
d 30 IGF-I, ng/mL	7.48	17.22
d 60 IGF-I, ng/mL	12.77	27.73
d 90 IGF-I, ng/mL	22.93	40.19
First 10 d average serum copper, mg/kg	0.95	0.55
d 30 serum copper:zinc	0.53	0.56
d 90 serum copper:zinc	0.86	0.71
Bovine leukocyte adhesion deficiency[4]	0	0.2

[1]Average of the values from all the calves in the bottom 2/5th percentile of growth (bottom 40%).

[2]Average of the values from all the calves in the top 2/5th percentile of growth (top 40%).

[3]Bull calf = 0, heifer calf = 1.

[4]CD18 homozygous negative = 0; CD18 heterozygous positive = 1.

niques, dairy calves may experience less malnutrition and greater growth rates, thus increasing the economic returns to the producer.

It is well documented that excessive prepubertal growth rates reduce future milk production (reviewed in Mourits et al., 2000). In fact, in large-breed dairy cattle, the optimal prepubertal growth rate has been modeled at 0.7 kg/d. Although this rate increases calf rearing time, thereby increasing calf costs, the reduction in future milk produced would not compensate for this short-term cost savings (Mourits et al., 2000). However, recent studies have shown that growth rate per se does not differentially affect mammary parenchymal development (Daniels et al., 2009). Thus, replacement heifer producers must balance a fine line between optimal prepubertal growth and suboptimal growth caused by malnutrition. The present study, however, focused on the need to identify, during the early neonatal life, calves susceptible to malnutrition and its associated impaired growth and immune function.

To our knowledge, prospective cohort studies of this magnitude studying IGF-I in newborn calves to d 90 of life have not been reported previously in cattle, although a positive association between IGF-I and growth has been reported for older beef cattle (Davis and Simmen, 2006, 2010; Lancaster et al., 2008) and dairy heifers (Brickell et al., 2009a,b,c). This study described changes in serum IGF-I from birth to d 90 in Holstein calves, accounting for sex and twin status. This study also found a relationship between birth weight, serum IGF-I, serum zinc, and serum copper and BW, length, and height at d 30, 60, and 90 of age. The data indicate that lighter birth weight, reduced serum IGF-I, and inflammation may be important causes of poor growth in neonatal Holstein dairy calves. This study targeted dairy calves; however, it is likely that the information from this trial can be used to some degree to interpret human infant malnutrition. Whether similar patterns of change and associations between growth and serum IGF-I occur in human infants requires further study and definition.

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Pathology of subclinical paratuberculosis (Johne's Disease) in Awassi sheep with reference to its occurrence in Jordan

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ABSTRACT: In this study, the pathological lesions and occurrence of subclinical Johne's disease in Awassi sheep is investigated using histopathological (HP) and immunohistochemical (IHC) examinations, Acid Fast Staining (AFS) of tissue sections, direct smears from tissues and culture. Histopathological examination of 202 ilea and the corresponding mesenteric lymph nodes (179) was conducted. In addition, IHC examination, using rabbit polyclonal antiserum, of 134 ilea and 123 mesenteric lymph nodes was also conducted. The occurrence of the disease was observed in 50% and 93% of the ilea examined using histopathology and IHC techniques, respectively. Fifty nine percent of lymph nodes were positive by IHC. The histopathological lesions were graded from I–IV, I being the least severe, based on the type of cellular infiltrate (lymphocytes, macrophages and epithelioid cells) and the severity of the lesions. Grades III and IV (SP) were considered positive while I and II were considered suspected. Analysis of the results also revealed that the majority of suspected cases (grades I and II) reacted positive with the IHC. Furthermore, the IHC reactions were classified into three categories depending on the number of stained cells and the intensity of the staining (I-mild, II-moderate and III-strong). Direct smears, and tissue sections obtained from the ilea and stained with AFS revealed that out of 202 tissue samples, 53 (26%) and 22 (11%) were positive, respectively. Results of the culture revealed that 22 (11%) out of 202 were positive. These results showed that the occurrence of paratuberculosis (Johne's disease) in Awassi sheep is very high in Jordan and needs further study in order to develop rational methods of control effective for the Jordanian sheep population.

Keywords: sheep; paratuberculosis; histopathology; acid fast stain; ileum; immunohistochemistry

Paratuberculosis (Johne's disease) is an infectious disease of ruminants, considered by many researchers to be one of the most serious infectious diseases currently plaguing the world's sheep, cattle and goat industries (Hope et al., 2000). An increasing number of publication highlights the importance of this animal infection with a zoonotic potential (Kaevska and Hruska, 2010). The disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a gram-positive acid-fast organism shed in the faeces of infected animals, is characterized by chronic granulomatous enteritis (Jubb et al., 1993; Radostits et al., 1994; Adams et al., 1996).

Contaminated feed and water, bedding and soiled udders are thought to be the major routes for spread of the organism and young animals less than six months of age are thought to be the most susceptible to infection. Cattle become infected early as young calves via faecal-oral transmission of the infectious agent (Stabel et al., 2002). Intrauterine infection in cattle has also been well documented (Martin and Aitken, 1991; Whittington and Windsor, 2009). Isolation of *M. paratuberculosis* from a sheep foetus has been confirmed, and antibodies to *M. paratuberculosis* have been demonstrated in colostrum-deprived lambs (Jones et al., 1997; Lambeth et al., 2004).

Focus on paratuberculosis has been intensifying as it is becoming an economically important disease for the livestock sector. It was reported that

the economic losses due to Johne's disease for the control and eradication program in one flock with a few cases would average $650 with most of that cost going for serological tests. The cost of disease for ovines is approximately $90 per clinical case and $250 per bovine clinical case (Menzies, 2010). The economic effect of Johne's disease consists in the restriction of livestock marketing and contamination of land. In Iceland, for example, measures to be taken for the control of paratuberculosis are expensive, involving the fencing of hundreds of kilometres to restrict sheep movement from the infected area. Laboratory tests used to detect infected animals are also costly and notwithstanding all these expenses it is very difficult to eradicate the disease (Fridriksdottir et al., 2000).

In addition, consumers of animal products are becoming increasingly sensitive to the possible effect of livestock disease and residues on product quality and human health. The potential link between Crohn's disease (CD) and *M. paratuberculosis* is an example of such a potential relationship (Kennedy and Allworth, 2000; El-Zaatari et al., 2001). Recent data have strengthened the etiologic association between *M. paratuberculosis* and CD (Naser et al., 2000). Milk has been suggested as a possible vehicle of transmission of *M. paratuberculosis* to humans as *M. paratuberculosis* has been detected by PCR in row milk as well as from pasteurized milk from cattle, sheep and goats, repeatedly in England (Millar et al., 1996; Ellingson et al., 2005; Hruska et al., 2005; Zoi Dimareli-Malli, 2010).

It has been suggested that the diagnosis of subclinical cases of paratuberculosis is still a problem (Kurade et al., 2004). It was also reported that the diagnosis of subclinical cases of paratuberculosis is more difficult by histopathology as lesions may be subtle and organisms may be low in number (Buergelt et al., 2000). In sheep, on the other hand, it has been reported that histopathology was found to be a better indicator of paratuberculosis than bacteriology (Kurade et al., 2004). Furthermore, it was suggested that histopathological studies of tissues from infected animals can be considered as the gold standard method for the diagnosis of Johne's disease and should be used to confirm the disease instead of other methods (Hope et al., 2000). However, expertise and diagnostic criteria may vary between institutions (Huda and Jensen, 2003). Therefore, accurate subclinical diagnosis needs more than one test to complement the histopathological diagnosis.

To estimate and understand the occurrence of Johne's disease in sheep, we conducted a field study of twenty sheep flocks, ranging from 50–500 heads and found that 80% of the flocks had a history of untreatable emaciation, bottle jaw and diarrhea which were associated with animal losses. Therefore, this investigation was carried out to study the associated pathological lesions of Johne's disease in the ilea and their corresponding mesenteric lymph nodes, and its prevalence in apparently healthy young Awassi sheep in Jordan, using gross/histopathological, immunohistochemistry (IHC), Acid Fast Stain (AFS) examinations and culture.

MATERIAL AND METHODS

Animals/Awassi sheep

Awassi sheep included in this study were located at 36°66'E, 32°04'N at about 616–686 m above sea level. During the period of this study, annual rainfall varied between 159 and 380 mm. Rainfall usually starts in November and ends by April. No precipitation occurs between May and November. Daily temperature is moderate with mean monthly maxima ranging between 12 and 32 °C and mean monthly minima between 2 and 14 °C. The mean monthly relative humidity ranged between 44 and 80%. The raising system of sheep in Jordan is mainly semi-intensive; farmers usually feed their animals chopped straw, barley and wheat bran during the winter season (October to February). During spring and summer seasons, the animals are maintained on grazing. Some farmers provide concentrate supplementation in seasons when the grazing grass is not adequate. The Awassi sheep breed is raised for meat, milk and wool production and it represents almost 98% of the sheep breeds in the country (Ministry of Agriculture, 2000).

Two hundred and seventy nine apparently healthy Awassi sheep of both sexes, 8–24 months of age, raised in different flocks in Jordan, were slaughtered and examined in the slaughterhouses of the Central (represents 32% of the national flock of 1.9 million sheep) and Northern (represents 45% of the national flock) parts of the country (Ministry of Agriculture, 2000). The animals had no signs of emaciation, bottle jaw and diarrhea, alone or in combination. The site for animal selection in the slaughterhouses was based on the sheep populations and distribution in Jordan. Animal movement

is free and the sheep are bought from the sheep farmers in local animal markets by the local butchers or animal traders twice a week; on Mondays and Thursdays. Sheep are either sent directly to the slaughter house or fattened for some time beforehand. Different sheep farmers bring their animals from different sites of the country; in some cases they transport them about 150 Km or more to bring them to the animal markets. In some cases, sheep traders visit sheep farmers and buy sheep from the farms directly before sending them to the slaughter house or for slaughter in local butcheries.

Tissue sample collection

During a period of five months (August to December 2001), a total of 279 apparently healthy Awassi sheep were subjected to postmortem examination in slaughterhouses where small and large intestines were examined thoroughly. The slaughterhouses were visited weekly to collect the samples. Before slaughtering, complete information about the animals regarding age, sex, significant clinical signs and health status were recorded. The age of the sheep ranged from 8 to 24 months. The majority of the sheep examined were males.

Gross examination and samples transportation

Samples from small and large intestines and respective lymph nodes of the 279 animals were thoroughly examined for any apparent gross lesions. Significant gross lesions including mucosal thickening, mucosal corrugations, prominent lymphatics and intestinal discolorations were recorded. The size, shape and discoloration of the respective lymph nodes were carefully described. Out of the 279 animals examined, 202 animals with some gross changes were sampled and tissues were collected from their ilea, ileocecal valves and respective lymph nodes based on tissue changes seen in these organs. In the few cases in which gross lesions were observed in the large intestine, tissue samples from the cecum and colon were collected. Tissues samples were trimmed to a smaller size (4 mm to 1 cm thickness), fixed in 10% buffered formalin, and subjected to histopathological examination (202 ilea and 179 lymph nodes) and immunohistochemical staining (134 ilea and 123 lymph nodes). Other tissue samples were transported on ice to the laboratory for direct smear (202 ilea) and for bacterial culture (202 ilea). Samples for culture were kept at –20 °C until needed.

Direct smear staining with acid fast stain

Scrapping of the intestinal mucosa and lymph nodes parenchyma were carried out using scalpel blade and smears were done on microscopic slides. Slides were dried by air, fixed with methanol (100%) and stained with AFS method according to Coles (1986). Each slide was examined for 30 min using oil immersion. The findings were registered according to the bacteria appearance, in which observation of bacteria's in clumps taken as positive, in dispersed form as suspected and as negative if neither of the two forms observed.

Culture

Media preparation. Middle Brook 7H10 agar base M199 (Himedia Laboratories, limited Mumbai (Bombay) 400086, India) was used with a supplement as a slant in tubes of 20 ml. The media was prepared as described in the OIE (2000). To 450 litres of distilled water, 9.73 g was added. Then, 5 ml of glycerol was added to this mixture which was boiled, autoclaved at 121 °C, 15 psi, for 15 min, and then cooled at 45 to 50 °C in a water bath (GFL 1083, Germany). Middlebrook OADC growth supplement (FD 018, India, 50 ml/l) was then added. For the suppression of contaminants, Penicillin (1000 000 IU) and the anti-fungal agent Nystatin (50 mg) were added to the media. The media containing mycobactin was prepared by adding mycobactin J 2 mg/l (Allied Monitor IAC, Fayette, MO, USA) of the medium dissolved in 4 ml of ethyl alcohol with the agar base before autoclaving.

Sample preparation and inoculation. Approximately 3–5 g of scraped intestinal mucosa and lymph node parenchyma was taken and ground together using mortar, and incubated with 0.5% trypsin at 4 °C overnight at pH = 7.2 to 7.4 (adjusted using 4% NaOH). After 16 to 8 hours, the mixture was filtered using a gauze (folded), and centrifuged at 400 × g for 20 min (PK110, ALC, Italy). The supernatants were decanted and decontaminated and 5% oxalic acid was added to the pellet. The samples were then left for 24–30 h at room temperature.

Finally, 0.1ml (100 µl) of inoculum was taken carefully from the bottom of the tube and evenly distributed on the media. Each sample was inoculated in three tubes (one tube without mycobactin J and two with mycobactin J) and incubated at 37 °C (Binder GmbH, Germany) with a loss screw and inclined to facilitate the evaporation of excess moisture and inoculum fluid for one week. After one week tubes were returned to the vertical position with a tightened screw and incubated for 16 weeks.

Culture reading. Starting from eight weeks of inoculation, cultures were observed weekly for the presence of any growth. At 16 weeks, smears were taken from cultures that showed growth, and stained by AFS as described previously (Prophet et al., 1994). A culture was considered positive when white spot colonies were seen and this was confirmed by AFS. Slides were observed under 100 × objectives for the detection of acid fast bacilli. Results were recorded considering the long incubation period, the colony appearance, and acid-fastness of the bacteria. Nine tubes with micro-fungal growth and contamination were discarded.

Histopathological examination

All the trimmed samples were processed routinely for histopathological examination according to a protocol described previously (Bancroft and Stevens 1990). Sections (4–5 µm) were cut and stained with Haematoxylin and Eosin (H&E). Staining of tissue sections by AFS was performed as described previously (Prophet et al., 1994).

Grading criteria for histopathological lesions

The H&E stained sections from 202 ilea and the corresponding lymph nodes (179) were observed under 4×, 10×, and 40× objectives. Pathological lesions were recorded. The lesions were classified and graded into grades I, II, III and SP, depending on the type and amount of the cellular infiltrates (lymphocytes, macrophages and epithelioid cells). The intestinal tissue sections were considered positive for paratuberculosis when there were macrophage infiltrations or epithelioid cells obvious in the lamina propia of the villi and between crypts, and there was involvement of the Peyer's patches as demonstrated by the presence of starry sky macrophages/tingible body macrophages or micro-granuloma. In cases where there was no microgranuloma, but macrophages and some epithelioid cells were present, these were considered suspected cases by histopathological criteria. The scoring of tissue lesions was as shown in Table 1. Therefore, grades I and II were considered suspected while grades III and SP were considered positive.

Immunohistochemical (IHC) staining

Tissue samples from paraffin-wax embedded blocks were sectioned at 2–3 µ prior to immunohistochemical staining. The sectioned tissue samples were laid on Vectabond (DAKO A/S.Glostrup, Denmark) coated slides, air-dried and then heated in an oven at 55 °C for two hours. Tissue sections were deparaffinized in xylene and hydrated by sequential immersion of slides in decreasing concentrations of ethanol (100%, 95% and 70%) for one minute each. Tissue sections were washed in distilled water for 5 min, then washed in PBS, followed by immersion in citrate buffer solution (pH= 6, 10mM). Antigen retrieval was carried out by autoclaving the tissue section at 120 °C, 15 psi for 15 min (Express, Italy). The sections were cooled at room temperature and washed in PBS for 5 min. All of the subsequent incubations were performed at room temperature, and all washes were in PBS (pH 7.4, 3×; 5 min each).

Table 1. Criteria used for grading pathological lesions found in the ilea and lymph nodes

Grade	Type of inflammatory cells				
	lymphocytes	macrophages	epithelioid cells	PP prol. and crypts replacement	microgranuloma
I	++++	++	+	–	–
II	+++	++++	+++	moderate	yes/no
III	+	+++	++++	severe	yes
SP	+	++++	++++	calcification, caseous necrosis in the LN	granuloma with giant cells

SP = special, PP prol. = Peyer's patches proliferation, LN = lymph nodes

Endogenous peroxidase was inactivated by immersion of the slides in a solution of 15% hydrogen peroxide in methanol for 30 min. After washing, non-specific adherence of proteins to tissue sections was blocked by incubating with 1% bovine serum albumin (BSA), (Sigma Chemical Co., St.Louis, MO) for 2 h. The solution was drained from the slides and the rabbit polyclonal *M. paratuberculosis* antiserum # 270, diluted 1 : 500 in PBS, was incubated on the slides for 2 h. The rabbit antibody was kindly provided by Dr. Stabel from the National Animal Disease Center, Ames, IA, USA. This was followed by further washing and incubation with biotinylated anti-goat, anti-rabbit, and anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark), diluted at 1 : 20, as the secondary antibody for 15 min. After washing, streptavidin-biotinylated peroxidase conjugate (DAKO, A/S, and Glostrup, Denmark) was applied, and incubated on the tissue section for 15 min. The slides were washed and incubated with chromogen (3,3 diamino- benzidin-4HCL; DAB, DAKO) at 1 mg/ml in PBS supplemented with hydrogen peroxide (10 ul of 3% hydrogen peroxide for 2 ml of DAB). Samples were incubated at room temperature for 3–5 min, and slides were then washed in distilled water for five minutes. Slides were then counter stained in haematoxylin for 2–3 min. The sections were washed in water and dehydrated in graded alcohol and cleared in xylene and mounted using DPX for further observation.

Slides were observed using 4×, 10× and 40× objectives. Sections were considered positive according to the color observation that is an indication of antibody-antigen reaction, and manifested by intra-cytoplasm or extra-cellular brown coloration in different areas of the stained tissue section. The findings were registered by counting the number of reactions at 10×, accordingly starting from one cell reaction recorded as positive; 1–10 as + (mild), more than 10 but less than 50% cells reaction as ++ (moderate), reaction in 50% cells from one tissue section was graded as +++ (strong as in Figure 6). And more that 50% reaction in the macrophages of one section as ++++, but because only one sample was ++++, it was added to the +++ group. Additionally the intensity of the reaction was considered and in all cases only a strong brown color was recorded as a positive reaction. If the above criteria were found in at least one field, it was considered positive. At least one slide from each tissue section from the ilea sampled from each animal was tested. In some cases, more slides were taken from each tissue preparation.

In all cases, positive and negative control slides were processed together from the same known group of tissue sections, in order to avoid false positive and negative reaction. The positive control tissue was obtained from a sheep clinically suffered from MAP as diagnosed by histopathology, AFS staining, IHC and culture. The negative control was obtained from a three-month old lamb which was healthy and negative by all the four tests.

RESULTS

In this study, gross/histopathological, immunohistopathological examinations, and AFS (tissue sections and scrapping) of the ilea and corresponding lymph nodes of 202 clinically healthy Awassi sheep, which were slaughtered during the study period, were used to assess, describe and grade the histopathological picture and examined the tissue

Table 2. Grade of histopathological findings, from ilea and corresponding lymph node H&E tissue sections in apparently healthy Awassi sheep aged 8 to 24 months in Jordan, 2002

Type of tissue	Samples processed	Grade of the lesions					
		I	II	III	SP	Total	
						+ve	–ve
Ileum	202	23	70	98	4	195	7
	%	(11)	(35)	(48)	(2)	(97)	(3)
Lymph nodes	179	10	35	76	4	125	54
	%	(5.5)	(19.5)	(42)	(2.2)	(69)	(31)

The percentage was taken by considering the decimal number > 5, adding one in all the results SP = special lesion, numbers in parentheses are percentiles

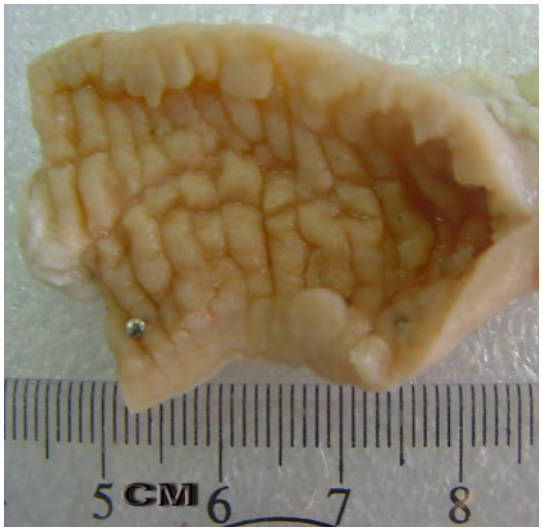

Figure 1. Gross pathology of an ileum section from apparently healthy sheep. Corrugations and thickening of the mucosal wall are prominent

reaction regarding the presence of *Mycobacterium avium* subps. *paratuberculosis* for the purpose of diagnosing the disease in its subclinical presentation. Culture was also used to confirm the diagnosis.

When the intestines and the corresponding lymph nodes were examined grossly in the slaughterhouses, we found that 202 intestines or lymph nodes out of the 270 animals examined harboured some sort of pathological changes. These changes ranged from very mild to severe congestion in the mucosa and thickening in the wall of the ilea. The wall of the ilea was very thick with corrugation in 13 animals (Figure 1). The surrounding mesenteric lymph nodes were enlarged and oedematous in few cases, and they were connected with each other as a result of their tissue reaction and appeared as cords.

Histopathological examination of H&E tissue sections from the 202 ilea and corresponding lymph nodes revealed that 195 (97%) ilea had variable increases in the thickness and congestion of the mucosa due to inflammatory cell infiltrations (Table 3). The mucosa and less frequently the sub mucosa were infiltrated primarily with variable numbers of macrophages, lymphocytes and plasma cells (Figures 2 and 3). Occasional numbers of epithelioid cells were also seen. They were present either as a scattered form in the lamina propria of the villi and between the crypts or as nests. Multinucleated giant cells were rarely seen in the affected tissues. In some cases the cellular infiltrates were associated with caseous necrosis and/or mineralization. Variable degrees of Peyer's patch hyperplasia were noted in many tissue sections with prominent starry sky macrophages/tingible body macrophages (Figure 3). In most cases, granulomatous lymphangitis with or without dilated lacteals were present (Figure 4). The villi exhibited different changes including: villous distortion and thickening by inflammatory cell infiltrations, villous atrophy and fusion. The lymph node changes consisted primarily of lymphofollicular hyperplasia and infiltration of mononuclear cells predominately with epithelioid cells (Figure 5). The most severely affected lymph nodes were characterized by granulomas formation with mineralization. In less severely affected lymph nodes, epithelioid cells mixed with

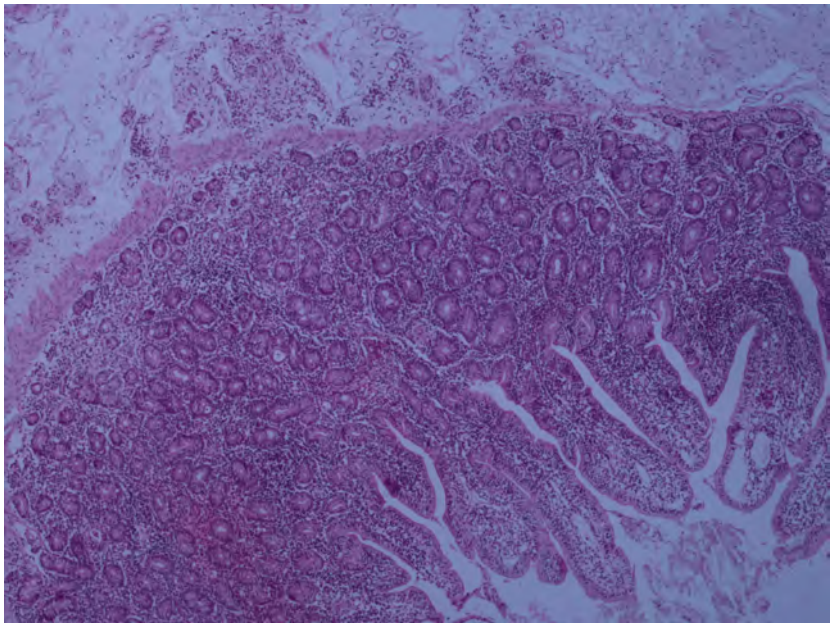

Figure 2. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. Payer's patches lymphoid hyperplasia is seen throughout the section. H&E stain, 4×

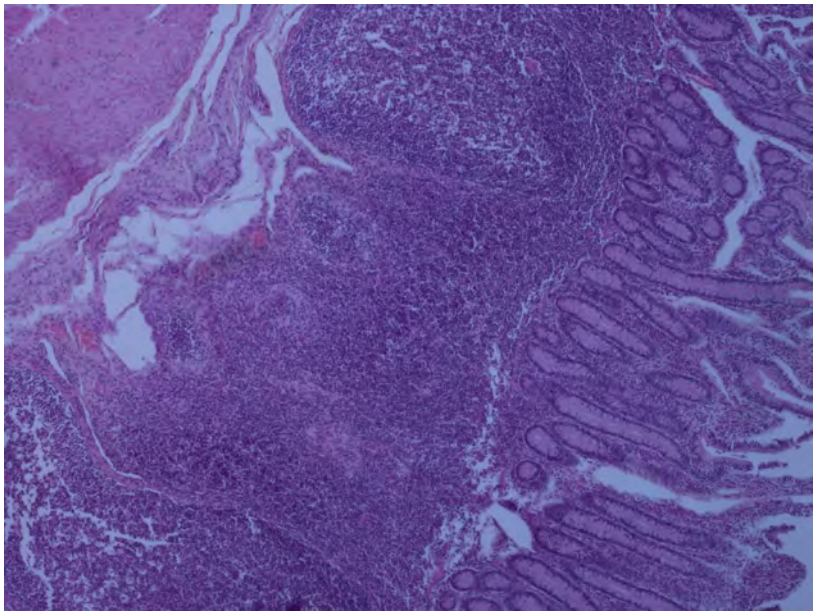

Figure 3. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. Mononuclear cells infiltrate primarily the mucosa, and less frequently the submucosa. Dilation of some of the lacteals in the villi is also seen. H&E stain, 4×

other mononuclear cells and a few neutrophils were scattered throughout the lymph node sections.

Table 2 shows the grading of the lesions seen in the intestine and the lymph nodes. Out of the 202 intestinal H&E tissue sections examined, 23 (11%), 70 (35%), and 98 (48%) were graded as I, II and III, respectively, based upon the grading criteria described; namely the degree of lymphocyte, macrophage, and epithelioid cell infiltration; cellular proliferation within the Peyer's patches and crypts; and the presence of microgranuloma. Four cases were considered as a special category where calcification with severe reaction and typical granulomatous reaction with multinucleated Langhans giant cells were observed in the lymph nodes. The cases in grades III and SP (IV) were considered positive while the cases in grades I and II were considered suspected for the disease by histopathological evaluation.

To further understand the cellular reaction in the intestine and in the respective mesenteric lymph nodes and to investigate whether this reaction was associated substantially with the presence of *M. paratuberculosis,* immunohistochemical examination was used. A positive reaction was manifested by the presence of brown colour around the macrophage nucleus (perinuclear and intracytoplasmic reaction) (Figures 6 and 7). Table 3 shows the grading of the lesions seen in the intestine and the lymph nodes as the tissue samples were examined by immunohistochemistry. We examined only 134 ilea tissue sections and 123 tissue sections from the corresponding lymph nodes, and because of the limitation in the reagents, we did not examine all tissue specimen. We found that 124 (93%) tissue sections from the ilea were positive, ranging from mild (+) moderate (++) and strong (+++); 108 (81%), 9 (7%), 6 (4%) and 1 (1%) were graded as +, ++, +++

Table 3. Immunohistochemical stain results from intestinal samples and lymph nodes in apparently healthy Awassi sheep, 8 to 24 months, in Jordan 2002

Type of tissue	Number of samples processed	Immunohistochemical stain				
		+	++	+++	total	
					+ve	–ve
Ileum	134	108	9	7	124	10
	%	(81)	(7)	(5)	(93)	(7)
Lymph node	123	55	10	7	72	51
	%	(44)	(8.1)	(5.6)	(58.5)	(41.4)

Numbers in parentheses are percentiles

Table 4. Distribution of Histopathological (HP) (H&E), Immunohistochemical (IHC) and Acid Fast Stain (AFS) used on histopathological tissue sections (AFS/HP) and AFS Direct Smears (AFS/DS) and Culture results from intestinal specimens of apparently healthy Awassi sheep, 8 to 24 months, in Jordan 2002

Type of test	Number of samples			Total
	positive	suspected	negative	
HP/H&E	102	93	7	202
%	(50)	(46)	(4)	(100)
IHC	124	0	10	134
%	(92)	(0)	(8)	(100)
AFS/HP	22	0	180	202
%	(11)	(0)	(90)	(100)
AFS/DS	53	99	50	202
%	(26)	(49)	(25)	(100)
Culture	22	0	180	202
%	11	0	89	(100)

and ++++, respectively, based upon the number of cells which reacted positively with the antibody (Table 3). Because only one sample was in grade 4, it was added to grade 3. Out of the 123 tissue sections from the lymph nodes tested by IHC, 72 (58.5%) sections reacted positive (Table 3). Fifty five (44%) out of 123 lymph node tissue sections showed mild 1+, 10 (8%) moderate 2+ and 7 (6%) strong 3+. Interestingly, seven lymph node samples which were negative by IHC were graded as 2+ and 3+ in the corresponding intestine by IHC. In either tissue sections of the ilea or their corresponding lymph nodes, if the tissue section shown 1+ or more, the tissue section (sheep) was considered positive for Johne's disease by IHC.

The IHC staining of the tissue sections from the ilea was studied also to find the distribution of the stained macrophages in different histological parts

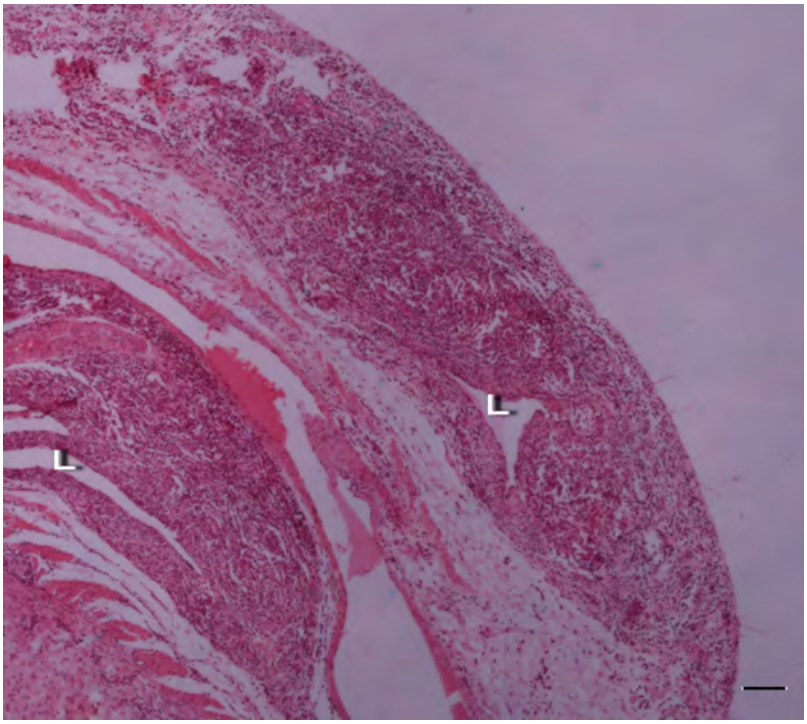

Figure 4. Histopathological section of the ileum (muscularis and serosa) from apparently healthy/subclinically infected sheep. Granulomatous lymphangitis is shown where mononuclear cells infiltrate the wall of the lymphatics. H&E stain, L = lymphatic vessels, bar = 100 μ

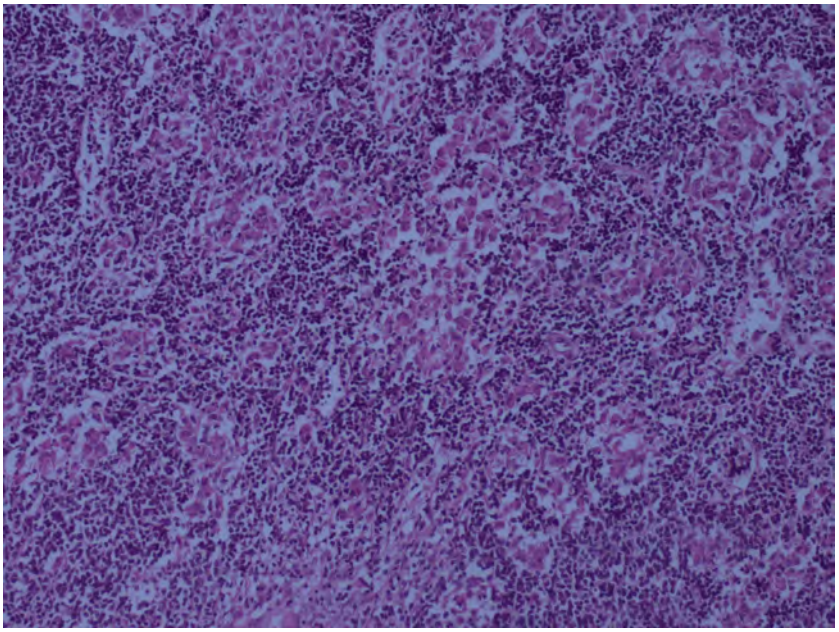

Figure 5. Histopathological section of the lymph node from apparently healthy/subclinically infected sheep. The lymph node is heavily infiltrated with mononuclear/epithelioid cells. H&E stain, 10×

of the tissue. In the intestinal tissue sections, the reaction was observed more in the macrophages infiltrated in the lamina propria between the crypts. The Peyer's patches showed less staining than the LP, while reactions were rarely seen in the LP of the villi. The reactions in the lymph nodes were commonly observed in the paracortex area dispersed between the cortex and medulla.

Analysis of the results pertaining to the AFS of direct smears from the 202 intestine samples revealed that 53 (26%) tissue samples were positive as the bacteria appeared in clumps. About 99 tissue samples (49%) were suspected, where the bacteria were seen in the dispersed form and 50 (25%) slide smears were negative. When the tissue samples from the ilea (202 samples) were sectioned and stained by AFS, acid fast bacilli were seen only in 22 (11%) samples. The bacilli were seen either inside the cytoplasm of the macrophages or free outside the cells. Furthermore, out of the 202 intestine samples cultured, only 22 (11%) were positive. When these samples were traced, we found that they were in grade II and III by histopathological examination and 1+ by immunohistochemical staining.

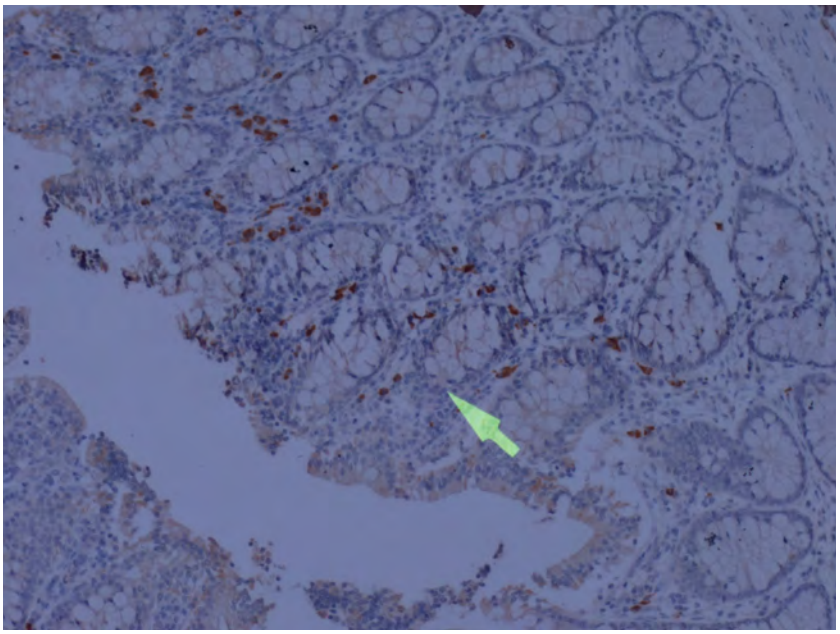

Figure 6. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. The section was stained using an immunohistochemistry staining technique with a rabbit polyclonal anti-body against *M. paratuberculosis*. Bacterial antigens where detected in the cytoplasm of macrophages as dark brown staining. IHC staining, 10×

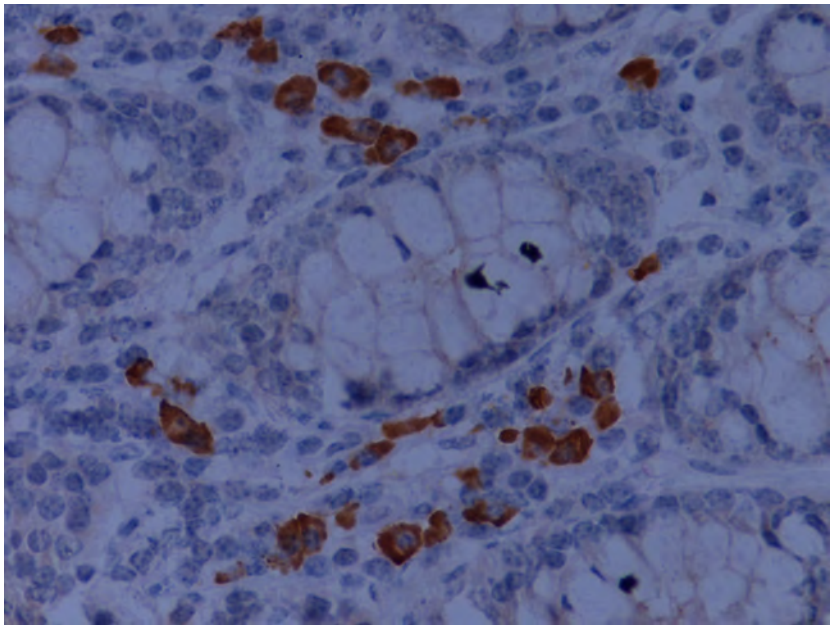

Figure 7. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. This is a higher magnification view of figure six. IHC staining, 40×

DISCUSSION

In the present study, the pathological lesions and the occurrence of subclinical Johne's disease was studied in apparently healthy Awassi sheep between the ages of 8 to 24 months and with no history of clinical Johne's disease using multiple diagnostic tests, histopathological and immunohistochemical examination, AFS and culture. Studies reporting previously on subclinical cases of Johne's disease have demonstrated the importance of tissue sample selection from different sites and it was found that sampling from limited foci can influence the results (Fraser et al., 1999; McDonald et al., 1999). Therefore, the selection of tissues from different sites along with the adjacent lymph nodes should be done to confirm the diagnosis of subclinical Johne's disease, with the ileocecal valve as the first site to be selected. It was also reported that histopathological examination should be considered as the first option for the diagnosis of subclinical cases. In our study, the last portion of the ileum and the ileocecal valve with their adjacent lymph nodes were utilized as the sample site and were used for all the implemented tests in this study.

Examination of the ilea and the corresponding lymph nodes from the clinically healthy Awassi sheep in the slaughter houses revealed that seventy-two percent (202 out of 279 examined) of them showed very mild to severe thickening and congestion in the intestine, especially in the last portion of the small intestine (Figure 1). Inflamed, oedematous, enlarged and corded mesenteric lymph nodes around the ileum and ileocecal valve were also evident. The histopathological findings such as the infiltration of the mucosa and submucosa with lymphocytes, macrophages, replacement of the crypts with macrophages, Peyer's patches proliferation extending towards the mucosa and the presence of microgranuloma is partially in agreement with the results of others (Perez et al., 1996; Watkins et al., 2002). The grade 1 lesions noted in our study (11%; dominated by lymphocyte infiltration) is in agreement with the asymptomatic form and to some extent with the paucibacillary form, while grades 2 and 3 (83%) are in agreement with the multibacillary form previously reported, keeping in mind that about 30% and 70% of the last two forms affected clinical cases, respectively (Watkins et al., 2002). This is in contrast to our study where the Awassi sheep were young and apparently healthy with no clinical signs in spite of the presence of marked macrophage infiltration and granulomatous reactions (42% of ilea) and with granuloma, Langhan giant cell formation and calcification (2% of animals) (Table 2). It has been reported that the paucibacillary form of the disease occurs particularly in sheep and in such animals *M. paratuberculosis* cannot be detected in the tissue microscopically and usually cannot be cultured (Hermon-Taylor, 1998). The grade 1 lesion in this study is close to the type 1 lesion in a study reported from South Africa although that study did not focus on lymphocyte infiltration and concentrated more

on the epithelioid cell nest in the mucosa and submucosa (Micheal and Bastianello, 2002).

Paratuberculous lesions have been classified into three types (1, 2 and 3a, 3b, 3c) disregarding the clinical signs (Perez et al., 1996, 1999). In these classifications, epitheloid cells were not encountered, and lymphocyte infiltrations were associated mainly with lesion type 3c. This classification was based mainly upon the presence of macrophages and acid-fast bacteria. In our study, only 22 (11%) of histopathological tissue sections revealed the presence of acid-fast bacilli and 26% were positive on the direct smears (Table 4). Most of the 22 samples were grade 3 and a few were grade 2, with one, two or three intra-cytoplasmic bacilli observed within the macrophages. In some cases, extracellular acid-fast bacilli were also observed. In clinical cases it was reported that the presence of acid fast bacilli might be demonstrated intra- and extracellularly using the AFS stain (Kahn, 1997). However, in subclinical cases, it was reported that it may not be possible to find bacilli in tissues. Each tissue section was examined for a minimum of 30 min for the presence of bacilli as reported by others (Carbonell, 1998). Also, it was reported that it is very difficult in sub-clinical cases to detect bacteria by light microscopy due to the possible changes in the cell wall during the tissue preparation and processing (Micheal and Bastianello, 2002).

In a previous study, polyclonal antiserum which was raised by inoculating heat-killed *M. paratuberculosis*, was shown to be useful for the detection of Johne's disease in infected bovine tissue (Stabel et al., 1996). The specificity and the sensitivity of the immunohistochemical stain were high and can be used as a diagnostic tool. It was also evaluated for cross-reactivity with *M. bovis* antigens by immunohistochemical staining of tissues from infected pigs and cows. Tissues were devoid of positive reactivity when evaluated at the same dilutions that demonstrated positivity in *M. paratuberculosis*-infected tissues. In addition, cross-reactivity of the polyclonal antibody with *M. avium* infected tissue in pigs showed little positive staining. Furthermore, *M. avium* infection does not generally target the intestinal tract in ruminant species. Since we used a polyclonal antibody, it is unfair to consider this antibody completely specific for *M. paratuberculosis*. This is the first study where we use this polyclonal antibody in Awassi sheep and the tissues are from young and apparently healthy animals; it would be very interesting to find out how this antibody reacts with tissues from sheep clinically infected with MAP. The 22 animals which were found by culture to be positive were also positive 1+ with this antibody by IHC. In tissues sections of ilea from apparently healthy cattle however, we found a significant correlation between the results of histopathological and IHC examinations; 66% of the ilea examined had lesions compatible with Johne's disease and 65% reacted positive with the polyclonal antibody (Hailat et al., in preparation). Furthermore, others have also reported that polyclonal and monoclonal antibodies could be used for the diagnosis of paratuberculosis in tissue sections (Coetsier et al., 1999). Thus, it was concluded that the production of a polyclonal antibody to cell wall proteins of *M. paratuberculosis* resulted in a highly sensitive, species-specific tool for the detection of paratuberculosis in tissue sections. In addition, it was suggested that histopathological and immunohistopathological methods produced evidence of infection, which can be comparable with bacteriological examinations (Sigurdardottir et al., 1999).

In the present study, we used the same antisera, where a high percentage of the sheep tissue sections were reactive within the infected macrophages and antibody reaction was also seen extracellularly (Table 3 and Figures 6 and 7). These results indicate that it is appropriate to use an immunohistochemical stain with a specific antiserum for the detection of *M. paratuberculosis*. The ultimate goal in determining the etiologic relationship of an organism to a disease state is to demonstrate the association of the organism with the lesion. In our study, 93% of the ilea tissue sections (81% 1+) and 58% of the lymph node tissue sections (44% 1+) reacted with the polyclonal antiserum. Interestingly, 97% of the tissue sections from the ilea had mononuclear cellular infiltrates (50% grades III and SP) strongly suggesting an etiological relationship of the organism to the intestinal and lymph nodes lesions of Johne's disease. In this study, it is also very obvious that the presence of an antigenic reaction by immunostaining in 108 (75%) out of 134 samples demonstrated by 1+, was characterised by high cellular infiltration in the LP and between the crypts with Peyer's patches proliferation involving its border towards the mucosa. This indicates that at early stage of the disease or in subclinical cases, cell-mediated immune reactions could start with only few bacteria present in the intestinal mucosa, as demonstrated by our results in these young animals.

We conclude that the occurrence of paratuberculosis (Johne's disease) is very high in Awassi sheep in Jordan. About 50% of clinically healthy Awassi sheep, 8 to 24 months of age, tested positive as they had histopathological lesions compatible with the disease. In addition, these cases were tested positive by IHC using a specific rabbit polyclonal antibody to *M. paratuberculosis* antigens. Thus, we consider paratuberculosis (Johne's disease) to be a significant problem in Awassi sheep in Jordan and believe there is a great need for further studies on the prevalence and epidemiology of the disease in order to develop rational methods of control effective on the Jordanian sheep population.

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Editor-in-Chief quotes from the reviewer's final recommendations

"I do believe that if the authors wish to pursue further work in this area, either further work to determine the specificity of this polyclonal antibody or additional confirmatory tests should be done to convince readers that their test results do identify MAP infected animals. Since they have cultured MAP from some sheep, it would be useful for them to try to determine the strain type (or types) that are represented."

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Proteolytic Products of the Porcine Reproductive and Respiratory Syndrome Virus nsp2 Replicase Protein[▽]

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The nsp2 replicase protein of porcine reproductive and respiratory syndrome virus (PRRSV) was recently demonstrated to be processed from its precursor by the PL2 protease at or near the $G^{1196}|G^{1197}$ dipeptide in transfected CHO cells. Here the proteolytic cleavage of PRRSV nsp2 was further investigated in virally infected MARC-145 cells by using two recombinant PRRSVs expressing epitope-tagged nsp2. The data revealed that PRRSV nsp2 exists as different isoforms, termed nsp2a, nsp2b, nsp2c, nsp2d, nsp2e, and nsp2f, during PRRSV infection. Moreover, on the basis of deletion mutagenesis and antibody probing, these nsp2 species appeared to share the same N terminus but to differ in their C termini. The largest protein, nsp2a, corresponded to the nsp2 product identified in transfected CHO cells. nsp2b and nsp2c were processed within or near the transmembrane (TM) region, presumably at or near the conserved sites $G^{981}|G^{982}$ and $G^{828}|G^{829}|G^{830}$, respectively. The C termini for nsp2d, -e, and -f were mapped within the nsp2 middle hypervariable region, but no conserved cleavage sites could be definitively predicted. The larger nsp2 species emerged almost simultaneously in the early stage of PRRSV infection. Pulse-chase analysis revealed that all six nsp2 species were relatively stable and had low turnover rates. Deletion mutagenesis revealed that the smaller nsp2 species (e.g., nsp2d, nsp2e, and nsp2f) were not essential for viral replication in cell culture. Lastly, we identified a cellular chaperone, named heat shock 70-kDa protein 5 (HSPA5), that was strongly associated with nsp2, which may have important implications for PRRSV replication. Overall, these findings indicate that PRRSV nsp2 is increasingly emerging as a multifunctional protein and may have a profound impact on PRRSV replication and viral pathogenesis.

Replicase polyprotein maturation is a highly orchestrated and precisely regulated process, which plays a very important role in the life cycle of positive-stranded RNA viruses. Its products are critical for the downstream assembly of viral replication complexes and are often antagonists of host innate immunity. The proteolytic cleavage of these replicase polyproteins is usually carried out by viral proteases, with occasional action by cellular proteases (10, 27). The focus of this report is the maturation of porcine reproductive and respiratory syndrome virus (PRRSV) replicase protein. PRRSV causes reproductive failure (e.g., abortions, mummies, stillbirths) in sows and respiratory distress (e.g., interstitial pneumonia) in young pigs, leading to millions of dollars of losses every year in North America (25) and even more in other regions (e.g., Southeast Asia) in recent years (36, 41). PRRSV is a positive-stranded RNA virus with a genome of about 15.4 kb and is a member of the family *Arteriviridae* in the order *Nidovirales* (3). PRRSV replication generates two replicase polyprotein precursors, pp1a and pp1ab, specified by ORF1a and ORF1a/b, respectively (28, 38). pp1a is made directly from ORF1a, whereas pp1ab stems from the translation of ORF1a/b via frameshift reading of ORF1 (28, 38). These polyproteins, either cotranslationally or subsequently, undergo extensive proteolytic maturation; at least 14 mature replicase subunits have been predicted according to studies of equine arteritis virus (EAV) (37, 42), the prototype of the family *Arteriviridae*. The proteolytic processing is thought to involve four virally encoded proteases within ORF1a, including two papain-like cysteine proteases (PCP1α and PCP1β), a cysteine protease (PL2), a picornavirus 3C-like serine protease ($3CL^{pro}$), and possibly cellular proteases (8, 30–32, 42).

The studies of EAV support a general model for PRRSV replicase maturation: nsp1 is released from the precursor by PCP1α and PCP1β; PL2 of nsp2 then cleaves the site at the nsp2–nsp3 (nsp2/3) junction; and the maturation of the remaining pp1a and pp1ab is executed by a 3C-like serine protease contained in nsp4. Lines of evidence have shown that nsp1 is processed *in vitro* into nsp1α and nsp1β by *cis*-active PCP1α and PCP1β, respectively, and that this processing occurs cotranslationally and rapidly (8, 30, 34). The nsp2 protein of PRRSV strain VR-2332, situated immediately downstream of nsp1, is a large multidomain protein of about 1,197 amino acids (aa) containing an N-terminal PL2 cysteine protease, a 500- to 700-aa middle hypervariable region, a putative transmembrane (TM) domain, and a C-terminal tail (13, 15). The PL2 protease is active both in *cis* and in *trans* and mediates the processing of nsp2 into one predominant product in CHO cells (14). *In vitro* mutagenesis studies of strain VR-2332 have revealed that the nsp2/3 cleavage is sensitive to mutations at the $G^{1196}|G^{1197}$ site (14). For example, even a conserved G1197A

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TABLE 1. Primers used in this study

Primer[a]	Genome position[b]	Sequence[c]
Construction of pV7-*myc* and pV7-HA-*myc*		
VR-1051U27/	1051–1077	5′ TCGCCATGCTAACCAATTTGGCTATC
/VR-2430L24	2407–2430	5′ TTGGCATGAGCCCATATTCTTCTC
/VR-1859L33	1827–1859	5′ ATGCTCGAGTTATCAGCTAGGCAGGTGCATCAC
VR-1824U24/	1824–1847	5′ TTGACCGGCTGGCTGAGGTGATGC
/VR-3349L26	3324–3349	5′ GCGTAGCAGGGTCATCAAGCTTAGTC
dVR-67U22/	2167–2187	5′ CGCCCGCCACGCGTAATCGACA
/Nsp2Δ324-434-*myc* 2306L64	c-*myc*	5′ GATCTTCTTCTGAAATCAACTTTTGTTCCAGATCTTCTTCAGAGA TGAGTTTCTGCTCGCTAGC
Nsp2Δ324-434-*myc* 2344U64/	c-*myc*	5′ ACAAAAGTTGATTTCAGAAGAAGATCTGGAACAGAAGCTCATCT CTGAGGAAGATCTGCCTAGG
Nsp2Δ324-434-*myc* 2387U43/	c-*myc*	5′ TCTGAGGAAGATCTGCCTAGGCCAAAAGTTCAGCCTCGAAAAA
VR-HA2 1387U65/	*HA*	5′ TCCAGATTACGCTTACCCATACGATGTCCCTGATTACGCAGTTCG TGAAACCCGGCAGGCCAAG
/VR-HA2 1350L65	*HA*	5′ GACATCGTATGGGTAAGCGTAATCTGGAACATCGTATGGGTAAC AAGAGCGTGCTTTTCTTGCTC
Construction of nsp2 truncation mutants and cloning of HSPA5		
Nsp2 PL2 1U37/	1373–1387	5′ AGCT***AAGCTT***GCCACC**ATGGTG**GCGACTGCTACAGTC
/VR-3755L33	3732–3755	5′ TAC***TCTAGA***AGAGCCGGCGCCACCTGTGCCTGCC
/VR-4258L31	4237–4258	5′ CTA***TCTAGA***GCCCAGTAACCTGCCAAGAATGG
/VR-4898L33	4875–4898	5′ CCC***TCTAGA***TGAAGGCTTGGAAATTTGCCTGATT
/VR-5583L35	5558–5583	5′ CTA***TCTAGA***AGACCCAAGCTGGGACGGGGTAAACAA
HSPA5 1U74/	HSPA5	5′ CCC***AAGCTT***GCCACCATGGGAGAACAAAAACTCATCTCAGAAGA GGATCTGAAGCTCTCCCTGGTGGCCGCGAT
/HSPA5 1988L38	HSPA5	5′ ATG***CTCGAG***TTACTACAACTCATCTTTTTCTGCTGTAT

[a] Forward primers are indicated by a slash after the designation; reverse primer designations are preceded by a slash.
[b] Based on GenBank accession number U87392 (VR-2332).
[c] Boldface italics indicate engineered restriction sites, and boldface indicates the engineered start codon for nsp2 expression.

substitution abolishes nsp2/3 proteolysis in CHO cells. Similar mutations also abolished the production of PRRSV progeny virions (14). These studies have pointed to the conclusion that the PL2-induced nsp2/3 cleavage most likely takes place at or near the $G^{1196}|G^{1197}$ dipeptide. In contrast to that in transfected CHO cells, nsp2 protein processing in PRRSV-infected cells is not yet understood. The goal of the experiments described here was to further assess the proteolytic products of nsp2 in the context of infection with the PRRSV type 2 strain VR-2332.

A major hindrance to the understanding of PRRSV replicase maturation has been the lack of antibodies of good quality. Here, by utilizing the genetically flexible nature of nsp2, two recombinant PRRSVs expressing nsp2 derivatives tagged with foreign epitopes were constructed and then utilized to further investigate the proteolytic processing of nsp2 in PRRSV-infected MARC-145 cells. We showed that nsp2 existed as several isoforms with apparently different C termini during PRRSV infection. Total proteolysis of PRRSV nsp2 likely involved both the PL2 protease and other, unknown viral or cellular proteases. The processing was rapid, and the cleaved products were relatively stable and finely balanced. Additionally, a cellular protein chaperone named HSPA5 was found to interact with the nsp2 protein and could be specifically coimmunoprecipitated by anti-nsp2 antibodies.

MATERIALS AND METHODS

Plasmids and antibodies. The plasmids used in this study, including pNsp2-3, pNsp2-3 C55A, pNsp2-3 G1197P, and pPL2, have been described previously (14). The genes specifying nsp2 polypeptides comprising aa 12 to 813, aa 12 to 981, and aa 12 to 1196 were amplified from plasmid pNsp2-3 and cloned into the site between BamHI and XbaI in plasmid pcDNA/HA-FLAG (GenBank accession number FJ524378) to generate the new plasmids pNsp2(12-813), pNsp2(12-981), and pNsp2(12-1196), respectively.

The antibodies used in this study include anti-c-*myc* monoclonal antibody 9E10 (Developmental Studies Hybridoma Bank at the University of Iowa), rabbit polyclonal anti-c-*myc* antibodies (Abcam), mouse anti-hemagglutinin (anti-HA) antibodies (Covance), mouse anti-FLAG antibodies (M2; Sigma), horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Southern Biotechnology, Inc.), and a rabbit anti-HSPA5 antibody (Santa Cruz Biotechnology). Mouse monoclonal antibodies D3A4 and E5F8 are gifts from Hanchun Yang (China Agricultural University) (40). The rabbit polyclonal antibody V was raised against a peptide containing PRRSV strain VR-2332 nsp2 aa 1078 to 1094 (SEKPIAFAQLDEKKITA) (Covance) and has been described in a previous report (14).

Construction of PRRSV deletion mutants. Plasmid pV7-nsp2Δ324-434-GFP has been reported previously (13). To generate a recombinant virus tagged with the c-*myc* epitope, the green fluorescent protein (GFP)-encoding gene was replaced by 3 c-*myc* epitopes (ASEQKLISEEDLEQKLISEEDLEQKLISEED) to produce plasmid pV7-nsp2Δ324-434-*myc* (pV7-*myc*) by overlapping PCR as described previously (GenBank accession number FJ524377) (13). To generate the HA–c-*myc* double-tagged virus pV7-HA-*myc* (GenBank accession number FJ524376), two copies of the influenza A virus HA epitope tag (YPYDVPDYA) replaced nsp2 aa 12 to 24 in plasmid pV7-*myc*. A similar strategy was applied to create nsp2 deletion viruses based on plasmid pV7-*myc*, and the new plasmids were designated pV7-*myc*-nsp2Δ543-632, pV7-*myc*-nsp2Δ633-726, and pV7-*myc*-nsp2Δ727-813. The sequences of the primers used for the construction of these viruses are given in Table 1.

Immunostaining of nsp2 protein. V7-*myc* was monitored for nsp2 protein expression using antibodies to c-*myc*. At 20 h postinfection, V7-*myc*-infected MARC-145 cells at passage 3 were labeled using monoclonal antibody 9E10 against a c-*myc* epitope and an Alexa 568-conjugated secondary antibody (red) (Molecular Probes). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue).

Transient expression. MARC-145 (ATCC) and CHO cells (Invitrogen) were maintained in Eagle's minimal essential medium (EMEM) (SAFC Biosciences)

supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. CHO cells were transiently transfected by using Lipofectamine 2000 (Invitrogen) as described previously (14). RNA transfection of MARC-145 cells was performed as described previously (13).

Viral growth assays. MARC-145 cells in T25 flasks were infected at a multiplicity of infection (MOI) of 0.1 with either parental or mutant viruses at passage 3. After 1 h of attachment at room temperature with gentle mixing, unbound viruses were removed; the monolayers were washed three times with serum-free EMEM; and the medium was replaced with 7 ml complete medium. Samples were collected from the medium at different time points after infection and were titrated by viral plaque assays on MARC-145 cells.

Immunoprecipitation and Western blotting. Transfected CHO cells or infected MARC-145 cells were rinsed twice with cold phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and 100 μl/ml protease inhibitor cocktail [P8340; Sigma]) on a platform shaker for 30 min at 4°C. The cell debris was removed by centrifugation at 13,000 rpm for 20 min. The supernatants were precleared by protein G agarose or protein A agarose and were then incubated with selected antibodies as well as protein G PLUS-agarose (Santa Cruz Biotechnology) or protein A agarose (Roche) at 4°C overnight. The immunocomplexes were washed twice with cold RIPA buffer, once with 0.1% sodium dodecyl sulfate (SDS) in RIPA buffer, and once with PBS. After being heated for 5 min at 100°C, the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and were then electrophoretically transferred to a nitrocellulose membrane. For Western blotting, the membrane was blocked with 5% milk powder in PBST (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20) for 1 h and was then incubated with the appropriate primary antibodies diluted in PBST–5% milk overnight at 4°C. After three washes with PBST for 30 min, the blot was incubated with an appropriate secondary antibody diluted in PBST for 1 h. The membrane was again washed and was then developed with the ECL Western blot analysis system (Pierce).

Radiolabeling and pulse-chase analysis of the nsp2 protein. MARC-145 cells in 60-mm-diameter petri dishes were infected with V7-*myc* (MOI, 0.1) at passage 3 and were incubated in 5 ml EMEM with 10% FBS at 37°C. For pulse-chase analysis, at 12 to 18 h postinfection, MARC-145 cells were washed with Dulbecco's modified essential medium (DMEM) deficient in methionine and cysteine (Met/Cys) and were starved for 30 min. The cells were then labeled with 4 ml of DMEM with 100 μCi/ml [^{35}S]Met-Cys. After 5 to 6 h of labeling, MARC-145 cells were washed twice with DMEM; then they were incubated with 5 ml DMEM with 10% FBS. At various times (0, 15, 30, 60, 120, 180, and 240 min), one dish was removed from the incubator; the cells were washed with cold PBS and were then lysed in RIPA buffer. Alternatively, MARC-145 cells were infected with PRRSV at an MOI of 50. At 13.5 h postinfection, 60-mm-diameter dishes were each labeled with 350 μCi/ml [^{35}S]Met-Cys in 1 ml DMEM for 20 min; the plates were washed; and DMEM with excess amounts of unlabeled methionine and cysteine (2 mM each) was added. Individual cell monolayers were lysed with RIPA buffer at 0, 5, 10, 15, 20, 25, 30, 60, 90, and 120 min. The cell lysates were cleared by centrifugation and were immunoprecipitated using suitable antibodies.

Tandem affinity purification and mass spectrometry. MARC-145 cells were infected with the recombinant PRRSV V7-*myc* (passage 3) at an MOI of 0.1 and were then incubated in EMEM with 10% FBS at 37°C. The cells were harvested at 36 h postinfection and were lysed in RIPA buffer. nsp2-associated complexes were purified by two rounds of immunoprecipitation with mouse anti-c-*myc* monoclonal antibodies and rabbit polyclonal antibody V. The complexes were washed twice with RIPA buffer, once with RIPA buffer with 0.1% SDS, and once with PBS and were then eluted by boiling in SDS-PAGE loading buffer with 5% 2-mercaptoethanol. The complexes were separated by SDS-PAGE on a 4 to 12% NuPage gel (Invitrogen) and were visualized with Coomassie blue or Sypro Ruby (Invitrogen). Sypro Ruby-stained bands were excised, trypsin digested, extracted, and analyzed by liquid chromatography and tandem mass spectrometry (LC-MS-MS) at the University of Minnesota Mass Spectrometry Center.

RESULTS

Construction and recovery of c-*myc*- and HA-tagged PRRSV. The nsp2 regions comprising aa 12 to 35 and aa 324 to 434 of PRRSV strain VR-2332 have been shown to be dispensable for viral replication in cell culture, and a recombinant PRRSV with a GFP-encoding gene in place of nsp2 aa 324 to 434 is viable (13). We accordingly replaced the deletions of PRRSV nsp2 aa 12 to 35 and aa 324 to 432 with foreign epitope tags in order to facilitate analysis of the nsp2 product(s) in PRRSV-infected MARC-145 cells utilizing high-quality commercial antibodies. To generate a c-*myc*-tagged virus, the GFP-encoding gene in the PRRSV infectious clone pV7-nsp2Δ324-434-GFP was replaced with 3 consecutive c-*myc* epitopes to generate the new plasmid pV7-nsp2Δ323-434-*myc* (pV7-*myc*) (Fig. 1A). To produce a double-tagged virus, two copies of an HA epitope were engineered into plasmid pV7-*myc* to replace nsp2 aa 12 to 24, and the new plasmid was designated pV7-nsp2Δ12-24-HA-nsp2Δ323-434-*myc* (pV7-HA-*myc*) (Fig. 1A). The plasmids were linearized and were transcribed *in vitro*. The RNA transcripts were transfected into MARC-145 cells. A virus-induced cytopathic effect (CPE), characterized by cell rounding, clustering, and detachment, was readily detected 4 to 5 days posttransfection (data not shown). The two mutants displayed growth kinetics indistinguishable from that of the parental virus VR-V7 in MARC-145 cells (Fig. 1B), suggesting that the insertion of one to two small foreign epitopes into the nsp2 region did not affect PRRSV replication. Mutant V7-*myc* was stable for at least 10 passages, as confirmed by sequence analysis of the nsp2-coding region (data not shown). Immunostaining with anti-c-*myc* antibodies revealed a typical perinuclear localization pattern of nsp2 (Fig. 1C), as reported previously (11, 13, 40). Mutant V7-HA-*myc* was stable for at least 3 passages, as confirmed by sequence analysis of the nsp2-coding region (data not shown). However, anti-HA antibodies failed to stain nsp2 in V7-HA-*myc*-infected cells, perhaps due to inaccessibility of the HA epitope under native conditions.

Detection of nsp2 products in PRRSV-infected MARC-145 cells. To detect nsp2 products, MARC-145 cells were infected with V7-*myc* at an MOI of 0.1. The cells were lysed with RIPA buffer at 24 to 36 h postinfection and were cleared by centrifugation. Following immunoprecipitation with mouse monoclonal antibodies (9E10) against the c-*myc* epitope and separation by SDS-PAGE, the nsp2 protein was probed with mouse monoclonal antibody E5F8, which recognizes the nsp2 region comprising aa 77 to 87 (40). Six specific products, with estimated sizes of 120, 100, 80, 51, 43, and 41 kDa, were detected and designated nsp2a, nsp2b, nsp2c, nsp2d, nsp2e, and nsp2f, respectively (Fig. 2A, first lane). To rule out the possibility that the nsp2 products were being generated during sample preparation or during immunoprecipitation, V7-*myc*-infected cells were pelleted after being washed with cold PBS three times and were then boiled in SDS-PAGE sample buffer. The proteins were separated, transferred to a nitrocellulose membrane, and probed with anti-c-*myc* antibodies. Again, we detected all 6 nsp2 species (data not shown). A similar processing pattern was also observed in V7-HA-*myc*-infected MARC-145 cells probed with anti-HA antibodies after immunoprecipitation with anti-c-*myc* antibody 9E10 (Fig. 2B, first lane). Since the HA epitope is in place of nsp2 aa 12 to 24 and the recognition site of antibody E5F8 is very near the N terminus of nsp2 (Fig. 1A), we conclude that these products most likely have the same N terminus.

Mapping of the C termini of the nsp2 species. To differentiate the C termini of the nsp2 products, we initially attempted to use antibody probing to distinguish the nsp2 isoforms.

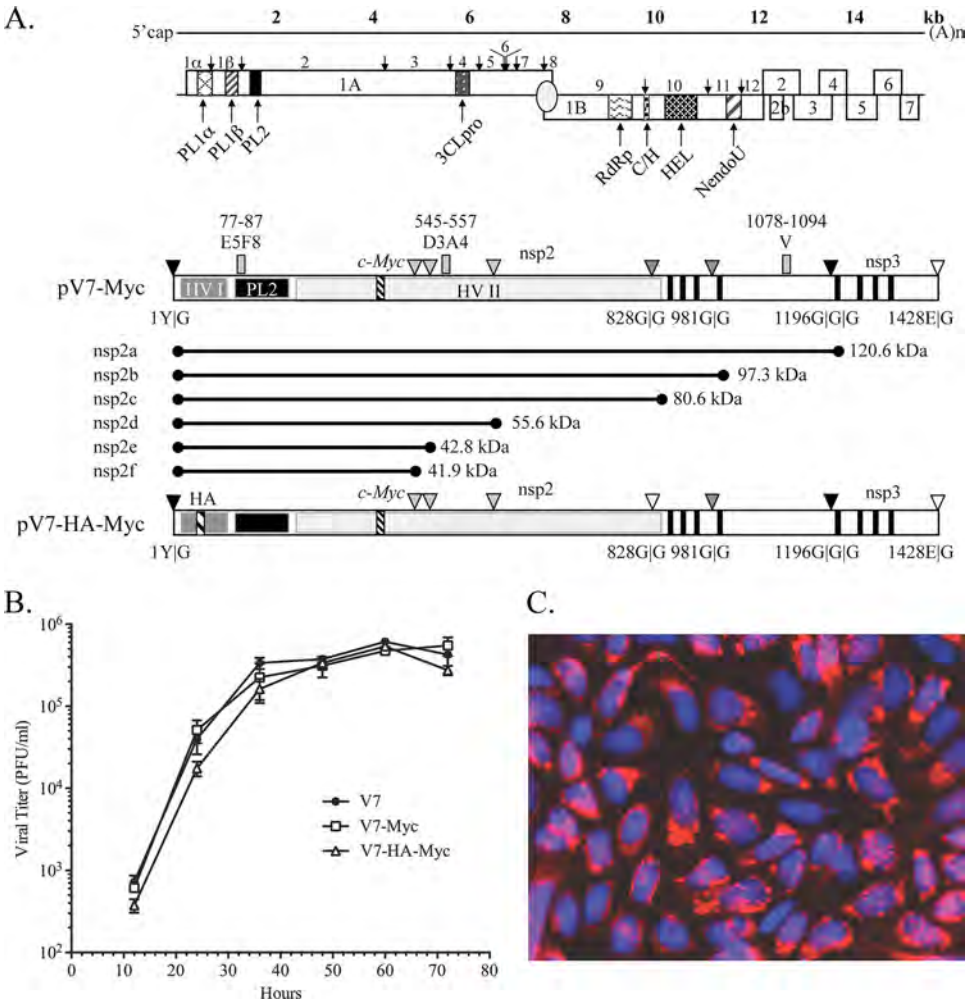

FIG. 1. Characterization of foreign-epitope-tagged PRRSV. (A) PRRSV genome annotation. The genome of PRRSV is shown with all identified open reading frames. ORF1A and ORF1B are posttranslationally cleaved by virally encoded papain-like proteases (PCP1α and PCP1β), a cysteine protease (PL2), and a poliovirus 3C-like serine protease (3CL). The following polymerase signature regions are indicated: RNA-dependent RNA polymerase (RdRp), cysteine and histidine rich (C/H), helicase (HEL), and nidovirus uridylate-specific endoribonuclease (NendoU). Below the genome schematic are expanded diagrams of nsp2-3 and the construction of recombinant PRRSVs expressing c-*myc*- or c-*myc*- and HA-tagged nsp2. nsp2 contains an N-terminal small hypervariable region (HV-I), a PL2 protease domain, a middle hypervariable region (HV-II), a putative transmembrane domain (filled vertical bars), and a C-terminal domain. The nsp2 PL2 protease is predicted to cleave at G|G dipeptides; there are 10 such dipeptides in type 2 strain VR-2332, some of which are not conserved in other strains. The major conserved predicted cleavage sites discussed in this report are represented by dark shaded triangles; potential cleavage sites that are not conserved are shown as light shaded triangles; and the cleavage sites of nsp1|nsp2 and nsp3|nsp4 are shown as filled and open triangles, respectively. Three consecutive c-*myc* epitopes were inserted in place of nsp2 aa 324 to 434, based on strain VR-2332 full-length cDNA clone pVR-V7, to generate mutant pV7-*myc*. In the bottom construct, an HA epitope replaced nsp2 aa 12 to 24 of pV7-*myc* to generate the double-tagged mutant pV7-HA-*myc*. (B) Growth kinetics of mutants V7-*myc* and V7-HA-*myc* and of parental virus VR-V7 at passage 3. The viruses were used to infect MARC-145 cells in T25 flasks at an MOI of 0.1. The virus-infected cell supernatants were collected every 12 h and were titrated by a viral plaque assay. The mean results were plotted; error bars indicate standard deviations. (C) Immunostaining of nsp2 protein. At 20 h postinfection, the nsp2 protein in V7-*myc*-infected MARC-145 cells was labeled using monoclonal antibody 9E10 against a c-*myc* epitope and an Alexa 568-conjugated secondary antibody (red) (Molecular Probes). Nuclei were stained with DAPI (blue). The fields were merged using Photoshop, version 8.

Mouse monoclonal antibody D3A4 recognizes the nsp2 peptide comprising aa 545 to 557 (40) and reacted with nsp2 segments a, b, c, and d but not with segments e and f (Fig. 2A, third lane). Since the proteins had initially been precipitated with an anti-c-*myc* monoclonal antibody recognizing the epitope located just before nsp2 aa 434, the antibody recognition pattern signified that the C termini of nsp2e and nsp2f are located between nsp2 aa 434 and aa 545 (Fig. 2A, third lane).

The fact that nsp2d reacted with antibody D3A4 suggests that nsp2d is cleaved after residue 557. To further map the cleavage site, three nsp2 deletion mutants based on V7-*myc* (V7-*myc*-nsp2Δ543-632, V7-*myc*-nsp2Δ633-726, and V7-*myc*-nsp2Δ727-813) were generated (Fig. 3A). The mutants were viable and showed growth titers comparable to those of V7-*myc* and the parental virus VR-V7 (data not shown). By using these mutants in analyses similar to those described above, nsp2d could be distinguished from nsp2c and nsp2e by the deletion of the nsp2 hypervariable region comprising aa 543 to

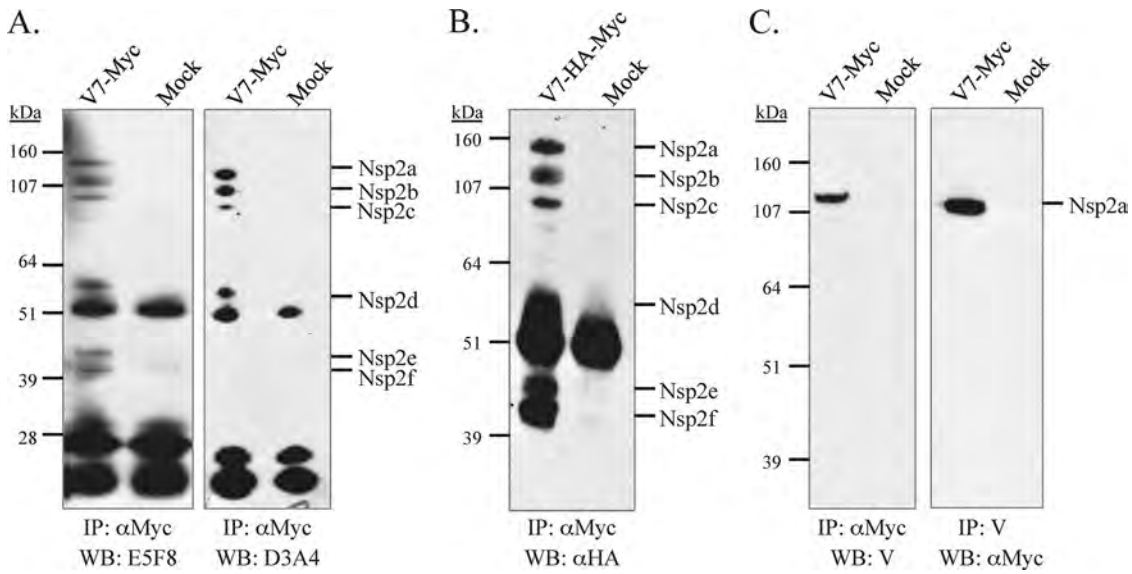

FIG. 2. Identification of nsp2 products in PRRSV-infected MARC-145 cells. (A) MARC-145 cells either were mock infected or were infected with V7-*myc* (MOI, 0.1) at passage 3. At 24 to 36 h postinfection, the cells were lysed, and nsp2 proteins were immunoprecipitated (IP) by mouse monoclonal antibody 9E10 recognizing the c-*myc* epitope. The samples were analyzed by reducing SDS-PAGE on a 4 to 12% polyacrylamide gel followed by Western blotting (WB) with the nsp2-specific antibody E5F8 or D3A4. (B) Analysis of nsp2-associated products in V7-HA-*myc*-infected MARC-145 cells. The nsp2 products were first immunoprecipitated by anti-c-*myc* antibody 9E10, then separated by SDS-PAGE, and finally analyzed by WB with a mouse monoclonal antibody to the HA epitope. (C) V7-*myc*-infected cell lysates were immunoprecipitated with anti-c-*myc* antibody 9E10 or rabbit antibody V, recognizing a peptide near the C terminus of nsp2; then they were separated by SDS-PAGE and analyzed by immunoblotting with antibody V or rabbit anti-c-*myc* polyclonal antibodies. The use of different antibody combinations for IP and WB frequently detected nonspecific proteins (25 kDa and 50 kDa, respectively), which are likely immunoglobulin light and heavy chains, respectively, that reacted with the secondary antibody.

632, because no nsp2d product was detected after the region was deleted (Fig. 3B), suggesting that the cleavage site of nsp2d is located between nsp2 aa 557 and aa 632.

In contrast to that of nsp2d, the detection of nsp2c was not affected by the deletion of nsp2 aa 543 to 632, aa 633 to 726, or aa 727 to 813 (Fig. 3B). Thus, the cleavage site of nsp2c lies downstream of residue Gly-813. nsp2b and nsp2c could not be differentiated by deletion mutagenesis, since further deletion of the putative transmembrane domains (aa 876 to 898, 911 to 930, 963 to 979, and 989 to 1009) or their upstream flanking sequence (aa 814 to 845) is lethal to the virus (13). To assess the relative sizes of nsp2b and nsp2c, peptides corresponding to nsp2 aa 12 to 813 and nsp2 aa 12 to 981 were *in vitro* expressed in CHO cells by cloning the respective nsp2 fragment into pcDNA3/HA-FLAG. The nsp2 C-terminal glycine residues (aa 813 and 981) were chosen because they resided at or near potential alternative cleavage sites for PL2, which has been shown to prefer G|G dipeptides (14, 31). As shown in Fig. 3C, nsp2c and nsp2b showed comparable migration rates with nsp2(12-813) (82 kDa) and nsp2(12-981) (100.3 kDa), respectively, suggesting that they have different C termini.

Rabbit antipeptide antibody V recognizes the nsp2 region comprising aa 1078 to 1094 (14). As shown in Fig. 2C, antibody V detected nsp2a but not other nsp2 species, consistent with the previous result that antibody V recognized the processed nsp2 in CHO cells (14). In Fig. 3C, nsp2a had a migration rate similar to that of nsp2 processed from transfected construct expressing nsp2-3 that was cleaved at or around the $G^{1196}|G^{1197}$ site in CHO cells, as demonstrated by site-directed mutagenesis, but migrated faster than the uncleaved precursor nsp2-3(C55A) (14). In addition, nsp2a had a migration rate similar to that of the *in vitro*-expressed polypeptide corresponding to nsp2 aa 12 to 1196 (123.6 kDa). Furthermore, mutations at the $G^{1196}|G^{1197}$ site that block the nsp2-3 cleavage *in vitro* are lethal to PRRSV (14). Therefore, we conclude that nsp2a is the equivalent of the nsp2 product processed in CHO cells.

nsp2d, nsp2e, and nsp2f are not essential for viral replication in cell culture. The experiments described above suggested that the cleavage sites of nsp2d, nsp2e, and nsp2f lie in the middle hypervariable region of nsp2. The deletion mutagenesis in this study, combined with the findings of a previous report (13), demonstrated that deletion of either nsp2 aa 324 to 525, containing putative cleavage sites for nsp2e and nsp2f, or nsp2 aa 543 to 632, containing the putative cleavage site for nsp2d, did not disrupt viral replication in cell culture. In addition, a 400-aa deletion (aa 324 to 726) of the nsp2 hypervariable region that contains the proposed cleavage sites of nsp2d, nsp2e, and nsp2f generated a virus that was viable but exhibited delayed growth kinetics, reduced cytotoxicity, and diminished plaque formation (13). Thus, consistent with the poor conservation of the respective possible G|G cleavage sites among PRRSV strains (Fig. 1A), nsp2d, nsp2e, and nsp2f are not essential for viral replication in cell culture.

We concluded previously that the cleavage of nsp2a most likely occurs at or near residues $G^{1196}|G^{1197}$ (14). By using reverse genetics, we had also shown that mutations that block the cleavage at this site are lethal to the virus (14). Thus, the proteolytic generation of nsp2a is critical for viral replication. The cleavage sites that generate nsp2b and nsp2c were mapped

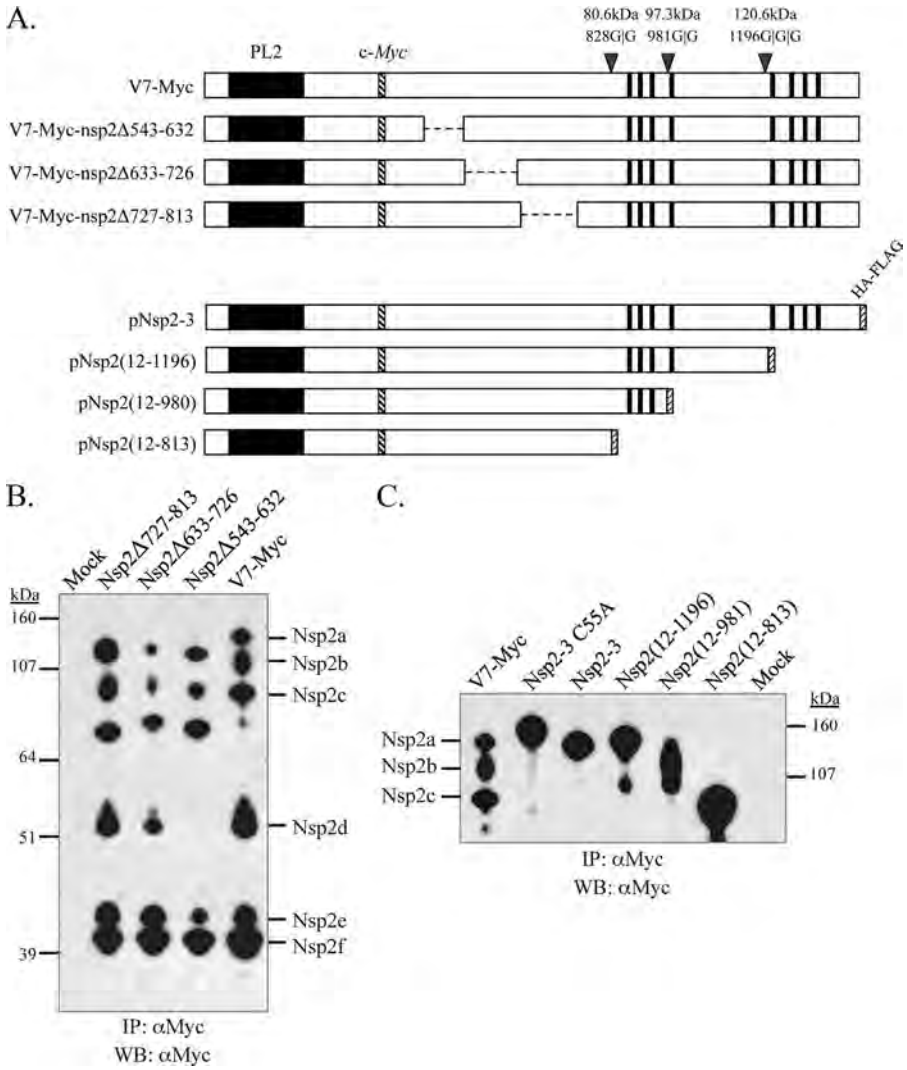

FIG. 3. Mapping of the relative positions of the nsp2 isoforms. (A) V7-*myc* is shown in schematic form with PL2 cleavage sites and the calculated molecular sizes of predicted proteins that included the nsp2 N terminus. V7-*myc* nsp2 regions comprising aa 543 to 632, aa 633 to 726, or aa 727 to 813 were deleted to generate new full-length infectious cDNA clone mutants pV7-*myc*-nsp2Δ543-632, pV7-*myc*-nsp2Δ633-726, and pV7-*myc*-nsp2Δ727-813, respectively. Polypeptides corresponding to nsp2 aa 12 to 813, aa 12 to 981, and aa 12 to 1196 were cloned into pcDNA3 to generate plasmid constructs pNsp2(12-813), pNsp2(12-981), and pNsp2(12-1196). The HA-FLAG epitope was attached to the C terminus of each polypeptide. The predicted molecular sizes for the corresponding shortened polypeptides were calculated and are given in the text. (B) MARC-145 cells were infected with nsp2 deletion mutant V7-*myc*-nsp2Δ543-632, V7-*myc*-nsp2Δ633-726, or V7-*myc*-nsp2Δ727-813. At 24 to 36 h postinfection, the cells were lysed and immunoprecipitated (IP) with the anti-c-*myc* monoclonal antibody 9E10, separated by SDS-PAGE on a 4 to 12% NuPage gel, and subjected to Western blotting (WB) with anti-c-*myc* rabbit polyclonal antibodies. (C) CHO cells were transfected with plasmids expressing either nsp2-3, nsp2-3(C55A), which does not undergo PL2 proteolysis (14), or one of the nsp2 truncation mutants. The cells were lysed after 48 h of transfection; then they were immunoprecipitated with anti-c-*myc* monoclonal antibody 9E10 and analyzed by Western blotting with an anti-c-*myc* rabbit polyclonal antibody. The nsp2 proteins immunoprecipitated from V7-*myc*-infected MARC-145 cells served as a control. The extra bands in the lanes for truncation mutants Nsp2(12-1196) and Nsp2(12-981) may represent degraded products, possibly due to cellular proteases.

to the region around the transmembrane (TM) domains. We could not directly determine the importance of nsp2b and nsp2c at this point, since deletion of either the TM domain or its upstream (aa 814 to 845) or downstream sequence is lethal to PRRSV (13).

Accumulation, stability, and turnover of the nsp2 isoforms. We wanted to study when and how the nsp2 species emerge during PRRSV infection. Accordingly, V7-*myc* was used to infect MARC-145 cells at a low MOI of 0.1 in order to observe the accumulation of nsp2 species. As shown in Fig. 4, the nsp2 proteins emerged almost simultaneously in the early stages of PRRSV infection, from 6 to 12 h, especially for the larger nsp2 isoforms, and accumulated to peak levels at 36 to 42 h postinfection, consistent with the viral growth curve (Fig. 1B). To investigate the processing kinetics of nsp2, radioimmunoprecipitation assays were carried out with anti-c-*myc* antibody 9E10 (Fig. 5A and B) or the rabbit antipeptide antibody V (Fig. 5C and D). In line with the results described above,

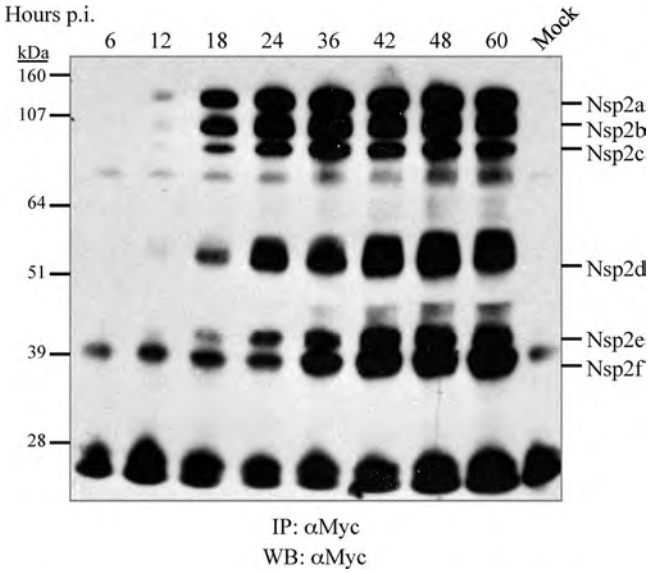

FIG. 4. Accumulation of the nsp2 isoforms during virus infection. MARC-145 cells in 60-mm-diameter petri dishes were infected with V7-*myc* at an MOI of 0.1. At different time points after infection, as indicated, the cells were lysed, immunoprecipitated (IP) with monoclonal antibody 9E10 against the c-*myc* epitope, separated by SDS-PAGE on a 4 to 12% NuPage gel with 5% β-mercaptoethanol, and subjected to Western blotting (WB) with rabbit polyclonal antibodies to c-*myc*.

products with sizes similar to those of the six nsp2 species were recognized in the radioimmunoprecipitation assay by anti-c-*myc* antibodies (Fig. 5A). As expected, of the six nsp2 cleavage products, antibody V immunoprecipitated only nsp2a (Fig. 5C). Interestingly, one product of host or viral origin with an estimated size of 70 to 80 kDa was markedly coimmunoprecipitated by both anti-*myc* and V antibodies. Two specific bands with molecular masses of about 14 kDa and 39 kDa were immunoprecipitated only by antibody V. The origins of these bands were not clear, but they could represent downstream cleavage products from nsp2b and nsp2c proteolysis (Fig. 5C). In addition, the large band precipitated by antibody 9E10 only in mock-infected cells was of unknown origin (Fig. 5A).

The stability of nsp2-associated products was analyzed by pulse-chase assays. Initially, MARC-145 cells were infected with the V7-*myc* virus at an MOI of 0.1 and were metabolically labeled with [^{35}S]methionine-cysteine for 5 h at 18 h postinfection. The nsp2 species accumulated to a maximal level after a pulse for 5 to 6 h, as revealed by immunoprecipitation with anti-c-*myc* antibodies (Fig. 5B, left). After a 5-h pulse, the ^{35}S-labeled nsp2 species were chased for various periods and were immunoprecipitated either with anti-c-*myc* antibodies for as long as 240 min (Fig. 5B, right) or with antibody V for as long as 180 min (Fig. 5D). Bands consistent with all six nsp2 isoforms were identified when immunoprecipitation was performed with anti-c-*myc* antibodies. When antibody V was used for immunoprecipitation, nsp2a was easily detected, as well as the 14-kDa and 39-kDa PRRSV-specific products seen in Fig. 5C. The cleavage of nsp2 from polyprotein pp1a occurred rapidly; no obvious precursor proteins were detected (Fig. 5B and D). Additionally, the ratio among different nsp2 isoforms appeared to be relatively constant (Fig. 5B).

The lack of an obvious precursor-product relationship prompted us to examine the possibility that the amounts of the individual nsp2 products may have reached a plateau during the extended time of [^{35}S]methionine labeling before the completion of the chase. To rule out that possibility, we performed infection with PRRSV V7-*myc* at a high MOI of 50, shortened the labeling time to 20 min, and then chased for various times. Immunoprecipitations were performed using an anti-c-*myc* antibody and antibody V (Fig. 5E and F). Again, we observed a similar processing pattern. The failure to establish a clear precursor-product relationship indicated that nsp2 appeared to be processed primarily in *cis* and that this processing occurred cotranslationally and rapidly.

Coimmunoprecipitation of heat shock 70-kDa protein 5 with the nsp2 replicase protein. The assays described above also revealed that a protein band with an apparent molecular mass of 70 to 80 kDa was strongly coimmunoprecipitated with nsp2 by either anti-c-*myc* antibodies or antibody V against nsp2 (Fig. 5B and D) but was not detected when the assays were subsequently analyzed by Western blotting (Fig. 2 to 4). In order to identify the protein, we performed two rounds of immunoprecipitation, with anti-c-*myc* antibody 9E10 and antipeptide antibody V, respectively. The immunocomplexes were eluted and resolved by SDS-PAGE (Fig. 6A). The 70- to 80-kDa specific band coimmunoprecipitating with nsp2 was visualized, excised, digested with trypsin, extracted, and subjected to LC-MS-MS analysis. The recovered peptides highly matched heat shock 70-kDa protein 5 (HSPA5, or GRP78; 72 kDa) (Fig. 6B). The coverage was approximately 32%, and a total of 19 unique peptides that matched the HSPA5 sequence of the rhesus monkey, a cousin to the African green monkey, from which the MARC-145 cell line is derived, were recovered (Fig. 6B). The identity of the protein as HSPA5 and its association with nsp2 were further confirmed by Western blotting with rabbit anti-HSPA5 specific antibodies after nsp2 immunoprecipitation with anti-c-*myc* antibodies (Fig. 6C). The Dana-Farber Cancer Institute *Sus scrofa* Gene Index (SsGI) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=pig) was used to identify a protein sequence of swine GRP78 (TC395795) that was 98.9% identical to rhesus monkey HSPA5, illustrating that the association was not merely an aberrant result of PRRSV growth in nonhost cells (data not shown).

DISCUSSION

The experiments described in this report revealed new information regarding the processing of PRRSV nsp2 in the context of viral infection. This protein is often discussed as though it consisted of only one proteolytic product. There now exists clear evidence that type 2 PRRSV strain VR-2332 nsp2 is processed into multiple species. Specifically, the nsp2 species that were identified in this report share the same N terminus but differ in their truncated C termini. This finding is consistent with the observation that EAV nsp2, the counterpart of PRRSV nsp2, undergoes internal cleavage, but in a cell type-dependent manner (29). Whether this finding will apply to other PRRSV strains or cell types (macrophages) remains to be determined.

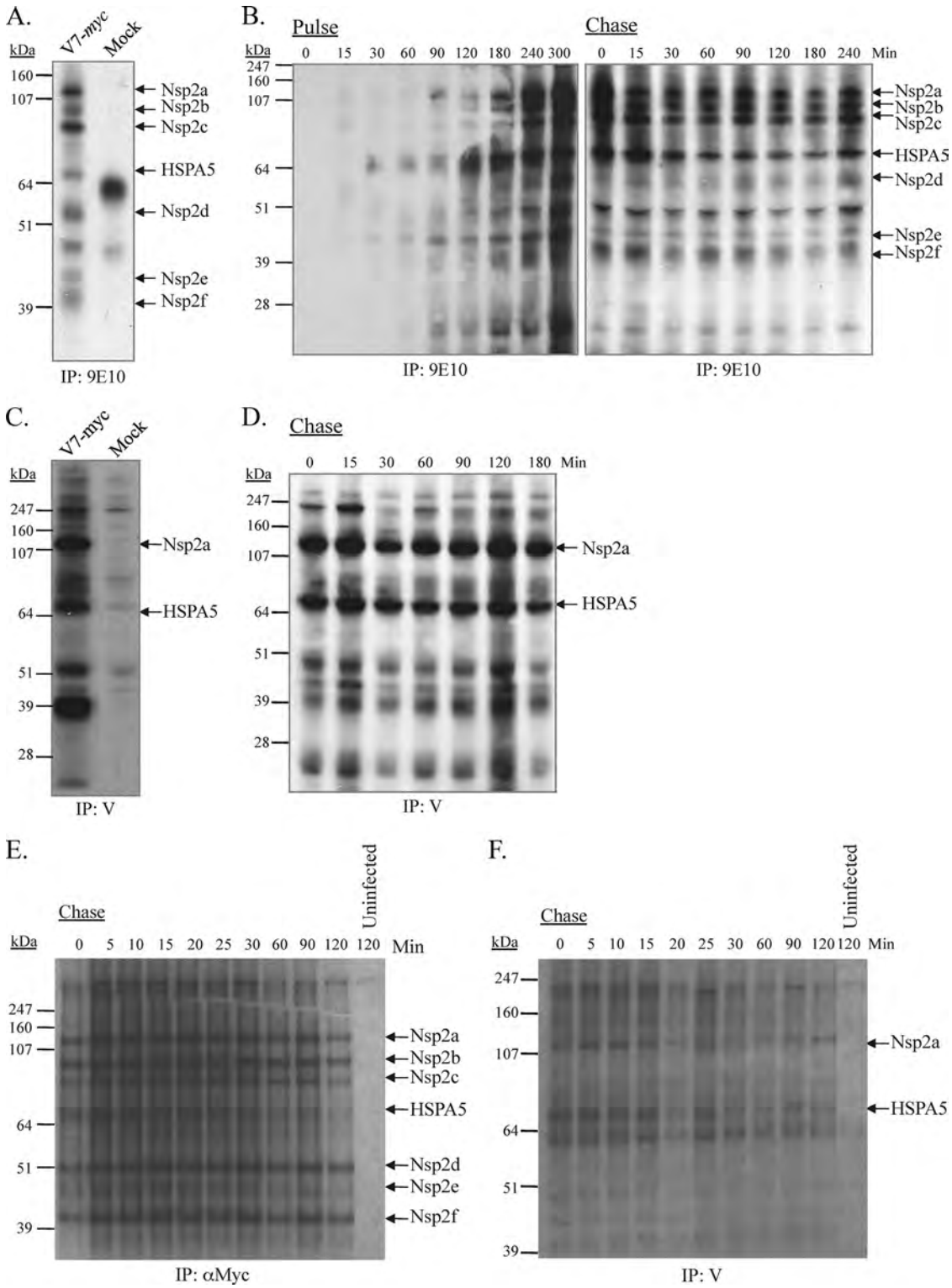

FIG. 5. Pulse-chase analysis of the nsp2 isoforms. (A to D) MARC-145 cells in 60-mm-diameter petri dishes were infected with V7-*myc* at an MOI of 0.1. At 20 h postinfection, the cells were labeled with [^{35}S]methionine-cysteine. Cells were lysed and immunoprecipitated (IP) with either anti-c-*myc* antibody 9E10 (A) or rabbit antipeptide antibody V (C). For pulse-chase analysis, the cells were pulsed for 5 h and then chased for as long as 4 h. nsp2 proteins were immunoprecipitated with anti-c-*myc* monoclonal antibody 9E10 (B) or rabbit antipeptide antibody V (D). (E and F) In an effort to elucidate the precursor-product relationship, cells were pulsed for 20 min and chased for as long as 2 h. nsp2 proteins were then immunoprecipitated with anti-c-*myc* monoclonal antibody 9E10 (E) or rabbit antipeptide antibody V (F). The proteins were separated by 4 to 12% SDS-PAGE with 10% dithiothreitol. The gel was dried, and radiolabeled nsp2 proteins were detected by autoradiography.

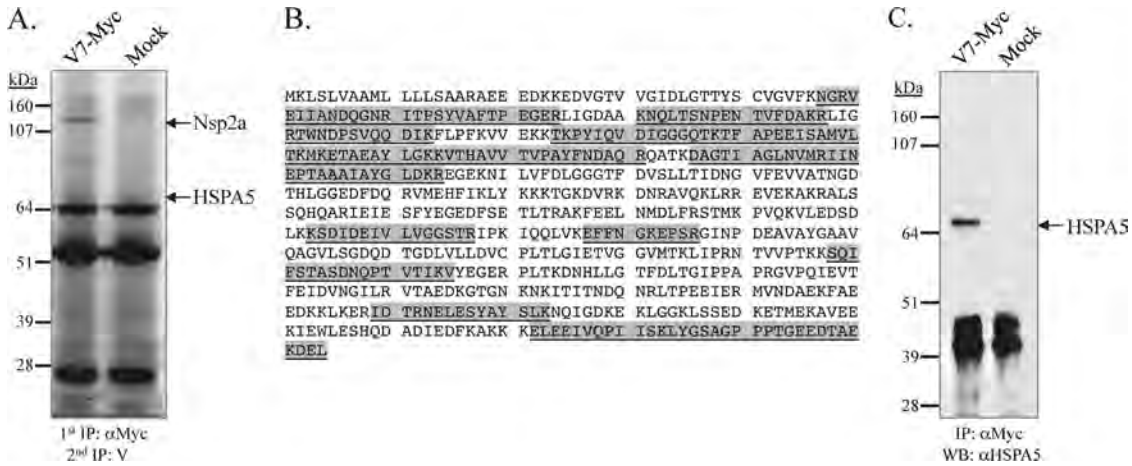

FIG. 6. Coimmunoprecipitation of HSPA5 with nsp2. (A) MARC-145 cells were infected with V7-myc at an MOI of 0.1. At 24 to 36 h postinfection, the cells were lysed and subjected to two rounds of immunoprecipitation (IP), with anti-c-*myc* monoclonal antibody 9E10 and rabbit antipeptide antibody V, respectively. The proteins were resolved on a 4 to 12% NuPage reducing gel and were stained with Coomassie blue. (B) The separated proteins were then stained with Sypro Ruby, excised, digested with trypsin, extracted, and subjected to LC-MS-MS analysis. The HSPA5 (GRP78) amino acid sequence of *Macaca mulatta* (rhesus monkey; GenBank ID 109112231) is shown, with the recovered sequences, sometimes overlapping each other, highlighted in gray. (C) Immunoblot analysis of the immunoprecipitated proteins with a rabbit polyclonal antibody to HSPA5.

Functional implications of multiple nsp2 species. The observation that PRRSV nsp2 has several isoforms has parallels in DNA and RNA viruses. For instance, herpes simplex virus always encodes full-length and truncated versions of the same protein that carry overlapping or distinct functions, although various mechanisms are employed to generate the isoforms, such as alternative splicing, proteolytic cleavage, or translation reinitiation (1, 21, 22, 26). The most salient examples are herpes simplex virus protein pairs $U_L26/U_L26.5$, $ICP22/U_S1.5$, and $U_S3/U_S3.5$ (21, 22, 26). For arteriviruses, besides EAV nsp2, it was recently reported that EAV nsp7 contains an internal cleavage site for $3CL^{pro}$ (37). Mutational analysis with an infectious clone of EAV demonstrated that both isoforms are critical for viral replication (37). Additional representative examples from RNA viruses include measles virus V/P, infectious bursal disease virus VP2/VPX, and Sendai virus C/C′ proteins (2, 7, 39). By analogy, the PRRSV nsp2 isoforms may serve different functions in the replication cycle of PRRSV. One benefit of adopting such a strategy, for a positive-strand RNA virus such as PRRSV, is to help maximize coding capacity. Other reasons for generation of isoforms may be to dictate protein abundance, to regulate protein-protein interactions, or to control protein trafficking.

Potential cleavage sites of nsp2 species. Our finding raises an important question regarding the mechanism for nsp2 cleavage. The PL2 protease displays a preference for a G|G dipeptide and mediates a single nsp2/3 cleavage at or around $G^{1196}|G^{1197}$ in transfected CHO cells (14). This preference resonates with the deubiquitinating activity of the PRRSV PL2 protease (12, 23, 35). It has been shown that several currently known viral deubiquitinating enzymes (DUBs) recognize and cleave after a G|G dipeptide in ubiquitin conjugates (33). Thus, the preference for a G|G dipeptide may represent a general feature of substrate recognition by the PRRSV PL2 protease.

We propose that $G^{828}|G^{829}|G^{830}$ and $G^{981}|G^{982}$ may serve as potential cleavage sites for nsp2c and nsp2b, respectively, based on the substrate recognition property of the PL2 protease and the fact that these sites are fairly conserved among PRRSV strains (15). Additional indirect evidence that nsp2b is cleaved at or near $G^{981}|G^{982}$ is as follows: (i) an *in vitro*-expressed polypeptide corresponding to nsp2 aa 12 to 981 had a gel migration rate similar to that of nsp2b (Fig. 3C), and (ii) the G981A and G981P substitutions were lethal to the virus (data not shown). Similarly, there is additional indirect evidence to support $G^{828}|G^{829}|G^{830}$ as the site for nsp2c cleavage: (i) nsp2c was processed after the residue Gly-813 and had a gel migration pattern similar to that of the polypeptide corresponding to nsp2 aa 12 to 813, expressed in CHO cells (Fig. 3C), and (ii) deletion of nsp2 aa 727 to 813 does not affect viral viability, while deletion of nsp2 aa 727 to 845 is lethal to PRRSV (13). The deleterious effect may be due to the elimination of the nsp2c processing site. Thus, the region comprising aa 813 to 845, containing the putative cleavage site $G^{828}|G^{829}|G^{830}$, appears to be crucial for viral replication. It should be noted that $G^{828}|G^{829}|G^{830}$ is highly conserved only in North American strains. Therefore, generation of nsp2c may be type or strain specific, as with hepatitis C virus, in which the internal cleavage of HCV NS3 takes place only in group 2a strains (19). The puzzling fact that the PL2 protease mainly recognizes the nsp2a cleavage site ($G^{1196}|G^{1197}$) but not other sites ($G^{828}|G^{829}|G^{830}$ and $G^{981}|G^{982}$) in CHO cells, in contrast to its behavior in PRRSV-infected cells, suggests that the processing of nsp2b and nsp2c species may need additional cofactors of either host or viral origin. Cofactor binding may alter the overall folding of the nsp2 precursor so that the cleavage sites would be exposed due to the conformational change, or it may change the substrate binding specificity.

The nsp2 region comprising aa 324 to 632, which contains the cleavage sites of nsp2d, nsp2e, and nsp2f, is highly heterogeneous among PRRSV strains (15). From an evolutionary point of view, it is unlikely that the virus would choose such a

highly variable region for its substrate. In addition, no dipeptides in VR-2332 nsp2 aa 324 to 632 could be identified as conserved among PRRSV strains and thus able to serve as potential sites that would satisfy the cleavage properties of the PL2 cysteine protease. However, there are several nonconserved G|G dipeptides that could serve as proteolytic substrates for the production of products of the estimated sizes of nsp2d to nsp2f in a strain-specific manner (Fig. 1A). Alternatively, these nsp2 species could be due to polypeptide degradation or translational attenuation, i.e., the ribosomes falling off the viral template during translation. However, we could not rule out the possibility that the PL2 protease recognizes residues other than the G|G dipeptide, or that other viral or host proteases may be involved in nsp2 cleavage.

Processing mechanism of nsp2. Another important issue concerns the regulation of nsp2 processing. In our pulse-chase assays, we did not observe a gradual decrease in the level of larger nsp2 species and an increase in the level of small nsp2 products, such as would be assumed for a standard precursor-product relationship. Instead, the results revealed that the nsp2 species showed up rapidly and had low turnover (Fig. 5B, D, E, and F) and that the ratio among them, in particular for nsp2a, nsp2b, and nsp2c, remained relatively constant during 2 to 4 h of chase (Fig. 4B and E). Therefore, we propose that the nsp2 species are likely processed in *cis* instead of *trans* and that this processing occurs cotranslationally and rapidly, in a manner similar to that of nsp1 (8). This hypothesis also makes sense for explaining the processing of nsp2a and nsp2b, the cleavage sites of which are predicted to be located on opposite sides (cytoplasmic versus lumen) of intracellular membranes. In addition, the well-established ratio balance among nsp2 species indicates that the processing of nsp2 is highly regulated and finely tuned in such a way that additional cleavage sites may not be accessible to the PL2 protease once they are processed.

Potential role of HSPA5. HSPA5 (also known as GRP78, or BiP) is a member of the heat shock 70-kDa protein family and is constitutively expressed in the lumen of the endoplasmic reticulum (ER) (6). This cellular protein is known to be associated with a variety of folding and assembly intermediates of cellular or viral membrane proteins (4, 5, 9, 24). In this report, we unexpectedly found that HSPA5 strongly coimmunoprecipitated with the nsp2 protein (Fig. 5 and 6). Although the details of the interaction need to be further analyzed, the observation has several important implications, as follows. (i) HSPA5 may play an important part in assisting nsp2 folding for proteolysis by either PL2 or host cell proteases, or both. It has been reported that the proteolytic activity of bovine viral diarrhea virus (BVDV) NS2 protein depends on a cellular cofactor, namely, a chaperone protein termed Jiv (J-domain protein interacting with viral protein) or its 90-aa fragment Jiv90 (20). It is possible that the interaction of HSPA5 with nsp2 could change the conformation of nsp2 and expose additional cleavage sites to PL2 protease activity. (ii) HSPA5 may be involved in the regulation of viral replication. The production of viral progeny depends on the successful recruitment of host cellular components for viral replication, protein synthesis, and virion assembly. During viral replication, a massive amount of proteins is synthesized in a relatively short time, and protein folding can thus become a limiting step (24). The assembly of viral replication complexes on intracellular membranes involves large numbers of viral and/or host proteins. The recruitment of HSPA5 to viral replication complexes may facilitate the folding of the PRRSV macromolecular replication complex. (iii) HSPA5 may be involved in immune evasion. Sequestration of molecular chaperones may lead to unfolded protein responses (UPS) or ER stress (16), leaving many host proteins incorrectly folded. The unfolded proteins, including those involved in host antiviral responses, are sent to the ubiquitin-proteosomal system (UPS) for degradation (16–18). In addition, ER stress also causes attenuation of host translation (16–18). Future work may be directed toward dissecting the detailed interaction between PRRSV nsp2 and HSPA5 and toward defining the role of HSPA5 in the PRRSV replication cycle.

In summary, we report the presence of nsp2 isoforms with apparently different C termini in PRRSV-infected MARC-145 cells and the interaction of nsp2 with the host chaperone HSPA5. These findings indicate that PRRSV nsp2 is increasingly emerging as a multifunctional protein and may have important roles in viral replication and pathogenesis.

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Evolution of the CD163 family and its relationship to the bovine gamma delta T cell co-receptor WC1

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Abstract

Background: The scavenger receptor cysteine rich (SRCR) domain is an ancient and conserved protein domain. CD163 and WC1 molecules are classed together as group B SRCR superfamily members, along with Spα, CD5 and CD6, all of which are expressed by immune system cells. There are three known types of CD163 molecules in mammals, CD163A (M130, coded for by *CD163*), CD163b (M160, coded for by *CD163L1*) and CD163c-α (CD163L1 or SCART), while their nearest relative, WC1, is encoded by a multigene family so far identified in the artiodactyl species of cattle, sheep, and pigs.

Results: We annotated the bovine genome and identified genes coding for bovine CD163A and CD163c-α but found no evidence for CD163b. Bovine CD163A is widely expressed in immune cells, whereas CD163c-α transcripts are enriched in the WC1+ γδ T cell population. Phylogenetic analyses of the CD163 family genes and WC1 showed that CD163c-α is most closely related to WC1 and that chicken and platypus have WC1 orthologous genes, previously classified as among their CD163 genes.

Conclusion: Since it has been shown that WC1 plays an important role in the regulation of γδ T cell responses in cattle, which, like chickens, have a high percentage of γδ T cells in their peripheral blood, CD163c-α may play a similar role, especially in species lacking WC1 genes. Our results suggest that gene duplications resulted in the expansion of CD163c-α-like and WC1-like molecules. This expanded repertoire was retained by species known as "γδ T cell high", but homologous SRCR molecules were maintained by all mammals.

Background

The CD163 family includes genes encoding CD163A (also known as M130, HbSR, and coded for by *CD163*), CD163b (also known as M160, and coded for by *CD163L1*) and CD163c-α (also known as CD163L1 and SCART). This family is a subset of the scavenger receptor cysteine-rich (SRCR) super-family, an ancient super-family defined by the presence of 100-110 amino acid domains [1]. Members of the CD163 family are group B SRCR proteins distinguished by the 6-8 cysteines in their SRCR domains resulting in 3-4 disulfide bonds; in comparison, group A SRCR proteins have only 6 cysteines and 3 disulfide bonds in their SRCR domains. SRCR domains, like Ig domains or epidermal growth factor-like domains, are thought to be involved in protein-protein interactions, although the known ligands of these domains vary widely.

CD163A is a receptor for haptoglobin-hemoglobin complexes, and is inducibly expressed on monocytes, macrophages and a subpopulation of hematopoietic progenitors [2-6]. CD163A protects against oxidative damage by mediating the endocytosis of haptoglobin-hemoglobin complexes [7]. Ligation of CD163A by haptoglobin-hemoglobin complexes induces the secretion of the anti-inflammatory cytokine IL-10 [8]. In addition, proteolytically-cleaved CD163A shed into serum inhibits phorbol ester-induced T cell proliferation [9]. The third SRCR domain of CD163A mediates its interaction with haptoglobin and TWEAK (TNF-like weak inducer of apoptosis) [10,11]. CD163A interacts with a molecule expressed on erythroblasts and with bacteria via its second SRCR domain [12,13]. The related group B SRCR molecule CD6 also binds to bacteria via one or more of its SRCR domains, through interactions with the bacterial

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non-peptiditic products lipoteichoic acid (LTA) and lipopolysaccharide (LPS) [14].

CD163A transcripts display alternative splicing of the extracellular and cytoplasmic coding regions, potentially increasing the diversity of its function [15,16]. The CD163A form with a short cytoplasmic domain predominates and mediates ligand internalization and degradation but the function of the CD163A form with a long cytoplasmic domain is unknown [17]. Cross-linking of CD163A induces inositol triphosphate and cytokine production [18]. The CD163A cytoplasmic domain is phosphorylated by casein kinase II and protein kinase C-α (PKC-α) and these phosphorylation events are tied to cytokine production induced by CD163A cross-linking [19]. Casein kinase II activity and anti-inflammatory cytokine production in macrophages is stimulated by CD163A binding to only one of the two alleles of haptoglobin, although both bind to CD163A with high affinity [20]. The non-stimulatory haptoglobin allele is correlated with increased susceptibility to cardiovascular disease [21,22].

CD163b is also expressed on macrophages, with two cytoplasmic domain variants of 71 and 39 amino acids, resulting from alternative splicing [23]. Little is known about the function or ligand of CD163b. Thus far, the gene encoding CD163b has only been found in the genomes of primates and the horse.

Unlike CD163A, which is encoded by one gene across eutherian mammals, several species possess multiple genes encoding CD163c-α molecules. There are two CD163c-α genes in mice: SCART1 and SCART2. SCART1 is expressed in the lymph node, trachea and lung; SCART2 is expressed on murine γδ T cells that secrete IL-17 [24,25]. The presence of multiple genes encoding the SRCR transmembrane receptor CD163c-α is similar to that of another set of group B SRCR proteins, WC1. Genes encoding WC1 have been found in the artiodactyl species cattle (*Bos taurus*), sheep (*Ovis aries*) and swine (*Sus scrofa*) [26-30]. WC1 molecules are encoded by a family of fifteen genes in the bovine and, like SCART2, are expressed on γδ T cells [26,27,30-34]. WC1 contributes to the γδ T cell response to *Leptospira* (Wang F, Herzig CTA, Hsu H, Chen C, Baldwin CL, Telfer JC: Scavenger receptor WC1 contributes to the gamma delta T cell responses to *Leptospira*, submitted) and WC1-mediated potentiation of T cell activation requires the phosphorylation of a tyrosine in its cytoplasmic domain [35]. Moreover, expression of different molecular forms of WC1 on bovine γδ T cells is correlated with differential response to bacteria, suggesting that WC1 functions as a pattern recognition molecule similar to the related SRCR molecules CD163A, CD5, CD6, Spα and DMBT1 [13,14,36-40]. No WC1 homologues have thus far been identified in human or murine γδ T cells, leading to the question of whether CD163 family members, particularly CD163c-α molecules, have evolved to serve functions equivalent to WC1 in mammals other than the artiodactyls.

In our recent annotation of the bovine genome we found the gene encoding CD163A embedded within the region coding for WC1 genes [31]. To determine the extent of the CD163 family in artiodactyls, we annotated the bovine genome to identify CD163 family genes in cattle. We found genes encoding both CD163A and CD163c-α, but not CD163b, and evaluated their expression profile in γδ T cells and other tissues. Incorrect assignment of genes belonging to the CD163 and the WC1 families from many species has created substantial confusion in naming and categorizing these genes. To appropriately categorize the genes identified both by us and by others, we undertook phylogenetic analyses of CD163 and WC1 family members. Here, we show the relationship between CD163A, CD163c-α and WC1 family receptors, all expressed in γδ T cells of artiodactyls, that WC1 orthologues are present in the chicken and platypus, and correlate the conservation over evolutionary time of a diverse array of these receptors with the presence of a high level of γδ T cells in the peripheral blood.

Results

Exon-intron structure of bovine CD163 family members

Annotation of the bovine genome indicated that cattle have a gene coding for CD163A, which was found on chromosome 5 within one of the two loci coding for the large WC1 family of genes [31]. A gene coding for bovine CD163c-α was also found but could not be placed in the bovine genome due to insufficient scaffolding. Schematics of the exon-intron structure of these two genes are shown in Fig. 1. Both CD163A and CD163c-α contain a cytoplasmic domain coded for by two exons, one of which also encodes the transmembrane domain. Interestingly, like WC1, both CD163A and CD163c-α contain an exon that encodes interdomain sequence (Fig. 1, ID) [31]. The gene encoding CD163A spans approximately 30 kbp; that of CD163c-α spans approximately 12 kbp.

The CD163c-α amino acid sequence generated by automated prediction lacked a transmembrane region that was correlated with a gap in the genomic sequence at the expected location of the exon encoding the transmembrane domain (Fig. 1B). We investigated whether bovine CD163c-α has a transmembrane domain by amplifying and sequencing cDNA template from peripheral blood mononuclear cells using primers designed from known sequences in the genomic sequence, which bracketed the putative transmembrane region. We obtained transmembrane domain sequence in the same frame as known sequence upstream and downstream (Fig. 2B), indicating

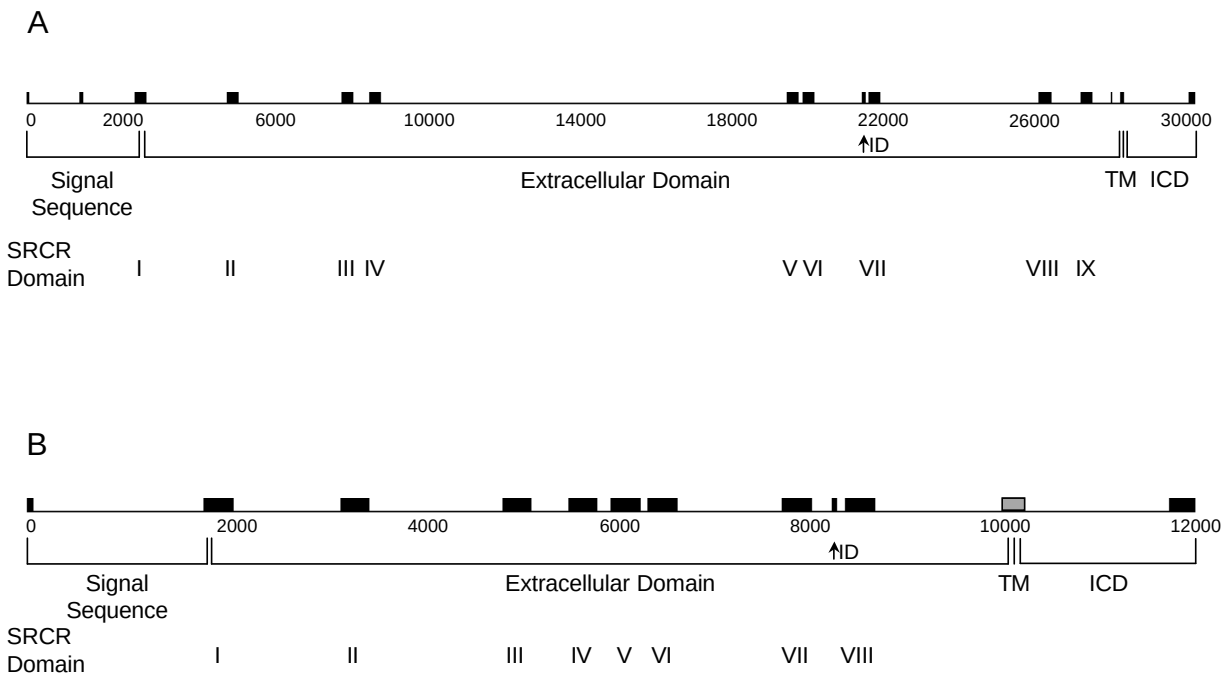

Figure 1 Schematic representation of bovine CD163 exon-intron structure. Exon-intron structures of (A) CD163A and (B) CD163c-α were determined based on annotation of the CD163 genes in the bovine genome assembly Btau_3.1. Proposed placement of the exon encoding CD163c-α transmembrane region is shown in grey and could not be confirmed due to a gap in the genomic sequence assembly. SRCR domain numbers are indicated by roman numerals. Scale is shown in base pair increments beneath the schematic. Abbreviations are as follows: ID, interdomain sequence; TM, transmembrane region; ICD, intracytoplasmic domain.

that the bovine CD163c-α is a transmembrane receptor. The bovine homologue of the human gene encoding CD163b was not found in the bovine genome assembly Btau 3.1, which could reflect its absence or a relatively large gap in the sequenced bovine genome.

Bovine CD163 sequences

Based on the manual annotation of the bovine CD163c-α and CD163A gene sequences (Fig. 1), we designed primers to amplify CD163A and CD163c-α transcripts. Deduced amino acid sequences based on the obtained cDNA sequence of CD163A (Fig. 2A) and on both the annotated genomic sequence and obtained cDNA sequence (shaded) of CD163c-α (Fig. 2B) were used to evaluate their predicted protein structures. Bovine CD163A has nine SRCR domains and bovine CD163c-α has eight SRCR domains in their extracellular regions, which are underlined in the predicted protein (Fig. 2). Most of the SRCR domains conform to the eight-cysteine consensus of SRCR group B. The exceptions are the eighth SRCR domain of bovine CD163A and the fifth SRCR domain of bovine CD163c-α. These SRCR domains lack the second and seventh cysteines, which form a disulfide bond in most, but not all, other SRCR group B domains. Estimates of the evolutionary divergence between SRCR domain amino acid sequences confirm that bovine CD163A is most similar to human CD163A (Table 1) and that bovine CD163c-α is most similar to human CD163c-α (Table 2) in both the order of SRCR domains and sequence identity. Bovine CD163A is identical to human CD163A in its SRCR domain organization, and highly similar in its sequence with 82% overall sequence identity. Bovine CD163c-α is identical to human CD163c-α in its SRCR domain organization and 68% identical in overall sequence. The evolutionary divergence between bovine CD163A or bovine CD163c-α SRCR domains and bovine WC1 SRCR domains is greater (Table 3), indicating that the genes we have identified as bovine CD163A or bovine CD163c-α are not WC1 genes. The sequences of the cytoplasmic domains of CD163c-α and CD163A from cattle do not exhibit significant sequence identity to each other (data not shown), although they contain the tyrosine-based motifs of YEDI and YREM respectively.

Expression profiles of bovine CD163A and CD163c-α

A variety of tissues were examined for expression of bovine CD163 transcripts including mesenteric lymph node (MLN), lung, intestinal intraepithelial lymphocytes (IELs, containing approximately 20% γδ T cells, data not shown) and peripheral blood mononuclear cells (PBMC). PBMC were evaluated both as *ex vivo* cells and following

A

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                                                                                  SRCR I
MVLHDNSGSAGFKRCSVHFGPFTLAVVSVLYACLVTSALGGTDKELRLVAGQTKCSGRVEVKVQEEWGTV   70
CNTGWDLAAVSVVCKQLGCPSVIKATGWTNSSAGTGRIWMDHVSCRGNESALWDCKHEGWGKHNCTHQQD  140
                SRCR II
VGVTCSDGSDLEMRLMNGGNRCSGRIEIKFQGQWGTVCDDNFNLDHASVVCKQLGCGSAVSFSGSANFGE  210
                                                                SRCR III
GSGPIWFDDLVCHGNESALWNCRHEGWGKHNCDHAEDAGVICLEGADLSLRLVDGVTKCSGRLEVRFQGE  280
WGTVCDDGWDSHDAAVACQQLGCPTAITAVGRVNASEGTGHIWLDSVSCQGHESAVWQCRHHEWGKHYCN  350
                SRCR IV
HNEDAGVTCSDGSDLELRLKGGGSRCAGTVEVEIQKLIGKVCDRSWGLKEADVVCKQLGCGSALRTSYQV  420
                                                                SRCR V
YSKIQATNTWLFLNNCNGNETSIWDCKNWQWGGLSCEHYHEAKVTCSAHREPRLVGGDIPCSGRVEVKHG  490
DTWGTICDSDFSLESASVLCRELECGSVVSILGGAHFGEGNGQIWAEEFQCEGNESHLSLCPVAPRLDGT  560
                SRCR VI
CSHSKDIGVVCSRYTEVRLVGGNTPCEGRVEVKILGTWGPLCNSHWDMEDAHVLCQQLKCGVAASIPGRA  630
PFGKGSGQPWRHMFHCTGTEQHMGDCPVTALGASLCPEGQVASVICSGNRSQTLYPCNSSSSDPESSVVL  700
                SRCR VII
EENGVPCIGSGQLRLVNGGGRCAGRIEVYHEGSWGTICDDSWDLDDAHVVCRQLGCGVAINATGSAHFGE  770
                                                        SRCR VIII
GSGPIWLDEVNCNGKEPRISQCRSHGWGRQNCRHKEDAGVICSEFMSLRLISDSSSETCAGRLEVFYNGA  840
WGSVGKSDMSATTVGVVCRQLGCTDKGSIRPAPSDKVENRYMWVDNVRCPKGPETLWQCPSSPWKRRLAS  910
                SRCR IX
PSEETWITCADKIRLQEGTTNCSGRVEVWHGGSWGTVCDDSWDLNDAQVVCRQLGCGLALEAGKEAAFGQ  980
                                                                   TM
GTGPIWLNEVKCKGNESSLWDCPARSWGHSDCGHKEDASVKCSEIAESKGSVKAAGHSSTVALGILGVIL 1050
LAFLIATLLWIQRRRQRQRLAVSSRGENSVHEIQYREMNSCLKADDLDLYNSSGLWVLRGSIALGFRLVT 1120
AAEAERHST*  1130
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B

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                                                  SRCR I
MRLLLCLLGLGPLVLILQAAPPRAAPEEQLTIFLHISRCNGILLLRHQGQLGTACTDTWGFPEAAVVCRE   70
                                                                  SRCR II
LDCGAPLGAPKEVPGPEIMAQPWLHGLTCQGNESSIQECALGAWGPRPCPHDWVPAVMCMGGLEVVIKLV  140
GGRSPCAGVPGLQHSNQTLTSCDPQSLEAGTILCKELGCGSMLQAPSPLPETEGPRKGQQFVGCEGSEPT  210
                                SRCR III
VLNCKIDWANFKPCVSNDEVICSGHTEARLVGGEHSCAGRLEVRRGLTWGTVCDADLDLATAHVVCRELQ  280
                                                                SRCR IV
CGAAVSTPQGAHFGQGPGLVWAEAFRCAGNESLLFHCPREPGHRCGHGQDAGLRCSEFRLVNGSSACEGR  350
VELQVQGAWAPLCAAHWDLADATVLCHQLDCGNAVATPPGGHFGGGASAPWPDEVHCVGTEPYLWSCAVS  420
                                SRCR V
TLGAPACGPGDAAAAVCSGLPDALRLRDGQSRCDGRVEVSLDGVWGRVLDEAWDLRGAAVVCRQLGCGAA  490
                                                                SRCR VI
ERAYEAAAPARGAVPLGLSRVRCAGTEPRLTRCNVSAAALVSAGASRDAGVVCSGSLQVRLAAGPGRCAG  560
RVELLHAGEWGTVCDDGWDLRDAQVVCRQLGCGHALGAPGAAHFGAGAGRIWMDELACEGHEAALWRCPS  630
                                SRCR VII
RGWGRHDCGHKEDAGALCSESVALRLRGGAGPCAGWLDVFHNGTWGAVCSNALKDASLSIICQQLGCGER  700
GWLENRPGHTSLGTSWVDNIQCRRLRSSTLWQCPSAPWHPHSCTRGEEVWITCAGSSGTATQDSGEALNC  770
                SRCR VIII
SLMGSCPEEGELRVRGGEDRCSGRVELWHAGSWGTVCDDSWDLADAEVVCRQLGCGRAVDAVAGAAFGPG  840
                                                                   TM
SGPVWLDEVGCRGSEASLWGCPAQPWGRGDCGHKEDAGVRCAGDTGAVTPRSASGSPLVPAPALEAGTLP  910
MALGLALGTLLLVTSLVLGARWFRGRDACRGSGMSGSLPSEGVYEDIGVATAGEKDEAAGAAAAAVLEEE  980
YDDVAEPEPEEGDAEEGALLSPTGAQLCVVASTVLLLLYWCYAQGSGPPSPYI*  1034
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Figure 2 Amino acid sequences of bovine CD163. Translated amino acid sequence, based on cDNA sequences for (A) CD163A and based on annotated and determined cDNA (shaded) sequences for (B) CD163c-α. Individual SRCR domains, identified by comparison to consensus group B SRCR domain sequence, are underlined, domain numbers are indicated by roman numerals, and cysteines are boxed. Putative transmembrane regions (TM) were determined by the DAS transmembrane prediction server and are also underlined [70].

Table 1: Estimates of evolutionary divergence between human CD163A, bovine CD163A and bovine CD163c-α SRCR domains

			HsCD163A								
	SRCR domain		**h**	**i**	**j**	**k**	**b**	**c**	**d**	**e**	**d'**
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
BtCD163A	**h**	**1**	**0.179**	**0.462**	**0.410**	0.538	0.603	0.641	**0.474**	0.679	0.513
			(0.043)	(0.056)	(0.056)	(0.056)	(0.055)	(0.057)	(0.057)	(0.053)	(0.057)
	i	**2**	**0.436**	**0.167**	0.513	0.551	0.564	0.628	**0.436**	0.692	0.500
			(0.056)	(0.042)	(0.057)	(0.056)	(0.056)	(0.056)	(0.056)	(0.053)	(0.057)
	j	**3**	**0.372**	0.500	**0.115**	0.590	0.628	0.615	**0.449**	0.705	0.500
			(0.055)	(0.057)	(0.036)	(0.056)	(0.055)	(0.056)	(0.056)	(0.052)	(0.057)
	k	**4**	0.590	0.590	0.590	**0.115**	0.692	0.654	0.526	0.731	0.551
			(0.056)	(0.056)	(0.056)	(0.036)	(0.052)	(0.056)	(0.057)	(0.053)	(0.056)
	b	**5**	0.577	0.513	0.628	0.641	**0.090**	0.538	0.564	0.731	0.603
			(0.056)	(0.057)	(0.055)	(0.054)	(0.032)	(0.056)	(0.056)	(0.050)	(0.055)
	c	**6**	0.590	0.628	0.615	0.679	0.526	**0.167**	0.577	0.782	0.577
			(0.056)	(0.057)	(0.056)	(0.053)	(0.057)	(0.042)	(0.056)	(0.047)	(0.056)
	d	**7**	0.526	**0.474**	**0.474**	0.551	0.577	0.551	**0.090**	0.667	**0.474**
			(0.057)	(0.057)	(0.057)	(0.056)	(0.056)	(0.056)	(0.032)	(0.052)	(0.057)
	e	**8**	0.718	0.744	0.744	0.692	0.692	0.705	0.705	**0.231**	0.744
			(0.052)	(0.050)	(0.052)	(0.052)	(0.052)	(0.053)	(0.053)	(0.048)	(0.052)
	d'	**9**	0.551	0.538	0.551	0.526	0.603	0.538	**0.423**	0.692	**0.167**
			(0.056)	(0.056)	(0.056)	(0.057)	(0.055)	(0.056)	(0.056)	(0.052)	(0.042)
BtCD163c-α	**m**	**1**	0.705	0.718	0.679	0.615	0.718	0.692	0.641	0.808	0.615
			(0.052)	(0.051)	(0.053)	(0.055)	(0.051)	(0.052)	(0.054)	(0.045)	(0.055)
	l	**2**	0.667	0.718	0.679	0.692	0.654	0.667	0.692	0.821	0.718
			(0.053)	(0.052)	(0.052)	(0.052)	(0.054)	(0.053)	(0.050)	(0.043)	(0.051)
	b	**3**	0.577	0.564	0.641	0.590	**0.449**	0.577	0.538	0.731	0.551
			(0.056)	(0.056)	(0.054)	(0.056)	(0.056)	(0.056)	(0.056)	(0.050)	(0.056)
	c	**4**	0.667	0.654	0.628	0.628	0.615	0.526	0.590	0.731	0.615
			(0.053)	(0.054)	(0.055)	(0.055)	(0.055)	(0.057)0	(0.056)	(0.050)	(0.055)
	n	**5**	0.654	0.679	0.628	0.590	0.654	0.667	0.564	0.705	0.603
			(0.054)	(0.053)	(0.055)	(0.056)	(0.054)	(0.053)	(0.056)	(0.052)	(0.055)
	d	**6**	**0.474**	0.526	0.500	0.526	0.577	0.590	**0.397**	0.667	**0.436**
			(0.057)	(0.057)	(0.057)	(0.057)	(0.056)	(0.056)	(0.055)	(0.053)	(0.056)
	e	**7**	0.628	0.667	0.679	0.654	0.679	0.744	0.641	0.603	0.679
			(0.055)	(0.052)	(0.054)	(0.055)	(0.053)	(0.049)	(0.055)	(0.055)	(0.053)
	d'	**8**	0.538	0.526	0.538	0.538	0.564	0.628	**0.423**	0.654	**0.397**
			(0.056)	(0.057)	(0.056)	(0.056)	(0.056)	(0.055)	(0.056)	(0.054)	(0.055)

The number of amino acid differences per site from analysis between sequences is shown. All results are based on the pairwise analysis of 26 sequences. Standard error estimates are shown in parentheses and were obtained by using analytical formulas. Analyses were conducted using the amino p-distance model in MEGA4 [64]. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 78 positions in the final dataset. Estimates below 0.500 are in boldtype, and values on the diagonal corresponding to a high level of identity between sequences are underlined. SRCR domains are labelled with alphabetical designations as previously defined and by inferring the evolutionary relationships between SRCR domains using the Neighbor-Joining and Bayesian methods [1,65,69].

Table 2: Estimates of evolutionary divergence between human CD163c-α, bovine CD163A and bovine CD163c-α SRCR domains

			HsCD163c-α							
	SRCR domain		**m**	**l**	**b**	**c**	**n**	**d**	**e**	**d'**
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
BtCD163A	**h**	**1**	0.577	0.692	0.590	0.641	0.641	0.500	0.628	0.513
			(0.056)	(0.052)	(0.056)	(0.054)	(0.054)	(0.057)	(0.055)	(0.057)
	i	**2**	0.590	0.731	0.551	0.628	0.667	**0.462**	0.641	**0.474**
			(0.056)	(0.050)	(0.056)	(0.055)	(0.053)	(0.056)	(0.054)	(0.057)
	j	**3**	0.615	0.679	0.654	0.628	0.641	**0.474**	0.628	0.526
			(0.055)	(0.053)	(0.054)	(0.055)	(0.054)	(0.057)	(0.055)	(0.057)
	k	**4**	0.551	0.654	0.615	0.692	0.628	0.577	0.679	0.590
			(0.056)	(0.054)	(0.055)	(0.052)	(0.055)	(0.056)	(0.053)	(0.056)
	b	**5**	0.654	0.667	**0.462**	0.590	0.679	0.538	0.692	0.564
			(0.054)	(0.053)	(0.056)	(0.056)	(0.053)	(0.056)	(0.052)	(0.056)
	c	**6**	0.654	0.654	0.551	0.500	0.667	0.590	0.731	0.615
			(0.054)	(0.054)	(0.056)	(0.057)	(0.053)	(0.056)	(0.050)	(0.055)
	d	**7**	0.615	0.615	0.551	0.590	0.577	**0.372**	0.679	**0.436**
			(0.055)	(0.055)	(0.056)	(0.056)	(0.056)	(0.055)	(0.053)	(0.056)
	e	**8**	0.718	0.795	0.744	0.756	0.679	0.654	0.577	0.692
			(0.051)	(0.046)	(0.049)	(0.049)	(0.053)	(0.054)	(0.056)	(0.052)
	d'	**9**	0.513	0.679	0.526	0.590	0.590	**0.436**	0.667	**0.410**
			(0.057)	(0.053)	(0.057)	(0.056)	(0.056)	(0.056)	(0.053)	(0.056)
BtCD163c-α	**m**	**1**	**0.462**	0.641	0.667	0.718	0.692	0.654	0.808	0.667
			(0.056)	(0.054)	(0.053)	(0.051)	(0.052)	(0.054)	(0.045)	(0.053)
	l	**2**	0.705	0.577	0.692	0.705	0.744	0.731	0.756	0.744
			(0.052)	(0.056)	(0.052)	(0.052)	(0.049)	(0.050)	(0.049)	(0.049)
	b	**3**	0.603	0.692	**0.167**	0.577	0.679	0.526	0.667	0.526
			(0.055)	(0.052)	(0.042)	(0.056)	(0.053)	(0.057)	(0.053)	(0.057)
	c	**4**	0.603	0.667	0.590	**0.218**	0.641	0.615	0.756	0.615
			(0.055)	(0.053)	(0.056)	(0.047)	(0.054)	(0.055)	(0.049)	(0.055)
	n	**5**	0.615	0.641	0.603	0.654	**0.231**	0.538	0.731	0.538
			(0.055)	(0.054)	(0.055)	(0.054)	(0.048)	(0.056)	(0.050)	(0.056)
	d	**6**	0.538	0.667	0.538	0.551	0.590	**0.218**	0.654	**0.410**
			(0.056)	(0.053)	(0.056)	(0.056)	(0.056)	(0.047)	(0.054)	(0.056)
	e	**7**	0.692	0.731	0.641	0.718	0.744	0.615	**0.179**	0.654
			(0.052)	(0.050)	(0.054)	(0.051)	(0.049)	(0.055)	(0.043)	(0.054)
	d'	**8**	0.551	0.692	**0.487**	0.577	0.564	**0.423**	0.628	**0.141**
			(0.056)	(0.052)	(0.057)	(0.056)	(0.056)	(0.056)	(0.055)	(0.039)

Analyses were conducted as described in Table 1.

Table 3: Estimates of evolutionary divergence between bovine archetypal WC1.1, bovine CD163A and bovine CD163c-α SRCR domains

			BtWC1.1					
	SRCR domain		**a**	**b**	**c**	**d**	**e**	**d'**
			1	**2, 7**	**3, 8**	**4, 6, 9**	**5, 10**	**11**
BtCD163A	**h**	**1**	0.551 (0.056)	0.667, 0.654 (0.053, 0.054)	0.641, 0.654 (0.054, 0.054)	**0.462, 0.462,** 0.500 (0.056, 0.056, 0.057)	0.705, 0.692 (0.052, 0.052)	0.500 (0.057)
	i	**2**	0.526 (0.057)	0.577, 0.564 (0.056, 0.056)	0.590, 0.615 (0.056, 0.055)	**0.436, 0.410, 0.436** (0.056, 0.056, 0.056)	0.705, 0.667 (0.052, 0.053)	**0.487** (0.057)
	j	**3**	0.500 (0.057)	0.679, 0.654 (0.053, 0.054)	0.654, 0.679 (0.054, 0.053)	**0.449, 0.449, 0.449** (0.056, 0.056, 0.056)	0.705, 0.705 (0.052, 0.052)	0.500 (0.057)
	k	**4**	0.564 (0.056)	0.654, 0.641 (0.054, 0.054)	0.615, 0.615 (0.055, 0.055)	0.564, 0.551, 0.577 (0.056, 0.056, 0.056)	0.705, 0.692 (0.052, 0.052)	0.500 (0.057)
	b	**5**	0.551 (0.056)	**0.449, 0.436** (0.056, 0.056)	0.577, 0.603 (0.056, 0.055)	0.526, 0.590, 0.513 (0.057, 0.056, 0.054)	0.718, 0.692 (0.051, 0.052)	0.538 (0.056)
	c	**6**	0.590 (0.056)	0.615, 0.590 (0.055, 0.056)	0.513, 0.538 (0.057, 0.056)	0.513, 0.590, 0.526 (0.057, 0.056, 0.057)	0.769, 0.731 (0.048, 0.050)	0.603 (0.055)
	d	**7**	**0.487** (0.057)	0.641, 0.628 (0.054, 0.055)	0.590, 0.603 (0.056, 0.055)	**0.218, 0.321, 0.218** (0.047, 0.053, 0.047)	0.667, 0.641 (0.053, 0.054)	**0.474** (0.057)
	e	**8**	0.744 (0.049)	0.731, 0.705 (0.050, 0.052)	0.731, 0.731 (0.050, 0.050)	0.718, 0.744, 0.692 (0.051, 0.049, 0.052)	0.513, 0.500 (0.057, 0.057)	0.692 (0.052)
	d'	**9**	0.526 (0.057)	0.615, 0.590 (0.055, 0.056)	0.590, 0.590 (0.056, 0.056)	**0.423, 0.436, 0.397** (0.056, 0.056, 0.055)	0.679, 0.667 (0.053, 0.053)	**0.359** (0.054)
BtCD163c-α	**m**	**1**	0.654 (0.054)	0.756, 0.731 (0.049. 0.050)	0.679, 0.679 (0.053, 0.053)	0.654, 0.667, 0.654 (0.054, 0.053, 0.054)	0.795, 0.769 (0.046, 0.048)	0.628 (0.055)
	l	**2**	0.718 (0.051)	0.692, 0.705 (0.052, 0.052)	0.705, 0.718 (0.052, 0.051)	0.654, 0.654, 0.667 (0.054, 0.054, 0.053)	0.769, 0.795 (0.048, 0.046)	0.679 (0.053)
	b	**3**	0.551 (0.056)	0.538, 0.526 (0.056, 0.057)	0.577, 0.577 (0.056, 0.056)	0.513, 0.577, 0.526 (0.057, 0.056, 0.057)	0.718, 0.692 (0.051, 0.052)	0.526 (0.057)
	c	**4**	0.603 (0.055)	0.628, 0.615 (0.055, 0.055)	0.526, 0.538 (0.057, 0.056)	0.603, 0.615, 0.603 (0.055, 0.055, 0.055)	0.744, 0.744 (0.049, 0.049)	0.615 (0.055)
	n	**5**	0.513 (0.057)	0.679, 0.667 (0.053, 0.053)	0.654, 0.654 (0.054, 0.054)	0.564, 0.590, 0.564 (0.056, 0.056, 0.056)	0.744, 0.756 (0.049, 0.049)	0.564 (0.056)

Table 3: Estimates of evolutionary divergence between bovine archetypal WC1.1, bovine CD163A and bovine CD163c-α SRCR domains (Continued)

	d	**6**	0.500 (0.057)	0.577, 0.564 (0.056, 0.056)	0.577, 0.590 (0.056, 0.056)	**0.346, 0.423, 0.333** (0.054, 0.056, 0.053)	0.679, 0.667 (0.053, 0.053)	**0.487** (0.057)
	e	**7**	0.705 (0.052)	0.667, 0.679 (0.056, 0.056)	0.692, 0.718 (0.052, 0.051)	0.628, 0.641, 0.628 (0.055, 0.054, 0.055)	0.526, 0.526 (0.057, 0.057)	0.615 (0.055)
	d'	**8**	0.526 (0.057)	0.577, 0.577 (0.053, 0.053)	0.615, 0.615 (0.055, 0.055)	**0.436, 0.423, 0.397** (0.056, 0.056, 0.055)	0.615, 0.615 (0.055, 0.055)	**0.321** (0.053)

Analyses were conducted as described in Table 1.

activation by Con A. Based on expressed sequence tag (EST) analysis in the NCBI UniGene database http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene, it was predicted that bovine CD163c-α is expressed in the intestine and that bovine CD163A is expressed in the intestine and mesenteric lymph node (MLN). Interestingly, we found CD163A to be expressed in all tissues evaluated (Fig. 3A) and not just in intestine and MLN. Surprisingly, we found that CD163A is also expressed on WC1$^+$ and γδ TCR$^+$ as well as WC1$^-$ and γδ TCR$^-$ cells sorted from *ex vivo* PBMC (Fig. 3B) indicating that the expression of bovine CD163A is not restricted to monocytes and macrophages, as is the case in humans [5,41].

We found that CD163c-α transcripts were relatively higher in PBMC, MLN and IELs, than in lung (Fig. 3A), consistent with expression in leukocytes. The CD163c-α family member SCART2 has been shown to be expressed in murine γδ T cells [25]. To test the hypothesis that bovine CD163c-α is expressed on a T cell subset separate from the WC1$^+$ γδ T cell subset, we evaluated the expression of bovine CD163c-α on WC1$^+$, WC1$^-$, γδ TCR$^+$ and γδ TCR$^-$ cells sorted from *ex vivo* PBMC (Fig. 3B). We found that CD163c-α was preferentially expressed in the

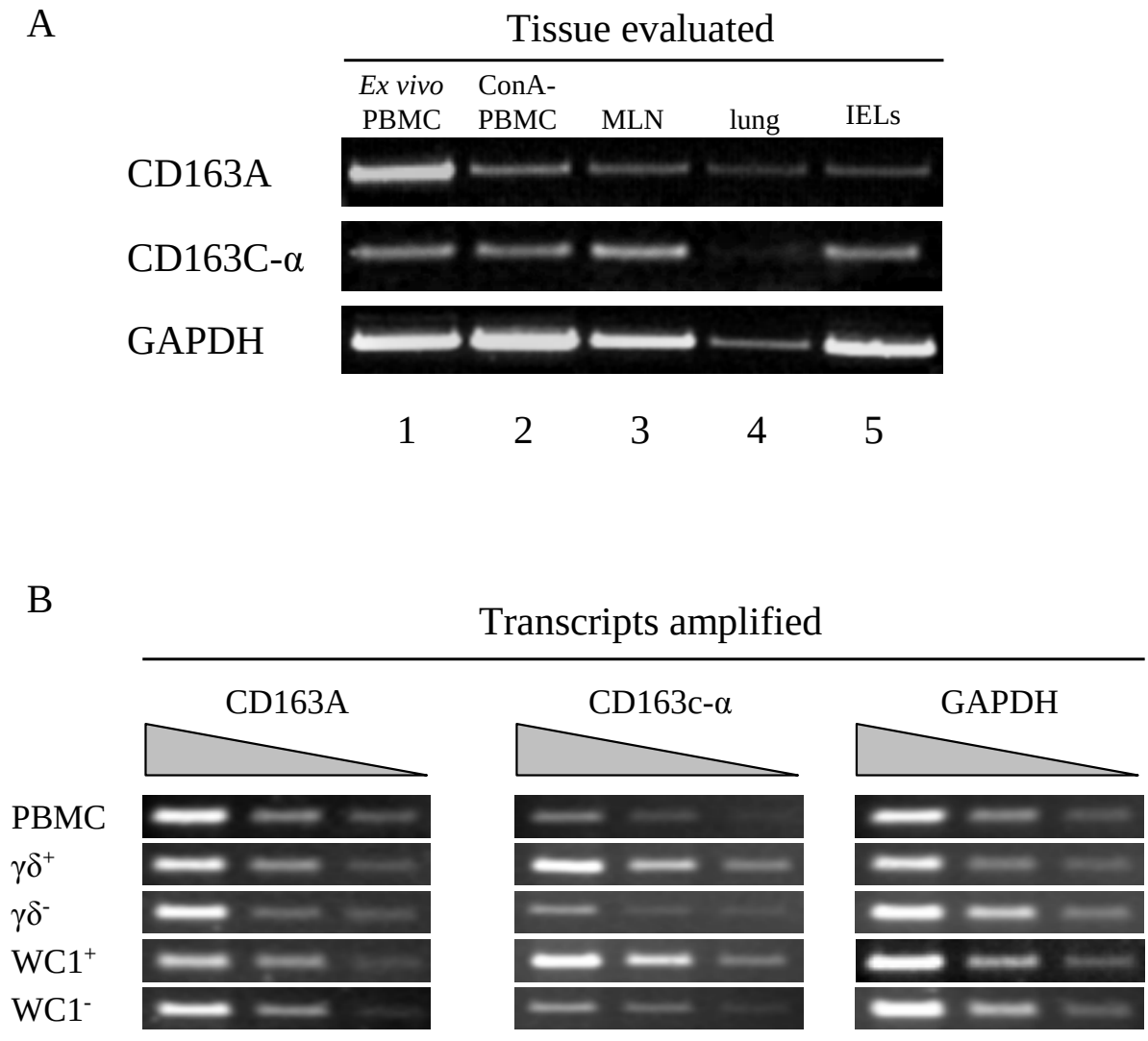


Figure 3 Expression profiles of bovine CD163 genes. (A) Bovine *ex vivo* PBMC (lane 1), ConA-activated PBMC (lane 2), mesenteric lymph node (MLN, lane 3), lung (lane 4) and intestinal epithelial lymphocytes (IELs, lane 5) were evaluated for expression of CD163A and CD163c-α by RT-PCR. GAPDH is shown for comparison. These results are representative of reactions (n = 3) performed for at least two animals. (B) Bovine total PBMC, γδ TCR$^+$ cells, γδ TCR$^-$ cells, WC1$^+$ cells, and WC1$^-$ cells were evaluated for CD163A, CD163c-α, and GAPDH expression by RT-PCR. Templates were serially diluted by a factor of three. These results are representative of two reactions.

WC1+ or γδ TCR+ cell populations in PBMC, suggesting that it increases the potential receptor repertoire of WC1^{+}γδ T cells.

Analysis of CD163 and WC1 SRCR domains

We undertook a phylogenetic analysis of CD163 family members and their closest molecular relative, WC1, with the goal of understanding the evolution and function of WC1 and CD163 family members. We performed multiple alignments of individual SRCR domains in bovine CD163c-α and bovine CD163A with defined SRCR domains from bovine WC1 and human CD163A [1]. The SRCR domains from bovine WC1 and human CD163A have previously been assigned an alphabet letter designation "a", "b", "c", "d", "d' ", "e", "h", "i", "j", or "k" [1]. SRCR domains in bovine CD163c-α and bovine CD163A that clustered in the same clades as these previously designated SRCR domains (Fig. 4A, indicated by asterisks) were assigned the same designation. SRCR domains that clustered in separate clades from the clades containing already designated SRCR domains were assigned the new alphabet letter designations "m", "n" and "l" (Fig. 4A,4B). Phylograms obtained using Bayesian analysis (Fig. 4A) showed that the first SRCR domain of bovine CD163c-α (BtCD163cSRCR1) and the first SRCR domains of CD163c-α-like molecules from the duck-billed platypus (OraCD163_4SRCR1, OraCD163_5SRCR1 and OraCD163_7SRCR1) are in the same clade as the first SRCR domain of primate, rodent and canine CD163c-α (designated "m"). The clade containing the fifth SRCR domain (designated "n") in bovine CD163c-α also includes SRCR domains from CD163c-α-like molecules from eutherian mammals, the duck-billed platypus and the chicken (Fig. 4A). The second SRCR domain of bovine CD163c-α (BtCD163cSRCR1) clusters in the same clade as other SRCR domains designated "l" from eutherian mammals and the duck-billed platypus (Fig. 4A). SRCR domains that we have designated as "d" are clustered in sister clades to defined "d" SRCR domains by Bayesian analysis: however, their designation as domain "d" is also supported by their position in the protein in reference to other SRCR domains (Fig.4B). Several SRCR domains from chicken CD163 molecules clustered apart from the "d" SRCR domain; although, their identification as "d" SRCR domains is suggested by their position in the protein (e.g. GgCD163_1 SRCR4). These SRCR domains were left undesignated (Fig. 4A,4B). The first six SRCR domains of human CD163b do not reproducibly cluster in the same clade as other defined SRCR domains and were also left undesignated (Fig. 4A,4B). Notably, CD163b has thus far been found only in primates and horses. It is also of note that SRCR domain "k", which appears to be diagnostic of CD163A or CD163b, does not appear in any of the annotated genes from the duck-billed platypus or the chicken, suggesting that CD163A or CD163b is not encoded in these animals' genomes. Thus, there appear to be at least 4 types of CD163 family members, not all of which appear in every species, and some of which are duplicated within a species: CD163A, CD163b, CD163c-α, and WC1.

Most of the CD163 molecules, especially those with a transmembrane domain, contain the usually membrane proximal "d' " and "e" SRCR domains (Fig. 4B). These domains from chicken and duck-billed platypus cluster together within each species, whereas CD163A "d' " and "e" SRCR domains from eutherian mammals are found in separate sub-clades from CD163b, CD163c-α, and WC1 "d' " and "e" SRCR domains from eutherian mammals (Fig. 4A).

Relationship of CD163c-α and WC1

The relationship of CD163c-α to WC1 is particularly intriguing since WC1 expression is restricted to γδ T cells and CD163c-α expression is enriched in WC1+γδ T cells (Fig. 3B). When we performed a multiple SRCR domain alignment and phylogenetic tree analysis of CD163 proteins across multiple species, we found that the SRCR domain structure and organization of bovine CD163c-α (m-l-b-c-n-d-e-d') is most similar to CD163c-α from other eutherian mammalian species, such as human, chimpanzee, dog, and mouse and rat. The SRCR domains "m" and "l" and "n" were unique to CD163c-α molecules and were not found to occur in bovine or swine WC1, nor did they occur in the other CD163 family members CD163A or human CD163b (Fig. 4A,4B). Domains "m", "l", and "n" clustered in separate clades than the WC1 domain "a", as well as from the first six SRCR domains from CD163b and SRCR domains "h", "i", "j", and "k" from CD163A (Fig. 4A,4B).

The chicken and the duck-billed platypus possess more CD163 genes than primates, dogs or rodents, displaying a diverse repertoire more similar to that seen with WC1 genes in the artiodactyls. The chicken possesses at least eighteen CD163 genes while the duck-billed platypus has at least ten CD163 genes. Seven of the chicken CD163 genes and three of the duck-billed platypus CD163 genes contained SRCR domains that clustered in the clade containing WC1 SRCR domain "a" (Fig. 4A). This is notable because domain "a" is the source of most of the diversity in WC1 family members and is thus thought to be the most likely SRCR domain to be responsible for WC1 isoform-specific function [31]. Chicken CD163_3 and platypus CD163_2 were the closest to bovine WC1 in extracellular SRCR organization, with SRCR domain organization of (a-b-c-a-b-c-x-e-d') and (a-c-d-d'), respectively, compared to the bovine WC1 SRCR domain organization of (a-b-c-d-e-d-b-c-d-e-d') (Fig. 4B). Six chicken CD163 proteins show a hybrid SRCR domain

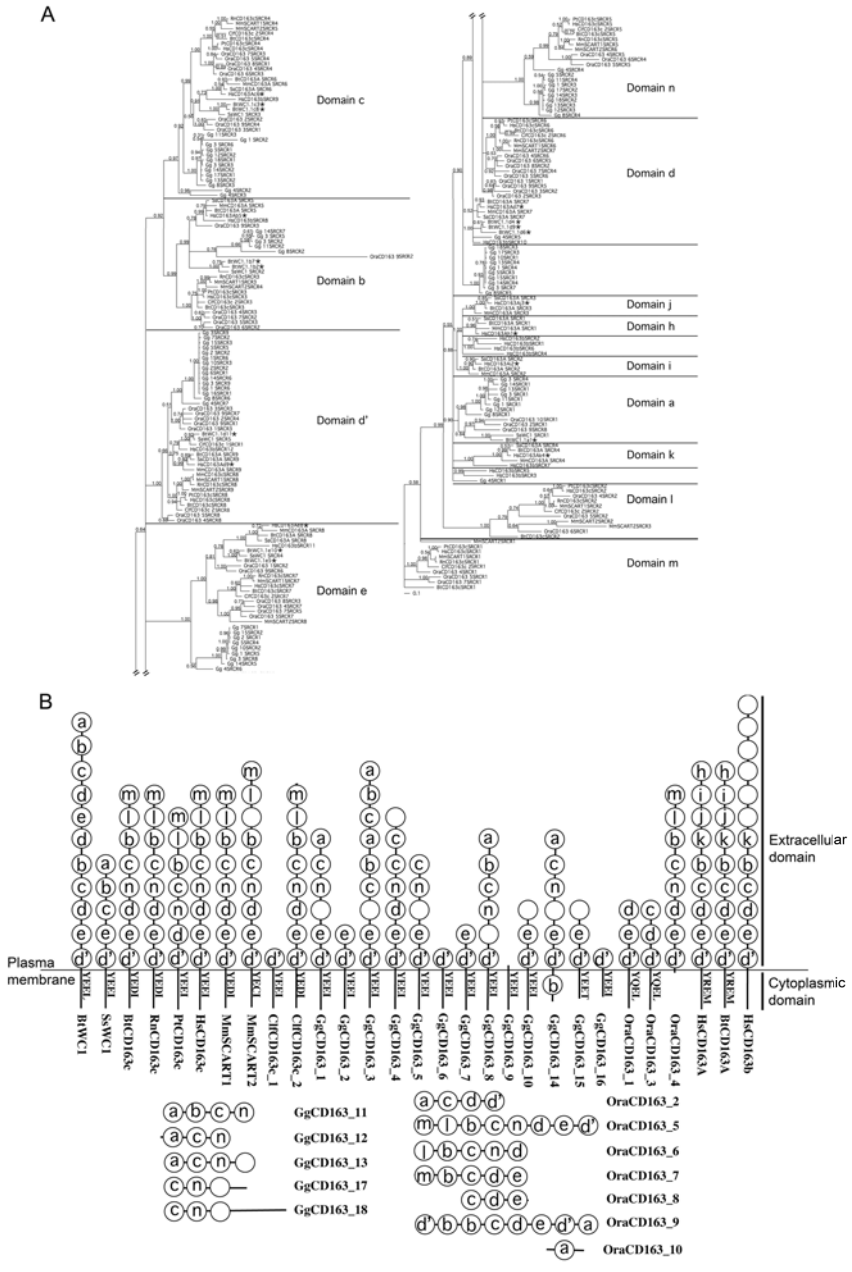


Figure 4 CD163 family member SRCR domain organization and cytoplasmic tyrosine-based motifs. >(A) CD163 family members from multiple species were identified by BLAST searches with bovine WC1, CD163A and CD163c-α SRCR and cytoplasmic domains. The evolutionary history of 242 taxa was inferred, using MrBayes3.2 to approximate the posterior probabilities of trees, shown at branch nodes [65]. SRCR domains clustering in a clade with pre-defined SRCR domains (asterisks) were identified and SRCR domains clustered together, but not in association with known SRCR domains, were assigned a new letter designation [1]. *Rattus norvegicus* (Rn) CD163c-α, *Pan troglodytes* (Pt) CD163c-α, *Canis lupus familiaris* (Clf) CD163c_1 and ClfCD163c_2, *Gallus gallus* (Gg) CD163_1 through GgCD163_18, and *Ornithorhynchus anatinus* (Ora) CD163_1 through OraCD163_10 are predicted from genomic sequence; *Bos taurus* (Bt) WC1, *Sus scrofa* (Ss) WC1, *Homo sapiens* (Hs) CD163c-α (partial cDNA, corrected from genomic sequence), *Mus musculus* (Mm) SCART1, MmSCART2, HsCD163A and HsCD163b cDNA have been isolated. (B) Summary of CD163 family member SRCR domain organization and cytoplasmic tyrosine based motifs. SRCR domain assignments were made as shown in Fig. 4A, with confirmation by analysis utilizing the Neighbor-Joining algorithm with the JTT model (data not shown). CD163 family members with transmembrane domains are shown with the plasma membrane and location of the transmembrane domains represented by the horizontal line. Tyrosine-based signalling motifs similar to that found in BtWC1 are shown [35]. CD163 family members without transmembrane domains are shown horizontally, with the N-terminus on the left.

organization that is intermediate between WC1 and CD163c-α, with the SRCR domain "a" diagnostic of WC1 and SRCR domain "n" diagnostic of CD163c-α. For example, chicken CD163_1 and CD163_8 possess a N-terminal domain "a" characteristic of WC1 but an SRCR domain cassette (c-n-d-e-d') characteristic of CD163c-α. In contrast, CD163 molecules from the duck-billed platypus contain either the WC1- diagnostic domain "a", or CD163c-α-diagnostic domains "m","l", or "n", but not both.

The consensus group B SRCR domain contains eight cysteines, with disulfide bonds formed between the first and fourth, the second and seventh, the third and eighth, and the fifth and sixth cysteines [42]. Some of the SRCR domains lack the second and/or the seventh cysteine, which results in a predicted SRCR domain with only three disulfide bonds rather than four disulfide bonds. However, MmSCART2_SRCR3 (missing C2, C4 and C7), Gg_1SRCR2 (missing C5, C7, and C8), Gg_4SRCR7 (missing C7 and C8), Gg_5SRCR7 (missing C7 and C8), OraCD163_3SRCR2 (missing C6 and C7), OraCD163_7SRCR4 (missing C1), OraCD163_9SRCR2 (missing C2, C6, and C7), and OraCD163_6SRCR4 (missing C2, C6, and C7) are missing additional cysteines resulting in SRCR-like domains with only one to three potential disulfide bonds. It is not known how these changes will affect SRCR domain structure and function.

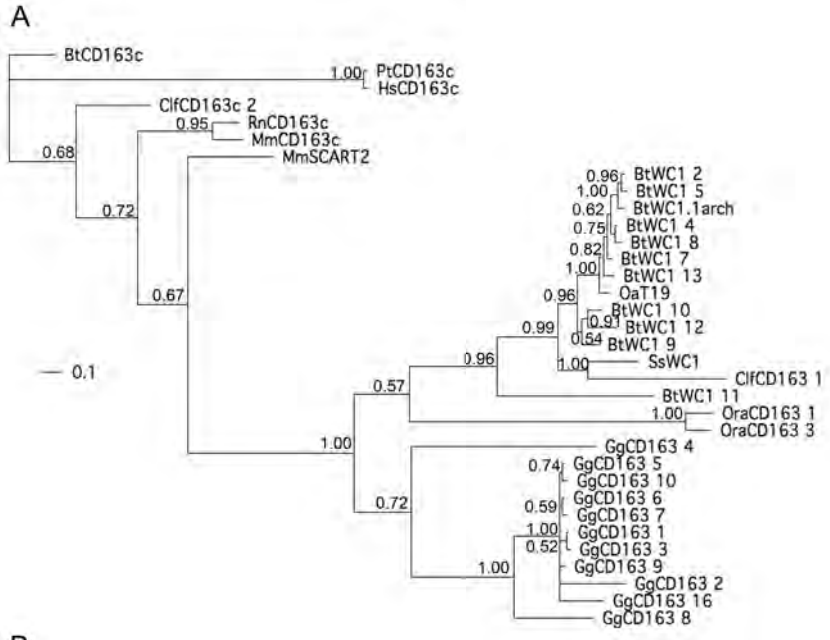
Relationship of cytoplasmic sequences

Several of the WC1-like or CD163-α-like molecules from chicken or duck-billed platypus do not have transmembrane or cytoplasmic domains; although, they are predicted open reading frames from genomic sequence and may not be complete. The cytoplasmic domain of chicken CD163_14, defined as being C-terminal to a transmembrane domain, is unusual in that it is not similar in sequence to other CD163 family members, but instead contains a SRCR domain (Fig. 4B). The cytoplasmic domains of bovine or human CD163A or human CD163b do not have any significant sequence identity with the cytoplasmic domains of CD163c-α-like or WC1-like molecules; although bovine and human CD163A have the tyrosine-based motif YREM in their cytoplasmic domains (Fig. 4B). We performed multiple amino acid alignment and phylogenetic tree analysis of the other cytoplasmic domains of CD163c-α-like and WC1 molecules and found that the cytoplasmic domains from canine ClfCD163_1, chicken and platypus CD163 molecules cluster more closely with the cytoplasmic domains of bovine and swine WC1, than with the cytoplasmic domains of canine ClfCD163_2, primate, bovine, and rodent CD163c-α (Fig. 5A).

Strikingly, all CD163c-α-like molecules from primate, canine, rodent, bovine, monotreme and bird species share a Y-(Q/E)-(D/C/E)-(I/L) motif with WC1, with the exception of chicken CD163_15, which has a threonine in place of the isoleucine or leucine (Fig. 5B). Phosphorylation of the YEEL tyrosine motif in WC1 is required for its potentiation of T cell activation, suggesting that WC1 and CD163c-α may signal via the same mechanism [35].

Discussion

Many genes that have been predicted to belong to the CD163 family have been erroneously classified as CD163A or CD163b homologues in their Entrez Gene reports. For example, ClfCD163_2, RnCD163c-α and MmSCART1 are referred to as similar to M160 or *CD163L1*, which are alternative names for CD163b, despite their greater similarity to CD163c-α in their SRCR identity and organization and in their cytoplasmic domain sequence. GgCD163_10 is referred to as CD5-like, despite showing greater sequence similarity to CD163c-α than CD5. Other genes from the chicken and platypus that are most similar to CD163c-α and WC1 are referred to as similar to CD163v2 and CD163v3, which are names for alternatively spliced isoforms of CD163A. It has been speculated that the plethora of CD163 molecules in the platypus are CD163A homologues important for regulating blood serum levels of free hemoglobin, instead of CD163c-α or WC1 homologues, which are more likely to be involved in the immune response [43]. In this study, we show that these genes encode CD163c-α, WC1, or CD163c-α/WC1-like proteins, based on the sequence of their SRCR and/or cytoplasmic domains. Genes coding for WC1-like proteins in non-artiodactyls have not been previously identified. SRCR domains "e " and "d' " (Fig. 4A) and cytoplasmic domains (Fig. 5A) that are common to many CD163 molecules across species cluster in sub-clades characterized by eutherian mammalian, platypus or chicken origin, suggesting that gene conversion in multi-gene families over millions of years of evolution has driven homogeneity [44]. Artiodactyls, platypus and chicken all display an expansion of WC1/CD163c-α-like genes (Fig. 6). The platypus and the chicken do not have a CD163A gene, suggesting that CD163A has evolved since the divergence of eutherian mammals from monotremes. In contrast, eutherian mammals have one CD163A gene and non-artiodactyl eutherian mammals have a maximum of two CD163c-α genes. This suggests that expression of multiple WC1/CD163c-α genes is the ancestral state and that eutherian mammals other than artiodactyls have lost multiple WC1/CD163c-α genes, since mammals diverged from the sauropsid lineage leading to birds and reptiles 315 million years ago [43]. In a similar finding, the platypus, sheep and cow share an expansion of the cathelicidin antimicrobial peptide gene family, whereas primates and rodents have only a single cathelicidin gene [45]. The pri-



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BtCD163c          RWFRGRDACRGSGMSSSLPSEGVYEDIGVATAGEKDEAAGAA
ClfCD163_2        QWYRGRRARRGSRMWSSLPSEGVYEDIGAVPMADQGEGPTVS
RnCD163c           WCHIRDASMGSGMLSNLQSDGVYEDIEVVPVMKKEE-NAIS
MmSCART1              RDASMGAGMLSHLQSEGIYEDIEVDPVPKKEE-NTVS
MmSCART2          QWCHSRAIGLGSGAVAELPSEVVYECIETVPMDEKEE-PAAS
BtWC1-9              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KGGLLGSP
BtWC1-10             RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEGLLGSP
BtWC1-12             RWRAERRALSSYEDVLAEAVYEELDYLLTQ-KEGLLGSP
BtWC1-1              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1-7              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1-2              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1-5              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1.1arch          RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1-4              RWRAERRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
BtWC1-8              RWRAERRALSSYSDALAEAVYEELDYLLTQ-KEG-LGSP
OaT19                RWRAECRALSSYEDALAEAVYEELDYLLTQ-KEG-LGSP
SsWC1                RWRAERRALSGLEGALAEAVYEEIDYLVTP-KEDLLGSP
ClfCD163_1           RWRAKQRAFPSFRDVLNEAEYEEIDHVVTT-KEDLLDNT
BtWC1-11             RWRAERRELSDFEAAVDEAVYQEIDDIIKPGKKDLLDSQ
OraCD163_1           QQRAQRRAASRRRDSLSEAVYQELEYALMG-EKDGLSFR
OraCD163_3           QQRAQRQAFTERRDTLSEAVYQELEYALMG-EKDGLSFR
GgCD163_6         RQMQSARARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_7         RQVQSARALHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_15        RQVQSARARHRDSRRSSEPFSEAVYEETKNSPMREKKMRFLLW
GgCD163_16        RQMQSARALHRDSRRSGEPFSEAVYEEISYSYMREKKMRFSFS
GgCD163_5         RQMQSARARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_9         RQMQSASARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_1         RQMQSARARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_3         RQMQSARALHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_10        RQMQSARARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_2         RQMQSARARHRDSRRSSEPFSEAVYEEIEYSPMREKKMRFSFS
GgCD163_8          QVQSAKAQRRDSRRNEESFPEVVYEEIRCSYAWEKQTMFVHS
PtCD163c             EVSPGEAIYDVISEMPLAGLYEEITEAEAVLQDEEDGSV
HsCD163c             EVSPGEAIYDVISEMPLAGLYEEIMEAEAVLQDEEDGSV
GgCD163_4              QRARAQQRGSYNSFNEAVYEEIDYKLMKEQQGMSGLS
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Figure 5 WC1 and CD163cα tyrosine-based motifs in the cytoplasmic domain. (A)Cytoplasmic domains were determined by ascertaining the transmembrane domain, using the DAS transmembrane server [70]. Proteins are labelled as described in Fig. 4, with the addition of bovine WC1 cytoplasmic domains [31]. The evolutionary history of 34 taxa was inferred using Bayesian analysis in MrBayes3.2 [65]. Markov chain Monte Carlo analysis was performed for 830,000 cycles, using 2 runs of 4 chains each, a temperature setting of 0.2, and an amino acid mixed model to approximate the posterior probabilities of trees, shown at branch nodes. The average standard deviation of split frequencies was 0.01, which was diagnostic of convergence at < 0.05. (B)Multiple alignment of the amino acids surrounding the tyrosine motif phosphorylated in WC1 (bold) with similar amino acid sequences from CD163 family members [35].

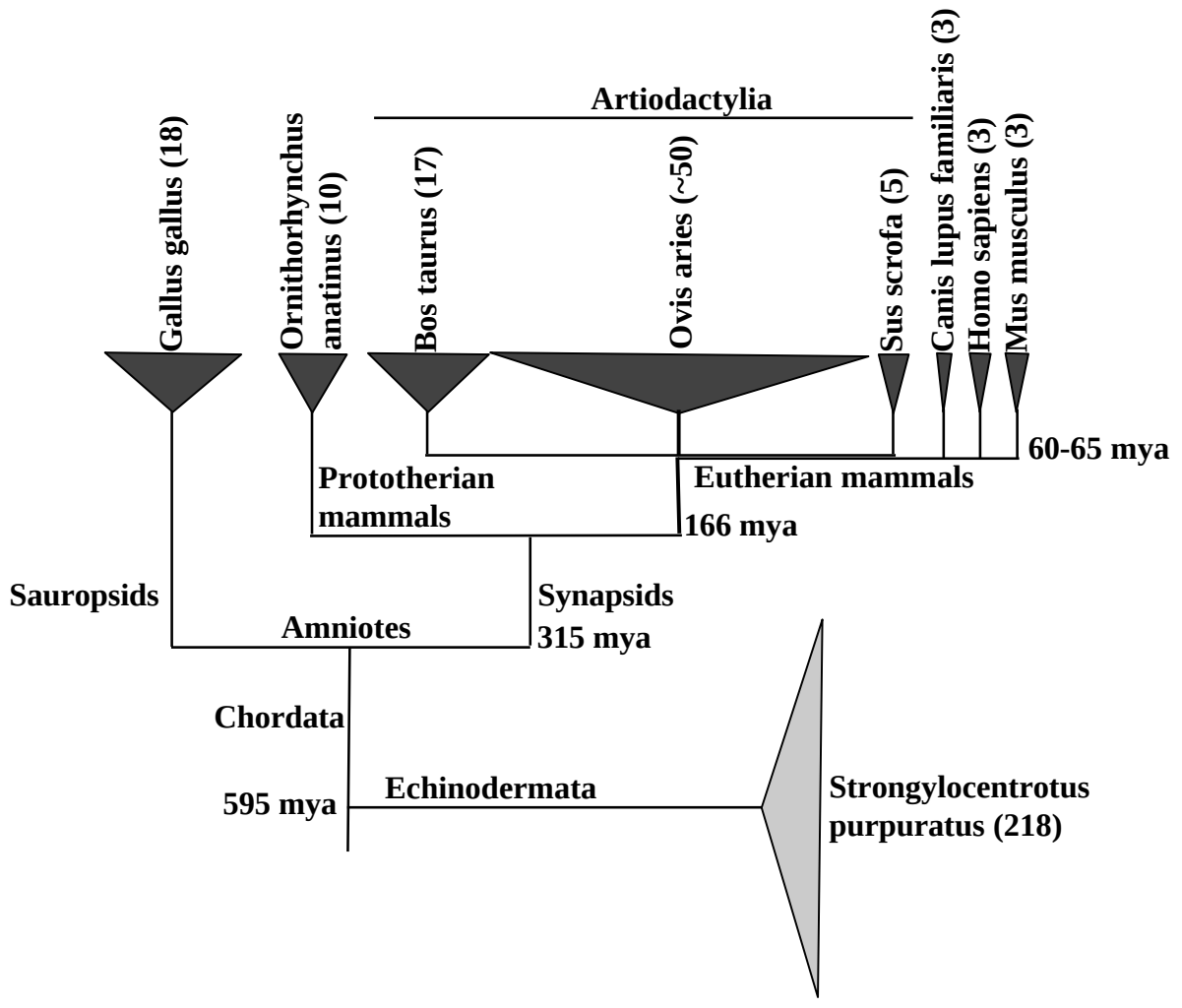

Figure 6 CD163 family members in multiple species. Numbers of group B SRCR CD163 family members in a representative of the sauropsid lineage, a prototherian mammal, and eutherian mammals are shown. The number of group A SRCR domain molecules in the echinoderm *Strongylocentrotus purpuratus* are shown [71]. Echinoderms diverged from chordates 595 million years ago; other divergence times are described on the Tree of Life web project http://tolweb.org[72].

mate or rodent cathelicidin must function equivalently to the plethora of cathelicidins in the platypus or ruminants; analogously, the one or two CD163c-α molecules in non-artiodactyl eutherian mammals may fulfil a function like that of WC1, in regulating the γδ T cell response.

Chickens, cattle and sheep are known to have a high percentage of γδ T cells in their peripheral blood. They are referred to as "γδ T cell high" species. In contrast, mice and humans have lower numbers of γδ T cells in their peripheral blood and are referred to as "γδ T cell low" species [46]. It is not known whether the duck-billed platypus is a γδ T cell high or γδ T cell low species, but it does possess γδ T cells [47]. We show that the chicken and platypus have multiple CD163c-α/WC1-like genes, a circumstance that is similar to the multiple WC1 genes in the genomes of the artiodactyls. We hypothesize that a diverse array of CD163c-α/WC1 SRCR transmembrane receptors conveys a selective advantage to the immune response to pathogens by γδ T cells and that WC1 and CD163c-α genes have been conserved because they play an important role in the response to pathogens.

Phosphorylation of the second tyrosine in the WC1 cytoplasmic domain is required for WC1-mediated potentiation of T cell activation though the TCR, thus, the conservation of this motif in WC1-like and CD163c-α-like genes over millions of years of evolution supports our hypothesis that CD163c-α plays a role similar to WC1 as a co-receptor to an activating receptor [35]. In contrast, it is unknown if the tyrosine-based motif YREM in CD163A of human or cattle is a phosphorylation target

or whether it participates in membrane proximal signaling by CD163A after ligation of this receptor. Phosphorylation of CD163A by the serine/threonine kinases casein kinase II and protein kinase C-α (PKC-α) is tied to cytokine production induced by CD163A cross-linking [19]. Detection of transcripts for bovine CD163A in sorted WC1 $^{+}$ and γδ^{+} T cells, as well as in tissues containing monocytes and macrophages, suggests that bovine CD163A may play a role in the regulation of the γδ^{+} T cell immune response, either through activation of transmembrane CD163A or proteolytic cleavage and release of soluble CD163A.

The characterization of the extracellular SRCR structure of WC1 and CD163 family members may help to isolate their ligands, which in turn would help us to better understand their role in the γδ T cell response. The role of WC1 and the proposed role for CD163c-α are consistent with other group B SRCR family transmembrane receptors expressed on T cells, such as CD5 and CD6. CD6 binds to the protein ALCAM/CD166 [48]. CD5 has been shown to bind to various proteins, including CD72, a 40-80 kDa glycoprotein expressed on murine B splenocytes and activated T cells, a human 150 kDa glycoprotein expressed on peripheral blood monocytes, certain IgV_H framework sequences and a bovine 200 kDa protein expressed on activated B cells [49-54]. Ligation of CD5 or CD6 affects responses through the T cell receptor and B cell signaling and lymphocyte activation [55,56]. It is known that SRCR molecules also bind to molecules other than proteins; for example, the group B SRCR molecules Spα, CD6, DMBT1 and CD163A bind to bacteria and Spα and CD6 specifically bind to the bacterial products lipoteichoic acid (LTA) and lipopolysaccharide (LPS) [1,13,14,39,57]. The SRCR domains in WC1 or bovine CD163c-α do not have the bacteria-binding motif found in multiple SRCR domains of DMBT1 [58]. However, the requirement for expression of specific WC1 receptors for the γδ T cell response to the bacteria *Leptospira* (Wang F, Herzig CTA, Hsu H, Chen C, Baldwin CL, Telfer JC: Scavenger receptor WC1 contributes to the γδ T cell responses to *Leptospira*, submitted) suggests that WC1/CD163c-α could recognize pathogen-associated molecules or a protein whose expression is induced by exposure to *Leptospira.* Although the ligands for WC1 and CD163c-α are unknown, domains 9 (d) and 11 (d') of bovine WC1 interact with an unknown molecule on the surface of macrophages or dendritic cells [59]. In contrast, another CD163 family member, CD163A, is known to bind haptoglobin-hemoglobin complexes and TWEAK through its third SRCR domain ("j") and to bacteria or a molecule on erythroblasts via its second SRCR domain ("i") [10-13]. Neither of these domains occurs in either WC1 or CD163c-α, suggesting that CD163c-α ligation could be mediated by its unique domains "m", "l", or "n", and that it does not bind to haptoglobin-hemoglobin complexes. Since most of the diversity of bovine WC1 is found in domain "a", it is a reasonable candidate for the ligand-binding domain of the WC1 multi-gene family, which confers diversity on the γδ T cell response to pathogens.

Conclusions

We have annotated the genes encoding CD163c-α and CD163A in cattle and shown that bovine CD163A is expressed in tissues containing monocytes and macrophages, as well as in sorted γδ T cells. The expression of bovine CD163c-α is enriched in WC1^{+} γδ T cells and it shares the conserved tyrosine motif necessary for WC1 signalling in T cells, implying that it functions similarly to WC1 in acting as a co-receptor for the γδ TCR. We have characterized CD163c-α and WC1/CD163c-α gene products in multiple species, correcting the incorrect assignment of many of them as CD163A gene products. The expansion of WC1, CD163c-α and WC1/CD163c-α genes is correlated with a high level of γδ T cells in the peripheral blood of species separated by approximately 300 million years of evolution, suggesting that a diverse array of these molecules conveys a selective advantage to the γδ T cell response. Future studies will address the significant questions of both the signalling potential of CD163c-α and CD163A in γδ T cells and the identification of ligands for CD163c-α/WC1 molecules.

Methods

Genome annotation

In conjunction with the Bovine Genome Sequencing Consortium http://genomes.arc.georgetown.edu/drupal/bovine/, manual annotation of the CD163 genes was performed using the Apollo Genome Annotation and Curation Tool, version 1.6.5 and the bovine genome assembly Btau_3.1 [60]. Putative CD163 genes were identified by performing a BLAST search with orthologous mouse and human genes against the Bovine Official Gene Set (called GLEAN). The predicted gene models identified from the search included GLEAN_00453 for CD163A and GLEAN_14183 for CD163c-α. These were analyzed using the Apollo software and annotated based on available EST evidence and BLAST search results.

Sequence analyses

Nucleotide sequences were aligned and consensus sequences were created using BioEdit version 7.0.5.3 [61]. Exon/intron structure schematics were based on alignments of cDNA and genomic DNA sequence using SIM4 and visualization with LalnView http://pbil.univ-lyon1.fr/software/lalnview.html[62].

Amino acid sequences used for comparison in phylogenetic analyses and their accession numbers are described

in Additional file 1, Table S1. SRCR domains were identified according to the group B SRCR consensus and compared to HsCD163A, HsCD163c-α, BtWC1, HsDBMT1, HsCD5, and HsCD6 [42]. Molecules containing group B SRCR domains clustering with CD163 family members were defined as CD163 family members; molecules containing group A SRCR domains or SRCR group B domains clustering with HsDBMT1, HsCD5 and HsCD6 were excluded from analysis. Genomic WC1 cytoplasmic domain sequences used for comparison here have been previously described [31]. Multiple sequence alignments were performed using Clustal X 2.0.8 [63]. Pairwise and multiple alignment penalties for gaps were 10.0, 0.10 for gap extension and Gonnet 250 for protein weight matrix. Alignments were refined in MacClade 4.08. Phylogenetic trees were created using the Neighbor-Joining algorithm in MEGA4 and Bayesian analysis in MrBayes3.2 [64,65]. For Bayesian analysis, 2 runs with 3 cold chains and 1 heated chain each were done. An amino acid mixed model was used to approximate the posterior probabilities of trees. The 242-taxa SRCR domain alignment was run independently three times, with temperature settings of 0.15, 0.2, and 0.25, for 2.6, 2.0, and 2.5 million generations respectively. Trees were sampled every 100 generations and the burnin fraction was 0.5. The convergence diagnostic used was the average standard deviation of split frequencies, which were < 0.05 (0.0427, 0.031, and 0.030) for the three runs. The consensus trees from the three runs had the same overall topology. The consensus tree from the run set to temperature 0.25 and 2.5 million generations (average standard deviation of split frequencies 0.030, average potential scale reduction factor 1.016) is shown in this paper. The 34-taxa cytoplasmic domain alignment was run once with a temperature setting of 0.2 for 830,000 generations. Trees were sampled every 100 generations and the burnin fraction was 0.25. The average standard deviation of split frequencies was 0.01 and the average potential scale reduction factor was 1.003. Phylograms were visualized using TreeView X 0.5.0 [66].

Animals, cells and tissues

Belted Galloway cattle of between 1 and 2 years of age were housed at the University of Massachusetts Amherst according to institutional and USDA guidelines. Animal protocols were approved by the University of Massachusetts Amherst institutional animal care and use committee. Blood was obtained via jugular venipuncture and collected into a solution of heparin. Peripheral blood mononuclear cells (PBMC) were isolated from blood via density gradient centrifugation over Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ) by standard techniques. Culture medium consisted of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 50 μM 2-ME. PBMC were cultured at 2.5×10^6 cells/ml with Concanavalin (ConA 1.0 μg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO_2 in air for 3 days where indicated. For evaluation of CD163 expression in intestine, intraepithelial lymphocytes (IELs) were isolated from the ileum of two-month old cattle using nonenzymatic methods [67]. IELs were stained for cell surface differentiation molecules, fixed in 1% paraformaldehyde, and analyzed via flow cytometry (LSR II, BD Biosciences, San Jose, CA) using the following primary monoclonal antibodies (mAbs): CC15 (pan-WC1; Serotec, Raleigh, NC), GB21A (δ TCR; VMRD, Pullman, WA), and IL-A12 (CD4;) [68]. Secondary antibodies used were isotype-specific polyclonal goat anti-mouse Ig conjugated with fluorescein isothiocyanate (FITC) (Southern Biotechnology, Birmingham, AL). Flow cytometric data was analyzed using FlowJo version 7.2.2 (Tree Star, Ashland, OR).

Purified γδ T cells and WC1 $^+$ γδ T cells were obtained from ex vivo PBMC by magnetic bead sorting (MACS; Miltenyi Biotec, Auburn, CA). Cells were suspended in PBS containing 6% heat-inactivated horse serum and incubated with monoclonal antibodies (mAbs) GB21A or CC15 for 20 min on ice, washed twice with PBS containing 0.5% bovine serum albumin and 2 mM EDTA and resuspended in the same along with goat anti-mouse IgG microbeads (Miltenyi Biotec) following the manufacturer's instructions. Cells were purified over pre-cooled MS-separation columns (Miltenyi Biotec); the positive and negative fractions were collected and each was passed over an additional separation column in order to optimize purity. Purity was assessed by flow cytometry and was found to be 99.6% for γδ T cells, 98.1% for γδ T cell-depleted cells, 95.5% for WC1$^+$ γδ T cells and 92.7% for WC1$^+$ γδ T cell-depleted cells.

RNA isolation and RT-PCR

Pelleted *ex vivo* or ConA-activated PBMC, sorted cells, and IELs were resuspended in TRIzol (Invitrogen). MLN and lung tissue samples frozen in TRIzol were thawed and homogenized. RNA was isolated according to the manufacturer's protocol. For RNA isolation from sorted cells, glycogen (Invitrogen) was added to the aqueous phase prior to RNA precipitation. Reverse transcription (RT) was performed using 1 μg of total RNA, oligo dT primers and AMV reverse transcriptase (AMV RT kit; Promega, Madison, WI). Prior to RT reactions, RNA derived from MLN and lung was treated using RQ1 RNase-Free DNase (Promega) according to the manufacturer's protocol. 2 μl of cDNA was used as template in subsequent PCR reactions.

Primer pair sequences used to obtain CD163A and CD163c-α cDNA sequences are as follows: (1) CD163Autr-for 5'-GAG TGG ACA AAC TCA GAA TGG TG and CD163A-rev2 5'-GAG GAA TTA TAT

AGG TCC AGA TCA TC; (2) CD163A2952-for 5'-CAT ATG GCT CAA TGA AGT GAA GTG and CD163Autr-rev 5'-GTG CAT CAC AGG CTT CTT ATT ATG; (3) CD163c2312-for 5'-GTG TGG AGC TCT GGC ACG CTG and CD163c-rev2 5'-CAA TGT CCT CAT AAA CAC CTT CTG; (4) CD163c1938-for 5'-CCT CTG CTC AGA GTC AGT G and CD163c2312-rev 5'-CAG CGT GCC AGA GCT CCA CAC using PCR Master Mix (Promega). Cycling parameters were 30 s at 94°C, 30 s at 50°C and approximately 1 min/kb at 68°C for 35 cycles. PCR products were ligated in the pCR2.1 vector (Invitrogen) and sequenced (GeneWiz, South Plainfield, NJ) using the T7 forward and M13 reverse primers, as well as the following sequence specific primers, where applicable: CD163Aseq2-for 5'-CCA ATC TGG TTT GAT GAT CTG GTA; CD163Aseq3-for 5'-TCT GAC TTC TCT CTG GAA TCG; CD163Aseq4-for 5'-CAG AAC TGC AGG CAT AAG GAG; CD163Aseq1-rev 5'-GCT GCC CCA AGC TCC GTT G; CD163Aseq2-rev 5' - CAT TCG TGA TGT CTG CAC TG; CD163A1824-rev 5'-CAT GTC CCA GTG AGA GTT GCA GAG.

For expression analysis PCR Master Mix (Promega) was used. Primer sequences were as follows: GAPDH-for 5'-GTCATCATCTCTGCACCTTCT; GAPDH-rev 5'-ACCACCTTCTTGATCTCATCAT; For CD163A and CD163c-α primer pairs (2) and (3) described above were used, respectively. Cycling parameters were 30 s at 94°C, 30 s at 55°C and 1 min at 68°C for 30 cycles (GAPDH) or 35 cycles (CD163A and CD163c). CD163A and CD163c-α PCR products were directly sequenced (GeneWiz, South Plainfield, NJ) in order to verify primer specificity.

Additional material

Additional file 1 Table S1. Accession numbers for amino acid sequences used for phylogenetic analysis.

Authors' contributions

CTAH annotated the bovine CD163 genes, performed the expression analysis and helped prepare the manuscript. WRW supplied the bovine tissues used for the expression analysis. CLB helped in the study coordination and manuscript preparation. JCT conceived of the study, performed the phylogenetic analysis and drafted the manuscript. All authors read and approved the final manuscript.

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SHORT REPORT Open Access

Detection of a novel porcine parvovirus, PPV4, in chinese swine herds

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Abstract

To determine whether the novel porcine parvovirus type 4 (PPV4) recently reported in America is prevalent in China, a set of specific primers was designed and used for molecular survey of PPV4 among the clinical samples collected from various provinces of China between 2006 and 2010. The results showed that PPV4 is present in Chinese swine herds at a rate of 2.09% (12/573) among the clinical samples examined and 0.76% (1/132) among the samples taken from healthy animals. We also noted that PPV4 was not detected in samples taken prior to 2009. Analysis of the coding sequences showed that the Chinese and American PPV4 genome sequences are closely related with greater than 99% nucleotide sequence identity. Similar to a previous study, viral genomes in head-to-tail configuration of various lengths of the non-coding region were detected. Our findings confirmed that PPV4 is a unique recently discovered virus in pigs. Phylogenetically, PPV4 is most closely related to bovine parvovirus 2 (BPV2, which is not a *Bocavirus* and is not assigned to any *Parvovirinae* genus) and shares limited ORF1 (33.6%) and ORF2 (24.5%) amino acid identity. With respect to genome structure and organization, PPV4 encodes an ORF3 in the middle of the viral genome that resembles the *Bocavirus* genus. However, the PPV4 ORF3 encoded protein shares minimal amino acid identity with the ORF3 encoded proteins of the *Bocavirus* genus.

Findings

The International Committee on Taxonomy of Viruses has classified the *Parvovirinae* subfamily of viruses into five genera: *Dependovirus*, *Bocavirus*, *Erythrovirus*, *Parvovirus* and *Amdovirus*. Recently, two new genera, *Hokovirus and Cnvirus*, have been proposed. *Hokovirus* includes a group of porcine and bovine isolates that clustered with the human PARV4 and PARV5 viruses [1,2] and *Cnvirus* includes the H-1 isolate from Myanmar [3] and a group of newly identified porcine isolates from China that exhibit a distinct sublineage from the *Hokovirus* [4,5]. In addition, there are two bovine isolates (BPV2 and BPV3) and one porcine isolate (PPV4) that do not cluster with any member of the *Parvovirinae* genera [6]. The genomes of the *Parvovirinae* members are linear single-stranded DNA of about 5 kilobases that contain terminal palindromic sequences. In general, the genome encodes two major open reading frames (ORFs) coding for the non-structural protein(s) located at the 5'-end and the capsid protein(s) located at the 3'-end. An additional ORF3 located in the middle of the viral genome was observed among viruses of the *Bocavirus* genus (bovine parvovirus [BPV1], canine minute virus [MVC], human bocavirus [HBoV], and, potentially, a partially sequenced porcine boca-like virus [Pbo-likeV]) [7,8]. PPV4 is unique in that its genome nucleotide sequence is most related to BPV2, but the coding capacity and genome organization are more related to viruses of the *Bocavirus* genus [6]. Limited amino acid identity, ORF1 (33.6%) and ORF2 (24.5%), exists between PPV4 and BPV2. The ORF3 amino acid identity among the three recognized *Bocavirus* members is 43.3-47.0%. Whereas the Pbo-likeV ORF3 shares 33.6-35.6% amino acid identity with the recognized *Bocaviruses*, the PPV4 ORF3 shares only 4.9-11.2% [6-8]. The ORF3 of Pbo-likeV and PPV4 share low nucleotide identity (45%) and amino acid identity (17.5%), which indicate Pbo-likeV and PPV4 are two distinct parvoviruses.

The aim of this study was to investigate whether PPV4 exists among swine herds in China by a PCR assay. A total of 705 animals were examined. A sample was collected from each pig (573 sick animals and 132 healthy animals) in 12 provinces of China from 2006 to 2010 (100 samples in 2006, 100 samples in 2007,100 samples

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in 2008,281 samples in 2009,124 samples in 2010). The sick pigs exhibited clinical symptoms that encompassed trembling, fever, testicular atrophy, abortion and death. The samples included 386 sera and 319 tissues from various organs. Briefly, viral DNA was extracted using a DNA/RNA extraction kit (catalog # DP315, Tiangen Biotech Inc., Beijing, China). Based on the PPV4 genomic sequence (GenBank accession numbers: GQ387499) [9], a pair of detection primers [PPV4-F (5'-GGGCGAGAACATTGAAGAGGT-3') and PPV4-R (5'-TTGTGAGTATGGGTATTGTGT-3')] targeting a 543 nucleotide sequence within ORF2 were used. The PCR reaction was performed according to manufacturer's instruction (catalog # KT201-2, Tiangen Biotech Inc., China). The cycling program was as follows: pre-denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 40 s, final extension for 10 min at 72°C. The PCR products were gel-purified and cloned into the PCR2.1T/A vector (Invitrogen Corporation, California, U.S.A.). Nucleotide sequences were determined using an AB-3730 automated DNA sequencer and the results obtained were analyzed with the BLASTx program http://www.ncbi.nlm.nih.gov/blast/. The results showed that 12 out of 573 clinical samples and 1 out of 132 healthy pig samples were positive for PPV4. PPV4-positive pigs were not detected among samples collected from 2006-2008. Eight of the positive samples were collected in 2009 and the other five were collected in 2010 and they came from five different breeding pig farms located in three provinces of China. Although the overall detection rate was low (13/705 = 1.84%), the rate in each positive farm was high: 37% (7/19), 25% (1/4), 40% (2/5), 20% (2/10) and 50% (1/2). Among the thirteen PPV4-positive samples identified, 10 samples were from adult pigs (7 sows about seven months and 3 boars about four months old), two from a dead piglet (about 8 weeks old) and one from a healthy piglet (about 4 weeks old). The adult pigs were diagnosed with reproductive failure, while the sick piglet had displayed fever and neurologic symptoms. Further analysis showed that PPV4 DNA existed in the heart, serum, lymph node, lung and kidney, with the heart containing the highest viral load (data not shown).

To explore whether there were any co-infecting viruses present in our PPV4-positive pigs, we investigated the presence of porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2), classic swine fever (CSFV), PPV1, PPV2, PPV3, pseudorabies virus (PrV) and porcine torque teno virus (genogroups 1 [PTTV1] and 2 [PTTV2]) according to the protocols previously described [1,3,9-11]. The results revealed that all 13 PPV4-positive samples were co-infected with both PTTV1 and PTTV2, while 4 out of 13 samples (30.7%) were co-infected with PCV2. The other viruses examined were not detected.

Table 1 Primers for the amplification of the full-length genome sequence of PPV4.

#	Primers	Sequences	Position*	Tm (°C)
1	PF356	5' TGACGCAGTACAGACCGACGAGA 3'	356	60
	PR1197	5' AATGCAAGTGCAAGCCACCTTTT 3'	1197	
2	PF1050	5' AGTAATCTGGTAATCGCTGTTCG 3'	1050	
	PR1942	5' ATGTTAGTCTTTCCTGTTGTGGC 3'	1942	
3	PF1568	5' GCTGGTGGATAACAACATCTGCT 3'	1568	
	PR2553	5' GTTTCTTCTTTCTCGGTGCTTCT 3'	2553	
4	PF2530	5' AAGAAGCACCGAGAAAGAAGAAA 3'	2530	
	PR3413	5' AAATCTAAGGGACAAGGCAAACG 3'	3413	
5	PF3363	5' AGATACTAAGAAAGACAAGG TGGAG 3'	3363	
	PR4263	5' AATAATAGAAGGTATAG CGTCTCCA 3'	4263	
6	PF3918	5' ACCTGCTCCTCCATCTTCTCCAC 3'	3918	
	PR4897	5' GGCCGTCATCATACATTCTGCTC 3'	4897	
7	PF4219	5' ACTTACTGTTCTATGATGTCTGGAG 3'	4219	
	PR5862	5' ATATCATCTGCGGTGTCTGGG 3'	5862	

*The position according to PPV4 clone C17 GenBank accession number: GQ387500) [6]

Three PPV4-positive pigs (2 sows and 1 boar) from two farms in two provinces: Jiangsu (JS0910 and JS0918, from the same farm) and Henan (HEN0922) were selected for full-length genome sequence determination. Seven pairs of primers (Table 1) (based on of GenBank sequence GQ387500) [6] were designed and used to determine the coding sequences, in the 5' to 3' orientation, from the beginning of ORF1, through ORF3, to the end of ORF2. The nucleotide sequences obtained were assembled using the Seqman software and aligned using CLUSTALW of alignment software and a phylogenetic tree was generated using MEGA 4.1 software [12] and the neighbor-joining method [13].

Sequence analysis revealed that the JS0910 sample yielded 2 variants, the JS0918 sample yielded 3 variants and the HEN0922 sample yielded 2 variants. These seven PPV4 genomes contained three major ORFs, which included a putative replicase (ORF1), an ORF3 of unknown function and a capsid gene (ORF2). The coding capacity of each ORF was identical to that of the American PPV4 [6]. Phylogenetic analysis showed that these Chinese PPV4 genomes clustered with the American PPV4 genomes. Among the Chinese isolates, the nucleotide sequence identity of ORF1 was 99.1- 99.3%, ORF2 was 99.2-99.8% and ORF3 was 99.2-99.3%. Between the Chinese isolates and the American isolates, nucleotide identity of ORF1 was 99.7-99.8%, ORF2 was 99.2-99.8% and ORF3 was 99.7-99.8%. Among the Chinese isolates, the amino acid identity of ORF1 was

98.9-99.3%, ORF2 was 99-100% and ORF3 was 99.3-99.6%. Between the Chinese isolates and the American isolates, the amino acid identity of ORF1 was 99.8-100%, ORF2 was 98.5-99% and ORF3 was 99.7-99.9%.

Previous work demonstrated that head-to-tail circular or concatameric PPV4 genomes exist in tissues of the infected animals [6]. Primers designed specifically to detect head-to-tail covalently linked sequences used in the previous study were employed to determined the head-to-tail junction sequences of the seven PPV4 genomes obtained. PCR experiments yielded head-to-tail products of four difference sizes. After sequence determination and assembly, it was noted that the sizes of the seven genomes ranged from 5400 to5644 nucleotides (nt) (GenBank accession numbers: GU978964-GU978968, HM031134-HM031135). In comparison with the genomes of the American PPV4 (5780-5905 nt), all the Chinese PPV4 genomes examined were smaller and the deleted sequence in each genome was located between nt 5964-595 in the non-coding region. The 5400 nt genome (GU978964, GU978968 and HM031134) was detected in all 3 pigs that came from 2 different provinces and the 5644 nt genome (GU978965 and GU978967) was detected in 2 pigs from the same farm. Interestingly, the nucleotide deletions of the three 5400 nt genomes were identical and the nucleotide deletions of the two 5644 nt genomes were also identical.

In conclusion, the results of this study demonstrated that PPV4 existed in some breeding pig farms in China, probably since 2009. Phylogenetically, the Chinese and American PPV4s are closely related and cluster in the same clade. They share greater than 99% nucleotide sequence identity in all 3 ORFs. The American PPV4 was first identified among swine suffering from an acute onset disease of high mortality in North Carolina, USA during late 2005 [14]. Examination of the farm records showed that the two PPV4-positive Chinese farms had purchased swine from a common source in North Carolina prior to the discovery of PPV4. Since the Chinese and American PPV4s are very similar with respect to nucleotide sequence and deletions in the non-coding region, it is possible that PPV4 was introduced into China through the importation of PPV4-infected pigs from the U.S. This study also confirmed previous study [6] that circular or head-to-tail concatameric PPV4 molecules were present in the the diseased animals. The head-to-tail configured PPV4 genomes are likely generated via circularization of the genome in which the left-end and right-end of the linear genome are covalently linked. It is not surprising that some terminal nucleotides of the linear genome may have been deleted during the circularization process, which is observed in this and previous studies. Similar to that of a *Dependovirus*, adeno-associated virus type 2, the presence of head-to-tail configured molecules in the infected animals suggests that PPV4 may be able to establish persistence in its host [15,16]. The fact that PPV4 was detected in diseased animals with other co-infecting viruses, further investigations are needed to determine the pathogenic capability of PPV4 and surveillance studies are needed to determine whether PPV4 could spread and become a serious emerging swine pathogen.

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Authors' contributions

LH, SLZ and HBZ obtained samples and extracted viral DNA. LH carried out the PCR and sequencing studies and drafted the manuscript. JXL, AKC and SSY conceived the study and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Primary transcriptomes of *Mycobacterium avium* subsp. *paratuberculosis* reveal proprietary pathways in tissue and macrophages

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Abstract

Background: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) persistently infects intestines and mesenteric lymph nodes leading to a prolonged subclinical disease. The *MAP* genome sequence was published in 2005, yet its transcriptional organization in natural infection is unknown. While prior research analyzed regulated gene sets utilizing defined, in vitro stress related or advanced surgical methods with various animal species, we investigated the intracellular lifestyle of MAP in the intestines and lymph nodes to understand the MAP pathways that function to govern this persistence.

Results: Our transcriptional analysis shows that 21%, 8% and 3% of the entire MAP genome was represented either inside tissues, macrophages or both, respectively. Transcripts belonging to latency and cell envelope biogenesis were upregulated in the intestinal tissues whereas those belonging to intracellular trafficking and secretion were upregulated inside the macrophages. Transcriptomes of natural infection and in vitro macrophage infection shared genes involved in transcription and inorganic ion transport and metabolism. MAP specific genes within large sequence polymorphisms of ancestral *M. avium* complex were downregulated exclusively in natural infection.

Conclusions: We have unveiled common and unique MAP pathways associated with persistence, cell wall biogenesis and virulence in naturally infected cow intestines, lymph nodes and in vitro infected macrophages. This dichotomy also suggests that in vitro macrophage models may be insufficient in providing accurate information on the events that transpire during natural infection. This is the first report to examine the primary transcriptome of MAP at the local infection site (i.e. intestinal tissue). Regulatory pathways that govern the lifecycle of MAP appear to be specified by tissue and cell type. While tissues show a "shut-down" of major MAP metabolic genes, infected macrophages upregulate several MAP specific genes along with a putative pathogenicity island responsible for iron acquisition. Many of these regulatory pathways rely on the advanced interplay of host and pathogen and in order to decipher their message, an interactome must be established using a systems biology approach. Identified MAP pathways place current research into direct alignment in meeting the future challenge of creating a MAP-host interactome.

Background

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes one of the most well documented chronic diseases of ruminants worldwide (Johne's disease (JD)) and yet the cues leading to its intracellular survival live in obscurity [1]. Major hindrances involved in examining gene regulation during MAP infection are the low amounts of bacterial RNA isolated from an infected host and the lack of an appropriate animal model [2]. In order to overcome the limited quantity of RNA, previous transcriptomic studies interrogating genes used in pathogenic mycobacterial infection were conducted utilizing mimetic conditions of infection in an in vitro environment (i.e. hypoxia, nutrient starvation, acid and nitric oxide (NO) stresses, etc.) [2,3].

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While these studies provided insight into a limited number of genes regulated by specific cues, it is not representative of natural infection since mycobacteria will encounter more than one stress at a time. Multiple stressors may change which genes are utilized as well as potential for gene:gene or protein:protein interactions that influence survival and dissemination in the host. Therefore, current investigations into the intracellular fate of MAP and host responses rely on in vitro macrophage models, specifically bovine and murine cells [3-8]. Studies from our laboratory using an in vitro bovine macrophage infection model in conjugation with selective capture of transcribed sequences (SCOTS) revealed upregulation of MAP genes involved in combating oxidative stress, metabolic and nutritional starvation and cell survival at 48 and 120 hrs post infection [9]. These results indicate that common sets of genes are required for MAP to persist within a multifaceted host environment. Furthermore, consistent with another study using SCOTS analysis with *Mycobacterium avium*, MAP expresses several genes involved in fatty acid degradation, which has been suggested as a universal theme used by pathogenic mycobacteria to successfully efface and invade macrophages and other cell types [10-12].

The utility of results from in vitro macrophage infections, as well as small animal models, is controversial as it is currently unknown if these applications faithfully reflect natural infection in MAP's preferred host. A recent study by Meyer-Barber et al. shows discrepant requirements for Toll expression between isolated murine bone marrow derived macrophages from in vitro and in vivo *M. tuberculosis* infection [10]. Since pathogens initiate and inhibit host signaling (i.e. recognition or evasion), there is also a potential for MAP regulatory networks to differ during in vivo infection. Additionally, a number of articles investigating host-MAP interactions use BOMAC (bovine macrophage) cells due to the advantage of having a cell line [13,14]. However, BOMAC cells are inherently dysfunctional; lacking several receptors and possessing an insufficient capability to phagocytose MAP [8,15]. Therefore, macrophage studies to date may 1) underestimate the speed of MAP responses and/or 2) may be serving as an apparition of rather than being an accurate representation of infection. More importantly, in vitro macrophage studies do not address the initial events that set the venue for MAP's transition into the macrophage. Prior to residing inside gut macrophages, MAP must first encounter the intestinal epithelium [1]. The intestinal epithelium represents a formidable fortress that actively secretes IgA and antimicrobial peptides, which is shielded by the glycocalyx and a thick layer of mucus, produced by intestinal goblet cells [16]. Therefore, it is of little surprise that most of the disease signs associated with JD (i.e. transmural inflammation, corrugation, and gross lesions) are inflicted upon the intestinal tissue. Despite MAP's successful siege against the intestinal barrier as evidence of its infiltration into lamina propria macrophages, the exact genes and pathways MAP employs within the intestinal epithelium remains a black hole in our understanding of overall pathogenesis [17]. Furthermore, it has been suggested that MAP processing by the epithelium may aid in efficiency of invasion in macrophages by pre-exposure to a hyperosmolar environment or expression of a MAP oxidoreductase (MAP3464) [17,18]. Thus, it seems short sighted to assume that no disparate mechanisms are used to survive in the intestinal tissue and macrophage given two different cell types with varying function. Furthermore, data compiled by the Immune Epitope Database and Analysis (IEDB) suggest that specific mycobacterial epitopes are present only within a given host. Studies using small animal models, such as the mouse, may not capture a comprehensive MAP epitope profile as well as transcriptome representative of the cow. The elucidation of host-specific epitopes and MAP genes required for survival during natural infection are expected to aid in the rational design of JD vaccines.

The aim of this study was to characterize the functional MAP transcriptional profiles in the ileum and mesenteric lymph node (MLN) of naturally infected cattle as well as an in vitro bovine monocyte derived macrophages (MDMs) infection model. We have employed advanced molecular techniques, computational and bioinformatic analyses to identify and characterize MAP gene expression during the natural infection process.

Results

Isolation and identification of MAP

Postmortem examination of two subclinical JD cattle revealed gross lesions and corrugation throughout the intestine indicative of chronic inflammation, especially within the ileum (Fig. 1A is a representative example). Histopathological sections of the ileum identified MAP by modified Ziehl-Neelson staining for acid-fast organisms (Fig. 1B), which was later confirmed by standard culture and PCR methods (Table 1). MAP was successfully isolated from intestinal lesion, mesenteric lymph nodes, liver and spleen of both subclinically infected animals. All isolates were genotyped by SSR analysis as >13G and 5GGT repeats, which was identical to MAP K-10 culture (15G and 5GGT) used for macrophage infection.

Gene expression of MAP during natural infection

Analysis of MAP from infected tissues showed differential expression of 2167 genes compared to broth

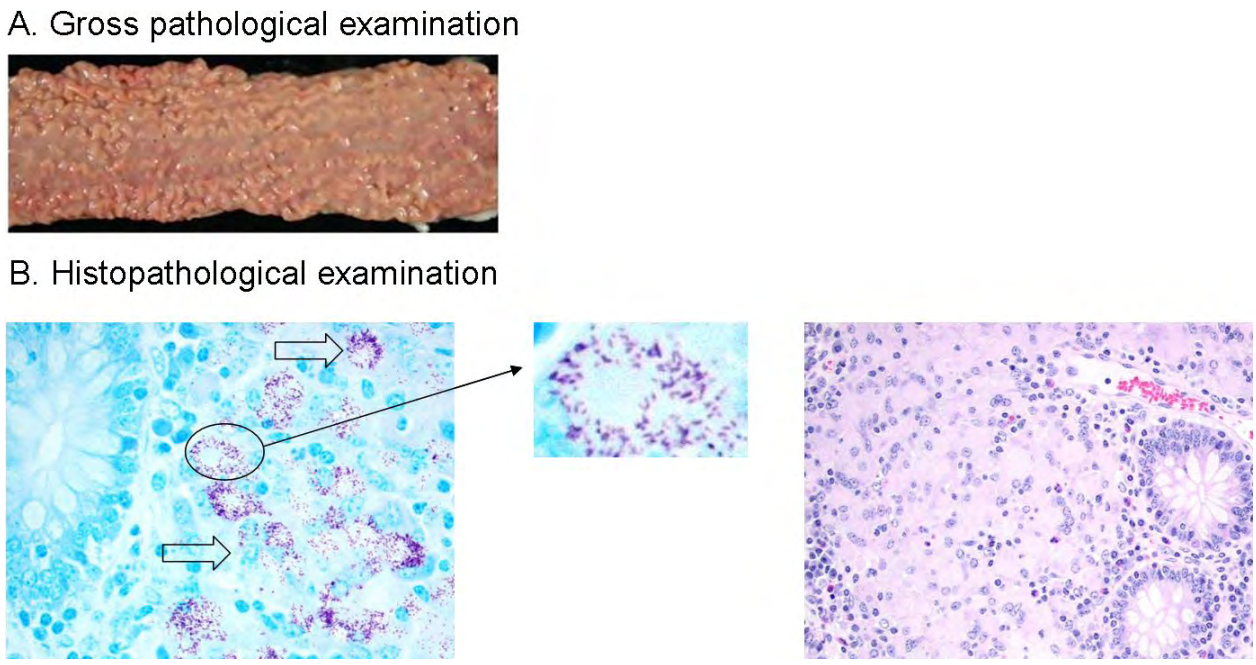

Figure 1 MAP infection in subclinically infected animals. (A) Section of bovine ileum infected with MAP: Longitudinal section of ileum showing inflammation and corrugated appearance of inner mucosal layer from a dairy cow with subclinical Johne's disease.(B) Histopathology of bovine ileum with MAP: Acid fast stain (400×) (left) and hematoxylin and eosin stain (100×) (right) of an ileal section of subclinical JD cow in Fig. 1A showing MAP organisms.

cultures. After multiple test corrections, 1795 genes were significantly different at $q \leq 0.05$ by unpaired t-test. Amongst these, 1684 genes were altered at ≥1.5 fold change and 1054 genes at fold change ≥2.0 compared with corresponding MAP isolates in broth culture. Table 2 shows a list of operons and Additional file 1 Tables S1, S2 and S3 show complete lists of genes differentially regulated during natural infection.

Shared and variable genes between the ileum and MLN are represented in Additional files 1, Tables S2 and S3. Genes were classified into various functional groups based on clusters of orthologous genes (COG) classification and the percent gene expression of each group was calculated. Functional groups enriched in both ileum and MLN belonged to virulence (i.e. MAP1575c, MAP3162c), unknown function or poorly described cellular pathways (i.e. MAP3812c, MAP4269c). Genes belonging to transcription (i.e.: MAP1736, MAP2418) and lipid metabolism and transport (i.e.: MAP0556c, MAP1451) were specifically enriched in the ileum, while energy production and conversion (i.e.: MAP1171, MAP2620c) and inorganic ion transport and metabolism (i.e.: MAP0982c, MAP3141c) were enriched in MLN (Fig. 2).

Table 1 Fecal culture results of MAP isolated from intestinal tissues

Animal ID	Organ	Colony Count	Test Result
386	Ileum	>100	positive
386	Mesenteric lymp node	>100	positive
39	Ileum	1-10	positive
39	Mesenteric lymp node	>100	positive

MAP organisms were grown in Herrold's egg yolk medium for 12 weeks at 37°C. The colonies were counted.

Gene expression of MAP in an in vitro macrophage infection assay

A total of 562 MAP genes were differentially expressed during macrophage infection compared with broth culture. Amongst them, 556 genes had a ≥1.5 fold change

Table 2 List of operons expressed in tissues

Operon	Function
MAP0150c-MAP0152c	Acetyl-coA dehydrogenase
MAP0232c-MAP0237c	Cell wall biosynthesis
MAP0564-MAP0569	MCE family
MAP1778c-MAP1780c	Lipid metabolism
MAP0107-MAP0116	MCE family
MAP2171c-MAP2177c	Mycobactin biosynthesis
MAP3464-MAP3465	ABC transporters
MAP2310c-MAP2314c	Fatty acid degradation
MAP1712-MAP1716	Fatty acid biosynthesis
MAP1522-MAP1523	Fatty acid biosynthesis
MAP2569c-MAP2571c	Glycosyl transferase

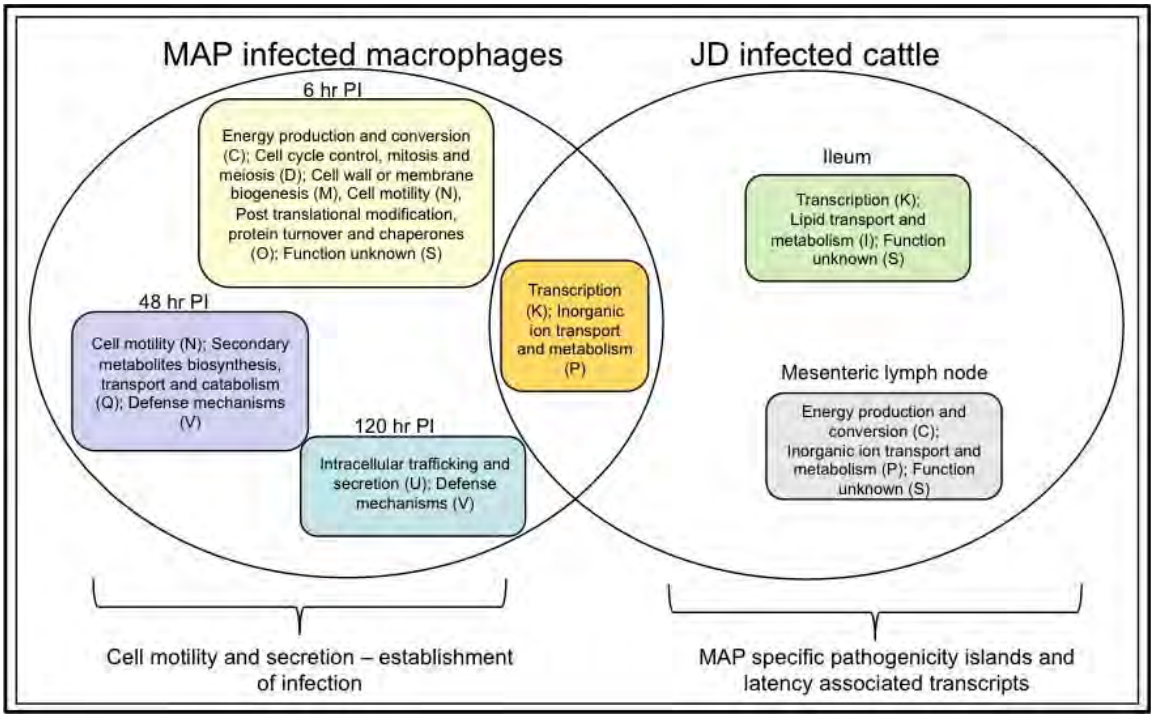

Figure 2 Classification of differentially expressed MAP genes into Clusters of orthologous genes (COG) groups. Differentially expressed genes in the tissues or infected macrophages were grouped based on clusters of orthologous genes (COG) classification. Significantly enriched COGs under each condition are represented in the Venn diagram. Shown in the parenthesis is the code for each COG category.

and 462 genes had ≥ 2.0 fold change ($p \leq 0.05$). At 6 hr post infection (PI), upregulated genes of significant interest included serine/threonine protein kinase, *pkn*B (MAP0016c), ATPase, AAA family protein (MAP0167) and PPE family protein (MAP1675). At 48 hour PI MAP upregulated PE family proteins (MAP0140; MAP0339, MAP1507), transcriptional regulators (MAP0475; MAP2428c) and *fad*D27 protein (MAP3156). Finally, at 120 hr PI MAP displayed higher induction of genes concerning major membrane protein, *mmp*L4 (MAP0076, MAP1240c), MCE-family proteins (MAP0566, MAP0759), PE-family proteins (MAP0140; MAP4076), oxidoreductase (MAP0444; MAP3507), lipase, *lip*E (MAP0248) and ABC transporters (MAP0563). A total of 55 genes were shared across different time points in the macrophage infection assay using MAP K-10 strain. Fig. 2 shows the distribution of the differentially expressed genes across three time points and Additional file 1, Tables S4, S5 and S6 shows the detailed list of genes.

Comparisons of gene expression profiles of naturally infected tissues and in vitro macrophage infection

While a total of 126 genes were commonly expressed between infected tissues and macrophages, 928 and 336 genes were specifically represented in tissues or macrophages, respectively (Fig. 2, Additional File 1, Tables S1 and S7). Functional categories belonging to transcription (MAP1631c, MAP1634, MAP3967) and inorganic ion transport and metabolism (MAP1110, MAP3773c, MAP4171) were represented both in tissues and macrophages (Additional Fig. 2). Macrophage specific gene expression represented functional categories belonging to cell cycle control (MAP2990c), cell wall biogenesis (MAP0670c), cell motility (MAP1506) and secretion (MAP1515). Tissue specific gene expression included genes categorized into virulence mechanisms and those that were not represented in any of the COG groups. Furthermore, MAP regulates expression of persistence related genes such as MAP0033c (WhiB family protein), MAP0038 (probable biofilm regulator), and MAP0075 (mycobacterium specific membrane protein) during natural infection.

Expression of MAP lineage specific genes during natural infection

Approximately 96 genes distributed in six loci (LSP 4, 11, 12, 14 and 15) were recently described as MAP lineage specific genomic insertions; majority of these genes were consistently upregulated (fold change > 2.0, $p < 0.05$) in the in vitro infected macrophages whereas downregulated in the tissues of both the animals (Additional File 1, Tables S8 and S9) [19]. Loci of interest include LSP 4 and 11, which carry putative prophages, transposons and unique

sequences with no hits in NCBI. MAP0858, located within LSP 4, has conserved domains resembling those of a virulence factor (proteophosphoglycan) belonging to *Leishmania*. LSP11 contains MAP2149c, which has conserved domains to that of SARP (**S**treptomyces **A**ntibiotic **R**egulatory **P**rotein) family transcriptional factor. Located within LSP 12 includes a mammalian cell entry (*mce*) operon (MAP2190 - MAP2194) which was downregulated in the tissues whereas MAP2189 (*mce*) and MAP2180c (a beta lactamase like protein) were upregulated in the macrophages. Downregulated genes located within LSP 14 belong to ABC transporter operon (MAP3731c - MAP3736c), siderophore biosynthesis operon (MAP3741 - MAP3746) and oxidoreductase (MAP3756c). However, an oxidoreductase (MAP3744), and ABC type transporter (MAP3739c) and a polyketides synthase (MAP3763c) belonging to LSP14 were all upregulated in macrophage infections. An ABC transporter operon (MAP3774c - MAP3776c), which is located on LSP 15, was downregulated in infected tissues. Interestingly, MAP3773c, a probable Ferric Uptake Regulator protein on LSP 15, was downregulated in the tissues and upregulated in experimentally infected macrophages. Lastly, located within the LSP specific for cattle MAP strains is an enzyme involved in xenobiotic biodegradation and metabolism (MAP1728c *yfnB*) was downregulated in the tissues whereas upregulated in the in vitro infected macrophages.

Real-time validation of microarray data

We selected seven genes for real-time PCR to validate microarray results. These genes were chosen based on their roles in diverse pathways. Selected genes included membrane protein (MAP0283c), inorganic ion transport (MAP0782, MAP2488), iron acquisition (MAP2173c), energy production and metabolism (MAP3898, MAP4120) and finally an LSP specific for cattle strains of MAP (MAP1728c). RNA extracts used for microarray analysis (ileum, MLN and macrophages) were also analyzed for their level of expression by real-time PCR assay with primers designed using universal probe library (Roche, Indianapolis, IN). The expression of these genes in the tissues of JD cows shows the same trend in microarray and the real-time analyses. MAP K-10 broth culture was used as a control to determine fold change. Fig. 3 demonstrates the fold change ratios of selected MAP genes in the microarrays as compared to their gene expression in real-time after normalization with a housekeeping gene *secA*.

Discussion

The hallmark of MAP infection is the subclinical manifestation of a persistent intestinal infection. Yet, surprisingly, there remains a paucity of studies investigating the intracellular lifestyle of MAP in the intestinal epithelium in comparison to research involving macrophage and/or lymphocyte models [4,9]. We sought to fill this critical knowledge gap by reporting the first transcriptome analysis of MAP in infected tissues and macrophages. Both the ileum and mesenteric lymph node have been suggested to act as potential MAP reservoirs within the host; therefore, it is critical to understand the MAP pathways that function to govern this persistence [1,20,21]. The current trend in MAP research is to isolate and analyze regulated gene sets given defined, in vitro stress related cues or during a particular infection stage using surgical methods and various animal species [3,22,23]. However, we have taken a more directed approach to uncover common and unique pathways utilized by MAP in intestinal tissues using the natural host under natural infection. Elucidation of the transcriptome active in local infection sites is expected to not only augment our knowledge of MAP pathogenesis, which will lend itself to the establishment of a host-pathogen interactome, as well as rational design of vaccines and/or antimycobacterial therapeutic modalities.

MAP residing within the ileum is primed for persistence in subclinical infection

Pathogenic mycobacteria have the uncanny ability to persist within the host for an indefinite period of time that can last several years [24,25]. Although the genes and signals that induce persistence remain unclear, mycobacteria entering this phase are characterized by a state of chronic or prolonged non-replication [24]. One cue that primes the cell to enter into the non-replication stage is the stringent response, which is characterized by the *relA* controlled production of hyperphosphorylated guanosine ((p)ppGpp) activated upon nutrient deprivation, hypoxia and oxidative stress [26,27]. Together *relA* and (p)ppGpp are able to combat hostile environments by negatively regulating bacterial "life" signals such as DNA and protein machinery. Interestingly, we have identified a unifying theme from naturally infected host tissue as the downregulation of several energy, carbohydrate, amino acid and lipid metabolism as well as transcriptional and DNA replication related genes. Similar attributes of the stringent response were found to be selectively upregulated within the ileum. For example, the RelA/SpoT domain-containing protein has a threefold upregulation in the ileum. Recently Geiger and colleagues have shown that the RelA/SpoT domain-containing protein, RSH synthetase/hydrolase enzyme, in *Staphylococcus aureus* is responsible for maintained production of (p)ppGpp and concomitant repression of genes regulating translational machinery [28]. Furthermore, a single metabolism gene regulating menaquinone biosynthesis and consequent production of vitamin K

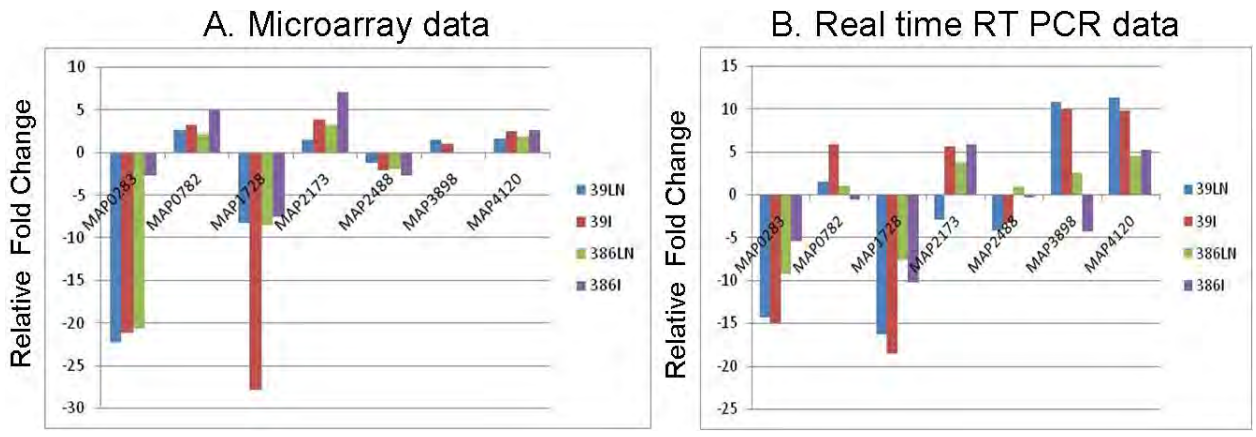

Figure 3 Comparisons of fold changes of selected genes by microarray and real time RT PCR. (A) Selected MAP genes that were differentially regulated (up or down) after subtraction with broth culture (data in linear scale). (B) These genes were validated for their expression pattern by real-time PCR to demonstrate similar trends in gene expression (data in logarithmic scale).

(MAP4052) was uniquely upregulated in the ileum [29]. In addition to initiating the synthesis of mycobactins, menaquinone biosynthesis genes have been shown to be a critical factor in maintaining non-replicating mycobacterial cell viability [30].

Stringent response priming of MAP cells is most likely due to host inflicted stresses, particularly nitric oxide resultant in DNA damage [26]. Previous studies examining MAP "scrapings" from the intestinal wall of JD clinical cattle show significant upregulation of *katG*, a bacterial catalase gene used to combat oxidative stress [31]. Furthermore, granulomatous lesions within the ileum or lymph node isolated from cattle naturally infected with either MAP or *M. bovis*, respectively, have enhanced immune-staining for natural resistance-associated macrophage protein 1 (NRAMP1) and inducible nitric oxide synthase (iNOS), which together synthesize nitric oxide [32,33]. Although we did not identify enrichment of *katG* in the ileum, we show upregulation of MAP2836, a LexA repressor, which is stimulated upon DNA damage and stress and results in the arrest of cell division and induction of DNA repair [34]. Similarly, increase in a LysR transcriptional regulator (MAP2442) within the ileum is indicative of an oxidative stress response [35]. These data suggest that during the early stages of infection, MAP is primed for persistence by the stringent response in order to avoid oxidative stress and DNA damage. This appears to be a "watershed moment" in the intracellular lifecycle of MAP as persistence during subclinical infection will ensure its survival and future dissemination into other organs.

MAP evades immune detection in the MLN

Similar to MAP pathways found in the ileum, the majority of MAP genes involved in energy, carbohydrate, inorganic ion, DNA repair, transcription and translation pathways are downregulated. However, there is a lack of stringent response as well as persistence-associated expression. The MLN contains populations of circulating effector cells, such as T and B cells; therefore, MAP may downregulate the aforementioned pathways to avoid detection by the host immune system [16]. Furthermore, common to both ileum and MLN, MAP upregulates several genes associated with cell envelope and outer member biogenesis (MAP1905c, MAP3019c and MAP3979). It is well established that mycobacterial cell wall components have immunomodulatory functions that enable pathogenic mycobacteria to escape immune surveillance by suppression of pro-inflammatory cytokines, phagosome-lysosome fusion and MHC class II presentation [5,36-38]. Thus, MAP may surround itself with complex cell wall associated glycolipids to prevent recognition and continue t unabated by the host immune system.

Expression of lineage specific large sequence polymorphisms (LSPs) during natural and in vitro macrophage infection

Comparative genomics of the *M. avium* complex (MAC) revealed that MAP evolved as a pathogen by acquiring large segments of DNA (i.e. pathogenicity islands) via horizontal gene transfer [19,39-41]. Our study is the first to directly show that some of these putative pathogenicity islands are associated with virulence. Contrary to expression found within the tissues, genes belonging to the LSPs were upregulated in macrophage infection. Qt-RT-PCR analysis also demonstrated that MAP1728c (*yfnB*), a gene involved in xenobiotic biodegradation and metabolism located within the LSP (deletion 2) specific for cattle MAP strains was downregulated in the tissues [19]. This is consistent with the regulation of other

MAP genes, which suggests that MAP transcriptional machinery remains silent in the tissues. Several iron related genes were downregulated in tissues including LSP15, a MAP unique pathogenicity island that encodes a ferric uptake regulator (MAP3773c), as well as the ABC transporter (MAP3731c - MAP3736c), and a possible siderophore biosynthesis operon (MAP3741-MAP3746) that contains a FUR binding box within the intergenic region [42]. This is of significant interest as part of this region (MAP3731c-MAP3736c) has previously been shown to be immunogenic and preliminary studies indicate its use as a potential vaccine candidate [43]. Furthermore, our genome analysis revealed that a type VII secretory system (esx3) was located immediately downstream of LSP15. Esx3 has recently been shown to be essential for mycobactin synthesis and we have identified its repression by the MAP iron dependent regulator (IdeR) in the presence of iron [44,45]. Taken together, transcriptional analysis suggests that LSP14, 15 and esx-3 form a major pathogenicity island that may play a potential role in maintaining iron homeostasis and hence survival within the macrophage.

Conclusions

MAP is an extremely resilient pathogen that employs a number of regulatory pathways to ensure its survival. Regulatory pathways that govern the lifecycle of MAP appear to be specified by tissue and cell type. While tissues show a "shut-down" of major MAP metabolic genes, infected macrophages upregulate several MAP specific genes along with a putative pathogenicity island responsible for iron acquisition. Despite differences in gene programs found within tissues and cell types, the overriding rule of MAP is to progress by deception either by entering a persistent state, shielding by complex cell wall components or hiding in the macrophage. Many of these programs rely on the advanced interplay of host and pathogen and in order to decipher their message, an interactome must be established using a systems biology approach [25,46]. Preliminary interactomes for the current study are reminiscent of those being developed for *S. pyogenes* and *H. pylori* and show promising networks that may aid in our understanding of overall pathogenesis as well as potential targets for novel vaccines and therapeutics [47,48]. The findings presented in this study will lend themselves in meeting this future challenge of creating a MAP-host interactome.

Methods

All cattle work in this study was performed according to institutional guidelines and approved animal care and use protocols at the University of Minnesota.

Sampling from subclinical JD cows

Two sub-clinically infected but apparently healthy dairy cattle, identified as low shedders by routine serological and fecal culture methods at the University of Minnesota Veterinary Diagnostic Laboratory, were purchased from a farmer and euthanized for this study. The infection status of the animals were established using standard serology for MAP-specific antibody (Idexx Laboratories, Inc., Westbrook, ME) [49] and fecal culture [50]. At necropsy, sections from affected portions of the intestines ileum, ileocecal junctions, and the surrounding enlarged mesenteric lymph nodes (MLN) were harvested, wrapped in aluminum foil and either snap-frozen in liquid nitrogen or fixed in formalin for RNA extraction and histopathological examination, respectively. All samples were stored at -80.0°C until RNA extraction. Furthermore organs were triturated and cultured for of the presence MAP using standard mycobacterial culture techniques. Sections of the MLN and ileum were taken for microscopy using hematoxylin and eosin staining and acid fast staining. A total of seven slides were created and imaged for each stain.

Genotyping of MAP

MAP colonies were sub-cultured in Middlebrook 7H9 broth (MB7H9) (DIFCO, Lawrence, KS) supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Fischer Scientific, Inc., Pittsburgh, PA) and mycobactin J (2 mg/L) (Allied Monitor, Inc., Fayette, Missouri) at 37°C with subtle shaking. MAP isolates were determined free of contaminant bacteria by absence of growth on Brain-Heart Infusion (BHI) agar at 37°C. Following genomic DNA extraction using a standardized protocol (Qiagen, Valencia, CA), isolates were confirmed for MAP specific IS*900* insertion sequence by PCR and agarose gel electrophoresis. MAP isolates from infected tissues as well as MAP cattle strain K-10 (MAP K-10) were genotyped based on short sequence repeats (SSR) from two polymorphic (G and GGT) loci as described [48,49].

Macrophage infection assay

Monocyte derived macrophages (MDMs) were prepared using a previously described method [4,51]. Briefly, blood was collected from the jugular vein of a JD-free healthy cow and mixed with an equal portion of acid-citrate dextrose to prevent coagulation. Blood was transferred in 40 mL aliquots into DNase/RNAse free conical tubes and centrifuged at 2,200 rpm for 20 min. at room temperature. Buffy coats were separated, washed in 1X D-PBS and layered on a 58% percoll gradient (Sigma-Aldrich, St. Louis, MO). Cells were collected from percoll, washed 1X PBS and expanded in RPMI containing

20 percent autologous serum at 37°C in 5 percent CO_2. MDMs were allowed to incubate for 4 days prior to seeding. MDMs were subsequently seeded at ~2.0 × 10^7 cells/flask in 25 cm2 flasks and allowed to adhere for 2 hr. A seed stock of MAP K-10 was sub-cultured and grown to mid-logarithmic growth phase (OD_{600} = 1.0) in MB7H9 broth (supplemented with OADC enrichment medium and 2 μg/ml of mycobactin J) at 37°C on a shaker set at 120 rpm. MAP K-10 was used at a 20:1 multiplicity of infection (MOI) in all infections. Infection was conducted in RPMI containing 2% autologous serum. Following infection after 2 hr,, MDMs were washed twice with fresh, pre-warmed serum-free RPMI 1640 (Gibco(r) Invitrogen, Inc., Carlsbad, CA) to remove non-adherent bacteria and the cultures were subsequently grown in RPMI 1640 with 2% autologous serum for 6, 48 and 120 hrs in duplicate at each time point.

Nucleic acid extraction

Prior to RNA extraction, all surfaces and equipment were treated with RNAse Away (Molecular Bioproducts, Inc., San Diego, Inc.). For total RNA extraction, ~30 mg of mesenteric lymph nodes and ileum were ground separately in liquid nitrogen using a mortar and pestle and dissolved in 600 μL of RLT buffer (Qiagen Inc., Valencia, CA). Total RNA from infected MDMs (6, 48 and 120 hrs p.i,) and MAP K-10 broth cultures were extracted by TRIzol reagent (Invitrogen Inc., Carlsbad, CA) per manufacturer's instructions. Samples were homogenized in a mini bead-beater (Biospec) with 0.3 ml of 0.1 mm sterile RNase-free zirconium beads for 4 min. followed by RNA extraction using RNeasy (Mini) kit (Qiagen Inc., Valencia, CA). All samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to eliminate genomic DNA contamination. The purity and yield of total RNA samples was examined using Nanodrop spectrophotometer (Thermo Scientific Inc., Wilmington, DE) and Agilent 2100E Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Purity of RNA samples were validated by the absence of MAP locus 251 amplification via PCR. All samples were stored at -80°C until later analysis.

Enrichment and confirmation of MAP transcripts

Total RNA obtained from naturally infected tissues and experimentally infected MDMs were processed to remove host RNA as well as ribosomal RNA. Similarly, the total RNA from broth cultures of tissue isolates and MAP K-10 were enriched for bacterial messenger RNA by removing ribosomal RNA. All samples were subjected to RNA amplification and analyzed on a regular denaturing agarose gel and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Furthermore, the presence of MAP specific genes was confirmed using RT-PCR, sequencing and BLAST analyses (data not shown) prior to use in microarrays.

Sample processing and microarray hybridizations

All microarray experiments were conducted using the minimal information about a microarray experiment (MIAME) guidelines. Polyadenylated host mRNA and bacterial rRNA were eliminated by processing the samples with MICROBEnrich and MICROB*Express* Bacterial mRNA Purification Kits (Ambion Inc., Austin, TX), respectively. RNA samples were amplified using MessageAmp(tm) II-Bacteria Kit for prokaryotic RNA amplification system (Ambion Inc., Austin, TX) and labeled with SuperScript(tm) Plus Direct cDNA Labeling System (Invitrogen Inc., Carlsbad, CA). MAP transcripts from infected tissues (two sections each for ileum and mesenteric lymph node) and macrophage infection assay (performed in duplicates) were combined individually with sheared genomic DNA of MAP K-10 labeled with BioPrime(r) Plus Array CGH Genomic Labeling System (Invitrogen Inc., Carlsbad, CA) and hybridized onto 70-mer oligonucleotide microarrays (obtained from Dr. Michael Paustian, NADC, Iowa). Every predicted open reading frame in the MAP strain K-10 genome is represented on this array. One 70-mer was designed for each gene with a total length of less than 4000 bp, while longer genes were split in half and one 70-mer oligo was designed for each half. Additional details of this microarray design can be found elsewhere [52]. RNA from MAP K-10 broth culture and tissue isolates was also processed in the same manner. After overnight hybridization, microarray slides were washed and scanned using the HP Scanarray 5000 (PerkinElmer Inc., Waltham, MA). Images were collected and stored for expression analyses. Microarray experiments were repeated twice for each sample.

Microarray data analysis

Numeric data was extracted from the two-channel hybridization images using the microarray image analysis software, BlueFuse (BlueGnome Ltd, Cambridge). Following normalization by global lowess, the gene expression data was analyzed by GeneSpring GX 10.0 (Agilent Technologies Inc., Foster city, CA). Two group T test was performed to identify the differentially expressed MAP genes (DEGs) and multiple test correction was applied to the T test. The DEGs in natural infected tissues (ileum and mesenteric lymph nodes) and in vitro infected macrophages were identified after normalizing the data with MAP in broth culture. The lists of genes obtained from the above were analyzed using Basic Local Alignment Search Tool (BLAST) algorithm in National Center for Biotechnology Information (NCBI) database against the MAP K-10 genome and the 11 mycobacterial genomes listed in the NCBI databank.

Table 3 Primer sequences used in Q-RT PCR

Gene and direction	Sequence
MAP0233c, F	ggggtagaaggacaggaagc
MAP0233c, R	agttctacgccagcatcgac
MAP0283, F	caatcttccgggtctaccac
MAP0283, R	gagccggtactgatggtga
MAP0782, F	ttcgtgtgcctgtgcaac
MAP0782, R	gcgacttcgttggtggtc
MAP1728, F	cagccacaaatacgacatcc
MAP1728, R	gtgacgaaggctgtttgga
MAP2173c, F	gcagggtgcggtagtgac
MAP2173c, R	ccgagtatctggtcgaggtg
MAP2488, F	gccggttgctcaactacct
MAP2488, R	tcaggcagaacgtcaggaa
MAP3698, F	ccgtcgatgtaccaccagt
MAP3698, R	catcggctccttggtgat
MAP4120c, F	ggaaaccaagggatgtcgt
MAP4120c, R	acgagacgctgcaagagc
secA, F	ggcctgctccttgaggtt
secA, R	gcgcaaggtgatctacgc

Gene IDs were categorized into various functional groups based on Clusters of Orthologous Groups (COGs). Differentially regulated genes were also uploaded in Pathway Studio 6.0 (Ariadne genomics Inc., Rockville, MD) with the *M. tuberculosis* H37Rv database to explore the cellular context of differentially expressed genes by computational methods of protein network identification.

Quantitative Real-time PCR validation

Selected genes from microarray data were validated using two-step SYBR-green based quantitative real-time PCR (Roche, Indianapolis, IN) analysis in Roche Light-Cycler 480 II (Roche Inc., Indianapolis, IN). Primers were designed using web-based tools, Primer3 http://frodo.wi.mit.edu/primer3/ or Universal Probe Library (Roche Inc., Indianapolis, IN) and verified by BLAST searches to confirm their specific binding to target sequences (Table 3). The following cycle program was used: denaturation at 95°C for 15 min. and PCR at 95°C for 10 s, 65°C for 15 s, 72°C for 22 s for 55 cycles. RNA (ileum, MLN and macrophage) used in microarray analysis was also used in real-time PCR. MAP K-10 broth culture served as a control for all RNA samples. Test and control samples were normalized using the house keeping gene, *secA*, and relative expression was calculated by $2^{-\Delta\Delta CT}$ method [53]. Results are reported as fold change. Each sample was conducted in triplicate.

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Additional material

Additional file 1: MAP identified genes in ileum, mesenteric lymph node and in vitro infected bovine macrophages. Fold changes, putative functions and regulation of MAP genes uniquely identified and shared in the ileum, mesenteric lymph node and in vitro infected bovine macrophages. Tables: S1-S9. S1: Ileum specific MAP genes. S2: Mesenteric lymph node specific MAP genes. S3: Ileum and Mesenteric lymph node shared MAP genes. S4: Macrophage specific MAP genes (6 and 48 hrs PI). S5: Macrophage specific MAP genes (6 and 120 hrs PI). S6: Macrophage specific MAP genes (48 and 120 hrs PI). S7: Common MAP genes between natural and in vitro infection. S8: Expression of MAP lineage specific LSPs in the tissues of naturally infected cattle. S9: Expression of MAP lineage specific LSPs in the in vitro infected macrophages.

Additional file 2: Pathway analysis of COGs enriched in tissues and macrophages. COGs enriched in tissues or macrophages were used to identify interactions with other groups and their diverse roles in various cellular processes using Pathway Studio 6.0 (Ariadne genomics Inc., Rockville, MD). Pictorial representation of the interactions of (A) Lipid metabolism genes centered on *kasA* (MAP 1998), a cell wall biogenesis gene upregulated in the tissues and (B) Intracellular trafficking and secretion genes centered on PE_PGRS4, a PPE family gene upregulated in macrophages. kasA interacts with other proteins such as pknL (MAP1914) and plays a role in lipid metabolism and cell survival. PE_PGRS4 interacts with other proteins such as prrC, rpiA and plays a role in colonization and virulence. Green ovals indicate metabolites, red ovals indicate genes and gold rectangles indicate processes.

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Authors' contributions

SS conceived the idea. HKJ, EAL and SS analyzed the data and wrote the manuscript. SG performed experiments and analyzed the microarray data. WWX and JZT helped in microarray data analysis and bioinformatic analysis. JBP and SJW contributed to new reagents. JS performed necropsy, histopathology and microbiological culture. All authors read and approved the manuscript.

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Utility, limitations, and promise of proteomics in animal science

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ABSTRACT

Proteomics experiments have the ability to simultaneously identify and quantify thousands of proteins in one experiment. The use of this technology in veterinary/animal science is still in its infancy, yet it holds significant promise as a method for advancing veterinary/animal science research. Examples of current experimental designs and capabilities of proteomic technology and basic principles of mass spectrometry are discussed. In addition, challenges and limitations of proteomics are presented, stressing those that are unique to veterinary/animal sciences.

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1. Background: transition from genomic studies to proteomic studies

Proteomics has rapidly moved from a relatively new technology to a rapidly maturing essential tool in the omics age. Its existence is largely due to the success of the genome projects as well as rapid advancements in commercial mass spectrometers. The field of proteomics could not exist without the success of the genome projects. The genome projects of the various domestic animals will continue to increase our ability to associate desired traits with the necessary genes/proteins. Genome projects have given us the gene sequence and gene expression information with the use of techniques such as real-time PCR and microarray assays. However, understanding the genes is only the beginning of the biological story. Proteomics is giving us information regarding protein expression and post-translational modifications. Together, these techniques have brought animal research to a molecular level.

Proteomics is the large-scale study of protein expression, protein–protein interactions, or post-translational modifications (for more specific reviews see Cravatt et al., 2007; Gingras et al., 2007; Ong and Mann, 2005; Witze et al., 2007). Unlike other methodologies that analyze a few proteins at a time, proteomics can analyze thousands of proteins in a single experiment. This ability to analyze thousands of proteins gives the field of proteomics a unique capability to demonstrate how cells dynamically respond to changes in their environment. Therefore, a goal of proteomics is to identify new and potentially unexpected changes in protein expression, interaction or modification as a result of an experimental treatment. Generation of large proteomic data sets is expected to demonstrate the interdependence of cellular processes important for normal cell growth or a cell's response to abnormal or disease conditions. In essence, a proteomic approach enables an investigator to step back and without prejudice, view the whole picture of cellular functions instead of one particular action of one protein. This type of research enables the discovery of unexpected connections between cellular processes and can serve as a precursor to new hypotheses.

2. Utility: what questions does a proteomics experiment ask?

Proteins play many fundamental roles in all biological processes. Some functions of proteins include: structural building blocks, conduits of information, controllers of chemical reactions, and antimicrobial defense mechanisms. The functional abilities of cells are dynamic as cells respond to stimuli or stresses. Much of a cell's response to stimuli or stress is manifest by the alteration of the

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expression levels of various proteins. Identification and understanding of proteins involved in biological processes have been goals of scientists for decades. This ability to analyze thousands of proteins gives the field of proteomics a unique capability to demonstrate how cells can dynamically respond to changes in their environment.

Proteomic experiments can be directed towards detection of certain known proteins of interest, or an indirect or shotgun approach can be taken. In this review we will concentrate on the proteomic approach that has the goal of identifying the greatest number of proteins in a biological sample. This type of broad proteomic experiment is referred to as "shotgun" proteomics. The ultimate goal of a shotgun proteomics experiment is to identify all the proteins in a sample. However, the number of proteins in a sample, the dynamic range of expression of the various proteins in a sample, and the limitation of the mass spectrometers, make this a very difficult goal to achieve. Consequently, the preparation of the sample plays a significant role in the amount of protein information one can extract from a proteomic experiment. Currently a single proteomics experiment can yield hundreds, and at times thousands, of protein identifications (Aebersold and Mann, 2003).

Shotgun proteomic experiments can be divided into two groups, survey and expression. A survey proteomics experiment is an experiment designed to identify as many proteins in a sample as possible. Whereas expression proteomics adds quantification of proteins to the goal of identifying as many proteins as possible in a given sample, for the purpose of assessing the potential effects of an experimental treatment.

2.1. Survey proteomics

Survey proteomics data allows an investigator to accomplish two basic goals. First, to obtain an overview of the types of proteins identified in a specific cell or tissue, and to then organize them by biological process, cellular compartment, or molecular function. From this type of data general conclusions about the types of proteins that are important for that cell's function can be reached. For example, in a survey proteomics experiment of bovine neutrophils it was found that 25% of the proteins identified fell into two functional categories, immune functions and cellular mobility (Lippolis and Reinhardt, 2005). This observation is in harmony with the known function of neutrophils, which is to relocate themselves to the site of an infection and to release various antimicrobial agents important in the resolution of an infection. Alternatively, a survey proteomic experiment that identified proteins of the bovine milk fat globule membrane showed that nearly 60% of the proteins observed fell into three functional categories, membrane/protein trafficking, cell signaling, and fat transport and metabolism (Reinhardt and Lippolis, 2006). Again, this observation is in harmony with the known functions of the milk fat globule membrane and the secretory mammary epithelial cells (sMEC) from which they are derived. From these data one can quickly observe that a proteome is not a set of conserved proteins with a few proteins that distinguish various samples, but the protein profile observed from a sMEC is quite different from that observed from the membrane of a neutrophil.

A more specific example of the utility of survey proteomics can be illustrated by the following. The proteomic survey of the milk fat globule membrane found the toll-like receptor (TLR) 2 and 4 proteins (Reinhardt and Lippolis, 2006). In this report the authors determine that two TLRs and CD14 were present on milk fat globule membranes and therefore by extension on the apical membrane of sMEC. This observation helps to explain the mechanism of how sMEC respond to the presence of a pathogen and that these cells likely play an important role in the innate immune response.

2.2. Expression proteomics

In addition to the more general categorization of proteins, the second basic goal of a survey proteomics is to identify interesting proteins for further study. Expression proteomics uses experimental treatment with the dual goal of identifying proteins and measuring experimentally induced changes in the expression of those proteins. Most often the type of quantitation is relative, meaning that two or more samples are compared and one of the samples acts as a reference to which the others are measured (Bantscheff et al., 2007; Lippolis and Reinhardt, 2008; Ong and Mann, 2005). For example, an experiment that compared the effect of growth of *Escherichia coli* in whole milk compared each protein identified to the same protein from the same *E. coli* grown in laboratory media (Lippolis et al., 2009). The result was information about the relative expression of 1000 proteins and any changes of protein expression due to a change in growth media. It has been shown that growth rates of various strains of *E. coli* in an infected gland prior to the immune response have been correlated with final severity of the infection (Kornalijnslijper et al., 2004). The interrogation of this type of proteomic data can yield important clues about protein function and in this case the proteins involved in the adaptation of *E. coli* for growth in milk and subsequently the bacteria's ability to cause an infection. Of the 1000 proteins that were identified with expression data, 20% were up regulated in *E. coli* grown in milk compared to laboratory media and 8% were down regulated.

One group of proteins that were up regulated in *E. coli* grown in milk was those whose function involved iron transport. Iron is an essential element for the growth of bacteria and the availability of iron has been linked to the pathogenicity of the bacteria (Klebba, 2003). Lactoferrin is a milk protein that binds and is part of the body's innate mechanism to sequester iron to deprive invading pathogens of this necessary nutrient (Legrand et al., 2005). *E. coli* grown in milk were shown to up regulate four outer membrane siderophore receptors (FecA, FepA, FhuA and Fiu), suggesting that the bacteria are reacting to the milk environment by increasing their ability to remove iron from their environment. An attempt has been made to block iron uptake by *E. coli* by generating an immune response to one of the siderophore receptors (FecA). The researchers were successful in generating antibody but unsuccessful in changing the clinical severity of

an infection (Takemura et al., 2002). This proteomic data has demonstrated the redundancy siderophore receptors, and may explain the lack of effect on the blocking of only one of a series of siderophore receptors in *E. coli*. The proteomic data further suggests that any successful blocking of iron uptake for the purpose of moderating an infection would require blocking multiple iron acquisition pathways (Lippolis et al., 2009).

3. Limitations: what are some difficulties of a proteomic experiment?

The promise of proteomics does come with a number of difficulties that must be addressed or acknowledged to reduce the limitations of this technology. Some of the factors that limit proteomics are the quality of the genomic databases, the complexity and dynamic range of proteins in a sample, the capabilities of various mass spectrometers, and the cost of these experiments.

3.1. Quality of the genomic database

Proteomics owes its existence, in part, to the Human Genome Project. Completed in 2003, this 13-year project had as two of its goals to identify all of the genes in human DNA and determine the sequence of the 3 billion chemical base pairs that make up human DNA. The success of this project has resulted in sequencing projects in other lab animal models, companion animals, and economically important agricultural animals and plants. However, the genomic databases of these species are not at the same level of completeness. As sequencing projects for these various species are completed the number and confidence of proteomic techniques to identify proteins will be increased.

Many proteomic software packages have been developed that align the data obtained from the mass spectrometer with various protein databases (e.g., NCBI non-redundant protein database, Swissprot). Two of the most common software packages (Mascot, Sequest) assume that the protein database contains all proteins of interest. If this assumption is not true then the software will likely identify a homologous protein from a species that has been more completely sequenced. However, we have found that the protein score will be reduced if mass spectrometry data is not matched to the correct species. A reduced score could lead to a misidentification or a lack of identification. To illustrate this point, the same mass spectrometry dataset was processed using a database from 2004 or 2007. Importantly, in 2006 the Bovine Genome Sequencing Center released an updated version of the genomic assembly, adding more genomic information to the bovine protein database. The data showed that twice as many proteins were identified as bovine using the 2007 database then using the 2004 database. Not only did the number of proteins identified as bovine increase but also the average protein score increased, indicating greater confidence in the identification. From these data we conclude that a more complete database results in a better data set as determined by the number of proteins of the correct species and the higher identification scores (Lippolis and Reinhardt, 2008).

The presence, absence or modification of a protein has limited value without knowledge of the function of the protein. The modulation of a protein with a known function can then be associated with a cellular compartment, biological process, or molecular function. In contrast, the modulation of a protein with an unknown function tells little to nothing about the biological, cellular, or molecular functions of the cell type. The challenge of proteomics is to sort through a mountain of data and find the information about protein changes that are critical to the cells response to changes in its environment. Continued efforts to categorize proteins according to their known or predicted functions are necessary and are currently underway. These efforts are critical to understand all the information that a proteomic experiment can yield. Consortiums such as The Gene Ontology (www.geneontology.org) and the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg) provide means to group proteins by function or into biological processes.

3.2. Mass spectrometers

The improvement in proteomic analysis is largely due to the advancement in the field of mass spectrometers. Many mass spectrometers can detect and identify peptides in the femtomole (10^{-15}) to attomole (10^{-18}) range (Moyer et al., 2003). In fact, investigators have been able to sequence as low as 10 amol of trypsin-digested cytochrome *c* (Martin et al., 2000). The level of sensitivity possible by some mass spectrometers allows investigators to identify proteins from a relatively small number of cells. For example, 160 fmol of protein is approximately 10^{10} molecules; if that protein were expressed in a cell at 1000 copies per cell, this protein would be detectable from 10^7 cells. This number of cells is easily obtainable from bacterial samples and eukaryotic cells. However, instrument sensitivity is not the only factor that is important to the detection and identification of proteins by mass spectrometry. Resolution, dynamic range, the ability to select and separate an ion of interest, and the ability to trap and store ions are all important factors that affect the utility of mass spectrometry in the field of protein chemistry. There are many types of mass spectrometers that can be used for proteomic studies, and each accomplishes the task of protein identification in a slightly different way (Domon and Aebersold, 2006; Han et al., 2008; Herbert and Johnstone, 2002; Steen and Mann, 2004; Yates, 1998, 2004). Therefore, the goals and budget of the research project must be aligned with the capabilities of the mass spectrometer.

The choice of which mass spectrometer to use will depend on several factors: the type of sample preparation, whether quantitation is expected, the detection of post-translational modification, and the ability to identify as many proteins as possible. First, sample preparation methods may affect the type of instruments used. Some preparation methods are generally associated with how the peptides enter the mass spectrometer. There are two predominant methods for ionization of peptides (Fig. 1): matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) (Yates, 1998). To use MALDI one mixes a protein or peptide sample with a UV-absorbing

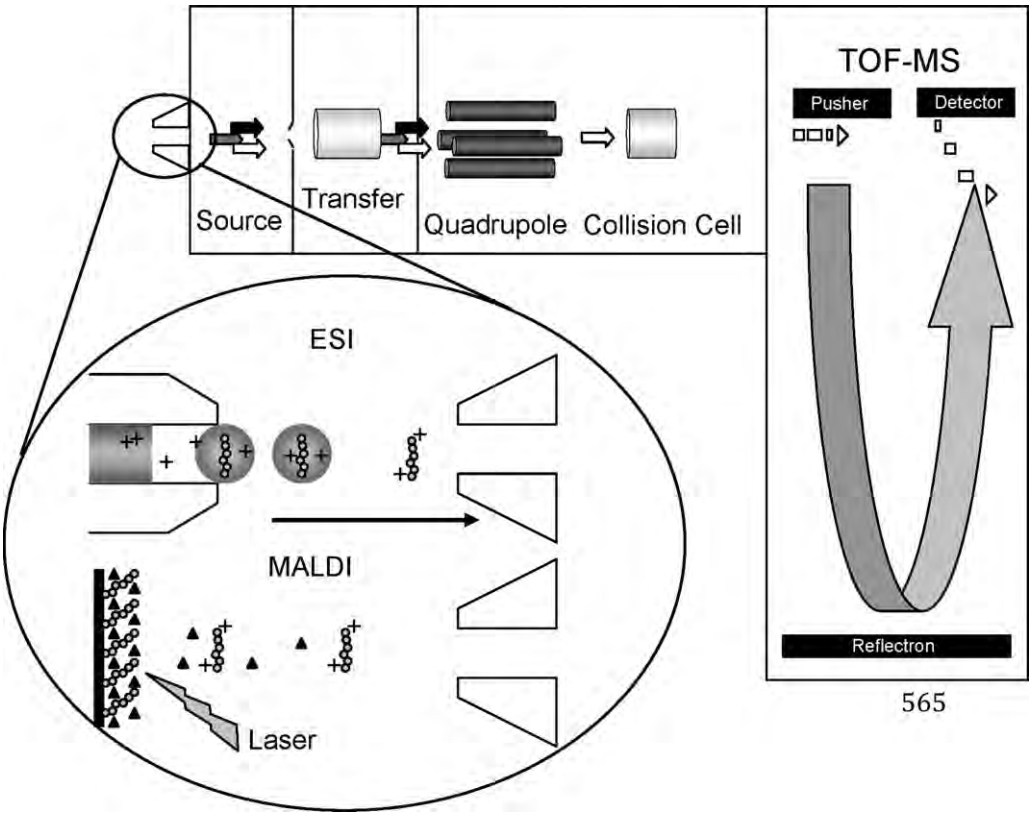

Fig. 1. Tandem mass spectrometer. Ionization of peptides is accomplished by one of two methods, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Charged gas-phase peptides are generated by ESI or MALDI when the acidic HPLC solution containing peptides evaporates or sublimation of peptides occurs when a peptide–crystal matrix is hit with a laser. Ionized gas-phase peptides are then drawn into the mass spectrometer. The qTOF is a tandem mass spectrometer that has a quadrupole mass analyzer in front of a Time-of-Flight mass analyzer. Ionized peptides travel in a constant stream through the instrument to the TOF. There, distinct packets of peptides are pushed orthogonally to their original flight path. A mass spectrum (MS) containing all the peptides in that package can be observed. To sequence a peptide the quadrupole is automatically set to allow only a single peptide to pass. The single peptide is then fragmented in the collision cell prior to entering the TOF, and the peptide's fragments are analyzed.

molecule (matrix) and allows the sample to dry into crystals. Striking the crystals with a UV laser causes rapid heating of the matrix, resulting in sublimation, proton transfer to the protein or peptide, and transfer of the protein or peptide into the mass spectrometer. Multiple laser strikes lead to more ionized sample, and the laser strikes can continue until the sample is consumed. MALDI is typically associated with gel-based forms of protein separation, as specific protein bands or spots from multi-dimensional gel electrophoresis are extracted from the gel and analyzed for content. In contrast, ESI is associated with liquid chromatography methods of protein or peptide isolation. Proteins or peptides in an acidified solvent are ejected from fused silica tubing into a large electrical potential difference between the tubing and the mass spectrometer inlet (Cole, 2000). This forms charged molecules in solvent droplets, where the droplets evaporate prior to the instrument inlet. ESI can be scaled down to solvent flow levels in the nanoliter per minute range in what is referred to as nanoESI. This allows small amounts (picomoles) of sample to be separated by reverse-phase HPLC columns with internal diameter of 75 μm, through tubing with internal diameter of 20–70 μm, out a spray tip as small as 3 μm.

Proteomic experiments with the goal of quantitation of proteins, identification of post-translational modifications, or the identification of as many proteins as possible can be achieved with several different types of mass spectrometers. However, each mass spectrometer has different strengths and weaknesses (Domon and Aebersold, 2006; Han et al., 2008; Herbert and Johnstone, 2002; Steen and Mann, 2004; Yates, 1998, 2004). Most mass spectrometers used in proteomic experiments have multiple mass analyzers in tandem, with the goal of capitalizing on the strengths of each analyzer. Mass analyzers such as quadrupoles (q) and ion traps, are often used in proteomic experiments to hold, filter, and collide ions. Examples of mass analyzers with higher resolving power and accuracy are Time-of-Flight (TOF), Fourier-transform Ion Cyclotron Resonance (FTICR) and Orbitraps; these analyzers allow the sensitivity, accuracy, and resolution necessary to sequence peptides with greater confidence. More sophisticated (and expensive) instruments typically use analyzers with lower resolving power and accuracy, but with the abilities to trap, filter and fragment ions prior to transfer to the high resolving, high accuracy, and high sensitivity analyzers that determine the mass of the ions. Combinations of mass analyzers to form specific instruments (e.g., qTOF is a

Table 1
Strengths of common mass spectrometers used in proteomics.

Instrument	Ion source	Identification	Quantification	Throughput	Detection of modifications
Ion-trap	ESI	+	+++	++	+++
TOF-TOF	MALDI	++	++	+++	+
qTOF	ESI/MALDI	++	+++	++	+
FTICR	ESI/MALDI	+++	++	++	+
Orbitrap	ESI	+++	++	++	++

The strengths of common mass spectrometers used in proteomic experiments are listed. The mass spectrometers are graded as follows: excellent (+++), good (++), and fair (+).

combination of a quadrupole and a Time-of-Flight) have different strengths as far as peptide identification, protein quantitation, sample throughput, and detection of post-translational modifications (Table 1). Optimal results can be expected when experiments are performed with the mass spectrometer best suited for the experiment.

A factor that affects the choice of instrument used, is the cost and availability of the instrument. Mass spectrometers are expensive to purchase, maintain, operate and, therefore, access can be somewhat limited. In addition, operation of a mass spectrometer requires a significant amount of training and expertise, and therefore requires dedicated personnel. Understanding of the physical limits of the instrument is an important factor in knowing what information the experiment can yield.

The time necessary for a mass spectrometer to scan a peptide is an important limitation. Many mass spectrometers have a number of sequential steps that are necessary for sequencing a peptide. For example, a qTOF instrument is a tandem mass spectrometer, meaning that it has two mass analyzers (Fig. 1). The first mass analyzer in a qTOF is a quadrupole (q), followed by the second analyzer the Time-of-Flight (TOF). The first type of scan that an instrument like this collects is a MS scan and this type of scan is detected by the TOF. The MS scan will indicate all the peptides that are entering the instrument at a given time (Fig. 2). If a peptide exceeds a user-defined threshold, the peptide is then sequenced through a process that causes its fragmentation and subsequent determination of all the fragments, this is called the MSMS scan. During a MSMS scan the quadrupole acts as a mass filter, only allowing a single peptide through. That single peptide is then fragmented in a process called collision-induced dissociation (CID). The peptide fragments are then detected by the TOF, and from that pattern, an amino acid sequence is deduced (Fig. 2). The problem with this type of analysis is that it takes time to complete this cycle and during part of this cycle only one peptide is being analyzed when there are potentially hundreds of other peptides entering the instrument at the same time. Depending on instrument settings, the cycle time to sequence 5 peptides can be as long as 25 s.

The problem of instrument cycle time can be illustrated by the following example. If a proteome that consisted of approximately 30,000 proteins was digested and each protein resulted in an average of 10 peptides there would be 300,000 unique peptides. If the peptides were injected onto a reverse-phase HPLC column that is inline with a qTOF, the peptides eluted off the column over a 60-min gradient, and the instrument cycle time to sequence 5 peptides was 25 s, then the number of peptides sequenced would be just over 700 of the 300,000. Mass spectrometers other than the qTOF may have faster cycle time, but some of these instrument sacrifice mass accuracy and/or resolution. Advancements in mass spectrometer are constantly improving mass accuracy, resolution and cycle time. Newer mass spectrometers can sequence multiple peptides at the same time. Although these instruments do not have the scan time issue, the complexity and dynamic range of the proteins in a sample affect the ability to identify proteins. In addition to advances in mass spectrometers, numerous separation techniques have been developed to reduce the number of proteins or peptides in a single sample, spreading them in multiple samples that are individually injected on a mass spectrometer.

3.3. Complexity and dynamic range

The Human Proteome Initiative has estimated that the 20,500 human genes could encode up to 1,000,000 different proteins (http://www.expasy.ch/sprot/hpi/). Proteome complexities are in part the result of post-translational modifications and alternate splicing of genes (Boeckmann et al., 2005; Harrison et al., 2002; Kettman et al., 2001).

In addition to the number of different proteins, the proteome is further complicated by protein expression differences that can range as much as 10 orders of magnitude in biological fluids, tissues and cells (Brunet et al., 2003; Panisko et al., 2002; Patterson and Aebersold, 2003). The outcome of this complexity and dynamic range is that high abundance proteins in proteomic surveys mask low abundance proteins of high interest, and require simplification of the proteome for robust mass spectrometry analysis. For example, nearly half of the protein in plasma is albumin, and the top ten proteins in plasma make up nearly 90% of the total protein (Cho, 2007). Various fractionation schemes have been used to enable a more complete identification of proteins (Brunet et al., 2003; Huber et al., 2003; Reinhardt and Lippolis, 2006, 2008; Zolotarjova et al., 2008).

3.4. Experimental design

Instrument limitations and the complexity of a sample necessitate experiments to be designed to fractionate and simplify protein samples to enable greater depth of the proteomic discovery. Simplification of a proteome can be achieved by subcellular fractionation, enrichment strategies, chromatography or gel electrophoresis (Stasyk and Huber, 2004). These separation strategies can be used individually or in combination in order to improve detection

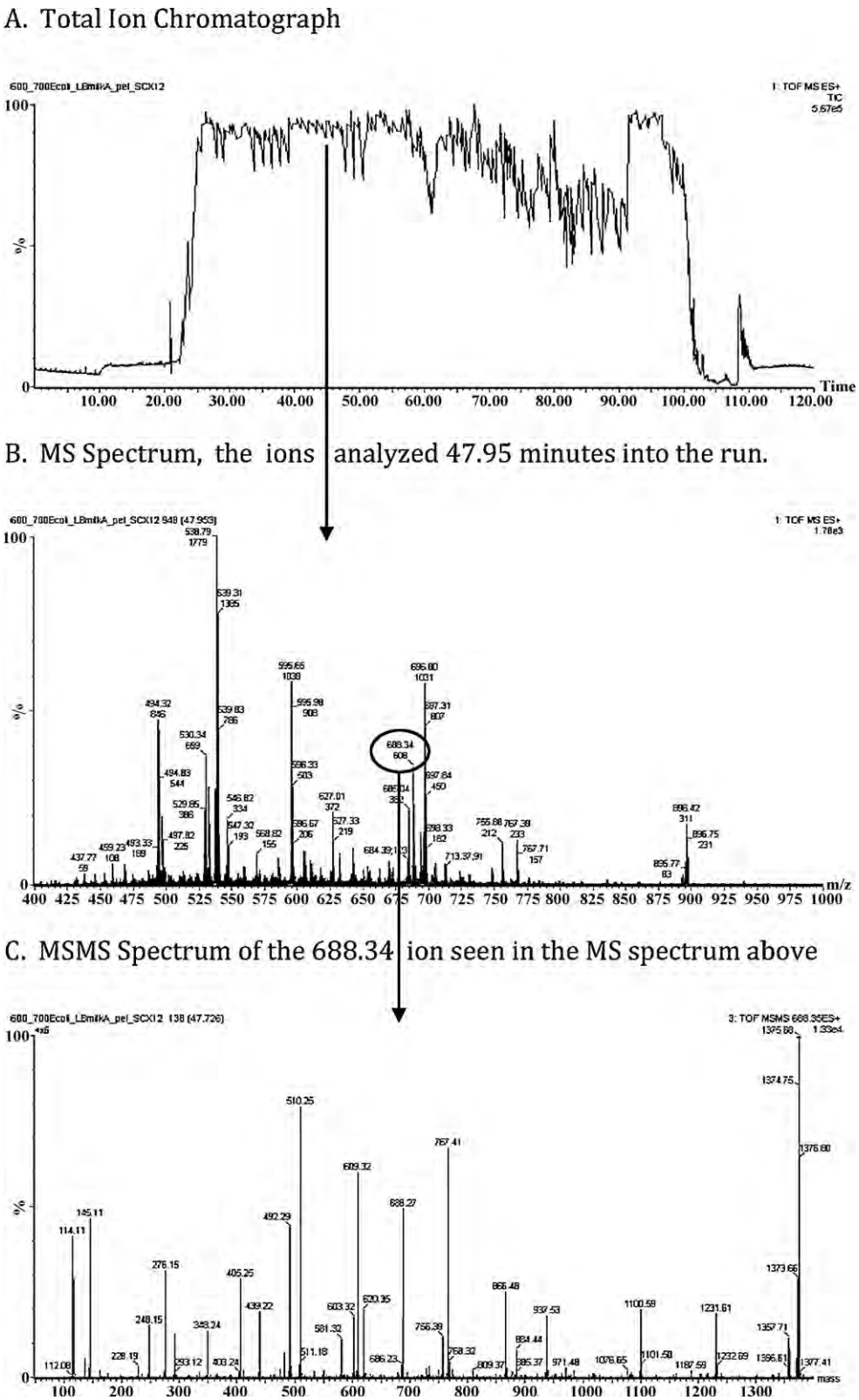

Fig. 2. Mass spectra. This data represents MS and MSMS data from a qTOF mass spectrometer. The top data panel contains a sample MS spectrum. This represents a single 1.5 s scan of the instrument in a 2 h experimental run. At this time point many peptides can be observed. The instrument computer software will select ions with sufficient abundance for subsequent MSMS analysis for sequencing. The bottom panel contains information that led to the sequencing of the 688.3 peptide seen in the top panel.

of low abundance proteins. The choice of which separation strategies to employ will depend on the nature of the sample and the goals of the experiment. For example, a common type of protein separation strategy is referred to as multidimensional protein identification technology (MudPIT), where two different column materials are packed into the same HPLC column (Washburn et al., 2001). In this type of experiment a strong cation exchange (SCX) column matrix is packed next to reverse-phase (RP) matrix. Peptides are injected onto the column and bind to the SCX material. Sequential solutions with different concentrations of salt will free a group of specific peptides from the SCX material and bind to the subsequent RP material. Between each increase in salt concentration, a RP gradient is run, which does not affect the peptides still bound to the SCX column, but will remove peptides bound to the RP column into the mass spectrometer for analysis. Using this technique investigators were able to identify 1484 proteins from *Saccharomyces cerevisiae*, whereas previous experiments using an alternate separation technique yielded just fewer than 300 protein identifications (Washburn et al., 2001).

However, ~1500 proteins identified by mass spectrometry are still well short of the tens of thousands of proteins in *S. cerevisiae*. One alternative is to isolate subcellular components for proteomic analysis, which would likely have a reduced protein complexity. An example of this approach can be seen in the proteomic analysis of bovine milk fat globule membranes (MFGM) (Reinhardt and Lippolis, 2006). MFGM represent the apical membrane of secretory mammary epithelial cells and are easily obtained in milk. As such MFGM contain the proteins necessary for transport of milk, milk fats and minerals such as calcium. However, proteomics of the MFGM is complicated by the fact that between 4 and 8 proteins comprise 60% of the total proteins by weight and 1 protein, butyrophilin, accounts for 30–40% of the total protein by itself (Mather, 2000).

Biological samples can contain a few proteins that make up the majority of the total protein content of the sample; MFGM and plasma are two such examples of this phenomenon. As stated earlier nearly half of the protein in plasma is albumin, and the top ten proteins in plasma make up nearly 90% of the total protein (Cho, 2007). The problem is that most mass spectrometers pick peptides to sequence on the basis of signal intensity; therefore the peptides with the highest signal intensity are more likely to be sequenced. In addition, peptides with higher signal intensity will be more likely to yield an unambiguous spectrum to deduce the sequence. Various solutions to this problem have been proposed, from depletion of abundant proteins to alternate separation strategies. Using a method to deplete abundant proteins, it has been possible to extend the detection of plasma proteins from μg/ml to ng/ml (Anderson and Hunter, 2006).

There are currently two major strategies to deplete overly abundant proteins (Polaskova et al., 2010). Antibody cocktails are commercially available that contain antibodies against the 12 highest abundance human plasma proteins. Thus, specific removal of the most abundant human plasma proteins has yielded greater depth of the proteomic dataset. However, it has also been observed that depletion of abundant proteins can result in the non-specific loss of low abundance proteins (Granger et al., 2005). A second method of depletion uses multiple affinity removal columns, which have random hexapeptides bound to a matrix (Thulasiraman et al., 2005). These hexapeptides provide non-covalent binding sites to capture proteins. Because each amount of each hexapeptide is limited, high abundance proteins are reduced at the same time low abundance proteins are concentrated. The goal of both of these techniques is to eliminate the signal suppression by high-abundance proteins and thus amplify the signal of low-abundance proteins. This later method precludes protein quantitation proteomics.

In addition to depletion strategies are those methods that use antibodies and metal ions as a means to enrich for a specific type of protein. For example, antibodies specific for a class of molecules called major histocompatibility complex (MHC) have been used to isolate these molecules away from other cellular proteins. A MHC molecule binds to a variety of peptide fragments both from proteins normally found in a cell and importantly from pathogens. The MHC–peptide complex is then present on the cell surface for detection by the immune system. MHC molecules and their associated peptides can be precipitated using monoclonal antibodies. The peptides are then separated from the MHC molecule using size exclusion filtration, and sequenced using mass spectrometry (Hunt et al., 1992; Lippolis et al., 2002). Examples of this type of proteomic approach include the determination of the nature of autoantigens that may be involved in the autoimmune disease type 1 diabetes (Nepom et al., 2001) and the nature of antigens involved in the response to diseases such as melanoma (Cox et al., 1994). In addition to the specific isolation of a molecule, antibodies can be used to isolate a class of molecules. Both antibodies and metal ions (immobilized metal ion affinity chromatography [IMAC]) have been used to enrich for phosphorylated proteins (Ptacek and Snyder, 2006). It is estimated that 30% of cellular proteins are phosphorylated, and phosphorylation often acts as an on/off switch for the protein's function. The isolation and subsequent identification of the phosphoproteome is an important new area of discovery.

Non-depletion strategies can also be used, but they also have their limitations. In the example of MFGM where 1 protein, butyrophilin, represents 30–40% of the total protein, investigators chose to isolate the butyrophilin away from the other proteins by SDS-PAGE (Reinhardt and Lippolis, 2006). In short, total MFGM proteins were loaded on a gel and proteins separated. The gel was cut into 37 gel slices, isolating various protein bands. The proteins were then digested and extracted from the gel for analysis by the mass spectrometer. Despite the clear butyrophilin band in the gel (Fig. 3), butyrophilin peptides were detected in every part of the gel. In subsequent analysis of the data we did show that in the area of the butyrophilin band, over 90% of the MSMS spectra in that slice of the gel are identified as butyrophilin peptides and in area distant to the butyrophilin bands a large number of MSMS spectra are identified as butyrophilin (Fig. 3). This shows that a single very abundant protein can cause major problems for

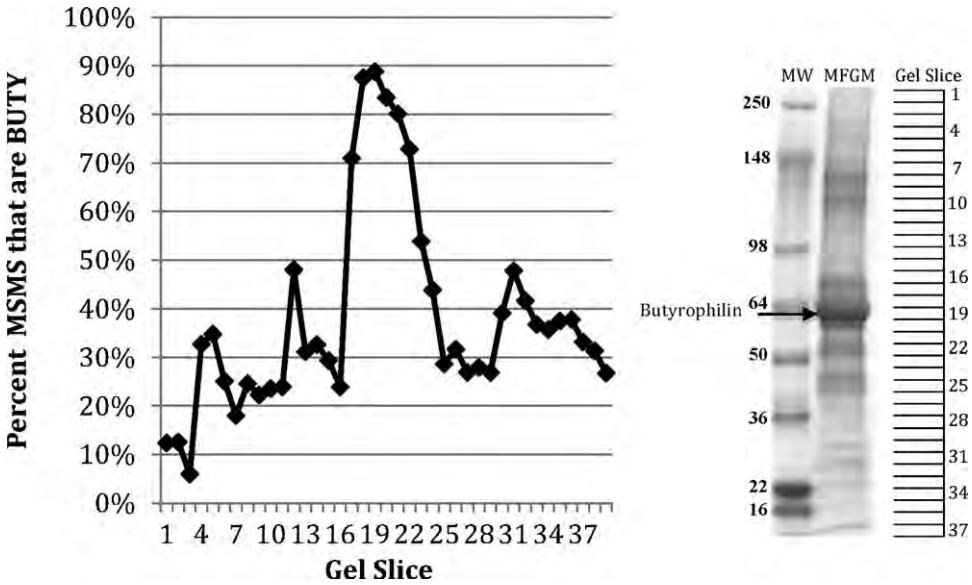

Fig. 3. Gel electrophoresis of proteome. Milk fat globule membrane proteome was separated by one-dimensional gel electrophoresis. The gel was sliced (37 times) and proteins were digested with trypsin in the gel. Peptides were extracted from the gel and the peptides from each gel slice were run on a HPLC column coupled to a tandem mass spectrometer (qTOF). The mass spectrometry data was analyzed using Mascot (Matrix Science) to predict protein matches. All MSMS spectra that were matched to butyrophilin were quantitated for each gel slice and graphed above.

proteomic analysis. In addition, separation techniques such as gel electrophoresis do not have the resolving power to mitigate the interference of an overly abundant protein.

3.5. Data validation

Proteomic experiments generate a large quantity of mass spectrometry data. A typical MudPIT proteomic experiment can yield tens of thousands of MSMS spectra, each requiring the determination of the peptide sequence. Gigabytes of information must be first distilled down to peptide sequences and those sequences matched to proteins, in a process that is termed computational proteomics (reviewed by Colinge and Bennett, 2007; McHugh and Arthur, 2008). Gone are the days of manually determining a peptide sequence from the MSMS spectrum. The number of tools available for computational proteomics has expanded greatly in the last decade. The pace that the field of proteomics will advance is dependent on the development of hardware, software, and data management tools (Table 2).

The first step in understanding the data obtained from the mass spectrometer is to align the data with proteins in the databases. Despite improved software, the continuing challenge for researchers who use proteomics as a tool is how to interpret the data and how much confidence one can have in the proteins identified. How many unique peptides are required to identify a protein? What is the false discovery rate? How are closely related proteins distinguished? These questions are presently a matter of debate in the proteomics field. However, these are the questions that one must address when writing and reading a proteomics paper (Carr et al., 2004; Quadroni et al., 2004; Wilkins et al., 2006).

There are many software packages that take different approaches to transform mass spectrometry data into pro-

Table 2
Various proteomic software packages.

Name	Function	Website
Mascot	Peptide and protein identification/quantitation	www.matrixscience.com
Sequest	Peptide and protein identification/quantitation	www.thermo.com
ProteinProphet	Validation of protein identification	http://proteinprophet.sourceforge.net/
X!Tandem	Peptide matching with MSMS spectra	www.thegpm.org
Scaffold	Peptide and protein identification and data organization	www.proteomesoftware.com
Trans-proteomic pipeline	Peptide and protein identification and quantitation. Tools for data visualization	http://tools.proteomecenter.org/wiki
The Gene Ontology	Protein functional database	www.geneontology.org
Kyoto Encyclopedia of Genes and Genomes	Protein functional database	www.genome.jp/kegg
PEAKS	De novo search program for peptide and protein identification	www.bioinformaticssolutions.com

A brief list of common proteomic software packages.

tein identification and expression data. In a study that compared four peptide identification algorithms to identify peptides from a complex protein sample, 608 peptides were identified by one or more search algorithm. Of the 608 peptides identified, only 335 peptides were correctly identified by all four search programs, and a range from 0 to 46 peptides were correctly identified by only one search program (Kapp et al., 2005). This data shows that there can be a significant number of differences in MSMS spectra interpretations of a dataset by various software packages, and that the use of multiple software packages may add to the confidence in the identification of the peptides.

Evaluation of mass spectrometry data is typically accomplished by the major software packages that interpret the dataset. Most of these software packages report a score on which the confidence of the mass spectrum solution is based. Based on the number of peptides and their scores, a protein score is calculated that can indicate the confidence that one can have in the identifications of proteins that exceed a threshold score. As discussed above, the various software packages can identify different subsets of proteins from the mass spectrometry data. Therefore, those that are identified by multiple software packages would have a higher confidence than those that were identified by a single software package.

In order to better determine the error rate of protein identification, methods have been developed to determine what is called the false discovery rate. In this method MSMS spectra are searched against a protein database of choice followed by a search against the same protein database where the sequences have been reversed, randomized, or shuffled (decoy database) (Elias and Gygi, 2007). The expectation is that there will not be any true matches to the decoy database. Therefore, any matches to the decoy database are defined as false positive matches. The false discovery rate is calculated by calculating the number of false positive matches divided by the sum of the number of true positive matches plus the number of false positive matches. This information can help the investigator alter the significance thresholds to optimize the data to limit the reporting of false positive protein identifications.

4. Promise: what will proteomics be able to do?

Despite the limitations that the field of proteomics currently has to deal with, it has become an extremely important tool in biological sciences. The first unique advantage of this technology is the fact that a fairly large number of proteins can be identified and quantitated at one time, without any prior knowledge that any specific protein might exist in a sample. Analyzing a proteomic dataset can often lead to surprising results, and the unexpected may be the most interesting observation. In fact, most shotgun proteomic experiments are not typical "hypothesis driven" experiments, but may be better considered experiments designed to find a hypothesis. In these experiments hundred of proteins can be identified whose expression is altered by a defined experimental condition. Some changes in protein expression may be expected and even well characterized. However, some may be unexpected or unknown and lead to new hypothesis for the connections between protein expression and cellular processes. For example, comparing the protein expression from *E. coli* grown in milk versus laboratory media, it is quite expected that proteins, such as beta-galactosidase, involved in lactose utilization would be up regulated in those bacteria grown in milk (Lippolis et al., 2009). In contrast, the protein S-rebosylhomocysteine lyase or the LuxS gene product was up regulated in bacteria grown in milk. This enzyme is critical for the synthesis of a bacterial hormone like compound called autoinducer-2 (AI-2) (Sperandio et al., 2003). AI-2 is involved in the regulation of hundreds of genes, many of which are virulence genes (DeLisa et al., 2001). Whereas the observation of beta-galactosidase perhaps was expected, the observations of the LuxS gene product was not and has lead to the generation of hypothesis from this unexpected result.

Other protein detection methodologies, such as western blots, rely on the availability of quality antibodies. Animal scientists are fully aware of the limited number of antibodies available to them when compared to those specific for human or rodent proteins. It is this independence from antibodies that makes proteomic experiments especially attractive to those wanting to identify proteins in domestic animals. Furthermore, the breadth of data generated by methodologies using antibodies would be less than a sweeping proteomic survey.

Although mRNA quantitation methods are very powerful tools there is the limitation that they do not measure the functional end product, which is the protein. Numerous studies have shown examples of a lack of correlation between mRNA and protein abundance (Griffin et al., 2002; Gygi et al., 1999; Ideker et al., 2001). For example, changing the carbon source for yeast resulted in a 500-fold increase in mRNA for a gene involved in sugar metabolism, whereas the corresponding protein only increased 10-fold. In addition, some genes showed no change in mRNA levels but showed significant increases in protein levels (Griffin et al., 2002). These examples of a lack of correlation highlight the importance of linking mRNA expression results with subsequent proteomic studies. In addition, mRNA expression experiments cannot detect protein post-translational modifications.

Proteomic experiments are also ideally suited for detection and identification of post-translational modifications on proteins. Antibody based detection of phosphorylated proteins are limited to detection of a whole class of phosphoproteins (e.g., proteins with a phospho-serine) or a specific phosphorylation event on a specific protein. Proteomics offers the as of yet not fully realized promise of analysis of an entire phosphoproteome. The ability to observe the effect of a stimulus of the entire phosphoproteome with time would be a very powerful tool in understanding the complex and integrated intracellular signaling mechanisms.

The ultimate goal of proteomics is to detect and quantify the tens of thousands of proteins that constitute a proteome. To be able to observe the entire cellular picture of protein expression, location, interaction, and modification under different experimental conditions would aid in the understanding of the molecular mechanism critical for cellular functions. This understanding of cellular functions

would be the basis for rational therapeutic designs to target pathogens and correct disease conditions.

Conflict of interest

Authors have no financial or other relationships that would inappropriately influence this work.

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Transcriptional Response of *Leptospira interrogans* to Iron Limitation and Characterization of a PerR Homolog▽†

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Leptospirosis is a globally significant zoonosis caused by *Leptospira* spp. Iron is essential for growth of most bacterial species. Since iron availability is low in the host, pathogens have evolved complex iron acquisition mechanisms to survive and establish infection. In many bacteria, expression of iron uptake and storage proteins is regulated by Fur. *L. interrogans* encodes four predicted Fur homologs; we have constructed a mutation in one of these, *la1857*. We conducted microarray analysis to identify iron-responsive genes and to study the effects of *la1857* mutation on gene expression. Under iron-limiting conditions, 43 genes were upregulated and 49 genes were downregulated in the wild type. Genes encoding proteins with predicted involvement in inorganic ion transport and metabolism (including TonB-dependent proteins and outer membrane transport proteins) were overrepresented in the upregulated list, while 54% of differentially expressed genes had no known function. There were 16 upregulated genes of unknown function which are absent from the saprophyte *L. biflexa* and which therefore may encode virulence-associated factors. Expression of iron-responsive genes was not significantly affected by mutagenesis of *la1857*, indicating that LA1857 is not a global regulator of iron homeostasis. Upregulation of heme biosynthetic genes and a putative catalase in the mutant suggested that LA1857 is more similar to PerR, a regulator of the oxidative stress response. Indeed, the *la1857* mutant was more resistant to peroxide stress than the wild type. Our results provide insights into the role of iron in leptospiral metabolism and regulation of the oxidative stress response, including genes likely to be important for virulence.

Leptospirosis is a widespread zoonotic disease caused by pathogenic species of the spirochete *Leptospira*. Human infection occurs via contact with infected animals or with soil or water contaminated with urine of carrier animals (1, 36). In tropical countries, large outbreaks of human leptospirosis have occurred following severe flooding, while in developed countries, cases usually occur via occupational contact or recreational activities (27, 35). Clinical manifestations of leptospirosis in humans are extremely variable, ranging from a self-limiting, influenza-like syndrome through to rapidly fatal forms involving multiorgan failure, with death occurring in 5 to 25% of severe cases (8, 49). Transmission of *Leptospira interrogans* from rodents to humans is usually indirect, as *L. interrogans* can survive for considerable periods of time in contaminated soil or water. Currently, little is known about pathogenesis mechanisms or transcriptional regulation in *Leptospira* spp.

Iron is an essential cofactor in many biological processes and is therefore required for the growth of most organisms, including *Leptospira* spp. (23), although some organisms, such as *Borrelia burgdorferi*, have eliminated the need for iron (59). In the animal host, the concentration of free iron is insufficient for bacterial growth, as the majority of iron is bound to high-affinity protein carriers such as transferrin, lactoferrin, and ferritin (12). The low availability of iron is therefore one of the first barriers that bacterial pathogens must overcome in order to survive and establish infection in the host.

To cope with changes in iron availability, bacterial pathogens have evolved response mechanisms for regulation of iron homeostasis and complex strategies for iron acquisition from the host. In many bacteria, transcriptional regulation of various genes involved in iron acquisition is under the control of the iron-dependent negative regulator, Fur (ferric uptake regulator) (4). When iron is abundant, Fur forms a complex with ferrous iron and blocks transcription of target genes by binding to conserved promoter regions termed Fur boxes. Under iron-limiting conditions, the Fur-Fe^{2+} complexes dissociate and Fur can no longer bind to target genes, allowing expression to proceed. This system enables a rapid response to changes in iron levels. While most genes are negatively regulated by Fur, some genes are positively regulated at the transcriptional level, for example, *pan1*, *norA*, and *nuoA* in *Neisseria meningitidis*, which are involved in anaerobic and aerobic respiration (19). Various *Escherichia coli* genes involved in iron storage, iron metabolism, and defense against oxidative stress are also positively regulated, but at the posttranscriptional level via repres-

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sion of a small RNA, RyhB (46). Virulence factors in various pathogens are iron regulated, for example, the Shiga-like toxin in *Escherichia coli*, diphtheria toxin, and the *Pseudomonas* exotoxin A (9, 13, 65). Various virulence-associated outer membrane proteins in *Vibrio* spp., *Neisseria* spp., and *Yersinia* spp. are also iron regulated (40).

Currently, very little is known about iron acquisition and regulatory mechanisms in pathogenic leptospires. Several outer membrane proteins have been shown to be iron regulated. For example, the TonB-dependent outer membrane receptor protein, LB191, is upregulated (6), while LipL36, LA0412 (pL24), and LA3469 (pL50) are downregulated under low-iron conditions (17). Processing of LipL32, the highly conserved major outer membrane protein of pathogenic *Leptospira* spp., has also been shown to be affected by iron availability, possibly due to iron-regulated proteases (17). Using random transposon mutagenesis with the saprophytic *Leptospira biflexa*, Louvel et al. (45) identified five hemin-requiring mutants; three of these had insertions in a gene encoding a protein with homology to FecA, the TonB-dependent ferric citrate receptor in *E. coli*, while the remaining two mutants had insertions in a FeoB-like protein. FeoB is conserved in many bacteria and plays a role in transport of ferrous iron (4). *L. interrogans* possesses a complete set of genes for *de novo* synthesis of heme (26) as well as a functional heme oxygenase (50) and is therefore capable of heme synthesis as well as using heme/hemoglobin as a sole iron source by scavenging iron from hemoglobin. Expression of genes involved in heme biosynthesis is likely to be regulated by a two-component system encoded between *hemE* and *hemL* (43). Heme (in the form of hemoglobin) is likely to be the main source of iron for pathogenic *Leptospira* spp. *in vivo*, and heme oxygenase is required for virulence and *in vivo* survival (52). *L. interrogans* can also bind to hemin via the hemin-binding proteins LB191 (also named HbpA) and LipL41 (6). The availability of genome sequences for *L. interrogans* has enabled identification of putative genes involved in iron acquisition and regulation (44, 54, 55, 60). The genomes of *L. interrogans* serovars (svs) Lai and Copenhageni encode four predicted Fur homologs; we have generated a transposon mutation (*la1857*) in one of these (51). Other bacterial species possess more than one Fur homolog. For example, *Bacillus subtilis* has three (Fur, PerR, and Zur) (11, 25, 68) which have significant sequence and structural similarities, but each responds to different environmental signals and regulates different target genes (24). All proteins in the Fur family are metalloregulatory proteins and include sensors of iron, zinc, nickel, and manganese, while other Fur family proteins, such as PerR, also bind to iron, but this converts PerR into an oxidation sensor rather than an iron sensor (37). Sensitivity of PerR to metal-catalyzed oxidation allows the protein to respond rapidly to low levels of hydrogen peroxide.

In the present study, we characterized the global transcriptional response of *L. interrogans* to iron limitation. Under low-iron conditions, 49 genes were downregulated and 43 genes were upregulated. However, 54% of differentially expressed genes were of unknown function. Some of these may encode unique leptospiral virulence factors or proteins necessary for *in vivo* survival. We also studied the transcriptional profile of the *la1857* mutant and found little overlap with the transcriptional response to low-iron conditions. Instead, LA1857 appears to play a role in the response to oxidative stress, consistent with our finding that the *la1857* mutant has an 8-fold increase in resistance to hydrogen peroxide. These data suggest that LA1857 functions as a PerR homolog, a member of the Fur family of regulators.

MATERIALS AND METHODS

Culture conditions. *L. interrogans* sv Manilae was grown in EMJH medium (33) at 30°C. For the *la1857* mutant, M776, kanamycin at a final concentration of 50 μg/ml was added. To study the effects of iron limitation, triplicate 25-ml cultures of the parent and *la1857* mutant were grown to mid-log phase (4×10^8 to 6×10^8 cells/ml), and then the bacteria were pelleted (4,000 × *g*, 15 min, room temperature), washed in 10 ml of EMJH base (Difco), and resuspended in 1 ml EMJH base. The culture was then transferred to 100 ml EMJH medium with or without preincubation with 40 μM 2,2′-dipyridyl (Sigma) and grown to mid-log phase before harvesting of bacteria for RNA isolation.

RNA purification. Leptospires were grown to a density of 2.5×10^8 to 6.5×10^8 cells/ml before harvesting of bacteria for RNA purification. The cell count was determined as described previously (2). Cultures were harvested and RNA was isolated from leptospires as described previously (41) and then further treated with a Turbo DNA-free kit (Ambion), according to the manufacturer's instructions. To confirm lack of contamination with genomic DNA, 0.5 μg of RNA was used as template in a PCR using primers toward *la0615*, encoding a conserved hypothetical protein in serovars Lai and Manilae (Table 1).

Microarray construction, hybridization, and analysis. The *L. interrogans* microarray was printed as described previously (41). Labeled cDNA probes were synthesized from 2 μg of total RNA using a 3DNA Array 900MPX expression array detection kit (Genisphere) and then hybridized with the microarray slides, as described previously (41). Microarray hybridizations were scanned using a GMS 418 array scanner (Genetic MicroSystems). The Cy3 and Cy5 images were aligned and then overlaid with a grid using ImaGene, version 5.1 (Biodiscovery), to allow accurate gene identification and quantification of fluorescence intensity. Spots were examined manually, and poor spots with very low signals or inconsistent morphologies were flagged for elimination from the analysis.

Three independent RNA samples (biological replicates) from parent and mutant strains of *L. interrogans* serovar Manilae grown with or without 2,2′-dipyridyl were compared. The orientation of Cy3 to Cy5 labeling was the same for replicates 1 and 3, while replicate 2 was a dye swap. Raw data from the comparisons were analyzed using the web-based program BioArray Software Environment (BASE) (62). Raw data from replicate arrays (3 for each comparison) were combined and used for further analyses, as described previously (41). Genes were considered to be differentially expressed if they were at least 2-fold up- or downregulated with a P value of <0.05.

Validation of microarray data by real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed as described previously (41). The primers used are shown in Table 1. Known concentrations of *L. interrogans* serovar Manilae genomic DNA were used to construct a gene-specific standard curve so that the concentration of template in each reaction could be determined. The gene encoding flagellum subunit B, *flaB*, was used as the normalizer for all reactions. Melting curve analysis confirmed that all PCRs amplified a single product.

Statistics for category comparisons. Fisher's exact test was performed for pairwise comparisons of the frequencies of various groupings of leptospiral genes affected by iron limitation (Fig. 1).

Phylogenetic analysis. Sequence alignment and phylogenetic analysis were performed using the software Geneious Basic, version 4.7.5, to compare similarity between each of the putative *L. interrogans* Fur homologs with other Fur, Zur, and PerR proteins.

Oxidative stress assays. Three biological replicates of mutant and wild-type cultures were tested in triplicate for sensitivity to hydrogen peroxide and cumene hydroperoxide. Hydrogen peroxide and cumene hydroperoxide were serially diluted using EMJH medium in a 96-well plate from 6.87 mM to 6 μM and 5 mM to 3 μM, respectively, in a 100-μl final volume. The mutant or wild-type cultures (5×10^6 cells in 100 μl EMJH medium) were added to the wells, and the plates were incubated overnight at 30°C. The minimum bactericidal concentration (MBC) was then determined by dark-field microscopy.

Microarray data accession number. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus database and are accessible through GEO series accession number GSE20422 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20422).

TABLE 1. Sequences of primers used in this study

Gene	Forward primer	Reverse primer
gyrB (*la0005*)	CCGACAAAAAAATTTCCACAA	CCCATGTAAACCCCCAGAA
la0615	CAAAATTGTATGAAAAGCGGACG	GAGAATATCGTTAAGGTCGTGTTC
la0695	GGTCCAACGGGAAGACAAGTT	GTGCCGTCGCTTCTACGGTA
la0706	CAAAGATTACCCGGAGCTCAGA	CGCCGTAATTCCAGGAAGAG
la1857	CTCACCGCCGATCAGGTTT	GCACGAGAAGCGTTTGGC
la2242	CCTTCCGTTGGTTTGATTGT	CGCTTGAGAACCCCTATGAA
la2641	TTTTGCTTCCGGTCTTGGAT	CGTTATCACCAGCGATTGAATC
la2824	CACGGACGAGTTGGTTAGGG	CAATTGCCCCCACCATAATC
la2887	TTTGCCTTACTCCGGTGGAA	GAATTGTGGGTTGTATAGAAACCGA
la3094	TTCAAGAACAAGCAGCAAGAGAA	TCCGTAAATGTTCAAACTATGACCTG
la3468	TACCGGAGTTGCTTCCTCTGA	TGACTTCGTGATCTCTTACGTTTCC
la3778	GAATATTACGGATTCGACATACATCG	CCTTGGATGGTTACAACGGATT
lb011	AGATTGGCTCGTTACTAGAGGCA	ACCAGTATGTTCCAAAGCGGG
lb183	TGAAATCCATCTTCCGGGAG	GATGGTGATGATGACGGCTTG
lb186	AATTATCGTACCGCGTTAGATTCC	GGCAAGAATGGATTGTTTTTCG
lb191	CTAAAAAGGACAACGGGAATGG	CACAATCTGTGATTCTTCCGGA
lb014	TCGTGCGATGTATCTTGCGA	CCGGAAAATCCTCACCTACAGA

RESULTS AND DISCUSSION

Iron limitation is one of the signals encountered by pathogens upon entry into the host. In order to characterize the global leptospiral response to iron limitation, *L. interrogans* sv Manilae cultures were grown in EMJH medium preincubated with 40 μM 2,2′-dipyridyl. The transcription patterns of these cultures were compared with those of cultures grown in normal EMJH medium. We used 40 μM 2,2′-dipyridyl, as this was the highest concentration at which growth was not severely inhibited. Genes which were up- or downregulated by at least 2-fold with 95% confidence were considered to be differentially expressed. The microarray data were validated by real-time RT-PCR analysis on 13 genes. A correlation coefficient (R^2) of 0.7287 was observed between the fold change values obtained by microarray and real-time RT-PCR analyses (data not shown).

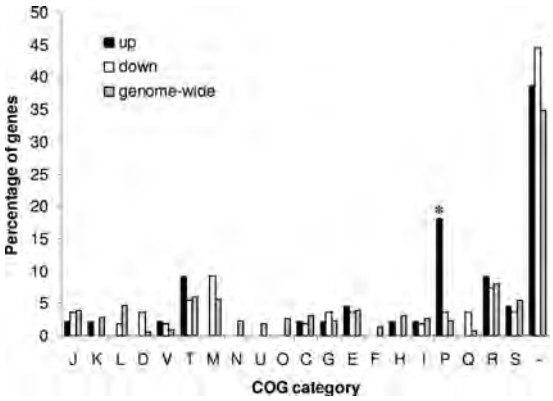

FIG. 1. Percentages of genes which were up- or downregulated under iron limitation and across the *L. interrogans* sv Lai genome in each COG category. The COG functional categories are as follows: information storage and processing (11% of coding sequences in *L. interrogans* serovar Lai genome) (includes J, translation; K, transcription; L, replication, recombination, and repair); cellular processes and signaling (20% of coding sequences in the serovar Lai genome) (includes D, cell cycle control, cell division, and chromosome partitioning; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall, membrane, or envelope biogenesis; N, cell motility; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, and chaperones); metabolism (19% of coding sequences in the serovar Lai genome) (includes C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism); poorly characterized (50% of coding sequences in the serovar Lai genome) (includes R, general function prediction only; S, function unknown; and −, not in COGs). The asterisk indicates that genes predicted to encode inorganic ion transport and metabolic proteins were overrepresented among genes upregulated by low-iron conditions (Fisher's exact test, $P < 10^{-5}$).

Effects of iron limitation on transcription. We identified 49 and 43 genes which were downregulated and upregulated in response to low iron, respectively (Table 2). While the microarray data were generated using *L. interrogans* sv Manilae, for gene nomenclature we have used locus tags from *L. interrogans* sv Lai (LA numbers), except where the gene is unique to sv Copenhageni (LIC numbers) and is present in our draft sequence of the sv Manilae genome. Therefore, while some genes have been predicted to be part of a transcriptional unit in sv Lai and/or sv Copenhageni, it is unknown whether sv Manilae has a similar genome arrangement.

To determine if any groups of genes were overrepresented, the differentially expressed genes were sorted into functional categories on the basis of clusters of orthologous groups (COG) (66) (Fig. 1). Consistent with the high proportion of uncharacterized genes (~50% of coding sequences in the *L. interrogans* genomes), the majority of differentially expressed genes encode proteins of hypothetical or uncharacterized function (56% and 53% of down- and upregulated genes, respectively, in COG categories R, S and no COG designation) (Fig. 1).

Growth under low-iron conditions did not affect expression of genes in COG categories F (nucleotide transport and metabolism), N (cell motility), O (posttranslational modification, protein turnover, and chaperones), or U (intracellular trafficking, secretion, and vesicular transport), suggesting little change to these cellular processes (Fig. 1). There were no upregulated genes in COG categories D (cell cycle control, cell division,

TABLE 2. *L. interrogans* sv Manilae genes differentially expressed under low-iron conditions, grouped by COG[a] category

Regulation and locus tag[b]	Mean fold change	*P* value	COG	Gene	Description of gene product	Predicted location[c]
Downregulated						
LA0055	−3.8	0.01	—[d]		Hypothetical protein	CYT
LA0135	−5.1	<0.01	—		Hypothetical protein	CYT
LA0137	−2.7	0.01	—		Hypothetical lipoprotein	NON-CYT
LA0573	−3.2	0.03	—		Hypothetical protein	CYT
LA0603	−5.2	<0.01	—		Methyltransferase	CYT
LA0983	−5.9	<0.01	—		Conserved hypothetical protein	CYT
LA1005	−10.1	<0.01	—		Aminopeptidase	UNK
LA1389	−6.2	<0.01	—		Hypothetical protein	IM
LA1472	−2.7	0.02	—		Hypothetical protein	CYT
LA1475	−3.4	0.01	—		Hypothetical protein	CYT
LA1478	−2.1	0.03	—		Conserved hypothetical protein	NON-CYT
LA1535	−7.4	<0.01	—		Hypothetical lipoprotein	NON-CYT
LA1536	−4.1	<0.01	—		Hypothetical protein	NON-CYT
LA1539	−4.3	<0.01	—		Conserved hypothetical protein	NON-CYT
LA1593	−7.1	0.01	—		Conserved hypothetical protein	CYT
LA1597	−3.5	<0.01	—		Conserved hypothetical protein	CYT
LA1601	−4.0	<0.01	—		Sugar isomerase	CYT
LA1759	−7.7	<0.01	—		Conserved hypothetical lipoprotein	NON-CYT
LA1796	−9.8	0.01	—		Hypothetical protein	CYT
LA2628	−2.1	<0.01	—		Conserved hypothetical protein	NON-CYT
LA2753	−3.0	<0.01	—		Hypothetical protein	CYT
LA2824	−5.0	0.02	—		Hypothetical protein	IM
LIC12649	−4.8	0.04	—		Hypothetical protein	NON-CYT
LA0630	−4.2	0.01	C		Oxidoreductase	CYT
LA0611	−4.8	0.01	D	*ftsA*	Cell division protein, actin-like ATPase	CYT
LA2459	−2.4	0.01	D		Conserved hypothetical protein	CYT
LA1481	−2.5	0.01	E	*proC*	Pyrroline-5-carboxylate reductase	CYT
LA0634	−8.5	0.01	EP		Permease component of an ABC transporter complex	IM
LA1457	−3.0	0.02	GM		Membrane protein of an ABC transporter complex	IM
LA0632	−2.7	0.01	GT	*ptsN*	Protein-*N*(pi)-phosphohistidine-sugar phosphotransferase, subunit A	CYT
LA1451	−2.0	0.01	I	*pssA*	CDP-diacylglycerol–serine *O*-phosphatidyltransferase	IM
LA1596	−6.1	0.01	J		Methionyl-tRNA formyltransferase	CYT
LA2691	−3.8	<0.01	L		*N6*-adenine-specific DNA methylase	CYT
LA1504	−2.3	0.02	M		Metallopeptidase	NON-CYT
LA1582	−2.1	<0.01	M		ADP-heptose synthase	CYT
LA1595	−2.0	0.05	M		Pyridoxal phosphate-dependent aminotransferase	CYT
LA1611	−2.0	<0.01	MJ		Nucleoside diphosphate-sugar pyrophosphorylase	CYT
LA2690	−3.1	<0.01	P	*bfr*	Bacterioferritin (cytochrome b_1)	CYT
LA0607	−3.3	0.02	Q		Substrate binding protein of an ABC transporter complex	PER
LA1555	−2.1	0.05	Q		Multicopper oxidase	NON-CYT
LA1081	−2.0	0.02	R		Permease of the major facilitator superfamily	IM
LA1476	−2.3	0.04	R		Dehalogenase-like hydrolase	CYT
LA1517	−2.1	<0.01	R		Host factor I-related protein	CYT
LA1592	−3.7	0.03	R		Methyltransferase	CYT
LA0557	−2.0	0.02	S		Conserved hypothetical protein	IM
LA0598	−2.0	<0.01	S		Conserved hypothetical protein	CYT
LA1526	−2.4	0.02	T		Signal transduction protein containing cyclic AMP-binding and CBS domains	CYT
LA1681	−2.0	0.03	T	*phoH*	Phosphate starvation-inducible protein	CYT
LA1600	−4.1	0.02	V		ATP-binding protein of an ABC transporter complex	IM
Upregulated						
LA0569*	3.9	<0.01	—		Conserved hypothetical protein	NON-CYT
LA0570*	5.1	<0.01	—		Conserved hypothetical protein	NON-CYT
LA0571*	3.2	0.01	—		Conserved hypothetical protein	IM
LA0589*	6.2	0.01	—		Conserved hypothetical protein	NON-CYT
LA1375*	2.3	0.01	—		Conserved hypothetical protein	NON-CYT
LA1767*	2.5	0.01	—		Hypothetical protein	CYT
LA1847*	2.7	0.02	—		Hypothetical protein	CYT
LA2060	2.1	<0.01	—		Conserved hypothetical protein	CYT
LA2240*	2.7	<0.01	—		Hypothetical lipoprotein	NON-CYT
LA2241	4.6	<0.01	—		Hypothetical lipoprotein	NON-CYT
LA2342*	2.1	0.01	—		Hypothetical protein	NON-CYT
LA2882*	2.7	<0.01	—		Hypothetical protein	CYT
LA3867*	2.1	<0.01	—		Conserved hypothetical lipoprotein	NON-CYT

Continued on following page

TABLE 2—*Continued*

Regulation and locus tag[b]	Mean fold change	*P* value	COG	Gene	Description of gene product	Predicted location[c]
LA3974*	2.2	0.01	—		Conserved hypothetical protein	CYT
LA4030*	2.0	0.02	—		Hypothetical protein	CYT
LB194	10.1	<0.01	—		Hypothetical lipoprotein	NON-CYT
LB225*	2.0	<0.01	—		Conserved hypothetical lipoprotein	NON-CYT
LA3470	5.6	<0.01	C		Thiol oxidoreductase	NON-CYT
LA2061	2.2	<0.01	E	*metX*	Homoserine *O*-acetyltransferase	CYT
LA2062	2.3	<0.01	E	*metY*	*O*-Acetylhomoserine aminocarboxypropyltransferase	CYT
LB187	2.5	<0.01	G		Permease	IM
LB191	5.8	<0.01	H		TonB-dependent outer membrane receptor	OM
LA0568	5.0	<0.01	I		Outer membrane transport protein	OM
LA0308	2.6	0.01	J	*hemK*	Methylase of polypeptide chain release factors	CYT
LA0572	3.1	0.02	P		TonB-dependent receptor	OM
LA0726	2.7	0.01	P		Sulfatase	IM
LA1155	6.4	<0.01	P		Substrate binding protein of an ABC transporter complex	PER
LA2242	2.7	<0.01	P		TonB-dependent protein	OM
LA3468	2.2	<0.01	P		Outer membrane receptor for Fe^{3+}-dicitrate/TonB-dependent receptor	OM
LA3469	4.6	<0.01	P		Iron-regulated lipoprotein	OM
LA4216	2.1	<0.01	P	*cysI*	Sulfite reductase (NADPH), alpha subunit	CYT
LB186	2.5	<0.01	P		Heme oxygenase	CYT
LA0605	8.9	0.01	R		SET family protein	CYT
LA3107	2.1	<0.01	R		Hydrolase/acyltransferase	UNK
LA3354	2.7	<0.01	R		Zn-dependent alcohol dehydrogenase	CYT
LA3471*	3.6	<0.01	R		Conserved hypothetical protein	NON-CYT
LA3020*	2.4	0.03	S		Conserved hypothetical protein	CYT
LA3410	2.2	<0.01	S		Conserved hypothetical protein	NON-CYT
LA3104	4.4	<0.01	T		Signal transduction protein	CYT
LA3234	2.1	0.01	T		Adenylate/guanylate cyclase	UNK
LA3909	2.0	0.04	T		Signal transduction protein	CYT
LB139	2.1	<0.01	TK		Regulator of sigma subunit	IM
LIC10929	3.4	0.03	V		Restriction endonuclease, type I	CYT

[a] COG categories are defined in the legend to Fig. 1.
[b] Locus tags from *L. interrogans* sv Lai (LA number) have been used, except where the gene is unique to sv Copenhageni (LIC number) and is present in sv Manilae. *, the gene is absent from *L. biflexa*.
[c] Predicted locations of proteins: noncytoplasmic proteins (NON-CYT); proteins located in the outer membrane (OM), periplasm (PER), inner membrane (IM), or cytoplasm (CYT); or unknown location (UNK).
[d] —, not in COGs.

and chromosome partitioning), M (cell membrane biogenesis), and Q (secondary metabolites biosynthesis, transport, and catabolism); but 16.7% of downregulated genes were in these categories (versus 7.1% across the genome; $P = 0.012$), possibly to divert cellular resources toward expression of essential proteins which are involved in iron acquisition and active transport. Genes assigned COG category P (inorganic ion transport and metabolism) were overrepresented in genes which were upregulated compared with the genome-wide frequency (18.6% and 2.4%, respectively; $P < 10^{-5}$), a finding consistent with COG category P, including proteins predicted to be involved in transport of iron.

***L. interrogans* genes involved in iron transport and utilization.** Expression of the gene encoding LA0572, predicted to be involved in transport of ferrienterobactin (55), and expression of the gene encoding LA2242, a desferrioxamine receptor homolog, were upregulated (Table 2), similar to what was found in *L. biflexa* (44). Expression of *la2242* is also induced by transition from low to physiological osmolarity conditions (48) and exposure to serum (57).

FecA is a surface protein which mediates transport of ferric citrate across the outer membrane (72). The gene encoding LA3468, a FecA homolog, was induced 2.2-fold in *L. interrogans* under low-iron conditions. In *E. coli*, the ferric iron transport genes (*fecABCDE*) are also upregulated under low-iron conditions (5). However, the ABC transport proteins involved in ferric citrate uptake by FecA in *L. interrogans* are unknown, since no other genes of the *fec* operon have been identified and the genes encoding the two-component signal transduction genes, *fecIR* (67), are also absent.

LB191 (or HbpA) from *L. interrogans* sv Lai is a hemin binding protein which is expressed in cultures grown under low-iron conditions (6). Our study confirmed upregulation of *lb191* transcription under iron limitation. Genes encoding a heme oxygenase (LB186) and a putative heme permease (LB187) were also upregulated. The genes appear to be arranged in an operon and together with *lb191* may be part of a heme acquisition and utilization locus. Since heme is likely to be the main source of iron in the host, upregulation of these genes was not surprising, especially given that heme oxygenase is required by *L. interrogans* for survival in the hamster model of infection (52). Genes encoding LB186 and LB187 were also upregulated in response to an increase in osmolarity (48) and exposure to serum (57), consistent with the importance of these genes for survival in the host.

Genes encoding predicted ferrous iron transport proteins, FeoA and FeoB (LA2578 and LA2579, respectively), were not differentially expressed under low-iron conditions, in contrast

to the downregulation observed in *L. biflexa* (44). This difference may be due to differences in metabolism or iron uptake mechanisms between pathogenic and saprophytic leptospires. Iron transporter proteins require an energy transduction complex, consisting of TonB, ExbB, and ExbD, for uptake of iron (3). There are two predicted TonB or TonB-like proteins encoded in the *L. interrogans* genome, five ExbD-related biopolymer transport proteins, and four ExbB- or TolQ-like transport proteins grouped in four distinct loci. TolQ is structurally similar to ExbB and can substitute for ExbB activity (10, 21). None of these was differentially expressed, although *la3246* (encoding a predicted ExbD-related biopolymer transport protein) was slightly upregulated (1.53-fold) under low-iron conditions. The constitutive expression of these genes suggests that the proteins are involved in transport of substances other than iron or their expression may respond to different iron sources. The gene *bfr*, encoding a putative bacterioferritin (LA2690), was 3.1-fold downregulated, consistent with the reduced need for iron storage proteins and other nonessential iron-containing proteins in order to release iron for crucial cellular processes. There was no change in transcription of genes encoding the other predicted bacterioferritins: Dps (LA3598), a DNA-binding ferritin-like protein, or LA4021, a predicted bacterioferritin-associated ferredoxin. In *E. coli*, Fur indirectly represses expression of bacterioferritin under iron-limiting conditions via derepression of RyhB, a small noncoding RNA which acts by hybridizing to the transcript and inhibiting translation (46). RyhB also negatively regulates a number of genes encoding iron-binding and metabolic proteins and thus, along with Fur, plays a major role in iron regulation in *E. coli* (47). Downregulation of bacterioferritin (LA2690) and other genes in *L. interrogans* may be regulated by a similar mechanism. However, there have been no small RNA species characterized in *Leptospira* spp. to date.

In some bacteria, Fur autoregulates its own transcription in response to iron levels (20, 30). This occurs via RyhB in *E. coli* (69). While transcription of the genes encoding Fur family proteins was not altered in *L. interrogans* under low-iron conditions (see Table S1 in the supplemental material), it is possible that their expression may also be regulated posttranscriptionally by small RNA species. Expression of the *la1857* ortholog in *L. biflexa* (LEPBIa2461) was likewise unaffected by the iron concentration, but the other three *fur*-like genes were downregulated at least 10-fold under iron limitation conditions (44). The reasons for this discrepancy are unknown but further suggest different regulatory mechanisms in pathogenic and saprophytic *Leptospira*.

Effect of iron limitation on membrane proteins and putative virulence factors. Various leptospiral outer membrane proteins are downregulated by iron, including LipL36 (LA0492), pL50 (LA3469), and pL24 (LA0412), a putative electron transfer flavoprotein (17). LA3469 (LruB) plays a role in equine recurrent uveitis and has an IrpA domain which is associated with iron metabolism regulation (70). Our results showed a 4.6-fold upregulation of *la3469* with iron limitation. Also, in contrast with the findings of the study of Cullen et al. (17), we detected no change in transcription of *la0412* or *lipL36*. The apparent discrepancy may be due to the differences in 2,2′-dipyridyl concentrations and the period of growth under iron-limiting conditions. However, we have observed previously that transcription levels do not necessarily reflect protein abundance (41, 42). LipL36, for example, is downregulated *in vivo* and during growth at 37°C (17, 28, 53), but our studies showed that *lipL36* transcription was not temperature responsive, suggesting posttranscriptional regulation (41).

LA0695 (also known as Lsa24 [7], LenA [64], or LfhA [71]) is expressed during mammalian infection and can bind to the complement regulatory protein factor H (71), laminin, collagen IV, and fibronectin (7), suggesting roles in resistance to complement-mediated killing and adhesion to host cells. LA0695 is one of five *L. interrogans* proteins (LenA-LenF) that are structurally similar to mammalian endostatins (64). Previous studies showed that the transcript of *la0695* was slightly upregulated by an increase in osmolarity (1.55-fold) (48), whereas there was no change in response to temperature (41). However, for unknown reasons, there was poor hybridization and therefore a low signal intensity of *la0695* spots on the microarray slides, resulting in inconclusive data for *la0695* transcripts. Given that LA0695 is likely to play a role in virulence, we selected this gene for real-time RT-PCR analysis and found that *la0695* was upregulated 2.8-fold. Genes encoding two other endostatin-like proteins, *lenB* (LA3103) and *lenD* (LA1433), were slightly upregulated (1.6- and 1.8-fold, respectively) by iron limitation, whereas *lenC*, *lenE*, and *lenF* (*la0563*, *la4324*, and *la4073*, respectively) were not differentially expressed (see Table S1 in the supplemental material). *lenD* is also upregulated under conditions of increased temperature and osmolarity (41, 48). LenB has factor H-binding ability, while both LenB and LenD can bind to laminin (64). The Len proteins appear to provide functional redundancy, whereby expression of different proteins performing similar roles may be regulated by different mechanisms and/or environmental signals, as suggested by results of this and previous microarray studies (41, 48). Functional redundancy and differential regulation suggest that the individual Len proteins may play other as yet undefined roles in different stages of pathogenesis.

Using iTRAQ and 2DGE analyses, Eshghi et al. (22) identified five proteins in *L. interrogans* sv Copenhageni which were upregulated under *in vivo*-like conditions (iron limitation combined with the presence of serum): the essential virulence factor Loa22 (61), a putative glycosyl hydrolase (LIC13050/LA0505), a putative coagulase (LIC13166/LA3961), a putative catalase (KatE or LIC12032/LA1859), and a TolC-like outer membrane protein (LIC12575/LA1100). However, the corresponding genes were not differentially expressed in our study, which examined iron-limiting conditions alone.

We identified 20 genes which encode proteins with no similarity to proteins found in other bacteria with increased transcription under low-iron conditions. Of these, 16 were absent from the genome of the saprophyte *L. biflexa* (Table 2) (58) and may encode unique leptospiral virulence factors.

Genes involved in iron acquisition are transcriptionally regulated by iron availability through the action of Fur in many species of bacteria. Louvel et al. (45) were not able to identify putative Fur boxes in the promoter regions of genes predicted to be involved in iron uptake. Likewise, we also were unable to identify a clear consensus Fur box for genes upregulated by low iron.

Genes with altered expression in the *la1857* mutant. The genome of *L. interrogans* encodes four predicted Fur ho-

TABLE 3. *L. interrogans* sv Manilae genes differentially expressed in the *la1857* mutant versus wild type, grouped by COG[a] category

Regulation and locus tag[b]	Mean fold change	*P* value	COG	Gene	Description of product	Predicted location[c]	Transcriptional response in parent strain under iron limitation[d]
Downregulated							
LA0951	−7.04	0.02	—[e]		Conserved hypothetical protein	IM	
LA2020	−2.05	<0.01	—		Hypothetical protein	NON-CYT	
LA2882	−4.61	0.04	—		Hypothetical protein	CYT	↑
LA0632	−4.18	<0.01	GT	*ptsN*	Protein-*N*(pi)-phosphohistidine-sugar phosphotransferase, subunit A	CYT	↓
LA1603	−2.45	0.02	M		Glycosyltransferase	CYT	
LA3247	−3.33	<0.01	M		TonB-related protein	NON-CYT	
LA1857	−18.4	<0.01	P	*fur*	Ferric uptake regulation protein, Fur	CYT	
LA3242	−2.42	0.03	P		TonB-dependent receptor	OM	
LA0598	−2.27	<0.01	S		Conserved hypothetical protein	CYT	↓
LA3244	−2.86	<0.01	U	*tolQ*	Transport protein, TolQ-like	IM	
LA3245	−2.47	<0.01	U		ExbD-related biopolymer transport protein	NON-CYT	
Upregulated							
LA0093	4.8	<0.01	—		Conserved hypothetical protein	CYT	
LA0430	2.0	<0.01	—		Hypothetical protein	NON-CYT	
LA0575	3.2	0.04	—		Conserved hypothetical protein	CYT	
LA1783	2.8	<0.01	—		Hypothetical protein	NON-CYT	
LA1833	8.9	0.01	—		Phage-related protein	CYT	
LA2588	5.0	0.02	—		Hypothetical protein	CYT	
LIC12653	3.6	0.01	—		Conserved hypothetical protein	UNK	
LIC12658	3.1	0.03	—		Hypothetical protein	CYT	
LA0630	5.0	<0.01	C		Oxidoreductase	CYT	
LA1209	2.7	0.04	E	*pepD*	Dipeptidase	NON-CYT	
LB011	2.4	<0.01	H	*hemCD*	Bifunctional porphobilinogen deaminase/uroporphyrinogen synthase	CYT	
LB012	2.0	<0.01	H	*hemB*	Delta-aminolevulinic acid dehydratase	CYT	
LB013	2.5	<0.01	H	*hemL*	Glutamate-1-semialdehyde aminotransferase	CYT	
LA0084	3.5	0.04	I	*plsC*	1-Acylglycerol-3-phosphate *O*-acyltransferase	CYT	
LA0932	4.8	0.03	I	*pldB-2*	Lysophospholipase	CYT	
LA0943	8.5	0.02	J	*infB*	Translation initiation factor 2 (IF-2; GTPase)	CYT	
LA1595	3.4	0.02	M		Pyridoxal phosphate-dependent aminotransferase	CYT	↓
LA0957	3.7	0.01	MU		Outer membrane efflux protein related to TolC	OM	
LA0666	5.7	<0.01	P	*mauG-2*	Cytochrome *c* peroxidase	NON-CYT	
LA1859	2.3	0.01	P	*katE*	Catalase	CYT	

[a] COG categories are defined in the legend to Fig. 1.
[b] Locus tags from *L. interrogans* sv Lai (LA number) have been used, except where the gene is unique to sv Copenhageni (LIC number) and is present in sv Manilae.
[c] Predicted locations are defined in Table 2.
[d] Transcriptional change effect from Table 2.
[e] —, not in COGs.

mologs, LA1857, LA2887, LA3094, and LB183; we have constructed a transposon insertion mutation in *la1857* (51) with the insertion 62 bp into the 438-bp gene. Global transcription of the M776 mutant strain was compared with that of the wild-type serovar Manilae parent, both of which were grown under iron-replete conditions. If LA1857 does indeed function as a ferric uptake regulatory protein, we expected that its inactivation would have the same effect as growth under iron limitation. However, our results showed that this was not the case. Genes differentially expressed in the LA1857 mutant did not correspond to the iron-regulated genes in the wild-type strain, with only two downregulated and one upregulated gene being common to both data sets. Therefore, LA1857 does not play a significant role in regulation of iron homeostasis. Repeated attempts to complement the mutation were unsuccessful, but given the >1-kb intergenic region between *la1857* and the next gene, there are unlikely to be downstream polarity effects.

We identified 11 and 20 genes which were down- and upregulated, respectively, in the mutant strain (Table 3). The microarray data were validated by real-time RT-PCR analysis of 13 genes, yielding a correlation coefficient (R^2) of 0.7638 (data not shown). In the *la1857* mutant, expression of LA1857 was negligible (Table 3) and likely represents nonspecific background fluorescence.

The Fur family of metalloregulatory proteins, Fur, Zur (**z**inc **u**ptake **r**egulator), and PerR (a peroxide stress regulator), are structurally similar and share high sequence similarity (29). Multiple Fur homologs have been described in other bacterial species; *Campylobacter jejuni* has two (Fur and PerR) and *B. subtilis* has three (Fur, Zur, and PerR) (11, 25, 68). In *B. subtilis*, while the paralogs are structurally similar, each per-

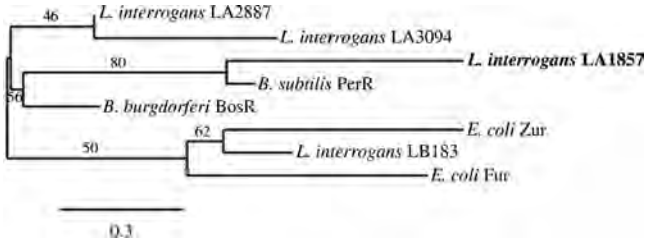

FIG. 2. Phylogenetic analysis of *L. interrogans* Fur homologs and other Fur family proteins generated by neighborhood joining with 100 bootstrap replicates and rooted at the midpoint. Branch support values are shown as percentages.

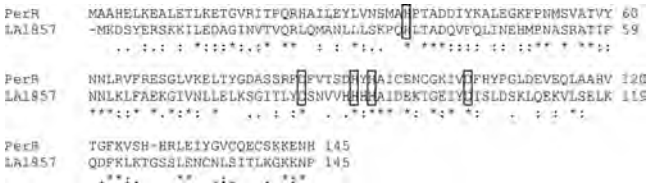

FIG. 3. Alignment of amino acid sequences of LA1857 and *B. subtilis* PerR showing identical residues (*), conserved residues (:), and semiconserved residues (.). Residues shown to be important for divalent metal binding (Fe^{2+} or Mn^{2+}) in *B. subtilis* PerR are also present in LA1857 (boxed).

forms distinct functions with very little or no regulon overlap (24). Likewise, the four predicted Fur homologs in *L. interrogans* may play distinct regulatory roles. *B. burgdorferi* does not require iron for growth, yet it possesses a Fur homolog, BosR, with 54.3% similarity to the *B. subtilis* PerR (59), which regulates *dps*, *hemA*, *katA*, and *mrgA*, all of which are involved in responses to oxidative stress and metal limitation (11, 14). In *C. jejuni*, PerR regulates the *katA* and *ahpC* genes (68). Therefore, on the basis of our microarray data (see below), LA1857 appears to be functionally more similar to PerR and is likely to function as a derepressor of genes encoding proteins involved in the oxidative stress response.

Catalase (KatA) and alkyl hydroperoxide reductase or peroxiredoxin (AhpC) are important bacterial enzymes in defense against oxidative stress. The *katE* gene, encoding a putative catalase, was upregulated 2.4-fold in M776. However, expression of *ahpC* (LA2809) was not altered. A gene encoding a putative cytochrome *c* peroxidase (*mauG-2* or *la0666*) was also upregulated in the mutant. Cytochrome *c* peroxidase plays a role in protection against peroxide killing in *Neisseria gonorrhoeae* (63). Resistance to oxidative stress is likely to be important in the early stages of infection, allowing pathogenic leptospires to persist in phagocytes and disseminate (18). Genes in the heme biosynthesis pathway (*hemCD*, *hemB*, and *hemL*) were also upregulated more than 2-fold in the mutant strain. The first gene in the putative heme synthesis operon, *hemA*, was slightly upregulated at 1.8-fold ($P < 0.001$) (see Table S1 in the supplemental material).

Phylogenetic analysis showed that LA1857 and LA2887 are indeed more similar to the *B. subtilis* PerR protein and BosR from *B. burgdorferi*, while LB183 and LA3094 are more similar to the *E. coli* Zur and Fur proteins, respectively (Fig. 2). *B. burgdorferi* BosR regulates the oxidative stress response and interfaces with the RpoS cascade and subsequent expression of known virulence factors; inactivation of BosR resulted in loss of infectivity in mice (31, 32, 56). However, the M776 mutant strain was not attenuated for virulence in the hamster model of infection (51), a finding that is consistent with derepression of oxidative stress genes potentially regulated by LA1857 and that indicates different roles for LA1857 and BosR.

In *B. subtilis*, the Fe(II)-bound form of PerR is highly sensitive to metal-catalyzed oxidation by bound ferrous ions, resulting in Fe release and loss of DNA binding activity, leading to derepression of oxidative stress genes (38). Accordingly, we also observed that expression of *katE* was further increased by 4.3-fold in the mutant versus wild type when both were grown under low-iron conditions (see Table S1 in the supplemental material). In many bacteria, expression of KatA and AhpC is regulated by OxyR in response to oxidative stress (15). OxyR is an activator of peroxide stress genes and is not a metalloprotein. Instead, hydrogen peroxide catalyzes disulfide bond formation in OxyR, allowing it to bind to and induce expression of peroxide stress genes (73). Since genes encoding OxyR and SoxRS are absent from *L. interrogans* genomes (54, 60), we hypothesize that LA1857 is a PerR-like regulator and a functional analog of OxyR, although they clearly have different modes of action. *C. jejuni* also lacks *oxyR*, and the peroxide stress response is regulated by PerR (68). However, unlike *C. jejuni*, *katE* expression in *L. interrogans* was not iron responsive (see Table S1 in the supplemental material), and given that there was little overlap in the low-iron and mutant microarray data sets, regulation of responses to iron limitation and peroxide stress appear to be distinct. Since LA1857 is a Fur-like protein, divalent metal ions would be required to activate DNA binding activity. A number of residues (H37, D85, H91, H93, and D104) have been shown to be important for divalent metal binding in the *B. subtilis* PerR (38). An alignment of LA1857 and the *B. subtilis* PerR showed that the metal binding residues are also present in LA1857 (Fig. 3).

While inactivation of *la1857* significantly increased the expression of *katE* under iron-replete conditions, expression of the *la1858* gene immediately upstream of *katE* was not altered in the mutant. The *la1858* and *katE* genes in serovar Manilae overlap by 1 nucleotide, suggesting transcriptional coupling. However, analysis of the sequence upstream of *katE* revealed a putative PerR binding box with 13/15 matches to the consensus sequence (24), consistent with our hypothesis that LA1857 is a PerR homolog and regulator of *katE*.

Inactivation of PerR in *C. jejuni* results in hyperresistance to peroxide stress (68). Since mutation of *la1857* resulted in derepression of *katE* and *mauG-2* (Table 3), to determine whether this also results in increased resistance to oxidative stress, sensitivity to hydrogen peroxide between the mutant and wild-type strains was compared. The mutant was 8-fold more resistant to hydrogen peroxide; the MBC for the *la1857* mutant was 430 μM, while the wild type was killed at 54 μM (Fig. 4). This finding is consistent with the higher levels of *katE* expression seen in the mutant, and therefore, LA1857 does indeed play a role in the oxidative stress response. However, we did not observe any difference in resistance to cumene hydroperoxide (Fig. 4). In *Streptococcus pyogenes*, PerR also regulates the peroxide stress response, but unlike *C. jejuni* and *B. subtilis*, the *S. pyogenes* PerR does not regulate *ahpC* (34). Glutathione peroxidase (GpoA) and

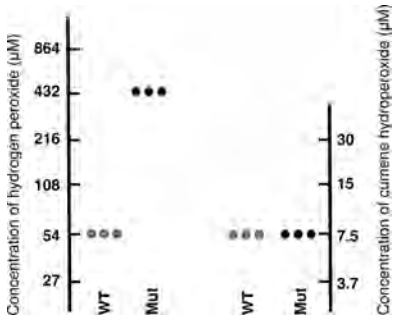

FIG. 4. Minimum bactericidal concentrations of hydrogen peroxide and cumene hydroperoxide for the LA1857 mutant (Mut) and wild type (WT). Three biological replicates were tested in triplicate, yielding identical results. The LA1857 mutant was 8-fold more resistant to killing by hydrogen peroxide, whereas there was no difference in resistance to cumene hydroperoxide.

AhpC are not required in the induced response to peroxide stress in *S. pyogenes* but do contribute to resistance against cumene hydroperoxide. Likewise, in *L. interrogans* PerR did not regulate *ahpC* (LA2809), which was not differentially expressed in the mutant versus the wild type. In addition, genes encoding other AhpC homologs (LA0734, LA0862, LA2312, LA2494, LA2809, LA3442, and LB117) and two GpoA homologs (LA1007 and LA4299) were also not differentially expressed in the LA1857 mutant, which may explain the lack of difference in resistance to cumene hydroperoxide between the LA1857 mutant and the wild type. The expression behavior of the *ahpC* and *gpoA* homologs in wild-type *L. interrogans* grown under peroxide stress conditions awaits further study.

It would be interesting to examine the roles of the other Fur homologs in *L. interrogans*. However, directed mutagenesis is extremely inefficient in pathogenic leptospires, with only two targeted mutations in a pathogenic species of *Leptospira* being reported to date (16, 39). Ongoing screening of our transposon library has not yet yielded mutations of the other Fur homologs. It is thus not possible to inactivate each of the homologs individually and/or in combination and study the subsequent effect on transcription patterns. This is the first report of peroxide stress regulation by a Fur-like protein in *L. interrogans*. However, since there are four predicted Fur homologs in *L. interrogans* svs Lai and Copenhageni, we suggest that each plays a distinct role in iron, metal, or stress responses.

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Detection of PrP^{Sc} in formalin-fixed, paraffin-embedded tissue by Western blot differentiates classical scrapie, Nor98 scrapie, and bovine spongiform encephalopathy

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Abstract. Transmissible, spongiform encephalopathies including bovine spongiform encephalopathy (BSE) and scrapie are fatal neurodegenerative disorders associated with the presence of an infectious abnormal isoform of normal mammalian proteins called prions. Identification of the prion protein associated with scrapie (PrP^{Sc}) in the central nervous system is typically based upon immunoassays including immunohistochemistry (IHC) using formalin-fixed tissues or Western blot (WB) assays using fresh and/or frozen, non–formalin-fixed tissues. Each assay can discriminate between BSE, classical scrapie, and a previously reported strain of scrapie recently identified in the United States named Nor98 scrapie. Different tissue samples are required from the same animal to run these 2 different immunoassays. This may result in inconsistent test results for the same animal. Sampling problems such as collecting insufficient volumes of fresh tissue or less than optimal anatomic location of brainstem for IHC can affect the ability of the test procedures to offer definitive and discriminatory results. Recently, a WB method using formalin-fixed, paraffin-embedded (FFPE) tissue to identify PrP^{Sc} was developed that successfully identified PrP^{Sc} in sheep affected by classical scrapie. In the current study, the use of this technique to produce discriminatory results identifying classical BSE in bovine tissue and both classical and Nor98 scrapie in ovine tissue using paraffin-embedded brain samples is described. Protein-banding patterns from WB using FFPE tissue were similar to protein-banding patterns produced by WB assays utilizing fresh tissues from the same animals, and results correlated well with the IHC PrP^{Sc}-positive staining present in the cerebellum and obex regions of brain samples from these animals.

Key words: Bovine spongiform encephalopathy; Nor98 scrapie; paraffin-embedded tissue; prion; scrapie; transmissible spongiform encephalopathy; Western blot.

Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders associated with the accumulation of abnormal forms of a natural host protein known as prion proteins (PrP^{Sc}), and include scrapie in sheep and goats and bovine spongiform encephalopathy (BSE), which primarily affects cattle. When manifested clinically, these diseases may be differentiated based on signalment, epidemiology, and clinical history.[14,17,25,26] It is difficult, however, to identify subclinical cases in the live animal. Serologic tests used to detect PrP^{Sc} are not available because specific immune responses are not recognized in TSE-affected animals. The infectious agent associated with TSE diseases has no known nucleic acid component, so nucleic acid–based tests are not useful. The common hallmark features of TSEs are detectible most consistently through postmortem findings including vacuolar degeneration of the central nervous system (CNS) as detected by histopathology and the associated presence of PrP^{Sc}; however, PrP^{Sc} can be detected in some cases prior to the occurrence of CNS lesions. In cases of subclinical classical scrapie, PrP^{Sc} may be detectable in lymphoid tissue alone. Diagnosis is typically defined by the detection of PrP^{Sc} in affected tissues through the use of immunoassays such as immunohistochemistry (IHC), Western blot (WB), and enzyme-linked immunosorbent assay (ELISA).[25,26] The ELISA serves most often as a screening test because results can be obtained in a short period of time and the sensitivity of the test is high; however, ELISA provides no discriminatory detail on the molecular nature or anatomic location of the prion agent. Immunohistochemistry and WB are typically used to confirm the presence of PrP^{Sc} as well as provide specific information on the disease agent.[25,26]

Multiple strains of scrapie agent have been identified through variation in incubation periods

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and neuropathology in rodent models.[4] In sheep, these various scrapie strains have also been found to differ in the distribution of PrP^{Sc} in host tissues, lesion profiles, and in prion protein conformation, glycosylation, protease resistance, and protein aggregation.[3,6,22] A distinct strain of scrapie, identified in Norway and called Nor98 scrapie,[2] has since been reported in sheep from Belgium, Germany, France, Ireland, United Kingdom, Sweden, Portugal, Netherlands, Lithuania, Italy, Estonia, Denmark, Falkland Islands, Switzerland, Iceland, and recently in the United States.[5,8,9,11–13,16,20,21] Nor98 scrapie has also been detected in goats from Switzerland, France, Spain, and Italy.[7,12,23] Classical scrapie and the Nor98 strain of scrapie can be differentiated based on epidemiology, signalment, clinical history, and genotype as well as through diagnostic assays including IHC and WB.[17] Definitive diagnosis of both classical and Nor98 scrapie is typically made with immunoassays that identify PrP^{Sc} in formalin-fixed tissue using IHC or in fresh or frozen, non-fixed tissues using the WB assay. Immunohistochemical staining of CNS tissues reveals differences in the distribution of PrP^{Sc} when comparing classical scrapie and Nor98 scrapie. In cases of Nor98 scrapie, regions of the brain including the cerebellum and spinal nucleus of the trigeminal nerve contain PrP^{Sc}, while areas typically affected in classical scrapie, such as the motor nucleus of the vagus nerve and lymphoid tissues, are spared. Western blot assays produce distinct protein band patterns for Nor98 scrapie that include at least 3 protein bands with an unglycosylated band measuring less than 15 kD. This differs from the banding profile of classical scrapie, which includes 3 protein bands ranging from 18–30 kD.[1] Bovine spongiform encephalopathy presents with diagnostic findings that are distinct from both classical and Nor98 scrapie. The earliest PrP^{Sc} deposition is found in the solitary nucleus and tract, making this the target area needed for identification of BSE by IHC. Results of WB assays for classical BSE include 3 protein bands with the molecular weight of the unglycosylated band being smaller than classical scrapie and greater than Nor98 scrapie.

With IHC, FFPE tissues are used, and results of these procedures provide information on the distribution of lesions and PrP^{Sc} within the various nuclei of brain tissue. Nuclei in the obex region of the brainstem, known to contain the earliest accumulation of PrP^{Sc}, must be present in histosections for samples to provide diagnostic and discriminatory results. Brain histosections that do not include the correct nuclei of the obex may not allow morphologic distinction between prion diseases.[10,25,26] Successful protocols for IHC detection of PrP^{Sc} may vary between diagnostic facilities, and one variation calls for immersion of tissue blocks in formic acid, post–formalin fixation prior to dehydration, and embedding in paraffin, which can affect prion epitope availability and inactivate prions. With WB assays, homogenization of the sample preempts the ability to document localization of PrP^{Sc} within appropriate nuclei. It does, however, provide the diagnostic capability of determining the molecular profile of the detected PrP^{Sc}. Routine WB assays require non-fixed tissue samples, which are often not available in sufficient quantities for testing. Retrospective studies can be limited by the difficulties of storing fresh, non-fixed tissues. Recently, a WB method utilizing immersion-fixed CNS samples was reported, which provided for PrP^{Sc} detection in homogenized, formalin-fixed tissue, creating the opportunity for WB diagnosis of prion diseases where fresh or frozen tissues were unavailable.[19] The FFPE tissue WB methodology used in the present study has been previously shown to identify PrP^{Sc} in sheep affected with classical scrapie.[15] The current study describes the use of this published technique to discriminate classical and Nor98 scrapie in ovine tissue and classical BSE in bovine tissue using paraffin-embedded brain samples (tissues from sheep with BSE and goats with Nor98 were unavailable).

Materials and methods

Archived FFPE tissue samples of brainstem (obex area) and cerebellum previously diagnosed by IHC as being positive for classical scrapie or Nor98 scrapie (case 1: Nor98-1, case 2: Nor98-2) were used in the current study along with bovine obex previously diagnosed by IHC as being positive for BSE. Matched samples of fresh tissue including obex and cerebellum for classical scrapie and Nor98-1, cerebrum for Nor98-2, and obex for BSE from the same animals were included for comparison. Formalin-fixed, paraffin-embedded ovine obex tissue, as well as fresh ovine obex tissue, from an animal determined to be negative for PrP^{Sc} by IHC were also included in the study as negative controls. Two additional cases of classical scrapie previously reported as PrP^{Sc} positive by IHC were included for a comparison of formalin-fixed tissue treated with formic acid prior to embedding in paraffin and non–formic acid-treated, paraffin-embedded samples from the same animal.

The archived samples that remained immersed in 10% neutral buffered formalin were embedded in paraffin wax and sectioned at 5-μm thickness. Formalin-fixed samples from the 2 additional positive classical scrapie cases were also placed in cassettes, incubated in 95% formic acid for 1 hr, and rinsed in tap water overnight prior to being embedded in paraffin wax and sectioned at 5-μm thickness. A total of 4 sections of each block were collected in individual 1.5-ml microcentrifuge tubes and processed for WB. A sequential fifth section from each block was

processed by an automated IHC method to confirm the presence of PrP^{Sc}.[16] The WB assay used in the present study utilizes a tissue extraction technique, originally developed for the purpose of DNA isolation from FFPE tissue, later applied to PrP^{Sc} detection in FFPE tissue.[15,18] This WB method with minor modifications was utilized for detection of PrP^{Sc}.[15] Briefly, 150 µl of 0.05 M Tris (pH 7.5), 1 mM ethylenediamine tetra-acetic acid, and 0.5% Tween 20 was added to each 1.5-ml microcentrifuge tube. The tube was placed in a 100°C water bath for 10 min followed by snapfreezing (ethanol on dry ice). The 10-min boil and snapfreeze cycle was repeated, followed by one more 10-min boil, and then the tube was immediately centrifuged 16,000 × *g* for 10 min to separate the paraffin while pelleting the tissue. An additional 10-min boil followed by a repeat of the 10-min centrifugation was performed if separation of the paraffin was not complete. The tissue pellet was drawn from beneath the paraffin layer by using a 16-gauge needle affixed to a syringe and transferred to a sterile 1.5-ml centrifuge tube with 150 µl of the Tris buffer added. Mechanical sheering with the needle tip was performed to reduce the tissue pellet to small particles. Further disruption of the tissue pellet was accomplished using a sonicator[a] at 60-sec intervals repeated 10 times in an ice bath with brief vortex mixing between sonications. Proteinase K[b] was added to each 1.5-ml tube to a final concentration of 100 mg/ml and incubated at 37°C for 1 hr. Pefabloc[c] was added to a final concentration of 0.1 mg/ml in order to stop digestion. An equal volume of sample buffer was added, and the samples were incubated at 99°C for 30 min. For WB, 15 µl of sample was loaded on a 12% commercially prepared sodium dodecyl sulfate–polyacrylamide electrophoresis gel[d] and run for 50 min. The samples were then blotted to a polyvinylidene difluoride membrane[d] and blocked with 3% bovine serum albumin. Detection of PrP^{Sc} by WB was accomplished by using mouse anti-PrP monoclonal antibody P4[e] at 1:10,000 dilution (0.1 mg/ml), L42[e] 1:2,500 dilution (1.0 mg/ml), or F99/97.6.1[f] at manufacturer's dilution as the primary antibody. Incubation in primary antibody occurred at 4°C overnight. A biotinylated sheep anti-mouse secondary antibody[g] at 0.05 mg/ml and a streptavidin–horseradish peroxidase conjugate[g] were used in incubations conducted at room temperature for 1 hr. A commercial detection system[g] allowed visualization using a digital imaging system.[d]

Results

Classical scrapie, Nor98 scrapie, and BSE were accurately identified in FFPE tissue sections by WB assays utilizing P4 and L42 antibodies. Negative control FFPE tissue sections produced no banding patterns in the same assays. Banding patterns for each of the positive FFPE tissue samples were compared with the banding produced using fresh tissue from the same animal. Molecular weights for each band were approximated based on results obtained from the digital imaging software.[d] The P4 antibody was used when examining classical and Nor98 scrapie cases (Fig. 1). For classical scrapie, fresh obex banding was 18, 23, and 27 kD (Fig. 1, lane 2). The corresponding FFPE tissue sample banding was 19, 24, 28, and 48 kD (Fig. 1, lane 3). Classical scrapie cerebellum produced no bands when fresh tissue was tested (Fig. 1, lane 4) and very weak bands at 24 and 27 kD for the corresponding FFPE tissue sample (Fig. 1, lane 5). For Nor98 scrapie cases, fresh cerebellum was available for Nor98-1. The protein-banding pattern for this fresh cerebellum sample was 6.5, 17, 20, 22, and 27 kD (Fig. 1, lane 9). The protein-banding pattern for the corresponding FFPE tissue sample from Nor98-1 was similar at 6.5, 18, 24, and 28 kD (Fig. 1, lane 10). Formalin-fixed, paraffin-embedded tissue WB results for Nor98-2 cerebellum were identical (Fig. 1, lane 13). Fresh and FFPE obex tissue from Nor98-1 had similar protein-banding patterns with multiple bands ranging from 6.5 to 28 kD (Fig. 1, lanes 7 and 8), but the bands were moderately weaker in intensity than those produced when evaluating cerebellum. Fresh cerebrum available for Nor98-2 produced 1 weak band at 6.5 kD (Fig. 1, lane 11). Formalin-fixed, paraffin-embedded obex tissue from this same animal produced no protein bands (Fig. 1, lane 12). Based on these results, FFPE tissue is a comparable diagnostic sample for cases of classical and Nor98 scrapie. Furthermore, cerebellum provides the most diagnostic results for cases of Nor98 scrapie when compared with obex from these animals, while for classical scrapie, the obex remains the diagnostic sample needed.

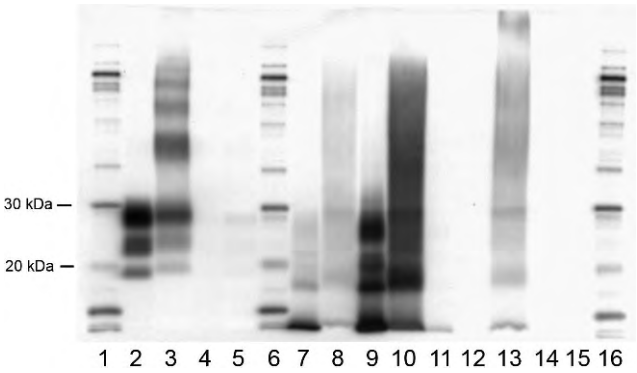

Figure 1. Western blot of paraffin-embedded tissues and fresh tissue from the same animals using P4 antibody, molecular weight marker (MWM),[h] diluted 1:100. Lane 1: MWM; lane 2: classical scrapie (CS) obex fresh; lane 3: CS obex formalin-fixed, paraffin-embedded (FFPE) tissue; lane 4: CS cerebellum fresh; lane 5: CS cerebellum FFPE tissue; lane 6: MWM; lane 7: Nor98-1 obex fresh; lane 8: Nor98-1 obex FFPE tissue; lane 9: Nor98-1 cerebellum fresh; lane 10: Nor98-1 cerebellum FFPE tissue; lane 11: Nor98-2 cerebrum fresh; lane 12: Nor98-2 obex FFPE tissue; lane 13: Nor98-2 cerebellum FFPE tissue; lane 14: negative control (NC) obex fresh; lane 15: NC obex FFPE tissue; lane 16: MWM.

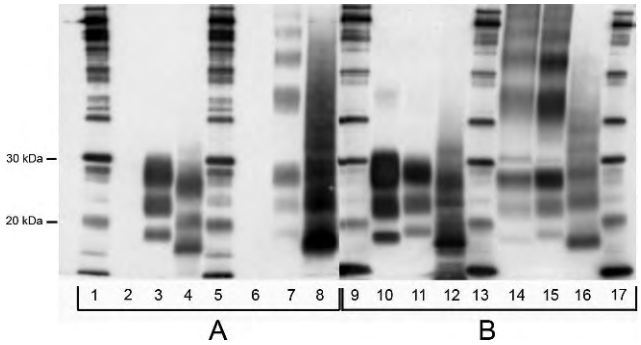

Figure 2. Diagnostic Western blot identifying classical scrapie, Nor98 scrapie, and bovine spongiform encephalopathy (BSE) using fresh tissue and formalin-fixed, paraffin-embedded (FFPE) tissue. **A**, P4 antibody, molecular weight marker (MWM),[h] diluted 1:50 (lane 1: MWM; lane 2: BSE obex fresh; lane 3: classical scrapie [CS] obex fresh; lane 4: Nor98 cerebellum fresh; lane 5: MWM; lane 6: BSE obex FFPE tissue; lane 7: CS obex FFPE tissue; lane 8: Nor98 cerebellum FFPE tissue; lane 9: MWM), and **B**, L42 antibody, MWM,[h] diluted 1:50 (lane 10: BSE obex fresh; lane 11: CS obex fresh; lane 12: Nor98 cerebellum fresh; lane 13: MWM; lane 14: BSE obex FFPE tissue; lane 15: CS obex FFPE tissue; lane 16: Nor98 cerebellum FFPE tissue; lane 17: MWM).

A second diagnostic comparison was made by FFPE tissue WB using classical scrapie–infected ovine obex, Nor98 scrapie–infected ovine cerebellum, and BSE-infected bovine obex (Fig. 2). Both P4 (Fig. 2, panel A) and L42 (Fig. 2, panel B) antibodies were used, and banding patterns were distinctive and diagnostic. The L42 antibody positively identified fresh and FFPE tissue samples of classical scrapie, Nor98 scrapie, and BSE. The P4 antibody, considered a discriminatory antibody, identified both classical and Nor98 scrapie with positive banding patterns, while, as expected, the fresh and FFPE tissue–positive BSE samples did not produce diagnostic banding (Fig. 2, lanes 2 and 6).[24] Classical scrapie and Nor98 scrapie cases produced bands similar in size to those described in Figure 1. Fresh obex from classical scrapie–infected animals produced 3 bands between 18 and 30 kD (Fig. 2, lanes 3 and 11). Fresh cerebellum from Nor98 scrapie–infected animals produced multiple bands with the smallest band measuring 6.5 kD (Fig. 2, lanes 4 and 12). Classical scrapie FFPE tissue banding included multiple bands, but the smallest 3 bands ranged from 18 to 30 kD (Fig. 2, lanes 7 and 15). Nor98 FFPE tissue also produced a banding pattern that approximated the fresh tissue samples. There were multiple bands, and the smallest measured less than 15 kD (Fig. 2, lanes 8 and 16). When using L42 antibody, fresh BSE-infected tissue produced 3 bands ranging from 17 to 30 kD (Fig. 2, lane 10) and BSE FFPE tissue produced multiple bands with the smallest 3 bands also ranging from 17 to 30 kD (Fig. 2, lane 14).

When brain samples from classical scrapie cases were treated with formic acid in the cassette prior to paraffin embedding, no bands were produced using the FFPE tissue for WB with P4, L42, and F99/97.6.1 as primary antibodies. Brain samples, from the same animals, not treated with formic acid did produce banding patterns typical for classical scrapie (data not shown).

Discussion

The WB assay using FFPE tissue differentiated classical scrapie, Nor98 scrapie, and BSE by producing distinctive molecular profiles for each of the prion proteins. The FFPE tissue WB technique also produced results that correlated well with the IHC PrP^{Sc}–positive staining present in cerebellum and obex regions of brain samples used in the study. Banding patterns were similar within each prion disease when comparing the fresh tissue WB results with the FFPE tissue WB results. Although more bands were typically present with the FFPE tissue WB, the molecular weights of the smallest bands matched well with the banding of the fresh tissue WB. Classical scrapie FFPE obex tissue produced 4–6 bands, while fresh tissue obex from the same animal produced 3 bands. In both instances, the smallest 3 bands ranged from 18 to 30 kD. Nor98 scrapie FFPE cerebellum tissue produced at least 4 bands for which the smallest bands had molecular weights that matched closely with the 3 bands produced using fresh cerebellum from the same animal (range: 6.5–28 kD). The diagnostic unglycosylated band measuring less than 15 kD was distinctly present in both Nor98 scrapie samples.

The diagnostic value of various regions of the brain differs depending on the strain of scrapie present. It is important to choose the most diagnostic samples to test when using FFPE tissue WB. High levels of PrP^{Sc} are not typically seen in the cerebellum of early classical scrapie cases. The FFPE tissue WB assay using cerebellum from classical scrapie cases produced weak to no banding, while this same location in Nor98 scrapie cases consistently produced diagnostic protein banding. So, while cerebellum is more diagnostic for Nor98 scrapie, obex is the preferred sample for classical scrapie when using the FFPE tissue WB technique.

The P4 antibody was chosen in the current study to evaluate classical and Nor98 scrapie cases. The banding results consistently differentiated these 2 strains of scrapie. Because P4 antibody is more selective for scrapie than for BSE,[24] a second antibody, L42, was chosen to confirm positive BSE

samples (Fig. 2). Banding patterns using L42 for all positive PrP^{Sc} cases examined in the present study were similar to those seen using P4, with one exception. Nor98 scrapie FFPE cerebellum tissue produced 4 bands that ranged in molecular weight from 38 to 14.5 kD. These bands matched well with the banding pattern produced using P4 and L42 on fresh cerebellum; however, the 6.5-kD band seen with the fresh cerebellum was not present in the protein-banding pattern produced by the FFPE cerebellum tissue.

Treating brain tissue from classical scrapie–affected animals with formic acid prior to paraffin embedding reduces the ability of the FFPE tissue WB to detect protein banding using parameters of the method described in the present study. Therefore, formic acid treatment of brain tissue prior to paraffin embedding is not recommended for FFPE tissue WB.

The ability to evaluate FFPE tissue for the presence of PrP^{Sc} through WB assays offers a diagnostic platform when fresh tissues are limited or not available. For cases with ambiguous results from other testing methods including nonoptimal regions of the brain used for IHC or low volume homogenates for ELISA or fresh tissue WB, FFPE tissue WB assay could potentially provide a definitive answer. There is also potential for numerous retrospective studies to be performed based on the presence of often large libraries of FFPE tissue blocks. Comparisons can be made between samples from different geographic regions, different breeds, different genotypes within breeds representing resistance or susceptibility to prion disease, and even from different locations of the brain within the same animal. This technique may provide an additional testing method for use in the diagnosis and characterization of TSEs.

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e. R-Biopharm, Darmstadt, Germany.
f. Anti-prion antibody (clone 99), Ventana Medical Systems Inc., Tucson, AZ.
g. ECL™ Plus, GE Healthcare Technologies, Piscataway, NJ.
h. Sigma-Aldrich, St. Louis, MO.

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One-step real-time RT-PCR for pandemic influenza A virus (H1N1) 2009 matrix gene detection in swine samples

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ABSTRACT

In the spring of 2009, a novel (H1N1) influenza A virus began to spread among humans worldwide. Although the 2009 H1N1 is related genetically to swine influenza viruses, human infection has not been connected to pig exposure. Because the virus is now circulating widely in the human population, swine herds are at increased risk of becoming infected. In order to investigate potential outbreaks of the 2009 pandemic virus in pigs, a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for the detection of the (H1N1) 2009 RNA in clinical specimens was developed. To evaluate the applicability of the test as a diagnostic tool in the screening of field specimens from swine, 64 field isolates of North American swine, 5 equine and 48 avian influenza viruses collected during diagnostic investigations were analyzed retrospectively as well as samples collected during an experimental *in vivo* infection with two novel H1N1 isolates, A/California/04/2009 (H1N1)v virus and A/Mexico/4108/2009 (H1N1)v. The sensitivity of the qRT-PCR was shown to be higher with respect to standard techniques such as virus isolation and the reproducibility was satisfactory. The present unique and highly sensitive assay is able to detect as little as 1×10^1 copies of RNA per μl of template and it represents a rapid and useful approach for the screening and quantitation of (H1N1) 2009 RNA in porcine specimens.

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1. Introduction

Swine influenza is an acute respiratory disease caused by influenza A viruses that belong to *Orthomyxoviridae*, a family of enveloped negative-sense, segmented, single stranded RNA viruses. Based upon the major differences within the hemagglutinin (HA) and neuraminidase (NA) proteins, 16 HA and 9 NA subtypes have been identified thus far (Rohm et al., 1996; Webster et al., 1992; Fouchier et al., 2005). It is recognized that influenza viruses evolve by reassortment and/or point mutation, thus giving rise to new viral subtypes with different host tropism. In April 2009, a novel swine-lineage influenza virus capable of rapid human transmission was reported, although infection with (H1N1) 2009 was not connected to pig exposure or to a contemporary infection in the swine population (Dawood et al., 2009). This novel pandemic H1N1 possessed a unique genome arrangement. Six genes, including PB2, PB1, PA, HA, NP and NS, cluster together with those belonging to the viruses identified as triple-reassortant swine influenza viruses of the North American lineage, whereas the M and NA genes are derived from Eurasian lineage swine influenza viruses (Dawood et al., 2009). Other than sporadic transmission to humans (Myers et al., 2007), classical swine influenza A viruses of the H1N1 subtype were historically distinct from avian and other mammalian influenza viruses based on host specificity, serotype, and/or genotype (Vincent et al., 2008). Swine influenza virus was first recognized as an agent of respiratory disease in pigs in 1928 (Shope, 1931), and the North American swine influenza virus-lineage genes of the pandemic virus have its genetic origins with this ancestral H1N1. Three predominant swine influenza virus subtypes are currently circulating in US swine following the emergence of the triple reassortant H3N2 in 1998: reassortant H1N1 (rH1N1), H1N2, and H3N2 and their drift mutant derivatives, all containing the triple reassortant internal gene cassette (TRIG) (for review see Vincent et al., 2008).

The novel (H1N1) 2009 is not known to be circulating widely among swine. Pigs have been shown to be susceptible to the human pandemic (H1N1) 2009 infection (Lange et al., 2009; Vincent, unpublished data). The chance of cross-species transmission may lead to serious consequences in terms of human risk of infection by increasing the reservoir of the virus in addition to dramatic costs for the pork industry. Swine have been shown to possess receptors for avian and human influenza viruses in the tracheal epithelium, leading to the suggestion that the pig is a mixing vessel

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Table 1
Primers and probe sequences for 2009 (H1N1) qRT-PCR. The probe was designed using the sequence of A/California/04/2009 (H1N1) (FJ96513).

Specificity	Primer	Sequence 5′–3′
(H1N1) 2009 Matrix Gene	M(76)-For	TCAGGCCCCCTCAAAGCCGA
	M(99)-Probe	FAM[a]-CGCGCAGAGACTGGAAAGTGTC-TAMRA[b]
	M(234)-Rev	GGGCACGGTGAGCGTGAACA

[a] 6-Carboxyfluorescein.
[b] Tetramethylrhodamine.

for the emergence of new subtypes with human pandemic potential (Ito et al., 1998; Scholtissek et al., 1993).

In order to recognize promptly the novel pandemic (H1N1) 2009 in swine, reducing the potential serious economic damage as well as exposure of humans to the virus, the development of a rapid and sensitive test capable of identifying and differentiating the pandemic strain from type A influenza viruses circulating in pigs is necessary. In this manuscript the development of a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using TaqMan technology for the rapid and sensitive detection of pandemic (H1N1) 2009 matrix gene and quantification of viral nucleic acid in diagnostic samples, is reported. For this purpose, field isolates of North American swine, equine and avian influenza viruses were analyzed retrospectively as well as samples (swabs and lavage fluid) collected during an experimental *in vivo* infection with A/California/04/2009 (H1N1)v and A/Mexico/4108/2009 (H1N1)v isolates. Data obtained by the qRT-PCR analysis were compared with those achieved from virus isolation of the clinical samples collected during the *in vivo* study.

2. Materials and methods

2.1. Oligonucleotide design and synthesis

The matrix (M) gene sequences of endemic swine influenza virus isolates, novel pandemic (H1N1) 2009, and sequences from a panel of human and avian type A influenza virus strains, including type A human seasonal strains, were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the DNAStar software package (DNAStar Inc., Madison, WI, USA). The primers were designed using the Geneious software (Biomatters, Ltd.) to amplify a conserved 159 bp within the aligned M genes. However, the probe was purposely designed using the sequence of A/California/04/2009 (H1N1) (FJ96513), in a conserved, yet lineage-specific region shared by all pandemic (H1N1) 2009 isolates sequenced thus far. The primers and probe were synthesized by IDT (Coralville, IA, USA). The TaqMan probe was dual-labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with tetramethylrhodamine (TAMRA) at the 3′ end. The position and sequence of primers and probe used for the assay are reported in Table 1.

2.2. Standard RNA for absolute quantification

To obtain a standard for the TaqMan assay, a 1022-bp RT-PCR product containing the full-length M gene of A/California/04/2009 (H1N1) virus was amplified using primer pair M+5 and M-1027 (Hoffmann et al., 2001), and the RT-PCR product was cloned into pGEM®-T easy vector system (Promega, Madison, WI, USA), then linearized and transcribed with RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI, USA), from the T7 promoter, according to the manufacturer's guidelines. After DNase treatment to remove residues of plasmid DNA, the transcripts were purified using a commercial column (RNeasy kit, Qiagen S.p.A., Germantown, MD, USA) and quantified by spectrophotometric analysis. Tenfold dilutions of the RNA transcript, representing 10^0 to 10^9 copies RNA μl^{-1} of template, were prepared in sterile water, and aliquots of each dilution were frozen at −80 °C. Each aliquot was used only once.

2.3. Field and experimental samples collection, preparation and virus isolation

To evaluate the applicability of the test as a diagnostic tool for the screening of field specimens, 64 field isolates of North American swine, 5 equine and 48 avian influenza viruses, collected during diagnostic investigations and 100 samples collected during an experimental *in vivo* study were examined. The *in vivo* study was conducted in two separate groups of 4-week-old pigs inoculated with two pandemic (H1N1) 2009 isolates, A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595), respectively, kindly provided by the Centers for Disease Control and Prevention (CDC). All pigs came from a herd free of swine influenza virus and porcine reproductive and respiratory syndrome virus (PRRSV). They were treated with ceftiofur crystalline free acid (Pfizer, New York, NY, USA) to reduce bacterial contaminants preceding the start of the study. The two groups were housed in individual isolation rooms at A-BSL3 and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA, USA) at the appropriate time during the course of the study. Thirty pigs, 15 per group, were inoculated intratracheally with 2×10^5 $TCID_{50}$ of A/California/04/2009 (H1N1)v and 2×10^5 $TCID_{50}$ of A/Mexico/4108/2009 (H1N1)v, both isolated and prepared on MDCK cells. Five pigs remained non-challenged as negative controls. The pigs were anesthetized by intramuscular injection of a cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg) and Telazol (6 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA, USA) followed by virus inoculation. Pigs were observed daily for clinical signs and sample collection. Nasal swabs were taken and placed in 2 ml minimal essential medium (MEM) on 0, 3, 5, and 7 dpi to evaluate nasal virus shedding and stored at −80 °C until the end of the study. Five inoculated pigs per group were euthanized on 3, 5, and 7 dpi and five control pigs were euthanized on 7 dpi. After euthanasia, each lung was lavaged with 50 ml of MEM to obtain bronchioalveolar lavage fluid (BAL fluid). Each nasal swab sample was subsequently thawed and vortexed for 15 s, centrifuged for 10 min at $640 \times g$ and the supernatant passed through 0.45 μm filter. Subsequently, 200 μl of the nasal swab sample was then placed on confluent MDCK cells in 24-well plates to incubate for 1 h. After 1 h of incubation the sample was removed and 400 μl MEM w/TPCK trypsin was added. The plate was checked at 24 and 48 h for cytopathic effects. After 48 h, 200 μl of cell culture supernatant from each well of the 24-well plate was subsequently passed onto a confluent 48-well plate after a freeze and thaw cycle. After 48 h evidence of cytopathic effects was evaluated and presence of virus antigen confirmed by immuno-cytochemical staining with an anti-influenza A nucleoprotein monoclonal antibody as described previously (Kitikoon et al., 2006). Tenfold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibi-

otics were made with each BAL fluid sample. Each dilution was plated in triplicate in 100 μl volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post-infection. At 72 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry as above.

RNA isolation was carried out from cell culture-grown virus isolates, nasal swab filtrates in MEM, and BAL fluid with the MagMAX™-96 Total RNA isolation kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Template RNA was eluted in 60 μl of buffer and stored at −80 °C prior to use.

2.4. One-step real-time RT-PCR

Duplicates of each RNA sample and standard were amplified by the qRT-PCR assay performed on a 7500 Real-Time PCR System (Applied Biosystem) with the AgPath-ID™ One-Step RT-PCR Kit (Ambion, Austin, TX, USA) with ROX added as passive reference dye. The 25 μl reaction volume for each sample contained 8 μl of extracted RNA, 12.5 μl of AgPath Kit 2× buffer, 1 μl of AgPath 25× enzyme mix, 500 nM of primers M(76)-For and M(234)-Rev, 100 nM of M(99)-probe, 1.67 μl of AgPath Detection Enhancer and 1.08 μl of ultrapure DNase–RNase-free distilled water. The thermal profile consisted of a single cycle of reverse transcription for 10 min at 45 °C and 10 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation. The amplification of cDNA was performed by 45 cycles including denaturation at 95 °C for 15 s, annealing for 1 min at 54 °C and extension at 72 °C for 15 s. The increase in fluorescent signal was registered during the annealing step of the reaction and the data were analyzed with sequence detector software (7500 System Software v.1.3.1, Applied). Data reported represent the average of the duplicates for each sample and standard.

2.5. Evaluation of qRT-PCR performance

In order to exclude cross-reactivity between pandemic (H1N1) 2009 and other viruses responsible for respiratory diseases of pigs, (H1N1) 2009 qRT-PCR test specificity was evaluated by analysis of the following: endemic swine influenza virus, avian and equine influenza viruses, coronaviruses (porcine respiratory coronavirus (PRCoV), transmissible gastroenteritis virus (TGEV)), PRRSV, porcine circovirus type 2 (PCV2), porcine adenovirus, porcine parvovirus, blue-eye paramyxovirus and pseudorabies virus. Nasal swab and BAL fluid samples collected from five uninfected pigs as well as sterile water were also included in the analysis as negative controls and no-template controls, respectively. To determine the detection limit of the (H1N1) 2009 qRT-PCR assay, 10-fold dilutions of a BAL fluid sample containing 1×10^7 copies of A/Mexico/4108/2009 (H1N1)v RNA μl^{-1} were made and subsequently analyzed. Serial 10-fold dilutions of standard RNA which contained from 10^1 to 10^9 copies of RNA transcript and the corresponding C_T values were used to plot the standard curve for the pandemic (H1N1) 2009 RNA absolute quantification.

Reproducibility of the assay was evaluated by testing several clinical samples containing A/California/04/2009 (H1N1)v RNA quantities that included the full range covered by the qRT-PCR. The intra-assay reproducibility was measured by testing the same samples 10 times in the same experiment, whereas the inter-assay reproducibility was confirmed by testing the same samples in 10 independent experiments. Coefficients of variation (CVs) were calculated by dividing the standard deviation of each tested sample by its mean and multiplying that result by 100 (Decaro et al., 2004, 2005).

2.6. USDA-validated qRT-PCR

Swine and equine influenza virus isolates and the clinical samples from pigs infected experimentally with 2009 (H1N1)v were subjected to the USDA-validated qRT-PCR procedure for the general detection of type A influenza virus RNA (matrix screening assay), following procedures described previously (Spackman and Suarez, 2008).

3. Results

3.1. Performance of the (H1N1) 2009 qRT-PCR assay

The no-template controls and 2009 (H1N1) negative specimens did not produce detectable fluorescence signal. The detection limit of the assay was assessed as 1×10^1 RNA copies μl^{-1}, whereas, in general, gel-based RT-PCR is limited generally to detect to 1×10^2 copies μl^{-1} of template. Tenfold dilutions of standard RNA were used to create a standard curve representing 10^1 to 10^9 copies of viral RNA standards and linearity was observed over the entire quantitation range (slope = −3.45). The coefficient of linear regression (R^2) was 0.998. In order to verify the reproducibility of the assay, intra-assay and inter-assay CVs were calculated and satisfactory results were obtained. Intra-assay CVs ranged from 22% (samples containing 5×10^7 RNA copies) to 44% (samples containing 2×10^2 RNA copies), whereas the inter-assay CVs ranged from 27% (2×10^3 RNA copies) to 51% (2×10^2 RNA copies). North American swine influenza virus isolates as well as equine influenza virus isolates were successfully detected by the USDA-validated qRT-PCR (data not shown). All endemic North American swine influenza virus isolates were negative for (H1N1) 2009 specific matrix gene RNA using the present qRT-PCR assay, whereas the (H1N1) 2009 strains used as positive control were positive. Cross-reactivity with other extant swine viral pathogens was not detected. Amplification of equine and avian influenza virus for (H1N1) 2009 remained below detection threshold, although weak cross-reactivity was observed at later cycle numbers. A single avian influenza isolate (A/Mynah/Mass/71 H4N8) was detected by (H1N1) 2009 qRT-PCR.

3.2. Isolation from clinical specimens

In the group of pigs infected with A/California/04/2009 (H1N1)v, 29/30 nasal swabs (Table 2) and 10/15 BAL fluids (Table 3) were positive for isolation on cell culture. One nasal swab sample as well as all BAL fluid samples at 7 dpi were negative by virus isolation. In the group infected with A/Mexico/4108/2009 (H1N1)v virus, virus was isolated from 18/30 nasal swabs and from 10/15 BAL fluid samples. Seven nasal swabs collected at 3 dpi, 5 collected at 7 dpi, and all BAL fluids collected at 7 dpi were negative for virus isolation.

3.3. qRT-PCR analysis of clinical samples

The qRT-PCR results from the clinical specimens from the *in vivo* study are summarized in Tables 2 and 3. When tested with the (H1N1) 2009 qRT-PCR assay, 78 clinical specimens were positive with similar C_T values as the USDA-validated qRT-PCR assays (data not shown). Briefly, 29/30 nasal swabs collected from the group infected with A/California/04/2009 (H1N1) virus and 19/30 nasal swabs collected from the group infected with A/Mexico/4108/2009 (H1N1) virus were positive by (H1N1) 2009 qRT-PCR. All BAL fluids samples were positive for both groups (15/15). The number of samples in agreement between the (H1N1) 2009 qRT-PCR and virus isolation (VI) were 63/78 including nasal swab and BAL fluid samples. In addition, 14 samples were positive by using (H1N1)

Table 2
Quantity of viral RNA copies per μl template extracted from nasal swabs collected at day (d) 3, 5, 7 p.i. from pigs infected with A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595). Nasal swabs collected at day 0 p.i. with negative results are not reported in the table as well as negative control groups. VI, virus isolation; NTC, no-template control; NS, nasal swab.

Pig number	VI	2009 (H1N1) qRT-PCR
551 d3	+	8.49×10^1
552 d3	+	1.06×10^2
553 d3	+	7.79×10^2
554 d3	+	2.66×10^2
555 d3	+	1.78×10^2
556 d3	+	6.92×10^2
557 d3	+	2.65×10^2
558 d3	+	1.27×10^2
559 d3	+	7.19×10^2
560 d3	+	–
561 d3	+	1.00×10^1
562 d3	+	1.00×10^1
563 d3	+	1.80×10^2
564 d3	+	1.00×10^1
565 d3	+	1.00×10^1
556 d5	+	6.87×10^3
557 d5	+	1.13×10^4
558 d5	+	9.51×10^3
559 d5	+	6.07×10^3
560 d5	+	2.50×10^4
561 d5	+	3.86×10^2
562 d5	+	2.01×10^3
563 d5	+	4.29×10^3
564 d5	+	9.14×10^3
565 d5	+	1.18×10^3
561 d7	+	1.00×10^1
562 d7	–	2.00×10^1
563 d7	+	1.00×10^1
564 d7	+	1.00×10^1
565 d7	+	2.98×10^1
NTC		–
581 d3	+	3.85×10^2
582 d3	+	1.30×10^2
583 d3	–	1.00×10^1
584 d3	–	1.00×10^1
585 d3	+	6.59×10^3
586 d3	–	–
587 d3	–	–
588 d3	–	–
589 d3	+	1.00×10^1
590 d3	–	–
591 d3	+	6.60×10^2
592 d3	+	9.01×10^2
593 d3	–	1.40×10^1
594 d3	+	–
595 d3	+	2.00×10^1
586 d5	+	1.61×10^2
587 d5	+	7.75×10^2
588 d5	+	2.10×10^2
589 d5	+	2.85×10^3
590 d5	+	–
591 d5	+	2.75×10^2
592 d5	+	2.16×10^2
593 d5	+	1.00×10^1
594 d5	+	1.59×10^1
595 d5	+	4.25×10^1
591 d7	–	–
592 d7	–	–
593 d7	–	–
594 d7	–	–
595 d7	–	–
NTC		–

2009 qRT-PCR and negative by using VI whereas only 3 samples were positive by VI and negative by (H1N1) 2009 qRT-PCR. The samples analyzed by the (H1N1) 2009 qRT-PCR contained a wide range of 2009 (H1N1) RNA copies per μl of template, from 2.56×10^2 to 1.14×10^7 (BAL fluid), and from 1×10^1 to 2.5×10^4 (nasal swabs).

Table 3
Quantity of viral RNA copies per μl template extracted from BAL fluids at day (d) 3, 5, 7 p.i. from pigs infected with A/California/04/2009 (H1N1)v (pigs 551–565) and A/Mexico/4108/2009 (H1N1)v (pigs 581–595). BAL fluid samples collected from the negative control group with negative results are not reported in the table. VI, virus isolation; NTC, no-template control.

Pig number	VI	2009 (H1N1) qRT-PCR
551 d3	+	2.90×10^6
552 d3	+	2.00×10^6
553 d3	+	9.67×10^4
554 d3	+	2.29×10^5
555 d3	+	1.48×10^6
556 d5	+	9.24×10^5
557 d5	+	2.65×10^5
558 d5	+	3.12×10^5
559 d5	+	9.00×10^4
560 d5	+	1.41×10^6
561 d7	–	1.50×10^4
562 d7	–	2.20×10^3
563 d7	–	4.78×10^3
564 d7	–	6.19×10^3
565 d7	–	5.71×10^5
NTC		–
581 d3	+	3.77×10^6
582 d3	+	1.73×10^5
583 d3	+	8.66×10^5
584 d3	+	2.08×10^5
585 d3	+	1.81×10^6
586 d5	+	1.21×10^5
587 d5	+	7.00×10^5
588 d5	+	5.00×10^5
589 d5	+	1.00×10^5
590 d5	+	1.14×10^7
591 d7	–	1.26×10^4
592 d7	–	1.77×10^3
593 d7	–	4.00×10^3
594 d7	–	3.50×10^4
595 d7	–	2.56×10^2
NTC		–

4. Discussion

The (H1N1) 2009 is of significant human and swine health concern and the future role of pigs in the ecology of this newly emerged virus remains unknown. There is an immediate and critical need for a rapid differential diagnostic method for pandemic (H1N1) 2009 virus detection in swine. Pandemic (H1N1) 2009 isolated from humans has had limited detection in the swine population so far (http://www.oie.int/eng/en_index.htm). However, pigs are susceptible to the infection, as demonstrated by the clinical signs and viral loads that found in nasal swabs and BAL fluids at day 3, 5, and 7 dpi. Similar results have been described by others (Lange et al., 2009). It is likely that the virus will continue to jump from humans to naïve pigs and may become established as an endemic infection in the swine population. In that case, two consequences will be obvious: first, a reservoir of H1N1 virus in the swine population poses an elevated risk for human infection via aerosol transmission from clinically ill pigs, and second, dramatic economic losses for the pork industry due to direct disease related costs as well as indirect market losses. The long-term consequence is the increased chance for novel reassortment between endemic swine influenza viruses and the novel H1N1 in the swine host, posing further human and animal health risks. It is apparent that pigs may be infected at least transiently with wholly avian and/or human viruses, allowing reassortment with swine viruses to acquire avian and/or human virus gene segments (Karasin et al., 2000; Ma et al., 2007). The (H1N1) 2009 underscores the potential risk to the human population of other influenza virus subtypes and genotypes with the swine influenza virus TRIG backbone. Increased surveillance and monitoring for the (H1N1) 2009 as well as other swine influenza virus in both the swine and human populations are critical to under-

stand the dynamic ecology of influenza A viruses in susceptible host populations.

In the event of the pandemic virus spread in the swine population, the assessment of a specific innovative diagnostic tool that permits a rapid identification of the pandemic (H1N1) 2009 in pigs is an absolute necessity. In fact, a sensitive and specific diagnostic test is critical for the implementation of response measures to outbreaks in swine to reduce human risk of infection. The qRT-PCR assay described is able to detect the new pandemic (H1N1) 2009 viral RNA with the ability to differentiate the new lineage from the extant swine influenza viruses circulating in the North American swine population. The assay was shown to be reproducible and linear over a range of 9 orders of magnitude, from 10^1 to 10^9 RNA copies, thus ensuring an accurate measurement of (H1N1) 2009 viral loads in clinical samples. If compared with the classical gel-based RT-PCR protocol, the processing time required by TaqMan RT-PCR is shorter, the contamination risks are lower because of the lack of post-amplification steps, and the specificity is enhanced by the probe hybridization. The specificity of the (H1N1) 2009 qRT-PCR was assessed against a set of viruses associated with respiratory disease in swine, including endemic North American swine influenza virus isolates, PRCoV, TGEV, PRRSV, porcine circovirus type 2, swine adenovirus, porcine parvovirus, blue-eye paramyxovirus and pseudorabies virus. Cross-reaction was not identified in swine specimens, thus providing evidence for high fidelity of the assay for the exclusive detection of (H1N1) 2009 in clinical samples from swine. There is a potential to detect matrix genes not of the classical swine lineage, especially those of avian lineage including the avian-like Eurasian lineage viruses. Since no Eurasian avian-like matrix genes have been reported in the US, any identification of avian-like or equine-like matrix genes would be a novel finding and should be investigated by further molecular diagnostics such as sequencing. Importantly, no endemic US swine influenza viruses tested here were shown to have the Eurasian swine influenza virus-lineage matrix gene, indicating the (H1N1) 2009 virus was not circulating in the US prior to 2009 based on current knowledge. Further testing of swine influenza virus repositories at veterinary diagnostic laboratories is warranted to rule out the existence of this lineage of viruses in North America prior to 2009.

The (H1N1) 2009 qRT-PCR was shown to be more sensitive with respect to VI in pigs inoculated experimentally over the duration of the shedding period. Indeed, 14 samples were demonstrated to be negative by VI but positive by qRT-PCR. However, 3 nasal swabs positive by VI were negative when tested by both qRT-PCR assays. The qRT-PCR may not be more sensitive than VI early in the course of infection when viral titers are extremely low, but it is more rapid and more specific and was more sensitive later in the course of infection. Additional testing in the diagnostic laboratory setting is necessary to compare further the (H1N1) 2009 qRT-PCR with VI. Indeed, the titers of the three 2009 qRT-PCR-negative samples were very low, $10^{0.5}$, $10^{0.7}$ and $10^{1.3}$ $TCID_{50}$/ml. VI is recognized as the gold standard for the detection of influenza viruses but is time-consuming and labor intensive, and lacks specificity; thus the qRT-PCR assay described here can be useful in (H1N1) 2009 outbreaks, experimental challenge studies, and vaccine trials as well. Although a large collection of (H1N1) 2009 virus isolates were not available in our laboratories for testing, analysis of published M-gene sequences of strains from worldwide geographical areas allowed us to pinpoint a novel lineage-specific nucleotide sequence for diagnostic development. The sensitivity of the (H1N1) 2009 qRT-PCR is comparable to the canonical USDA-validated type A influenza virus assay reported by Spackman and Suarez (2008), thus encouraging the use of both assays, first for influenza A screening, followed by differentiation and quantification of pandemic (H1N1) 2009 RNA in clinical samples as described here. In fact, while the type A influenza virus real-time RT-PCR matrix screening assay is able to detect all viral isolates tested in this study, the (H1N1) 2009 qRT-PCR selectively detects only the novel pandemic (H1N1) 2009 viral RNA. This assay can be a powerful tool in the diagnostic laboratory setting for specific simultaneous analysis of up to 96 samples on the same plate in minimal time.

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Influenza virus coinfection with *Bordetella bronchiseptica* enhances bacterial colonization and host responses exacerbating pulmonary lesions

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ABSTRACT

Influenza virus (Flu) infection and secondary complications are a leading cause of morbidity and mortality worldwide. The increasing number of annual Flu cases, coupled with the recent Flu pandemic, has amplified concerns about the impact of Flu on human and animal health. Similar to humans, Flu is problematic in pigs, not only as a primary pathogen but as an agent in polymicrobial pneumonia. *Bordetella* species play a role in mixed infections and often colonize the respiratory tract without overt clinical signs. Pigs serve as a valuable animal model for several respiratory pathogens, including *Bordetella* (Bb) and Flu. To investigate Flu/Bb coinfection pathogenesis, a study was completed in which pigs were inoculated with Flu-only, Bb-only or both agents (Flu/Bb). Results indicate that Flu clearance is not altered by Bb infection, but Flu does enhance Bb colonization. Pulmonary lesions in the Flu/Bb group were more severe when compared to Flu-only or Bb-only groups and Bb did not cause significant lesions unless pigs were coinfected with Flu. The type I interferon response was elevated in coinfected pigs, but increased expression of antiviral genes Mx and PKR did not appear to enhance Flu clearance in coinfected pigs, as viral clearance was similar between Flu/Bb and Flu-only groups. IL-1β and IL-8 were elevated in lungs of coinfected pigs, correlating to the days enhanced lesions were observed. Overall, Flu infection increased Bb colonization and enhanced production of proinflammatory mediators that likely contribute to exacerbated pulmonary lesions.

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1. Introduction

Influenza virus (Flu) infection and complications associated with Flu disease are a leading cause of morbidity and mortality worldwide. Recent increases in the annual number of Flu cases, coupled with the emergence of the novel H1N1 pandemic strain (pH1N1), have amplified concerns about the impact of Flu infection on human and animal health. Pigs are a natural host for influenza A virus, and suffer a similar clinical disease to that observed in humans [1]. Influenza disease manifests itself rapidly, with an incubation period of 1–3 days, followed with recovery beginning 4–7 days after the onset of symptoms. The acute stage of disease in pigs is characterized by fever, inactivity, decreased food intake, coughing, sneezing, and nasal discharge [1]. Although Flu is typically a self-limited infection characterized by high-morbidity and low mortality, secondary complications substantially increase flu-associated illness and death [2].

In humans, bacterial pneumonia secondary to Flu infection is often observed. The same phenomenon is also seen in pigs, as swine Flu is a key contributor to the porcine respiratory disease complex (PRDC), a multifactorial complex characterized by severe respiratory disease after infection with two or more agents. Bacterial pathogens associated with PRDC include *Haemophilus parasuis*, *Streptococcus suis*, *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica*. *Haemophilus influenzae* and *Streptococcus pneumoniae* are commonly associated with secondary bacterial infection in humans, with increasing isolations of *Staphylococcus aureus* being reported [3]. Recent reports indicate that pulmonary bacterial infections are occurring in individuals infected with pH1N1 influenza, similar to that observed with seasonal influenza [4,5]. However, the age-group most affected by pH1N1 is individuals less than 65-years-old, in contrast to the age-group most

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affected by seasonal influenza [6]. The majority of animal studies investigating Flu and bacterial superinfection use a sequential infection model in which the animals are first inoculated with Flu and subsequently challenged with a bacterial pathogen. This approach clearly shows that Flu predisposes to bacterial pneumonia, but clinical cases are often reported as coinfection and it is difficult to determine the order, if not simultaneous, of infection with Flu and bacterial pathogen [7,8].

Upon infection, the innate immune response is critical for controlling pathogen spread and initiating the adaptive immune response. Host cells recognize conserved motifs expressed by various pathogens and respond with the production of proinflammatory cytokines. Tracheal epithelial cells and other pulmonary cells, such as alveolar macrophages, play a critical role in pulmonary health by responding to invading microorganisms with the production of innate immune mediators. After detection of a pathogen, host cells produce proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and chemokines (e.g., IL-8, MCP-1, and RANTES) to activate cellular defense mechanisms and initiate the infiltration of additional immune effector cells, such as neutrophils. Type I interferons (IFN-α/β) are important mediators of the antiviral response by initiating the production of intracellular antiviral mediators, such as Mx-1 and dsRNA-dependent protein kinase R (PKR) [9]. The host antimicrobial response in the lung must be sufficient to combat the infection, but also regulated to prevent overt immunopathology that could impair gas exchange.

Animal models to study Flu pathogenesis include macaques, ferrets, chinchillas, guinea pigs, cotton rats, chickens, pigs and mice [10–15]. Mice have been used extensively to study the host response to Flu infection because of the availability of reagents and gene knock-out strains [16–19]. Though these studies have provided useful information, mice are not a natural host to influenza A virus and such studies require the use of mouse adapted strains of influenza. In addition, alveolar development in the mouse lung is different than that in humans and pigs [20] and mice, unlike pigs, do not express an ortholog to human IL-8 [21], though murine KC appears to be analogous to human and pig IL-8. Ferrets and pigs are useful models for studying lower respiratory tract Flu infection (with non-avian strains) because the host cells to which the virus attach are similar to those observed for humans [22]. Ferrets are a principal model for Flu pathogenesis studies, but lack of reagents has limited their use in studying the immune response to infection. An ideal animal model is one in which the animal is a natural host to the pathogen of interest, reagents are available for studying host response to infection, and the course of disease reproduces what is known about human disease.

Pigs are natural host to Flu and the clinical disease is similar to that of humans, making them very useful for Flu studies. The bacterial components of influenza-associated bacterial pneumonia can also be studied in pigs because the bacterial pathogens causing disease in humans are similar to those in pigs [23]. In the current study we investigated disease pathogenesis and host immune response following coinfection of pigs with Flu and *B. bronchiseptica* (Bb). The result of coinfection with Flu and Bb has not been previously studied, thus, we used a simultaneous inoculation strategy to establish if there is an enhancement of disease with coinfection.

2. Results

2.1. Flu/Bb coinfection does not affect influenza nasal shedding and minimally alters Bb nasal colonization

To determine if coinfection alters nasal shedding of Bb or Flu, groups of pigs were challenged with Flu, Bb, or both agents (Flu/Bb) and infectious load of each agent in the nasal cavity was evaluated. Results from nasal swab samples indicate that coinfection did not significantly alter shedding of Flu from the nasal passages nor did it alter the kinetics of clearance of virus from the nasal cavity (Fig. 1A). The average Flu titer in nasal swabs peaked between days 4 and 5 post-challenge for the Flu-only group and the Flu/Bb group, and, there was no statistical difference in titers between challenge groups. Flu titers in both groups decreased by day 6 post-challenge, with clearance by day 8 in all but one pig in the Flu-only group.

Bb burden in the nasal cavity was statistically similar between groups of Bb-only pigs and Flu/Bb pigs on all sample dates except day 8, with higher Bb CFU in the coinfected group (Flu/Bb) when compared to the Bb-only group (Fig. 1B). This trend continued on day 9 post-challenge, but by day 10 there was no statistical difference between challenge groups. Nasal Bb CFU increased gradually after inoculation in both groups, with an average peak in colonization occurring on day 5. Bb colonization decreased slightly thereafter and leveled off by day 10 following challenge.

2.2. Flu/Bb coinfection increases Bb burden but not Flu titers in the respiratory tract

To further evaluate the effect of coinfection on infectious load in the respiratory tract, four pigs from each group were euthanized on days 1, 5 and 10 following challenge to measure Flu titer and Bb CFU in the trachea and lung. Flu titers in the trachea and lung were not different between challenge groups (Flu-only versus Flu/Bb) on days 1, 5 or 10 following inoculation (Fig. 2A). On day 1 following inoculation Flu virus was recovered from the trachea of all coinfected pigs and 3 of the 4 pigs infected with Flu-only. By day 5 Flu virus was isolated from the trachea of all Flu-only and Flu/Bb infected pigs. Flu titers in the lung were lower than those observed in the trachea, though the kinetics of infection were similar between the 2 sample sites. By day 10 following inoculation Flu virus was not isolated from the trachea or lung of pigs in either challenge group (Fig. 2A).

Although Flu/Bb coinfection did not alter Flu titers in the trachea or lung (Fig. 2A), coinfection did significantly affect Bb colonization (Fig. 2B). On day 1 post-challenge Bb was isolated from the trachea of only 2 of the 4 pigs infected with Bb-only, but was isolated from the trachea of all 4 pigs in the Flu/Bb group (Fig. 2B). Trachea Bb CFU were also higher in the coinfected animals on day 1 post-challenge when compared to the Bb-only group ($p = 0.06$, Fig. 2B). By day 5 post-challenge Bb CFU in the trachea were not different between challenge groups and Bb was not recovered from the trachea of one pig in each group. On day 10 post-challenge pigs in both groups were colonized with Bb in the trachea and no significant difference in CFU was observed between groups. In the lung, a significant difference in Bb colonization was measured between the Bb-only group and Flu/Bb group on days 1 and 10 following inoculation (Fig. 2B). On day 1 following challenge Bb was not isolated from the lungs of any of the pigs in the Bb-only group, but Bb was isolated from all the pigs in the Flu/Bb coinfected group ($p < 0.001$). On day 5 following challenge lung Bb burden was not different between the 2 challenge groups, but on day 10 there was a significant difference in Bb lung colonization ($p = 0.04$). Overall, these results indicate that coinfection with Flu and Bb results in increased Bb colonization in the lower respiratory tract of pigs, primarily in the lung.

2.3. Flu/Bb coinfection leads to enhanced macroscopic and microscopic lung lesions

2.3.1. Macroscopic lesions

On day 1 post-challenge macroscopic lesions, characterized by dark, red-colored consolidation with well demarcated borders and a cranial–ventral distribution, were observed in all 4 coinfected pigs

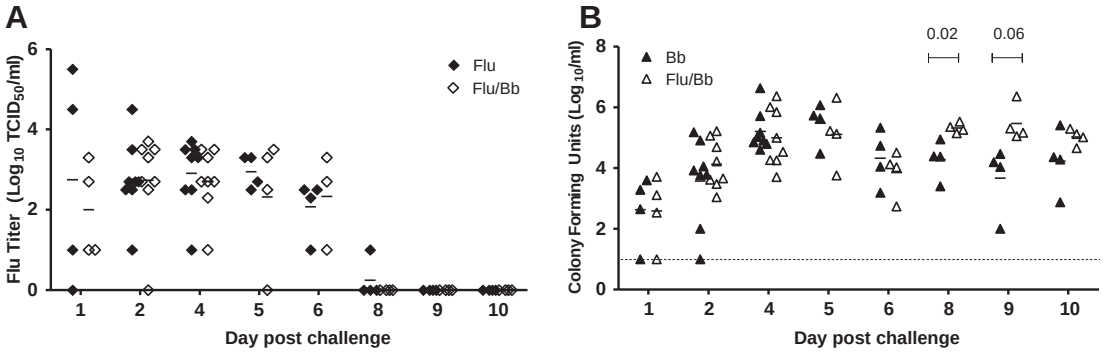

Fig. 1. Influenza virus titers and *B. bronchiseptica* CFU in the nose. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). Nasal swabs were collected at the indicated days post-challenge and (A) Flu titers and (B) Bb were enumerated for each pig. Flu was not isolated from Bb-only pigs and Bb was not isolated from Flu-only pigs (data not shown). Each data point represents a single pig with the bar at the average for the group. The number of samples collected at each time point is explained in Materials and Methods section. A student's *t*-test was performed for statistical analysis.

but none of the pigs infected with Flu-only or Bb-only (Table 1). On day 5 post-challenge 3 of the 4 pigs inoculated with Flu-only exhibited macroscopic lesions, whereas all 4 pigs in the Flu/Bb groups exhibited lesions. Enhanced pneumonia was also observed on day 10 post-challenge in the coinfected group, as all 4 pigs had macroscopic lesions with a greater percentage of the lung affected when compared to the single pig exhibiting lesions in the Flu-only group. Pneumonia lesions were not observed in any of the pigs inoculated with Bb-only or mock-infected on any necropsy date.

2.3.2. Microscopic lesions

Fig. 3 shows representative images depicting microscopic lung lesions appreciated in the different challenge groups at different days post-infection. On day 1 following challenge, 2 of the 4 pigs in the Flu-only group had no significant lesions and the other 2 had only mild peribronchiolar lymphocytic infiltration. All 4 pigs in the Flu/Bb group had moderate peribronchiolar lymphocyte infiltration as well as changes to the bronchiolar epithelium consisting of mild to moderate rounding and degeneration of epithelial cells

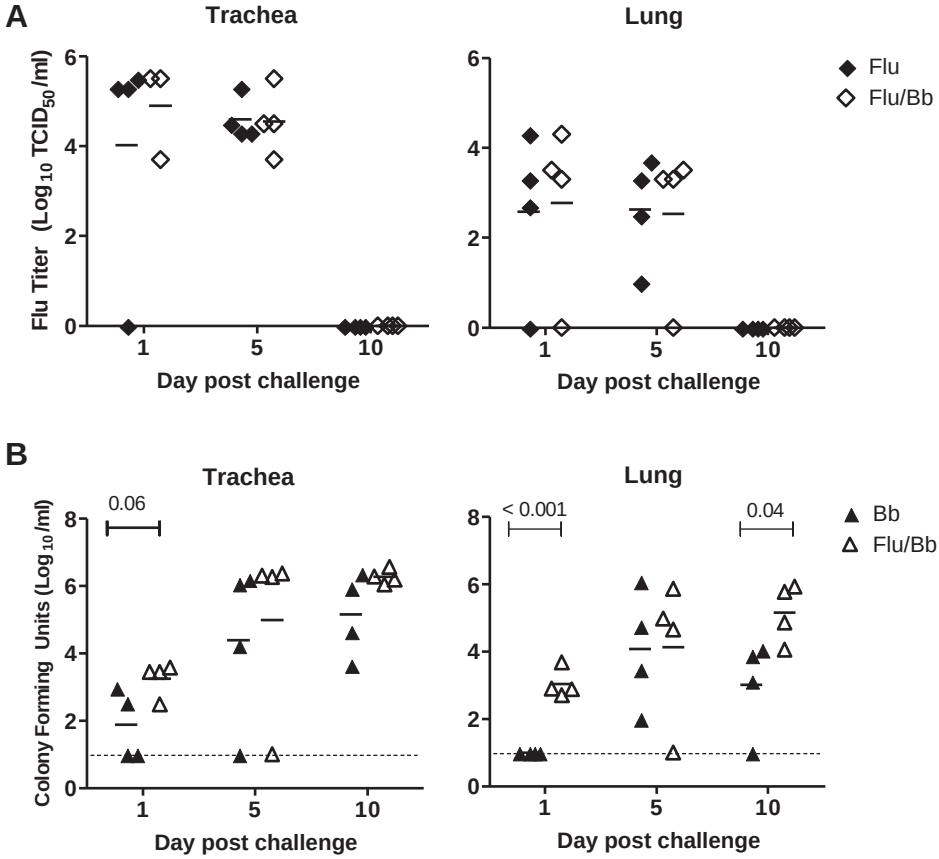

Fig. 2. Influenza virus infection altered pulmonary *B. bronchiseptica* colonization in the lower respiratory tract. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). On day 1, 5, and 10 post-challenge (A) influenza virus and (B) *B. bronchiseptica* were enumerated in the trachea wash and lung lavage. Flu was not isolated from Bb-only pigs and Bb was not isolated from Flu-only pigs (data not shown). Each data point represents a single pig with a bar at the average. A student's *t*-test was performed for statistical analysis.

Table 1
Mean percentage[a] of lung affected by pneumonia.

	Day 1	Day 5	Day 10
Sham	0 (0/4)[b]	0 (0/4)	0 (0/4)
Bb	0 (0/4)	0 (0/4)	0 (0/4)
SIV	0 (0/4)	3.19 (3/4)	2.38 (1/4)
SIV/Bb	2.38 (4/4)	4.69 (4/4)	4.75 (4/4)

[a] See Materials and Methods for calculation.
[b] Number of animals exhibiting lesions out of total number of animals in the group.

progressing to cuboidal to squamous metaplasia and intraluminal accumulation of neutrophils. On day 5 post-challenge, lesions were similar between Flu-only and Flu/Bb pigs, though only 3 of the 4 Flu-only pigs had lesions and all 4 coinfected pigs had lesions. Observed changes included peribronchiolar lymphocyte infiltration, attenuation and loss of the airway epithelium and intraluminal accumulation of neutrophils, alveolar epithelial cell necrosis and filling of alveoli with necrotic debris and mixed inflammatory cell infiltrates, and interlobular edema. None of the Bb-only pigs had significant lesions on day 1 or 5 post-challenge and only 1 of the 4 Bb-only pigs had lesions at day 10 characterized by a focal area of mild filling of the alveoli with macrophages and some syncytia. On day 10 post-challenge, 2 of the 4 Flu-only pigs had lesions, 1 with changes similar to those observed on day 5 only milder, and the other had lesions similar to day 5. On day 10 all 4 pigs in the coinfected group had significant lesions with characteristics similar to those observed on day 5.

2.4. Type I interferon response is enhanced in pigs coinfected with Flu/Bb

mRNA levels of IFN-α, Mx, and PKR in tracheal epithelial cells and lung were evaluated. On day 1 following challenge, transcription of IFN-α was significantly elevated in tracheal epithelial cells from coinfected (Flu/Bb) pigs compared to pigs infected with Flu-only or Bb-only (Fig. 4A). In addition, expression of antiviral mediators Mx and PKR was elevated in the trachea of coinfected pigs on day 1 post-challenge (Fig. 4B). On days 5 and 10 post-challenge, Mx and PKR mRNA levels were increased in both the trachea of the Flu-only and Flu/Bb groups, though no significant difference between the two groups was observed (data not shown).

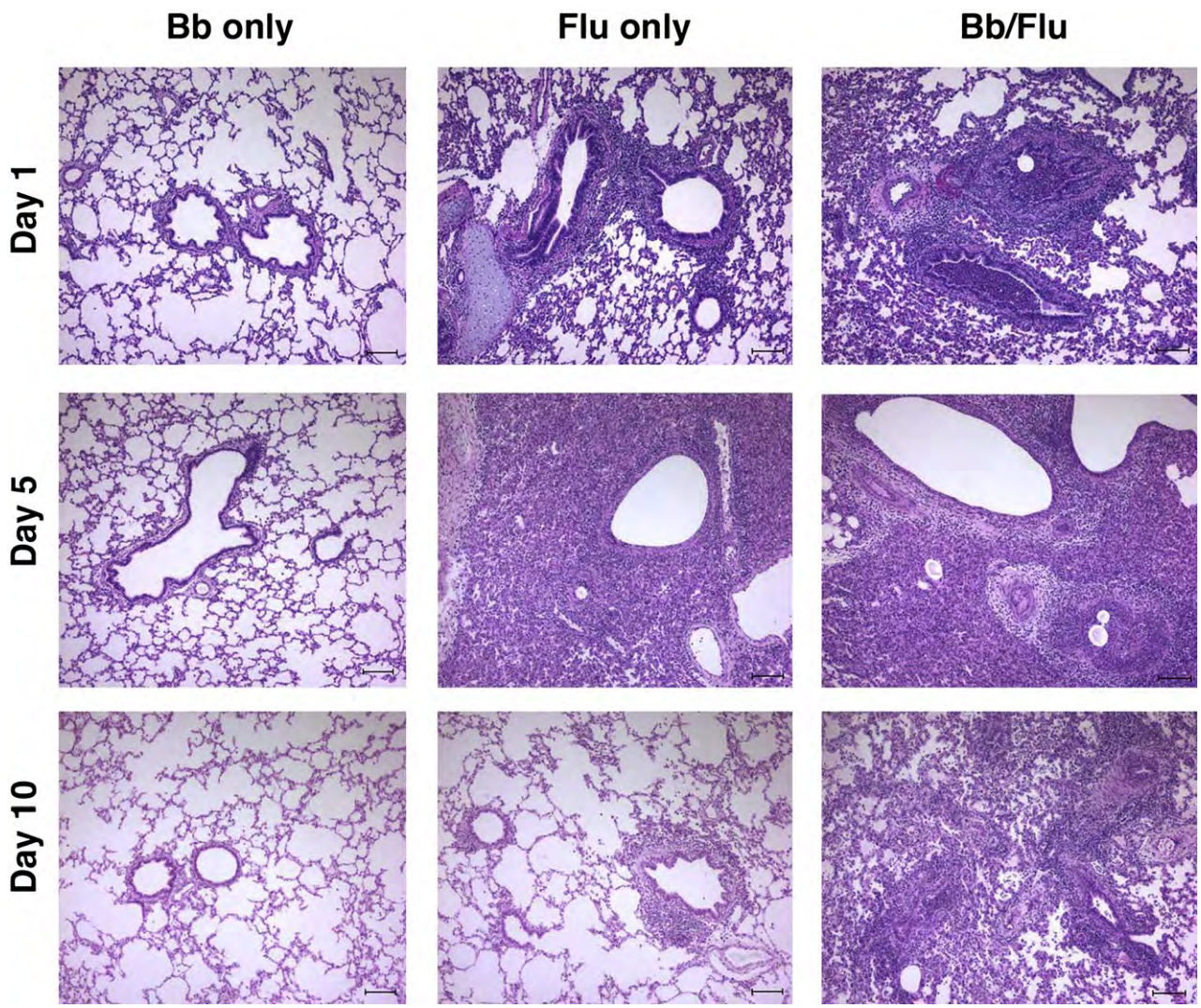

Fig. 3. Pulmonary microscopic lesions were more severe in Flu/Bb coinfected pigs on days 1 and 10 post-challenge. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). On days 1, 5, and 10 post-infection a section of the right cranial lobe was collected for histological examination. A representative image from a single pig in each challenge group is shown. All images are at 10× magnification.

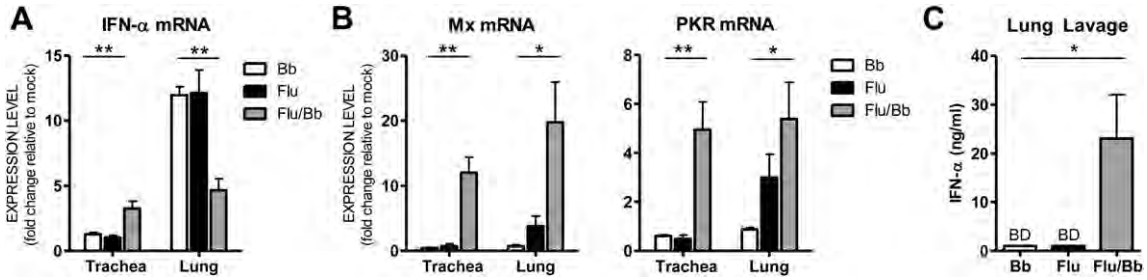

Fig. 4. Type I interferon response was enhanced in coinfected pigs on day 1 post-challenge. Groups of pigs were infected with influenza A virus (Flu), *B. bronchiseptica* (Bb), or coinfected with both agents (Flu/Bb). On day 1 post-challenge (A) mRNA levels of IFN-α, Mx-1, and PKR were measured in tracheal epithelial cells and lung by real-time PCR and (B) IFN-a protein levels in the broncho-alveolar lung lavage were measured by ELISA. Data are shown as mean ± SEM of four animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. *p*-values less than 0.05 are noted with a single-asterisk and *p*-values less than 0.01 are noted with a double-asterisk.

In the lung, IFN-α mRNA levels were similar between pigs in the Flu-only and Bb-only groups, both averaging approximately a 12-fold increase over mock-treated pigs (Fig. 4A). Coinfected pigs had approximately a 4-fold increase in IFN-α mRNA levels in the lung compared to mock-treated pigs, which was significantly less than the levels observed in pigs infected with Flu-only or Bb-only (Fig. 4A). The downstream genes Mx and PKR were not significantly elevated in the lungs of pigs infected with Flu-only or Bb-only, but were elevated in coinfected pigs (Fig. 4B). On days 5 and 10 post-challenge, Mx and PKR mRNA levels in the lung were elevated in the Flu-only and Flu/Bb groups, with no significant difference between the groups observed (data not shown). The amount of IFN-α protein in the lung was significantly elevated in the lungs of coinfected (Flu/Bb) pigs, but was not detected in pigs infected with either agent alone (Fig. 4C). IFN-α protein could not be detected in any samples taken after day 1 (data not shown).

2.5. Enhanced proinflammatory cytokine response in the lungs of pigs coinfected with Flu/Bb

The proinflammatory cytokine response is critical for recruiting effector cells to the site of an infection but elevated or prolonged production can also contribute to pathogenesis observed during disease. To determine if coinfection with Flu and Bb results in increased production of proinflammatory mediators IL-1β or IL-8, mRNA and protein levels in the lung were evaluated. Non-infected animals served as mock-treated controls. On day 1 following challenge, pigs in the Flu/Bb group had enhanced mRNA expression levels of both IL-1β and IL-8 compared to pigs infected with Flu-only or Bb-only (Fig. 5A). By day 5 post-challenge, IL-1β and IL-8 mRNA levels in coinfected pigs had decreased significantly from the elevated levels observed on day 1. The increased mRNA levels coincided with increased detection of IL-1β and IL-8 protein in the

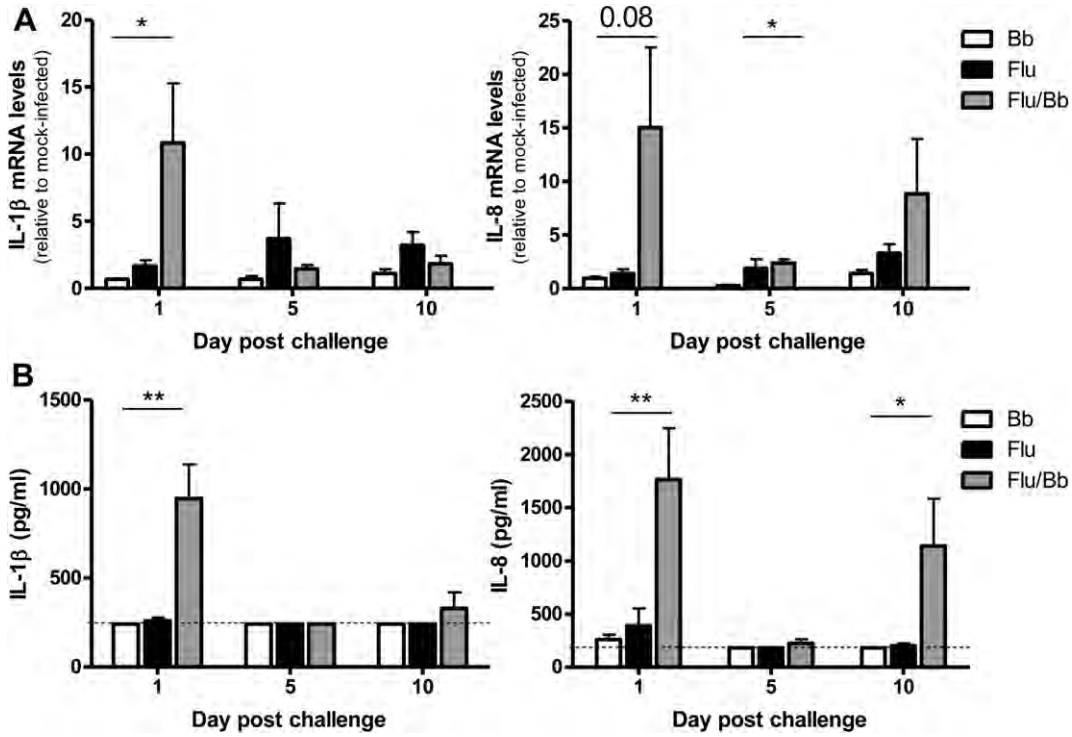

Fig. 5. Coinfection altered the proinflammatory cytokine response in the lung. Groups of pigs were infected with influenza A virus (Flu), *B. bronchiseptica* (Bb), or coinfected with both agents (Flu/Bb). On days 1, 5 and 10 post-infection (A) mRNA levels of IL-1β and IL-8 were measured in lung by real-time PCR and (B) IL-1β and IL-8 protein levels in the broncho-alveolar lung lavage were measured by ELISA. Data are shown as mean ± SEM of four animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. *P*-values less than 0.05 are noted with a single-asterisk and p-values less than 0.01 are noted with a double-asterisk.

lung on day 1 as well. IL-8 mRNA levels were significantly increased in both Flu-only and Flu/Bb pigs on day 5 following challenge, but this increase in mRNA did not result in differences in IL-8 protein (Fig. 5A and B). IL-8 mRNA levels were increased on day 10 following challenge in the Flu/Bb group which coincided with significantly increased levels of IL-8 protein in the lung on day 10. Taken together, proinflammatory cytokine responses in the lung were heightened in pigs coinfected with both Flu/Bb when compared to pigs infected with either agent alone.

3. Discussion

In pigs *B. bronchiseptica* (Bb) infection often results in chronic colonization, and depending on the age in which the animal becomes infected, bacteria can be routinely isolated from the respiratory tract without overt clinical signs of disease. Neonatal piglets (less than 2 weeks of age) that become infected often suffer a more severe disease than older pigs. In the current study, 4-week old pigs were used; therefore, Bb alone caused minimal disease, even though it was re-isolated from the lungs (Fig. 2). Flu infection in pigs causes an acute disease, with virus titers and lung lesions peaking on day 5 following infection, and viral clearance by day 7 [1]. In the current study, the course of influenza disease did not appear to be altered by Bb coinfection. Viral titers in the respiratory tract still peaked on day 5 and virus was cleared by day 10 (Fig. 2). However, the course of Bb disease was altered by Flu coinfection. Pulmonary lesions were observed by day 1 following coinfection, and lesions persisted in a more severe state in coinfected pigs than that observed in pigs infected with either pathogen alone. These data suggest a more rapid and heightened inflammatory response during coinfection that likely plays a role in the severity of pulmonary lesions.

There are several reported methods by which Flu infection predisposes to secondary bacterial infection, including compromising the respiratory epithelial barrier [24], increasing host expression of receptors for bacteria leading to increased colonization [25,26], and altering host immune responses [16,27]. It's likely that these mechanisms are not mutually exclusive, but instead, it is probable that Flu compromises host health in a variety of ways. For example, Flu can decrease tracheal mucociliary velocity, contributing to increased bacterial colonization, as shown with *S.pneumoniae* [28]. In the current study, Bb colonization was increased primarily in the lungs of coinfected pigs, suggesting that changes to the ciliated epithelium may not be the only mechanism in which Flu predisposes to secondary Bb infection. If this was the case, we would have expected enhanced Bb colonization in the trachea of coinfected pigs. Yet, tracheal Bb colonization was slightly different on day 1 following challenge, but not day 5 or 10 (Fig. 2B).

Another mechanism by which Flu predisposes to secondary infection is by impairing alveolar macrophage phagocytic function, subsequently hindering bacteria uptake and killing [29,30]. Also, Flu infection has been shown to alter the response of macrophages to secondary stimuli [16]. In the current study, increased numbers of Bb were isolated from the lung, indicating that Flu is likely causing a defect in the response and clearance of Bb from the lung, but it is not clear if this is due to a direct defect in macrophage responses. Production of IL-1β and IL-8 proinflammatory cytokines was increased in the lungs of coinfected animals; thus, it does not appear that Flu caused a decreased response to secondary stimuli, but instead a heightened response (Fig. 5). Didierlaurent et al. recently reported a desensitization of lung cells to TLR agonists following respiratory viral infection, which is in contrast to our findings [16]. The difference is likely due to the timing of stimulation, as we did simultaneous infection, whereas the previous report looks at responses several weeks following viral challenge [16]. A study in mice examining the host response to pneumococcal infection 7 days after Flu infection shows increased production of proinflammatory cytokines in the lung following bacterial challenge, more similar to our findings [27]. However, a recent report examining influenza pathogenesis in mice previously infected with an attenuated strain of *B. pertussis* indicate that prior bacterial inoculation can attenuate influenza pathogenesis depending on the bacterial dose and time of exposure [31]. Thus, the time of secondary insult is likely to have an effect on the host response and understanding the agonist, kinetics, and magnitude of the response will be important for developing therapeutics to combat bacteria/Flu superinfection. The work described here shows that simultaneous coinfection with Flu and Bb results in exacerbated disease and future work is aimed at looking at disease pathogenesis following sequential infection with influenza infection preceding or proceeding *B. bronchiseptica* infection.

There did appear to be a discord between the detection of IFN-α mRNA and protein in the lungs (Fig. 4) and the reason for this is not completely clear. The IFN-α mRNA expression level in coinfected pigs was elevated over mock-treated animals, but IFN-α mRNA expression levels were greater in the Flu-only and Bb-only pigs (Fig. 4A). It is possible that IFN-α mRNA levels in the coinfected pigs were higher before dpi 1, which would explain the detection of IFN-α protein and the transcription of the downstream antiviral mediators Mx and PKR on dpi 1 in this group. There is a feedback loop for regulating type I IFN production that may have decreased the signal for IFN-α mRNA in the coinfected group [32]. We did not sample the lungs between days 1 and 5 following challenge, so it's possible that IFN-α protein levels were elevated in the Flu-only and Bb-only pigs on one of these days. The type I IFN response is known for its role in the antiviral immune response; however, even with the differences we observed in IFN-α protein levels, and Mx and PKR mRNA levels, Flu clearance did not seem to be altered by coinfection. Coinfection may alter the kinetics of the type I IFN response and a more in-depth analysis with additional time points for sample collection would likely provide additional information.

In Flu/Bb coinfected pigs, the increased levels of IL-8 and IL-1β cytokine coincided with increased pulmonary lesions and changes were observed within a day following challenge and continued to be increased at day 10 (Fig. 5 and Table 1). One might speculate that the increased cytokine production was the result of increased bacterial burdens observed in the lungs of coinfected pigs when compared to Bb-only pigs. While this may be true for day 1, there was no significant difference in lung Bb CFU in the coinfected pigs between days 5 and 10 (Fig. 2). However, cytokine levels were minimal on day 5, and increased on day 10 in the coinfected group (Fig. 5). Thus, the number of Bb alone is not sufficient to explain the increase in cytokine responses observed at day 10 following coinfection. IFN-α has been shown to increase TLR responsiveness in macrophages and it is possible that influenza infection increases the expression of TLR that would enhance responsiveness to secondary bacteria stimulation [33]. A recent report on sequential infection of mice with Flu and *Bordetella parapertussis* shows that increased proinflammatory responses, as opposed to increased bacterial burdens, are detrimental to the host [34]. Overall, Flu/Bb coinfection resulted in an impaired clearance of Bb from the lower respiratory tract, and the heightened cytokine response did not correlate with Bb clearance. Instead, Bb persisted at higher levels in the lungs of coinfected animals and pulmonary lesions were exacerbated.

Bacterial pneumonia, with Flu infection, continues to contribute significantly to Flu-associated morbidity and mortality. With the recent emergence of the 2009 pandemic Flu, coupled with concerns of antibiotic resistance to bacterial pathogens, it is important to understand mechanisms in which Flu and bacteria act

synergistically in the lung and cause severe pneumonia. This will allow for a more targeted approach to treatment as well as prevention. Although mice serve a useful purpose in the study of disease pathogenesis, other animal models, such as the one described here, provide useful information in infectious disease research. Our results show that pigs are similarly affected by Flu as humans are, and Flu can be exacerbated by bacterial coinfection in pigs. While the emphasis has been on secondary bacterial infection following primary influenza infection, or results show that simultaneous coinfection with Flu and a bacterial pathogen can result in enhanced pneumonia.

4. Materials and methods

4.1. Influenza virus and B. bronchiseptica inocula

A classical, α-cluster H1N1 swine influenza virus isolate, A/Swine/Minnesota/37 866/1999 (MN99), was prepared in Madine–Darby Canine Kidney (MDCK) cells as previously described in Refs. [35,36]. Non-infectious cell culture supernatant from MDCK cells was used for sham inoculum. Pigs were inoculated intranasally with 2 ml (1 ml/nostril) of 2×10^6 $TCID_{50}$/ml of virus or sham supernatant. *B. bronchiseptica* strain KM22 is a virulent phase I swine isolate initially cultured from a herd with atrophic rhinitis [37]. To prepare the inoculum, *B. bronchiseptica* was cultured on Bordet-Gengou agar supplemented with 10% sheep's blood at 37 °C for 40 h. A culture suspension with an A_{600} of 0.42 was prepared in phosphate-buffered saline (PBS). This suspension has approximately 2×10^9 colony forming units (CFU)/ml, and a 1:2000 dilution of this suspension was made in PBS for generation of the final inoculum. Pigs were inoculated intranasally with 1 ml (0.5 ml/nostril) of the final inoculum. Cultured dilutions of the inoculum contained approximately 10^6 CFU/ml and one hundred percent of the inoculum colonies appeared to be in the Bvg^+ phase, based on colony morphology and presence of hemolysis. PBS was used for sham inoculum. The bacterial and viral inoculums were prepared and delivered separately.

4.2. Experimental design

Six pregnant sows were transferred to an isolation facility at the National Animal Disease Center (NADC) approximately 2 weeks prior to their farrowing due date from a herd negative for antibody to porcine reproductive and respiratory syndrome virus (PRRSV) and Flu and negative for *B. bronchiseptica* colonization. Piglets from these sows were early weaned by 7 days of age to reduce the transfer of bacterial respiratory pathogens colonizing the sow to the piglets. No medication or vaccinations was given to the piglets. Nasal swabs were obtained from all the piglets prior to starting the experiment and neither Flu nor Bb were isolated. At 4 weeks of age, 2 piglets from each sow were divided into 4 groups with 12 pigs per group. Pigs in group 1 were inoculated with Bb and non-infectious cell culture supernatant (Bb-only), pigs in group 2 were inoculated with PBS and influenza virus (Flu-only), pigs in group 3 were inoculated with influenza virus and *B. bronchiseptica* (Flu/Bb), and pigs in group 4 were inoculated with PBS and non-infectious cell culture supernatant (Sham). Inoculums were administered separately and not mixed. Groups were housed in individual isolation rooms and cared for in compliance within the guidelines of the NADC Institutional Animal Care and Use Committee. Four pigs from each group were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) on days 1, 5, and 10 following challenge. Nasal swabs were taken on days 0, 2, 4, 6, 8, and 9 of the experiment from all pigs remaining in each challenge group on those days.

After euthanasia, nasal swabs, nasal wash, tracheal wash and broncho-alveolar lavage (BAL) were performed. The nasal swab was collected in 1 ml of MEM media and the nasal wash was performed by flushing 5 ml of phosphate-buffered saline (PBS) into the nostrils and collecting the effluent. The tracheal wash was performed by placing a 5 cm portion of trachea, taken immediately caudal to the larynx, into a 15 ml conical tube with 5 ml of PBS and gently agitating for 30 s BAL was performed by pipetting 20 ml of PBS into each of the cranial, middle, and accessory lobes and aspirating as much as possible back from each location. An aliquot of BAL was frozen at −80 °C for virus titration and cytokine protein analysis.

4.3. Virus and bacterial isolation

Number of CFU of Bb per ml of swab fluid, tracheal wash, and BAL was determined by plating serial 10-fold dilutions on duplicate selective blood agar plates containing 20 μg/ml penicillin, 10 μg/ml amphotericin B, 10 μg/ml streptomycin and 10 μg/ml spectinomycin as previously described in Ref. [38]. For determining viral load, nasal swab, tracheal wash and BAL were processed as previously described in Ref. [39]. Briefly, 10-fold serial dilutions of each sample were made in serum-free MEM media supplemented with TPCK trypsin plus antibiotics. Each dilution was plated in triplicate onto PBS-washed MDCK cells grown to confluency in 96-well flat-bottom plates. Plates were evaluated for cytopathic effect between 48 and 72 h post-infection. A $TCID_{50}$/ml was calculated for each sample using the method of Reed and Muench [40].

4.4. Cytokine gene expression

After collecting tracheal wash, epithelial cells were collected from a trachea section. A longitudinal cut was made down the dorsal membrane to open the trachea. Using a sterile razor blade, the epithelial lining of the trachea was scraped from the cartilage and collected in a microcentrifuge tube. RNA collection buffer (RLT, Qiagen Mini RNeasy Kit) was added and samples stored at −80° for later RNA extraction. RNA extraction was performed according to manufacturer's recommendations (Qiagen Mini RNeasy Kit).

At necropsy, a 1 g piece of lung from the right cranial lobe was placed in RNA later and frozen until RNA isolation was performed. For RNA extraction, approximately 30 mg of the lung piece was taken and minced with a sterile razor blade while frozen. A mortar and pestle was used to further disrupt the tissue piece. Sample was kept on ice during homogenization steps. RNA collection buffer was added to the sample, which was transferred to a 15 ml conical tube. The sample was lysed by sonicating 3 times, 10 s each, chilling on ice in between each sonication. The sample was centrifuged and the supernatant used for RNA extraction according to manufacturer's recommendations (Qiagen Mini RNeasy Kit).

Reverse transcription for cDNA synthesis was performed using random primers and SuperScript II Reverse Transcriptase according to the manufacturer's recommendations (Invitrogen). SYBR green based real-time PCR was carried out for various mRNA targets (SYBR Green Master Mix, Applied Biosystems) as previously described in Ref. [41]. Levels of mRNA were calculated using the $2^{-\Delta\Delta C_t}$ method, which expresses mRNA from the cells of infected pigs relative to the cells from sham pigs after normalizing to β-actin [42].

4.5. Cytokine protein levels

Levels of IFN-α protein in the BAL were measured by ELISA using F17 monoclonal antibody, K9 MAb and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described in Ref. [43]. Recombinant porcine IFN-α (rIFN-α) was used as a standard and sample concentrations were calculated from a standard

curve. Levels of interleukin-8 (IL-8) and interleukin 1-beta (IL-1β) in the BAL were measured by ELISA using DuoSet ELISA reagents from R&D Systems according to the manufacturer's recommendations (Minneapolis, MN).

4.6. Pathological evaluation

At necropsy, an estimate of gross lesion lung involvement was assigned based on the percentage of each lung lobe affected and the percentage of total lung volume each lobe represented. Percentage of total lung volume of each lobe was estimated as 10% for the left and right cranial, left and right middle and accessory lobes and 25% for the left and right caudal lobes. Gross lung lesion scores are frequently used to evaluate pneumonia in pigs [44–46] and the method used in these studies was initially described by Halbur et al. [47].

Sections from the lung were taken for microscopic evaluation. All tissues were fixed in 10% neutral buffered formalin for 24 h and then placed in 90% ethanol. All sections were routinely processed and embedded in paraffin, sectioned and stained with hematoxylin and eosin. Sections were evaluated by light microscopy and the evaluating pathologist was blinded to treatment groups.

4.7. Statistical analysis

GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA) was used for all statistical analyses. A student's *t*-test assuming unequal variance was used to compare the number of Bb CFUs and viral titer of influenza in different tissues comparing the single-infected group to the coinfected group. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test was used for analyzing cytokine data.

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Identification and characterization of a highly virulent triple reassortant H1N1 swine influenza virus in the United States

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Abstract A highly virulent H1N1 influenza A virus, A/Swine/Kansas/77778/2007 (KS07), which caused approximately 10% mortality in finishing pigs, was isolated from herds in the Midwestern United States. Molecular and phylogenic analysis revealed this swine isolate was a triple reassortant virus, similar to an H1N1 virus that infected humans and pigs at an Ohio county fair in August 2007. A pig challenge model was developed to evaluate the pathogenicity and transmission capacity of the KS07 virus. The results confirmed that the KS07 virus is highly virulent in pigs and easily transmitted to sentinel animals. The KS07 virus failed to cross-react with a panel of H1-specific swine sera. Interestingly, the KS07 virus shed for a prolonged period up to 7 days in infected pigs, indicating that this virus can spread efficiently between animals. The highly virulent H1N1 swine influenza virus is further evidence of reassortment among avian, human and swine influenza viruses and justifies the need for continued surveillance of influenza viruses in swine.

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Keywords Swine · Influenza A virus · Pathogenesis

Introduction

Swine influenza is an acute infectious respiratory disease in pigs caused by influenza A virus within the family *Orthomyxoviridae*. Influenza A viruses are negative, single-strand RNA viruses containing eight segments in their genomes. The segmented nature of the influenza A virus genome allows for antigenic shift or reassortment, leading to generation of novel viruses. Influenza A viruses are divided into different subtypes based on two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are 16 HA and 9 NA subtypes of influenza A viruses that have been found from wild waterfowl and seabirds [1]. Only H1, H3, N1 and N2 subtypes have been consistently isolated from pigs [2–4]. The first swine influenza virus (SIV) isolate in the United States was identified in 1930 belonging to the H1N1 subtype; this SIV subtype still circulates in swine populations worldwide. With the exception of one isolation of human H3N2 virus from pigs in Colorado in 1977 [5], only the classical swine H1N1 (cH1N1) virus was isolated in U.S. swine prior to 1998. In 1998, a triple reassortant H3N2 virus emerged and became endemic in North American swine herds. This virus was composed of HA, NA and polymerase basic 1 (PB1) genes from human influenza viruses; matrix (M), nonstructural (NS) and nucleoprotein (NP) genes from classical swine viruses; and polymerase acidic (PA) and polymerase basic 2 (PB2) genes from avian viruses [6]. Reassortment between triple reassortant H3N2 viruses and cH1N1 viruses has produced a variety of novel reassortants. A consistent finding among all of these viruses is the conservation of the internal genes from the H3N2 triple

reassortant virus. This constellation of genes (PB1 gene of human virus origin; PA and PB2 genes of avian virus origin and the remaining internal genes [M, NS and NP] of swine virus origin [7]) has been referred to as the triple reassortant internal gene cassette (TRIG) [8, 9]. In combination with the TRIG, the reassortant H1N1 viruses contain the HA and NA from the cH1N1 virus; the H1N2 viruses contain the HA from the classical swine virus and the NA from human H3N2 viruses [10, 11]; the H3N1 viruses contain the NA from the classical swine virus, the HA and internal genes from the triple reassortant H3N2 viruses [12, 13]; and in the last few years reassortants between human H1N1 and H1N2 genes with the TRIG cassette have become established in North American swine. The recent emergence of the swine origin pandemic H1N1 influenza A virus in humans demonstrates the flow of genes and viruses between humans and swine is a two-way process [14]. Interestingly, this human virus has a modified TRIG, which has picked up a new matrix gene of a Eurasian swine lineage [14, 15]. Although there have been sporadic reports of swine infected with the human pandemic H1N1 virus following contact with infected humans [16], the significance of the modified TRIG for swine and human infections remains unknown. Therefore, the continuous surveillance of influenza viruses and genes in swine populations is necessary for the protection of swine and humans.

In December 2007, an outbreak of severe respiratory disease occurred in pigs at a commercial grow-finish swine farm in Kansas and caused approximately 10% mortality in finishing pigs. Influenza A virus was identified from pigs, and the isolates were characterized as swine H1N1 virus genetically similar to the H1N1 virus isolated from an Ohio county fair which infected pigs and humans [17]. Here, we report the genetic and biological characterization of the swine isolate A/Swine/KS/7778/2007 (KS07) in an experimental challenge and transmission study.

Materials and methods

Analysis of clinical samples

In December 2007, an outbreak of respiratory disease occurred in pigs at a commercial grow-finish swine farm in Kansas and caused approximately 10% mortality. At necropsy, the attending veterinarian observed gross lesions of pneumonia. Lung tissues from animals that succumbed to infection and 90 nasal swab samples from affected pigs were submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Bronchial swab samples from the lung tissue were suspended in 2 ml of phosphate-buffered saline and tested for a variety of suspected agents at the KSVDL using standard diagnostic techniques. Presence of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) was tested by PCR [13]. Real-time PCR was used to detect influenza A (matrix), porcine reproductive and respiratory syndrome virus (PRRSV; commercial Tetracore test) and PCR was used to detect porcine circovirus type 2 (PCV2) open reading frame 2.

Lung tissues (~5 g) were cultured aerobically for bacteria by inoculation on MacConkey, colistin-nalidixic acid, brilliant green, and blood agar plates with and without nicotinamide adenosine dinucleotide (NAD) factor (*Staphylococcus epidermidis* nurse colonies). In parallel, lung tissues (~10 g) were homogenized in Eagle's MEM containing 4% bovine serum albumin, 15 μg/100 ml trypsin, and an antibiotic mixture of ciprofloxacin, penicillin, streptomycin and Amphotericine B and cultivated on Madin-Darby canine kidney (MDCK) cells.

For virus isolation, nasal swab samples were passed through 0.45 μm filters to remove any bacterial contamination and were inoculated on monolayers of MDCK cells in 24-well plates. The MDCK cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 1 μg/ml TCPK-trypsin and 0.3% bovine albumin. The plates were incubated at 37°C in a CO_2 incubator and observed daily. After the appearance of cytopathic effects (CPE), cells were lysed by freezing and thawing and virus was stored for further analysis.

Hemagglutination inhibition (HI) assays

Sera from all experimental pigs before infection were tested by HI assay [18]. For HI assays, sera were heat-inactivated at 56°C, treated with a 20% suspension of kaolin (Sigma Aldrich, St. Louis, MO) to eliminate nonspecific inhibitors, and adsorbed with 0.5% chicken or turkey red blood cells. HI assay was performed to test antibodies against a panel of reference SIV strains including A/Swine/IA/1973 H1N1, A/Swine/Texas/98 H3N2 and A/Sw/NC/2001 variant H1N1 to confirm to be negative for SIVs. HI assays were also conducted for KS07 virus to determine seroconversion of infected and contact pigs and cross-reactivity with other H1 SIV anti-sera. To determine the KS07 virus cross-reactivity with other H1 anti-sera, the fold-decrease between the geometric mean titer of homologous virus/anti-serum and the geometric mean titer of KS07 virus/heterologous anti-serum was calculated with ≤4-fold decrease being cross-reactive; between 4- and 8-fold reduction being moderately cross-reactive; and ≥8-fold reduction being a considerable loss in cross-reactivity.

DNA sequencing, phylogenetic analysis and subtype determination

Viral RNA was prepared from 200 μl of virus suspension with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA)

according to the manufacturer's recommendations. Two-step RT-PCR was performed by using universal primers as previously reported [12, 19]. Each gene segment was amplified under standard conditions. PCR products were purified by using a QIAamp Gel extraction kit (Qiagen, Inc., Valencia, CA) and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). Multiple sequence alignments were made by using SeqMan (DNA-Star, Inc., Madison WI) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [20]. The sequences of influenza A virus published in GenBank were used in the multiple alignments and were identified by their accession numbers. A Megablast search of the Influenza Sequence Database was performed to determine the virus subtype based on the sequence.

Experiments in pigs

SIV and PRRSV-negative pigs were used in this study and all animal experiments were in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center (NADC). The inoculation protocol has been described previously [21]. Briefly, a group of twenty 4-week-old cross-bred pigs were inoculated intratracheally with 1×10^5 $TCID_{50}$/pig of KS07 virus prepared in MDCK cells. Five 4-week-old contact pigs were comingled with inoculated pigs on day 3 post-inoculation (p.i.) to study transmission efficiency. Twenty control pigs were inoculated with noninfectious cell culture supernatant. Nasal swabs were taken on days 0, 3, 5 and 7 p.i., placed in 2 ml MEM and stored at −80°C. Blood was collected from all inoculated, contact and control pigs on days 0, 3, 5, 7, and 14 p.i. Five pigs from the inoculated and control pigs were euthanized on days 3, 5, and 7 p.i., respectively. Bronchioalveolar fluid (BALF) was obtained by the lavage of each lung with 50 ml MEM. Blood was also collected from contact pigs on day 19 post-contact (p.c.), and from the remaining five inoculated and control pigs on day 21 p.i. and tested for HI antibody.

Viral load in BALF was determined in a 96-well plate as previously described [21]. Briefly, 10-fold serial dilutions of each sample were made in serum-free MEM supplemented with TPCK-trypsin and antibiotics. Each dilution (100 µl) was placed on PBS-washed confluent MDCK cells in 96-well plates. Wells were evaluated for CPE after 24 h. At 48 h, plates were fixed with 4% phosphate-buffered formaldehyde and immunocytochemically stained with a monoclonal antibody to influenza A nucleoprotein [22]. The $TCID_{50}$/ml was calculated for each sample by the method of Reed and Muench [23].

Samples from nasal swab were vortexed for 15 s, centrifuged for 10 min at $640 \times g$, and passed the supernatant through 0.45-µm filters to reduce bacterial contamination. An aliquot of 100 µl was plated on confluent, phosphate buffered saline (PBS)-washed MDCK cells in 48-well plates. After incubation for 1 h at 37°C, 500 µl of serum-free MEM supplemented with 1 µg/ml TPCK trypsin and antibiotics was added. All wells were evaluated for CPE after 48 h. At 72 h, plates were fixed with 4% phosphate-buffered formaldehyde and stained as described above.

BALF was tested for the presence of PRRSV and *M. hyopneumoniae* by diagnostic PCR assays. For PRRSV, the total RNA was isolated from each sample by using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). One microgram of the extracted RNA and a primer pair specific for open reading frame 7 of PRRSV were used in real-time PCR as described previously [13]. DNA was extracted from BALF for detecting *M. hyopneumoniae* as described previously [13].

Examination of lungs of experimental pigs

At necropsy, lungs were removed *in toto*. A single experienced veterinarian recorded the percentage of gross lesions on each lobe. A mean value was determined for the seven pulmonary lobes of each animal [21]. For histopathologic examination, tissue samples from the trachea, the right cardiac pulmonary lobe, and other affected lobes were fixed in 10% buffered formalin, processed and stained with hematoxylin and eosin. Lung sections were examined by a veterinary pathologist in a blinded fashion and given a score of 0 to 3 to reflect the severity of bronchial epithelial injury as described previously [21, 24].

Results

Analysis of clinical samples

The overall mortality in the herd was approximately 10% with the remaining pigs exhibiting severe clinical signs including respiratory distress, lethargy and anorexia. Lungs collected from dead pigs had moderate, subacute to chronic bronchopneumonia and interstitial pneumonia with bronchiolitis and peribronchitis. Pooled lung tissue (from 3 dead pigs) was negative for PCV2 and *M. hyopneumoniae*, but was positive for PRRSV, influenza virus, *Pasteurella multocida*, *Streptococcus suis* and *Streptococcus* sp. Ninety nasal swab samples were positive for influenza virus matrix RNA and 2 of them were also positive for PRRSV RNA. Nasal swabs were negative for PCV2 and *M. hyopneu moniae*.

Subtyping and molecular analysis of the KS07 virus

Nasal swabs with the highest matrix RNA concentration were submitted to the National Animal Disease Center

(NADC) for virus isolation, sequencing and subtyping. The isolate was identified as an H1N1 virus by nucleotide sequence and a BLAST search of the Influenza Sequence Database (www.flu.lanl.gov). The KS07 was repeatedly plaque-cloned, retested, and confirmed by sequencing as belonging to the H1N1 subtype. The 8 gene segments of KS07 (Genbank accession numbers: GQ484355-GQ484362) most closely matched A/Swine/OH/511445/2007 (OH07), an H1N1 virus which infected pigs and humans at an Ohio county fair [17]. Sequence comparison showed that the identity of 8 gene segments between the KS07 and OH07 viruses ranged from 99.7 to 99.9% at the nucleotide level (Table 1). OH07 virus is a triple reassortant virus which carries the surface protein HA and NA genes from swine virus origin and internal genes from human (PB1), avian (PB2, PA) and swine (NP, M, NS) influenza virus origin [17]. Based on the HA genes, the H1 SIV isolates in the U.S. cluster into three distinct genetic groups: H1α (cH1N1), H1β (rH1N1-like) and H1γ (H1N2-like) [11, 25]. Phylogenetic analysis showed that the HA gene of the KS07 isolate can be placed in the H1γ cluster of contemporary H1 SIVs. As expected, the KS07 virus is most closely linked to the OH07 isolate. The same cluster also contains the current pandemic H1N1 viruses (Mexico09 and CA09 H1N1 viruses) (Fig. 1a). The NA gene of the KS07 isolate can be placed in the North American swine N1 cluster. Again the KS07 is closely related to the OH07, but distinct from the NA gene of H1 pandemic viruses, such as Mexico09, which is of Eurasian origin (Fig. 1b).

Molecular comparison of the HA molecules revealed that the HA genes of the KS07 virus had 93.7–99.9% identity at the nucleotide level and 93.5–100% at the amino acid level to those of other H1 viruses in the same cluster H1γ. The HA of the KS07 H1N1 and other H1 viruses in H1γ cluster depicted in Fig. 1a contain the mammalian receptor binding motif, 190D and 225D (based on H3 numbering). The same motif is found in human H1 viruses. The HA of KS07 and OH07 viruses share putative antigenic determinant sites involving serine at position 71 and aspargine at position 162. These amino acid residues are linked to serological cross-reactivity between OH07 virus with anti-sera raised against viruses in the same genetic cluster [17]. The HA molecule of the current pandemic H1N1 virus (CA09 and Mexico09) contains the same serine at position 71, but a serine not an asparagine at position 162.

Molecular comparison of the NP, PB1 and M1 molecules of the KS07 H1N1 isolate with other H1N1 viruses revealed that they have 100% identity with the OH07 H1N1 virus at the amino acid level. The NS1 showed 99.5% identity between the KS07 and OH07 viruses and NS1 of KS07 differs from that of OH07 by one amino acid substitution (V54I). The NA, PB2 and PA showed 99.6% identity between the KS07 and OH07 viruses at the amino acid level. The PB2 of KS07 virus contained the conserved avian amino acid residue glutamic acid at position 627 as the currently circulating triple reassortant SIVs in North America. Interestingly, KS07, isolated 5 months later than OH07, had two additional substitutions (S35N and G76D) in the NA molecule, three (M344V, M402I and G678D) in the PB2 molecule and three (T32N, D216N and S632T) in the PA molecule when compared to the OH07 virus.

Table 1 Influenza A viruses with the highest and the second highest nucleotide sequence identity to H1N1 swine influenza virus (KS07) as determined by blast search in the Influenza Sequence Database (http://www.flu.lanl.gov/)

Gene	Identity (%)	Virus designation	Subtype	Genbank accession no.
HA	99.9	A/Swine/Ohio/511445/2007	H1N1	EU604689
	99.9	A/Swine/Ohio/02/2007	H1N1	FJ986621
NA	99.9	A/Swine/Ohio/511445/2007	H1N1	EU604690
	99.7	A/Swine/Ohio/24366/07	H1N1	EU409949
PB1	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604692
	99.7	A/Swine/Ohio/24366/07	H1N1	EU409945
PB2	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604691
	99.6	A/Swine/Ohio/24366/07	H1N1	EU409946
PA	99.8	A/Swine/Ohio/511445/2007	H1N1	EU604693
	99.1	A/Turkey/Ohio/313053/2004[a]	H3N2	DQ335776
NP	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604694
	97.8	A/Swine/Indiana/PU542/2004	H3N1	DQ150434
M	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604695
	99.8	A/Swine/Quebec/4001/2005	H3N2	EU826546
NS	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604696
	98.2	A/Turkey/Ohio/313053/2004[a]	H3N2	EU735822

[a] Triple reassortant virus

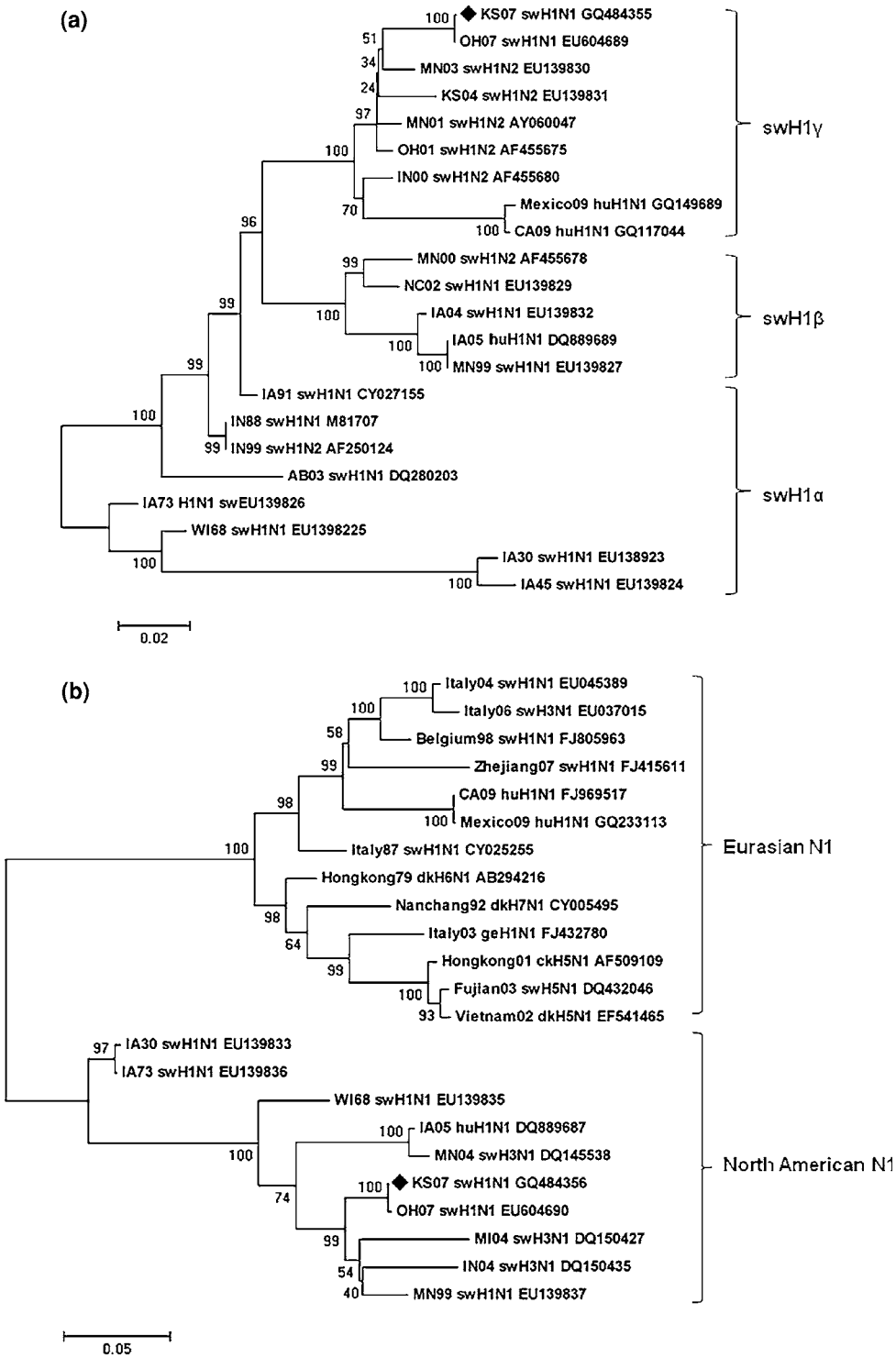

Fig. 1 Phylogenetic trees of HA and NA genes based on the nucleotide sequences of the ORFs from the KS07 and other influenza A virus sequences available from GenBank. **a** HA phylogenetic tree with three clusters of related viruses, H1α (cH1N1), H1β (rH1N1-like) and H1γ (H1N2-like). **b** NA phylogenetic tree with N1 subtype. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA 4. The reference viruses used in the analysis are abbreviated with their state and year of origin, subtype preceded by host abbreviation (*sw* swine, *hu* human, *dk* duck, *ck* chicken, *ge* goose) and GenBank accession number

Antigenic cross-reactivity with H1 SIV anti-sera

HI assays were conducted to investigate cross-reactivity between KS07 virus with various anti-sera raised against H1 SIV isolates, including viruses from the H1α, H1β and H1γ clusters. As shown in Table 2, the KS07 virus displayed the greatest cross-reactivity with H1 SIV anti-sera from the same genetic cluster. Reactivity was decreased with anti-sera from the H1α and H1β clusters. The least amount of reactivity was obtained using sera against IA/30 virus, the first SIV isolate in the United States. The results showed that the KS07 virus failed to cross-react with most of the other H1 SIV anti-sera presented in our panel (Table 2). As predicted, the highest antigenic cross-reactivity was with anti-serum from OH07

Table 2 Serological cross-reactivity with H1 reference anti-sera

Anti-sera							
H1α cluster			H1β cluster		H1γ cluster		
IA30	IA73	MN99	NC02	IA04	MN01	KS04	OH07
45[a]	8	6	16	32	3	4	1
5120/113[b]	905/113	1810/320	640/40	1280/40	160/56	1280/320	320/226

[a] Fold-decrease as compared to homologous virus/anti-sera geometric mean titer

[b] Geometric mean titers for homologous antigen and antisera/KS07 antigen with heterologous antisera

H1N1 virus due to 100% identity of HA molecules between KS07 and OH07 H1N1 viruses.

Pathogenicity and transmissibility of KS07 H1N1 swine influenza virus in pigs

To investigate pathogenicity of the isolate KS07 virus, we inoculated intratracheally twenty 4-week-old pigs with 1×10^5 $TCID_{50}$/pig of the KS07 virus. Twenty control pigs were mock-inoculated with noninfectious cell culture supernatant. Transmissibility was assessed by the introduction of five age-matched contact pigs with the inoculated pigs, starting on day 3 p.i. All pigs used in this study were seronegative for H1 and H3 SIVs. One inoculated pig died overnight due to complications from the challenge. Five inoculated pigs and five control pigs each were euthanized for necropsy on days 3, 5, and 7 p.i. The contact pigs and four remaining virus-inoculated pigs were serologically tested on day 21 p.i. Although no respiratory distress, lethargy and anorexia were observed in KS07 inoculated pigs, severe macroscopic lung lesions (plum-colored, consolidated areas) were observed in inoculated pigs at necropsy. The mean lung lesion score was 19.83, 20.91 and 23.14% for inoculated pigs versus 3.97, 1.17 and 0.57% for control animals at days 3, 5 and 7 p.i., respectively (Table 3). Lungs from inoculated pigs exhibited mild to moderate interstitial pneumonia and acute to subacute necrotizing bronchiolitis with slight lymphocytic cuffing of bronchioles and vessels. The histopathologic score (0–3) expressing the extent of damage to lung architecture was >2.5 in inoculated pigs between days 3 and 7 p.i. (Table 3).

Virus was determined in BALF fluid and in nasal swab samples. Virus titers in the BALF averaged $10^{6.20}$ $TCID_{50}$/ml on day 3 p.i., $10^{5.35}$ $TCID_{50}$/ml on day 5 p.i. and were negative on day 7 p.i. in the KS07 challenge group (Table 3). In the KS07 inoculated group, 89.5% (17/19) of pigs shed virus in the nasal swab samples on day 3 p.i. with an average titer of $10^{3.18}$ $TCID_{50}$/ml (Table 4).

Table 3 Virus titers in BALF, macroscopic lung lesion and microscopic pneumonia in pigs inoculated with KS07 H1N1 virus or mock-inoculated

Group	Virus titer in BALF $TCID_{50}$/ml[a]	Lung lesion score (%)[b]	Histopathologic score (0–3)[b]
KS07 day 3	6.20 ± 0.30	19.83 ± 5.02	2.80 ± 0.34
Control day 3	0.00 ± 0.00	3.97 ± 1.99	0.40 ± 0.29
KS07 day 5	5.35 ± 0.28	20.91 ± 4.69	2.50 ± 0.52
Control day 5	0.00 ± 0.00	1.17 ± 0.36	0.20 ± 0.12
KS07 day 7	0.00 ± 0.00	23.14 ± 4.82	2.80 ± 0.34
Control day 7	0.00 ± 0.00	0.57 ± 0.27	0.00 ± 0.00

[a] Numbers are $\log_{10}$ geometric mean BALF $TCID_{50}$/ml ± SEM

[b] Values are mean ± SEM

Table 4 Shedding and transmission of the KS07 virus

	Nasal shedding			Seroconversion
	Day 3	Day 5	Day 7	Day 19/21
Primary	17/19[a] (3.18 ± 0.23)[b]	12/14 (3.40 ± 0.27)	2/9 (1.00 ± 0.00)	4/4 (226)[c]
Contact	5/5 (3.15 ± 0.17)	5/5 (2.60 ± 0.47)	3/5 (1.75 ± 0.75)	5/5 (105)

[a] Number positive out of total number on days 3, 5, 7, 19 or 21 post-infection of post-contact

[b] Numbers in parentheses are $\log_{10}$ geometric mean nasal swab $TCID_{50}$/ml ± SEM

[c] Numbers in parentheses are the geometric mean reciprocal HI titer

On day 5 p.i., 85.7% (12/14) of nasal swabs were positive with an average titer of $10^{3.40}$ $TCID_{50}$/ml. The 22.2% (2/9) of the nasal swab samples were positive on day 7 p.i. with an average titer of 10^{1} $TCID_{50}$/ml (Table 4). The remaining KS07 inoculated pigs seroconverted by the end of the experiment (day 21 p.i.) with reciprocal HI titers ranging from 80 to 640 and a geometric mean reciprocal HI titer of 226 (Table 4). A few control pigs had an occasional small focus of mild interstitial pneumonia (Table 3), but were negative for SIV infection. In contrast to the inoculated group, 100% (5/5) of pigs in the contact group shed virus in the nasal swab samples on days 3 and 5 p.c. with average titers $10^{3.15}$ and $10^{2.60}$ $TCID_{50}$/ml, respectively (Table 4). On day 7 p.c., 60% (3/5) of the nasal swab samples were positive with an average titer of $10^{1.75}$ $TCID_{50}$/ml (Table 4). The contact pigs were analyzed for seroconversion at day 19 p.c. with reciprocal HI titers ranging from 80 to 160 and a geometric mean reciprocal HI titer of 105 (Table 4). Our results indicate that the KS07 H1N1 virus is virulent in pigs and is transmissible among pigs.

Discussion

The triple reassortant H1N1 influenza virus has been one of the major endemic subtypes in swine populations of North America. In this study, we characterized a highly virulent triple reassortant H1N1 virus, KS07, which caused approximately 10% mortality in finishing pigs and is genetically similar to the OH07 H1N1 virus. The OH07 H1N1 virus was isolated from infected pigs and humans who exhibited signs of influenza clinical illness [17]. The OH07-like H1N1 viruses did not disappear, but continued to be isolated from the U.S. swine herds. The epidemiological data indicates that OH07/KS07-like viruses have spread and are endemic in U.S. swine populations.

When compared to the OH07 H1N1 virus, the KS07 virus had similar pathogenicity and transmissibility among pigs [17]. Although 10% of mortality was reported in original herds where the KS07 virus was isolated, no obvious clinical signs were observed in experimentally infected pigs. There are several possible explanations for the different outcomes. The field pigs were significantly older and exposed to the virus under field conditions, which included several additional stressors. For example, the presence of PRRSV, *P. multocida*, *S. suis* and *Streptococcus* sp, circulating in the herd at the time of infection, may have also enhanced clinical disease signs. However, the KS07 virus induced detectable macroscopic and microscopic lesions in lungs, which were similar to the OH07 isolate [17] and more severe than lesions associated with other contemporary H1N1 SIVs under similar experimental protocols [25, 26]. The KS07 virus failed to cross-react with sera from previous H1N1 isolates similar as shown for the OH07 virus [17], suggesting that current vaccines might not provide efficient protection for swine herds.

An important difference between KS07 and OH07 viruses is that the OH07 virus infected humans. There have been no reports of clinically ill humans exposed to herds acutely infected with the KS07 virus. To date, it remains unknown whether the KS07 virus is able to infect humans; one likely possibility is the limited interaction of swine herds with humans due to increased biosecurity. As reported recently, the sequences of the OH07 swine and human isolates are identical [17]. The genome sequence of the KS07 virus was shown to be almost identical to the OH07 virus. Importantly, the amino acid sequence of the KS07 and OH07 HA are 100% identical. Two amino acid residues (E190D and G225D, H3 numbering) in the receptor binding sites have been described to have a fundamental role in avian and human receptor binding specificity of H1 viruses [27], and these two residues are conserved between human and swine H1 isolates. As predicted, these positions are mammalian-like D190 and D225 in the HA of both OH07 and KS07 H1N1 viruses. The PB2 gene has been shown to be crucial for interspecies transmission barriers for avian influenza viruses to infect mammals, especially the amino acid residue E627K [28]. Since the PB2 in the contemporary SIVs is of avian viral origin, it is of interest to evaluate the PB2 at this amino acid position. Interestingly, the avian type (627E) is present in both KS07 and OH07 PB2 genes and also in currently circulating triple reassortant SIVs' PB2 genes, indicating this site is not critical for SIVs. The KS07-like H1N1 viruses cause severe respiratory disease outbreaks in young to mature pigs and have continued to be identified in swine populations. Therefore, it is necessary to continue surveillance due to its high virulence in pigs and potential threat for humans.

Pigs have been considered to be a "mixing vessel" for human and avian influenza viruses because the epithelial cells in pigs have receptors for both influenza viruses [29]. Transmission of SIVs to humans has been documented. However, most transmission events are isolated events and do not lead to outbreaks in human populations [30, 31]. As mentioned above, pigs can be infected with wholly avian and/or human viruses, allowing swine viruses to acquire avian and/or human virus gene segments to generate novel SIVs [7, 32–36]. Further supporting the occurrence of such events is our recent identification of a swine and avian reassortant H2N3 virus [24], an HA subtype not seen in the human population since the 1957–1968 H2N2 pandemic. The TRIG cassette from the contemporary swine triple reassortant viruses seems to have offered currently circulating SIVs the ability to acquire new HA and NA genes as well as an increased rate of antigenic drift [9, 37]. This was

not previously observed with classical swine H1N1 viruses. The appearance of the new swine-origin pandemic influenza A H1N1 virus has demonstrated the promiscuity and cross-species adaptability of these SIVs. As of 6 August 2009, it has infected more than 177,400 people and caused the deaths of 1,462 persons in over 170 countries and territories worldwide [38]. The WHO raised the alert level for the pandemic H1N1 to the highest possible level 6. This entirely novel H1N1 virus has not circulated previously in humans. The genome of the pandemic H1N1 virus contains 6 genes (PB1, PB2, PA, HA, NP and NS) from currently circulating SIVs in North America and 2 genes (NA and M) from Eurasian SIVs [14, 15]; this indicates that pigs may have played a central role in the generation of this virus. So far the pandemic H1N1 virus has been found in several swine farms in Canada [39], Argentina [40] and Australia [41], indicating the potential for the infection of pigs. How and when this virus evolved is still unknown, but genetic analysis indicated the reassortment between North American swine triple reassortant viruses and Eurasia SIVs might have occurred years before emergence in humans and have been circulating undetected among swine herds somewhere in the world [14, 15] Notably, OH07/KS07-like viruses as well as the current pandemic H1N1 virus belong to the same HA cluster and share similar putative antigenic determinant sites compared to other H1 viruses in the same cluster. The OH07 virus was transmitted to humans, but seemed to not transmit among them. Therefore, it is critical to study transmission capacity of swine H1N1 viruses to elucidate the molecular mechanism by which the pandemic H1N1 virus can successfully transmit from human-to-human in order to prevent and control future pandemics.

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Expression and self-assembly of virus-like particles from two genotypes of marine vesiviruses and development of an ELISA for the detection of antibodies

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ABSTRACT

Sequences encoding the major and minor capsid proteins (VP1 and VP2) from two marine vesivirus isolates (Steller sea lion viruses V810 and V1415) were engineered for expression of virus-like particles (VLPs) in the baculovirus system. The resulting VLPs were morphologically similar to native vesivirus virions. Purified VLPs were probed in immunoblots with pooled antisera specific for nine San Miguel sea lion virus (SMSV) types, and a predominant protein of approximately 60 kDa was detected. An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies was developed in which the VLPs served as antigen. The VLPs were adsorbed to the wells of a microplate, and the specificity of the ELISA was established with hyperimmune sera raised against 24 serotypes of the genus *Vesivirus*. The ELISA was used to screen for the presence of vesivirus specific antibodies in the sera of free-ranging Steller sea lions. The ELISA results demonstrated that Steller sea lions that inhabit the Pacific Ocean waters of southeast Alaska are widely exposed to antigenically related marine vesiviruses, while no previous exposure could be demonstrated using VLP antigens in 17 Steller sea lions from the Aleutian Islands. The broad reactivity of these VLPs and their non-infectious nature will facilitate global sero-epidemiological studies aimed at determining the incidence and prevalence of marine vesiviruses in mammals that inhabit the Pacific and Atlantic oceans as well as susceptible terrestrial animals.

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1. Introduction

Members of the genus *Vesivirus* in the family *Caliciviridae* infect both marine and terrestrial organisms (Smith and Boyt, 1990). Among vesiviruses known collectively as the "marine vesiviruses" are 13 serotypes isolated from swine between 1932 and 1956 called vesicular exanthema of swine viruses (VESVs), which are considered foreign animal disease (FAD) agents in the USA because they sometimes cause a vesicular disease in swine clinically indistinguishable from foot-and-mouth disease. It has been proposed that the VESV strains were accidentally introduced into pig herds through the ingestion of feed contaminated with marine mammal and fish products

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harboring serologically related vesiviruses (Smith et al., 1973). Working with these viruses requires their handling in at least a BSL-2 plus or enhanced biosafety laboratory and special USDA-APHIS permits. Due to these restrictions, research on marine vesiviruses has been hampered and diagnostic reagents are not readily available to study the distribution and impact of marine vesiviruses in marine or terrestrial animals. The identification of marine vesiviruses initially relied on virus isolation in cell culture and virus neutralization (VN) using panels of specific antisera raised against different isolates (Smith and Akers, 1976; Smith and Boyt, 1990). Although more than 45 serotypes have been established with this approach, the extent of serotypic diversity among these viruses in not known. In recent years, molecular approaches have been developed to detect and genotype new isolates (McClenahan et al., 2008; Reid et al., 1999, 2007).

Although the genotype of new isolates can be determined by nucleotide sequencing and phylogenetic analyses (Berke et al., 1997; Ganova-Raeva et al., 2004; McClenahan et al., 2008; Neill, 1992), additional studies are needed to understand the relationship between genotype and serotype. Moreover, the availability of non-restricted (non-infectious) vesivirus serologic assays would facilitate studies of the natural history of marine vesiviruses in their vast ecosystem. In this context, virus-like particles (VLPs) are empty viral capsids that resemble the capsid of the native virus, but that do not contain the infectious viral RNA that characterizes all members of the family *Caliciviridae* (Ball et al., 1996; Bertolotti-Ciarlet et al., 2003; Di Martino et al., 2007; Jiang et al., 1992; Laurent et al., 1994; Noad and Roy, 2003). The generation of the first calicivirus VLPs was reported for the Norwalk virus, a member of the genus *Norovirus*, following co-expression of ORF2 and ORF3 in a single baculovirus (Jiang et al., 1992). In members of the genus *Vesivirus*, the complete capsid gene (ORF2) is translated as a precursor protein approximately 73 kDa that is cleaved by the viral protease encoded in ORF1 to generate a mature capsid protein (VP1) of approximately 60 kDa (Carter et al., 1992; Neill et al., 1991). Virus-like particles have also been produced from the vesivirus feline calicivirus (FCV) by expressing either the capsid precursor or mature capsid protein in a baculovirus expression system (DeSilver et al., 1997; Di Martino et al., 2007). However, although the development of marine vesivirus VLPs has not been reported, recombinant antigens of broader reactivity to detect marine vesivirus antibodies have been recently described; i.e., a recombinant antigen (D3A) of 293 amino acids corresponding to a capsid fragment of San Miguel sea lion virus 5 (SMSV-5) expressed in a bacterial system was reactive with antisera to more than 30 different serotypes of marine vesiviruses (Kurth et al., 2006a). The antigen was also used to detect vesivirus antibodies in 15% ($n = 693$) of serum samples from sick and healthy cattle (Kurth et al., 2006a), and in the serum of mares that had aborted; in which an increase in the frequency of antibodies from 47.1 to 88.2% was demonstrated (Kurth et al., 2006b).

We recently reported the isolation and characterization of two unique marine vesivirus genotypes from Steller sea lions (SSL-*Eumetopias jubatus*), SSL V810 and SSL V1415 (McClenahan et al., 2008). We report here the production of VLPs from these two genotypes by expression of their mature VP1 or VP1 and VP2 capsid proteins in a baculovirus system. The VLPs were utilized as antigens to develop an enzyme linked immunosorbent assay (ELISA) for the detection of serum antibodies to marine vesiviruses in animals from two free-ranging populations of SSL in Alaska.

2. Materials and methods

2.1. Viral RNA

Marine vesiviruses SSL V810 and SSL V1415 were isolated from Alaskan Steller sea lions in Vero and Madin Darby canine kidney (MDCK) cell cultures, respectively (McClenahan et al., 2008). After the fourth passage in Vero cell cultures, total RNA was extracted from the cell monolayers at the height of cytopathogenic activity using TRIzol-LS (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

2.2. Construction of recombinant baculoviruses

Oligonucleotide primers were designed to amplify the mature capsid gene (VP1) from the SSL vesiviruses V810 and V1415. Primers BacVFW-ORF2 (CACC*ATG*TCG-GATGGTCCAG) and BacVRV-ORF2 (TCCAAAATTTGCA-TAATTCAT) were used to amplify a fragment of approximately 1680 nucleotides (nt). During natural vesivirus infection, the mature capsid protein is produced post-translationally after cleavage of the full-length precursor of the capsid protein by the virus-encoded proteinase, so an ATG sequence was engineered into the forward primer. Separate baculovirus constructs were also created to express the VP1 and the protein product of ORF 3 (VP2) from a single template. BacVFW-ORF2 was used as the forward primer and BacVRV-ORF3 (GCAACCTACCAAT-TAACTAATTC) as the reverse primer to generate a PCR fragment of approximately 2170 nt.

All amplified PCR products were cloned into the Gateway vector, pENTR (Invitrogen) by Topo cloning and plasmids containing the desired inserts were verified by sequence analysis. Plasmid DNA was then added to a reaction mix containing baculovirus DNA (Baculodirect kit, Invitrogen), and the recombination reaction was performed according to the manufacturer's instructions. A transfection mixture was prepared that contained 250 ng recombinant baculovirus DNA, 6 μl of Cellfectin (Invitrogen), and 2 ml unsupplemented Grace's Medium (Invitrogen). Following incubation at room temperature for 45 min, the mixture was added to Sf-9 cells seeded at 8×10^5 cells per well in a 6-well plate. The transfection mixture and cells were incubated for 5 h at 28 °C, after which the transfection mixture was removed and replaced with 2 ml serum-free HyQ Medium (HyClone, Logan, UT) containing 100 mg/ml ganciclovir, as a negative selection agent, and supplemented with penicillin, streptomycin and Amphotericin B. The cultures were incubated at 28 °C for 5 days and the cells and supernatants were harvested as the first passage (P1) viral stock. The P1 viral stock (5 μl)

was added to a fresh Sf-9 monolayer in the well of a 6-well plate and maintained in the presence of 100 mg/ml ganciclovir for 120 h. The resulting P2 viral stock was verified as free of wild-type baculovirus with a β-gal kit (Invitrogen), and then used to infect a 100 ml suspension culture of Sf-9 cells at a multiplicity of infection (MOI) of 0.5 in the absence of ganciclovir. The resulting P3 viral stock was titrated by plaque assay on Sf-9 cell cultures in 6-well plates, and used to infect Sf-9 suspension cultures at a MOI of 3 for protein expression and purification.

2.3. Protein purification

Recombinant viruses were propagated in suspension cultures of Sf-9 cells grown in 150-ml of HyQ medium. Five days post-infection the cultures were harvested, frozen and thawed twice, and clarified by low-speed centrifugation. The supernatants were layered onto a 25% sucrose cushion and subjected to centrifugation in a SW28 rotor at 24,000 rpm for 4 h at 4 °C. The resulting pellets were dissolved in phosphate buffered saline (PBS) pH 7.5 and further purified through a cesium chloride (CsCl) gradient. The samples were mixed with 1.6 g/cm^3 of CsCl to produce a final density of 1.38 g/cm^3 as verified by refractometry. The samples were centrifuged in a SW55Ti rotor at 48,000 rpm for 18 h at 15 °C and visible bands were collected with a needle and syringe. The collected fractions were dialyzed against PBS to remove the CsCl using a Slide-A-Lyzer cassette (Pierce, Rockford, IL), and the protein concentration was determined with a commercial Bradford assay kit (Pierce).

2.4. SDS-PAGE and immunoblotting

The protein samples were mixed with sample loading buffer containing 2% sodium-dodecyl sulfate (SDS), 0.5 M Tris–HCl, pH 6.8, 25% glycerol, 0.5% bromophenol blue, and 2% beta-mercaptoethanol and heated at 95 °C for 5 min. The denatured proteins were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 4–20% gradient gels (Lonza, Basel, Switzerland) run at 125 V for 1.5 h with a protein standard (Invitrogen). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 7 min using the iBlot Gel Transfer Device (Invitrogen). In the immunoblot analysis, the PVDF membrane was blocked for 1 h at room temperature in Blotto buffer (5% dried non-fat milk in PBS). The membrane was then incubated with a mixture of polyclonal antisera specific for SMSV serotypes; 1, 2, 4, 5, 6, 9, 10, 11, and 13, diluted to 1:2000 in Blotto, incubated for 1 h with shaking and then overnight at 4 °C. The membrane was washed four times, 5 min each time, with Western Breeze wash buffer (Invitrogen) and incubated at room temperature for 1 h with goat anti-rabbit IgG-alkaline phosphatase conjugate (Pierce) (diluted 1:1000 in blocking buffer) in order to detect binding of the primary antibodies. The membrane was washed four times for 10 min before incubation with the substrate BCIP/NBT (Zymed lab, South San Francisco, CA) for 5 min at room temperature. The reaction was stopped with water and the membranes were dried prior to photography.

2.5. Electron microscopy

Electron microscopy was used to visualize the VLPs expressed from the recombinant baculoviruses. The purified VLPs were adsorbed onto Formvar-coated grids, stained with phosphotungstic acid (PTA), blotted dry, and examined with a FEI Tecnai 12 electron microscope.

2.6. Enzyme linked immunosorbent assay

The VLPs produced from recombinant baculoviruses expressing the mature capsid protein VP1 of vesivirus isolates SSL V810 and SSL V1415 were each employed as an antigen in the development of an ELISA modeled on previously described assays (Barajas-Rojas et al., 1993; Crowther, 2001; Ferris and Oxtoby, 1994; Kurth et al., 2006a,b). The VLPs were diluted in carbonate–bicarbonate buffer pH 9.5 (Sigma) to a final concentration of 1 μg/ml and 100 μl of the dilution was added per well of a 96-well MaxiSorp polystyrene plate (Nunc, Roskilde, Denmark). Following adsorption of the antigen at 4 °C overnight, the wells were washed once with 200 μl wash buffer (Tris-buffered saline with 0.1% Tween 20 [TBST]) and blocked with 200 μl of blocking buffer (5% non-fat dried milk in TBST) for 1 h at 37 °C. Rabbit polyclonal antisera against various SMSV and VESV serotypes and serum or plasma collected from free-ranging marine mammals (primary reagents) were titrated by performing serial two-fold dilutions in the plate beginning at a 1:50 dilution in TBST containing 1% non-fat dried milk (TBST-blotto). Each serum sample was also tested in at least three wells not coated with VLPs (no antigen wells) to determine its non-specific absorbance background. The plates were incubated for 1 h at 37 °C and subsequently washed six times with TBST buffer. The optimal dilution (1:3000) of the secondary reagent, protein A-peroxidase conjugate, was determined by checkerboard titration of a 0.5 μg/ml stock (Pierce) diluted in TBST-blotto, and 100 μl of the optimal dilution was added to each well. The plate was incubated at 37 °C for 1 h, followed by four washes with 200 μl of TBST per well. The ABTS (2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid; Kirkegaard & Perry Laboratories, Gaithersburg, MD) substrate (100 μl) was added to each well and the plates were incubated for 30 min at room temperature. The absorbance of each reaction was read at 405 nm on a Synergy plate reader (Bio-Tek, Winooski, VT). A serum sample was considered to contain antibodies to marine vesiviruses if two criteria were met simultaneously. First, the corrected absorbance value (VLP antigen absorbance minus no antigen absorbance) was 0.200 or greater, and second, if the index was equal or greater than 2.0 (VLP antigen absorbance divided by no antigen absorbance). Each serum sample was tested in triplicate in the blank wells. The titer for each serum sample was expressed as the reciprocal of the highest dilution that was positive by the two criteria above.

2.7. Serum and plasma samples assayed in the ELISA and virus neutralization test

Hyperimmune reference sera prepared in rabbits specific for the various SMSV and VESV strains (Seal

et al., 1995) were obtained from the National Animal Disease Center, USDA, Ames, Iowa, and were assayed for their reactivity in the ELISA. These included sera specific for SMSV 1, 2, 4, 5, 6, 7, 9, 10, 11, 13, VESV-A48, B51, C52, D53, E54, F55, G55, H54, I55, J56, K54, and 1934B. Hyperimmune sera raised against FCV and mink calicivirus (MCV), both members of the genus *Vesivirus* were also examined. Serum samples were obtained from free-ranging SSL, including 41 samples from Southeastern Alaska collected in 2004 and 17 SSL samples from the Aleutian Islands, Alaska, collected in 2005. Some sera were stored at 4 °C for several days before long-term storage at −20 °C or −70 °C. The virus neutralization (VN) test was performed as previously described (McClenahan et al., 2008).

3. Results

3.1. Baculovirus expression of vesivirus capsid proteins

Recombinant baculoviruses expressing the VP1 protein from SSL vesiviruses V810 and V1415 were constructed by inserting an ~1680-bp fragment of the vesiviruses VP1 gene into the baculovirus genome. A third recombinant baculovirus was constructed by insertion of an ~2170-bp fragment corresponding to the V810 VP1 + VP2 genes into the baculovirus genome. These recombinant viruses were propagated twice in Sf-9 cell cultures, in the presence of ganciclovir to screen out non-recombinant wild-type baculovirus. Recombinant baculoviruses expressing V810 VP1, V1415 VP1, or V810 VP1 + VP2 were titrated in a plaque-forming assay and exhibited titers of 2.3×10^7, 2.5×10^7, and 3.0×10^7 PFU/ml, respectively. Each of the three recombinant baculoviruses was used to infect 150-ml suspensions of Sf-9 cell cultures at a MOI of 3. A 500 μl sample was collected daily and the cell viability was determined. Cell viability was over 95% prior to infection and decreased over time, reaching 30% viability at day 5 in all three infected cell cultures. Daily samples collected on days 1 through 5 were analyzed by SDS-PAGE for protein expression. The presence of an approximately 60 kDa protein was apparent in extracts from the infected cell pellets and in supernatants harvested from all three recombinant baculoviruses, but not in the mock infected cell controls (Fig. 1A–C). The baculovirus-infected material was then subjected to isopycnic centrifugation as described in Section 2. A visible band corresponding to a density of 1.29 g/cm^3 was evident in the upper half of the CsCl gradient, consistent with the isolation of recombinant VLPs. The analysis of the material in this band by SDS-PAGE showed the presence of an expected 60 kDa protein

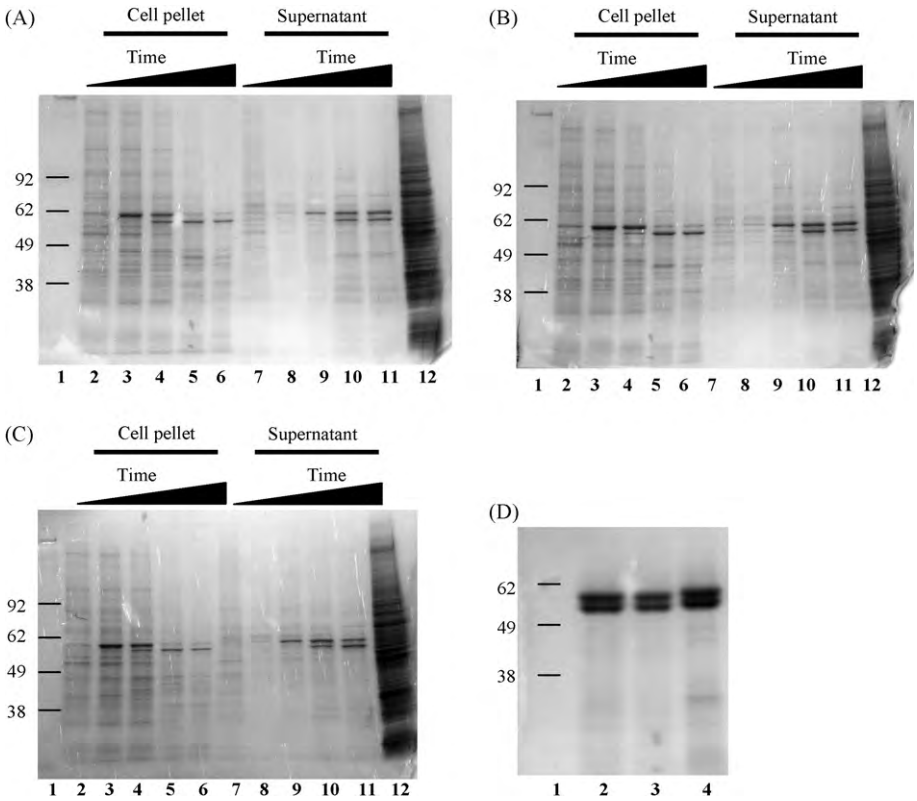

Fig. 1. SDS-PAGE analysis of SSL vesivirus proteins produced in the baculovirus expression system. Proteins were separated by SDS-PAGE and stained with Gel Code Blue (Invitrogen). (Panels A–C) Samples were harvested daily for 5 days from recombinant baculovirus-infected Sf-9 cell cultures. Lane 1: molecular weight marker, lanes 2 through 6: daily collections from cell pellets, lanes 7 through 11: daily collections from cell culture supernatant, lane 12: uninfected Sf-9 cell control. (Panel A) V810 VP1, (panel B) V1415 VP1, (panel C) V810 VP1 + VP2, (panel D) proteins purified from cesium chloride gradients of the supernatant from day 5 from the 3 recombinant viruses. Lane 1: molecular weight marker, lane 2: V810 VP1, lane 3: V1415 VP1, lane 4: V810 VP1 + VP2.

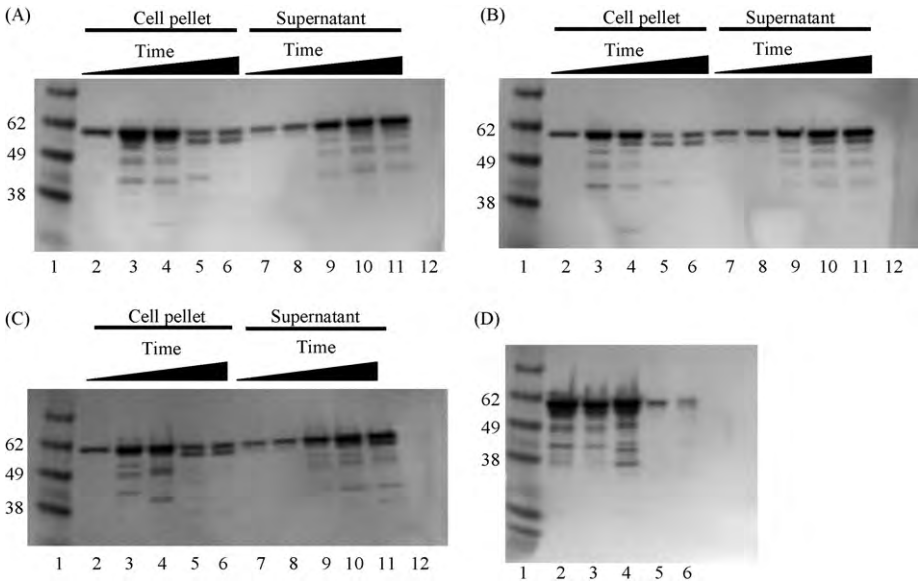

Fig. 2. Immunoblot analysis of SSL vesivirus VP1 proteins expressed in the baculovirus expression system. The panels are the same as described above for Fig. 1. The viral proteins were detected using a 1:2000 dilution of a mixture of type specific antibodies to San Miguel sea lion virus (SMSV) serotypes 4, 6, 9, 10, 11, and 13. A 60 kDa protein consistent with the positive control vesiviruses V810 and V1415 (panel D, lanes 5 and 6) was detected from the daily samples and from VLPs purified in cesium chloride gradients.

(Fig. 1D). A protein of approximately 58 kDa co-purified with the capsid protein in the CsCl gradient, but its identity was not determined (Fig. 1D), consistent with the appearance of "doublet" bands observed sometimes in capsid expression studies of the caliciviruses (Belliot et al., 2001; Kitamoto et al., 2002; Taube et al., 2005). Additional studies will be needed to determine whether the smaller 58 kDa vesivirus protein was generated by mechanisms such as proteolytic cleavage (degradation), alternative processing in the baculovirus system, or initiation from an internal methionine on the messenger RNA.

A time course analysis of VP1 expression in insect cells was examined by immunoblotting. Baculovirus-infected Sf-9 cell lysates in the blot were probed with a mixture of hyperimmune sera raised against SMSV strains as described in Section 2.4. A protein of approximately 60 kDa was detected that increased in intensity over time (Fig. 2A–C). This protein corresponded in size to the mature VP1 protein detected in lysates of Vero cell cultures infected with SSL vesiviruses V810 and V1415 (Fig. 2D).

3.2. Electron microscopy

Negative staining and electron microscopy of purified protein samples from both VP1 recombinants and the VP1 + VP2 recombinant baculoviruses revealed the presence of VLPs consistent in size (32–35 nm) and shape to native calicivirus virions (Fig. 3).

3.3. Detection of vesivirus antibodies by ELISA

3.3.1. ELISA antibody titers of SMSV and VESV antisera

The reactivities of a panel of rabbit hyperimmune sera directed against several SMSV and VESV strains of varying

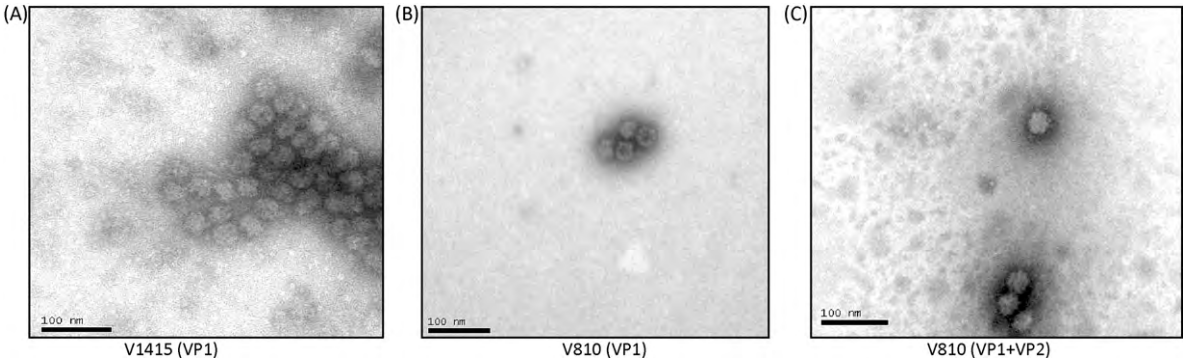

Fig. 3. Electron micrograph of virus-like particles (VLPs) expressed in the baculovirus expression system. Proteins were visualized by negative staining with phosphotungstic acid. Shown are representative images of VLPs derived from vesivirus strain: V1415 (VP1 only—panel A), V810 (VP1 only—panel B), and V810 (VP1 + VP2—panel C).

Table 1
Antibody titers obtained by virus neutralization (VN) and enzyme linked immunosorbent assay (ELISA). Specific antisera to various marine vesiviruses (SMSV) and vesicular exanthema of swine virus (VESV), as well as antisera to feline calicivirus and mink calicivirus VLPs were assayed by VN with infectious virus and by ELISA with VLP antigens. ND: not done.

Antiserum	V810 VLP ELISA titer	V810 VN titer	V1415 VLP ELISA titer	V1415 VN titer
SMSV-1	6,400	<4	3,200	<4
SMSV-2	12,800	<4	6,400	<4
SMSV-4	3,200	<4	3,200	<4
SMSV-5	≥25,600	<4	≥25,600	<4
SMSV-6	200	<4	200	<4
SMSV-7	≥25,600	<4	≥25,600	<4
SMSV-9	6,400	<4	6,400	<4
SMSV-10	12,800	<4	6,400	<4
SMSV-11	6,400	<4	6,400	<4
SMSV-13	≥25,600	<4	≥25,600	<4
VESV-A48	800	<4	1,600	<4
VESV-B51	3,200	<4	3,200	<4
VESV-C52	6,400	<4	≥25,600	<4
VESV-D53	≥25,600	4	≥25,600	4
VESV-E54	6,400	<4	12,800	<4
VESV-F55	1,600	<4	3,200	<4
VESV-G55	12,800	<4	12,800	<4
VESV-H54	12,800	<4	12,800	<4
VESV-I55	12,800	<4	12,800	<4
VESV-J56	3,200	<4	3,200	<4
VESV-K54	3,200	<4	3,200	<4
VESV-1934B	≥25,600	<4	≥25,600	<4
Feline calicivirus	≥25,600	ND	≥25,600	ND
Mink calicivirus	800	ND	1,600	ND

serotype were examined in the ELISA using SSL vesiviruses V810 and V1415 VLPs (Table 1). SMSV antiserum titers ranged from 200 to ≥25,600, while VESV antiserum titers ranged between 800 and ≥25,600 when tested against V810 VLPs. SMSV antiserum titers ranged from 200 to ≥25,600 while VESV antiserum titers ranged between 1,600 and ≥25,600 when assayed against V1415 VLPs. Specific antisera raised against other *Vesivirus* genus members including FCV and MCV virions also reacted with SSL vesivirus VLPs with titers as high as ≥25,600 for FCV antiserum, and 800 and 1600 for MCV antiserum (Table 1).

3.3.2. ELISA titers of marine mammal sera

A total of 58 serum samples obtained in 2004 from free-ranging marine mammals from Southeast Alaska, and in 2005 from the Aleutian Islands, Alaska, were tested in the V810 and V1415 ELISA for specific antibodies. When 41 sera collected from young free-ranging SSL from Southeast Alaska were assayed by ELISA, 36 (87.8%) were positive for antibodies reacting with the V810 VLPs and 38 (92.7%) were positive for the V1415 VLPs (Table 2). Antibody titers against isolate V810 VLPs ranged from 50 to 800 with a median of 200 while titers against isolate V1415 VLPs ranged from 100 to 12,800 with a median of 400 (not shown). Virus neutralizing antibody titers were demonstrated in 27 (65.9%) of 41 sera tested against isolate V810 and in 38 (92.7%) of 41 sera tested against isolate V1415 (Table 2). The median VN antibody titer was 16 against isolate V810 and 512 against isolate V1415 (not shown). Except for four borderline results, all 17 sera obtained in 2005 from SSL from the Aleutian Islands were negative for ELISA vesivirus antibodies. The same 17 SSL sera were also negative for VN antibodies against isolates V810 and V1415 (Table 2).

4. Discussion

The major capsid protein (VP1) of two recently characterized SSL vesiviruses; V810 and V1415, was expressed in the baculovirus system. The expressed VP1 (with or without the presence of VP2) self-assembled into VLPs. This is an advance in the study of marine vesiviruses because recombinant VLPs are nearly indistinguishable from native virions, and therefore have the potential for use as non-infectious antigens in diagnostics assays (Ball et al., 1996). In the host, after natural infection with caliciviruses, the most abundantly produced antibodies are directed against the capsid protein, and a significant proportion of these antibodies have type-specific neutralizing activity (Guiver et al., 1992; Matsuura et al., 2001; Neill et al., 1991; Tohya et al., 1991). The availability of recombinant VLPs should also facilitate the study of antigenic relationships among the marine vesiviruses by providing an unlimited supply of immunogens for the production of antisera against structural (capsid) proteins. Such sera would enable the rapid characterization of new vesivirus strains, and provide reference standards in the development and validation of diagnostic assays.

Although vesiviruses encode a precursor of the capsid protein in ORF2, we engineered the baculoviruses to express only the predicted processed form of the VP1 by engineering an ATG codon at the 5′-end of the VP1 gene cloned into the recombinant baculovirus. In addition, a recombinant baculovirus also yielded VLPs when the complete VP2 gene sequences encoded in ORF3 were included with those of the VP1. Like other caliciviruses, the VP1 gene of marine vesiviruses alone seems sufficient for assembly of the VLPs and the co-expression of VP2 is not necessary for capsid formation (Bertolotti-Ciarlet et al.,

Table 2
Antibody titers in the serum of Steller sea lions against marine vesiviruses as determined by virus neutralization (VN) using isolates V810 and V1415 and ELISA using VLPs from the same isolates as antigen. SSL: Steller sea lion; SE: Southeast Alaska; AL: Aleutian Islands, Alaska.

Serum sample	V810 VLP ELISA titer	V810 VN titer	V1415 VLP ELISA titer	V1415 VN titer
SSL2004-499SE	200	<4	800	128
SSL2004-500SE	100	<4	800	≥8192
SSL2004-501SE	100	4	800	512
SSL2004-502SE	<50	<4	<50	16
SSL2004-503SE	200	4	800	512
SSL2004-504SE	400	<4	400	8
SSL2004-505SE	400	<4	800	64
SSL2004-506SE	200	1024	200	512
SSL2004-507SE	400	4	400	1024
SSL2004-508SE	50	32	800	512
SSL2004-509SE	800	16	200	2048
SSL2004-510SE	200	8	400	256
SSL2004-511SE	200	16	800	4096
SSL2004-512SE	<50	8	200	≥8192
SSL2004-513SE	200	4	3,200	16
SSL2004-514SE	400	32	6,400	512
SSL2004-515SE	50	<4	400	512
SSL2004-516SE	100	<4	100	32
SSL2004-517SE	400	<4	12,800	1024
SSL2004-518SE	100	<4	800	4096
SSL2004-519SE	100	128	200	2048
SSL2004-520SE	200	64	1,600	2048
SSL2004-521SE	100	<4	100	32
SSL2004-522SE	400	16	800	8
SSL2004-523SE	200	4	200	8
SSL2004-524SE	800	64	3,200	2048
SSL2004-525SE	800	16	800	<4
SSL2004-526SE	100	32	200	8
SSL2004-527SE	800	16	400	<4
SSL2004-528SE	400	4	800	1024
SSL2004-529SE	50	4	200	<4
SSL2004-530SE	100	4	200	128
SSL2004-531SE	100	16	800	2048
SSL2004-532SE	50	64	200	64
SSL2004-533SE	200	8	3,200	≥8192
SSL2004-534SE	800	1024	400	8
SSL2004-535SE	<50	8	<50	128
SSL2004-536SE	100	<4	400	1024
SSL2004-537SE	<50	<4	200	1024
SSL2004-538SE	100	<4	200	512
SSL2004-539SE	<50	<4	<50	8
SSL2005-594AL	<50	<4	<50	<4
SSL2005-595AL	<50	<4	<50	<4
SSL2005-596AL	<50	<4	50	<4
SSL2005-597AL	<50	<4	<50	<4
SSL2005-598AL	<50	<4	<50	<4
SSL2005-599AL	50	<4	<50	<4
SSL2005-600AL	<50	<4	<50	<4
SSL2005-601AL	<50	<4	<50	<4
SSL2005-602AL	50	<4	50	<4
SSL2005-603AL	<50	<4	<50	<4
SSL2005-604AL	50	<4	50	<4
SSL2005-605AL	<50	<4	<50	<4
SSL2005-606AL	<50	<4	<50	<4
SSL2005-607AL	<50	<4	<50	<4
SSL2005-608AL	<50	<4	<50	<4
SSL2005-609AL	<50	<4	<50	<4
SSL2005-610AL	<50	<4	<50	<4

2003; Di Martino et al., 2007). The ORF3 protein product has an unknown function, but due to the basic characteristic of the VP2 protein, it has been proposed to interact with the viral RNA and possibly, to encapsidate the RNA of progeny viruses (Neill et al., 1991; Prasad et al., 1999). The VP2 protein has been shown to be a structural protein (Glass et al., 2000; Sosnovtsev and Green, 2000), and is necessary for infection of FCV (Sosnovtsev et al., 2005). Previous experiments with noroviruses indicated that VLPs expressed from ORF2 and ORF3 sequences had higher yields than when expressed from ORF2 alone, and the VLPs showed increased stability (Bertolotti-Ciarlet et al., 2003). In the present study, it appeared that the V810 vesivirus VLPs produced with both VP1 + VP2 genes exemplified the

traditional "cup-like" morphology of members of the *Caliciviridae*, but further experimentation will be needed to determine whether there are advantages to co-expressing VP1 and VP2 of these marine vesiviruses.

A new ELISA was developed using non-infectious VLPs expressed from recombinant baculoviruses as antigen coated on microtiter plate wells. Rabbit origin typing sera against 22 marine vesiviruses (10 serotypes designated SMSVs and 12 serotypes designated VESVs) were cross-reactive with VLPs produced from SSL vesivirus strains V810 and V1415. However, none of these antisera neutralized SSL isolates V810 and V1415, indicating that these two viruses did not belong to any of the known VESV or SMSV serotypes tested. These findings also indicated that the V810 and V1415 VLPs can serve as broadly reactive antigens, which recognize antibodies against many known marine vesivirus types and strains. Recently described ELISAs for the detection of antibodies against marine vesiviruses have in addition to the VLPs reported here, used both a bacterially expressed peptide, D3A and pooled whole viruses (SMSVs 5, 13, and 17) as coating antigens (Kurth et al., 2006a,b; Smith et al., 2006). These ELISA coating antigens were reported to be cross-reactive with more than 14 different marine vesivirus serotypes, which is similar to the cross-reactivity of the VLPs described here.

The VLP-based ELISA was used in a serologic survey of Steller sea lions living near southeast Alaska in 2004 and the Aleutian Islands in 2005. Each of the 58 SSL sera was assayed by VN against vesiviruses V810 and V1415, and by ELISA against VLPs produced by recombinant baculoviruses expressing VP1 from isolates V810 and V1415. Over 90% of the tested animals in Southeast Alaska were positive for one or both vesiviruses in the ELISA. In addition, ELISA and VN antibodies against V1415 VLPs and the 2005 viral isolate V1415, respectively, were present in higher titer and frequency than similar antibodies against the V810 VLPs and the 2002 viral isolate V810. These results may reflect a more recent exposure to vesivirus SSL V1415-like strains circulating in 2004 versus vesivirus SSL V810-like strains circulating in 2002 in the same population of SSL in Southeast Alaska. An interesting finding was the identification of 17 antibody-negative serum samples collected in 2005 from SSL in the Aleutian Islands (Table 2). Further screening of SSLs from this area in the near future will be needed to determine whether animals in this rookery have remained free of exposure to marine vesiviruses.

In summary, we report the development, expression and production of VLPs of marine vesiviruses from recombinant baculoviruses, in which either the mature capsid gene VP1 or both VP1 and VP2 sequences had been introduced. These VLPs were consistent in appearance with native virions both in size and structure, and the VLPs containing VP1 were successfully used to develop an ELISA for the detection of vesivirus antibodies. The ELISA was shown to detect antibodies in reference hyperimmune rabbit sera raised in the laboratory against 24 different vesiviruses, including 12 VESV serotypes that were isolated decades ago (1932–1956) and in the sera of free-ranging Steller sea lions undergoing natural vesivirus infection 4–7 years ago (2002–2005). The ELISA will make possible for the first time, the large-scale screening of marine mammals for infection with vesiviruses in sero-epidemiological studies both in the North Pacific Ocean waters, known to be a natural habitat of these viruses (Burek et al., 2005; Skilling et al., 1987; Smith et al., 1973), as well as in the Atlantic Ocean and Gulf of Mexico waters, from where marine vesiviruses have not yet been isolated. This ELISA will contribute also to the screening of terrestrial mammals for potential exposure to pathogenic marine vesiviruses or related vesivirus strains. Because certain marine vesiviruses (VESVs) are restricted agents by virtue of inducing clinical signs in swine indistinguishable from those of foot-and-mouth disease, these safe, non-replicating VLP antigens can now be used safely in conventional diagnostic virology laboratories and in basic calicivirus research.

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Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Pathogenicity and Molecular Characterization of Emerging Porcine Reproductive and Respiratory Syndrome Virus in Vietnam in 2007

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Summary

In 2007, Vietnam experienced swine disease outbreaks causing clinical signs similar to the 'porcine high fever disease' that occurred in China during 2006. Analysis of diagnostic samples from the disease outbreaks in Vietnam identified porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2). Additionally, *Escherichia coli* and *Streptococcus equi* subspecies *zooepidemicus* were cultured from lung and spleen, and *Streptococcus suis* from one spleen sample. Genetic characterization of the Vietnamese PRRSV isolates revealed that this virus belongs to the North American genotype (type 2) with a high nucleotide identity to the recently reported Chinese strains. Amino acid sequence in the nsp2 region revealed 95.7–99.4% identity to Chinese strain HUN4, 68–69% identity to strain VR-2332 and 58–59% identity to strain MN184. A partial deletion in the nsp2 gene was detected; however, this deletion did not appear to enhance the virus pathogenicity in the inoculated pigs. Animal inoculation studies were conducted to determine the pathogenicity of PRRSV and to identify other possible agents present in the original specimens. Pigs inoculated with PRRSV alone and their contacts showed persistent fever, and two of five pigs developed cough, neurological signs and swollen joints. Necropsy examination showed mild to moderate bronchopneumonia, enlarged lymph nodes, fibrinous pericarditis and polyarthritis. PRRSV was re-isolated from blood and tissues of the inoculated and contact pigs. Pigs inoculated with lung and spleen tissue homogenates from sick pigs from Vietnam developed high fever, septicaemia, and died acutely within 72 h, while their contact pigs showed no clinical signs throughout the experiment. *Streptococcus equi* subspecies *zooepidemicus* was cultured, and PRRSV was re-isolated only from the inoculated pigs. Results suggest that the cause of the swine deaths in Vietnam is a multifactorial syndrome with PRRSV as a major factor.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes reproductive failure of sows and respiratory disease of piglets and growing pigs. The disease occurs in most pig-producing countries worldwide and has a high economic impact on the global swine industry. The aetiological agent is a positive, single-stranded RNA virus of approximately 15.5 kb in length and is a member of the *Arteriviridae* family in the order *Nidovirales* (Cavanagh, 1997). Based on the genetic differences seen in the prototype strains Lelystad (Meulenberg et al., 1993) and VR-2332 (Nelsen et al., 1999), PRRSV has been historically classified in two major genotypes: the European-like strains (type 1; prototype Lelystad) and the North American-like strains (type 2; prototype VR-2332). The two major genotypes share about 60% identity on the nucleotide level, while individual PRRSV isolates within genotypes can vary up to approximately 20% in nucleotide sequence. PRRSV undergoes remarkable genetic alteration, perhaps related to its ability to undergo high frequency viral recombination (Yuan et al., 1999, 2000, 2001, 2004; van Vugt et al., 2001). The high degree of genetic and antigenic diversity adds to the complexity of control and eradication of the disease.

Porcine reproductive and respiratory syndrome virus is present in nature and causes a variety of clinical signs including reproductive problems (abortions, pre-mature birth and infertility), loss of appetite, coughing, high fever, respiratory distress and death in severe cases. Unlike typical cases of PRRSV infection that may cause high mortality rates in young pigs (Collins et al., 1992; Wensvoort et al., 1992, Meredith, 1993; Murtaugh et al., 1993, Wensvoort, 1993; Batista et al., 2002), the highly pathogenic PRRSV causes outbreaks leading to high mortality in adult animals (Feng et al., 2008; An et al., 2010). In 2006, an atypical, highly pathogenic form of PRRSV affected more than 2 million pigs causing deaths of at least 400 000 pigs from June to September 2006 in at least 10 Chinese provinces (Tian et al., 2007a). Despite efforts to control the outbreaks in China in 2006 through containment, slaughter and vaccination, the disease continued to spread throughout the country and across international borders.

Here, we describe a complete diagnostic investigation of samples from the 2007 outbreaks in Vietnam, the isolation and genetic characterization of the PRRSV isolates, and the disease pathogenicity in naïve pigs under experimental conditions.

Materials and Methods

Outbreak and sample collection

In March 2007, swine disease outbreaks causing respiratory distress, high fever, constipation and diarrhoea first occurred in Hai Duong (HD) province in northern Vietnam. Disease rapidly spread to six other provinces of the Red River Delta, namely Hung Yen (HY), Quang Ninh (QNi), Thai Binh (TB), Bac Ninh (BN), Bac Giang (BG) and Hai Phong (HP) with thousands of pigs affected (Fig. 1). In spite of placing strict control measures to prevent disease spread, several cases were reported in Quang Nam (QN) province in central Vietnam by June, and in surrounding provinces of Thua Thien Hue (TTH), Da Nang (DN) and Quang Ngai (QNg) with more than 32 000 pigs affected and 23% mortality. By July, the disease outbreaks spread to Long An (LA) and Ba Ria Vung Tau (BR-VT) provinces in the South. Lungs and spleens collected from three affected pigs from Thang Binh (TgB) district in the central province of QN, and lung and lymph node homogenates collected from four pigs from TB, BG, HY and Ha Noi (HN) were submitted for analysis to the National Veterinary Service Laboratories, (NVSL); Foreign Animal Disease Diagnostic Laboratory (FADDL), Greenport, NY USA; the Diagnostic Virology Laboratory and Diagnostic Bacteriology Laboratory in Ames, IA USA. This submission was organized and facilitated by the Food and Agriculture Organization (FAO) of the United Nations, Rome, Italy. Field clinical observations indicated an outbreak characterized by high fever, respiratory distress, blue ears, skin haemorrhages, stillbirths, abortions, high morbidity and increased mortality.

Diagnostic approach

Virus isolation (VI) in cell culture, polymerase chain reaction (PCR) tests, nucleic acid sequence analysis, electron microscopy (EM), standard bacteriological culture methods and serological assays were performed to rule out African swine fever (ASF), classical swine fever (CSF), foot-and-mouth disease (FMD), swine vesicular disease (SVD), porcine circovirus type 2 (PCV2), porcine teschovirus (PTV) and PRRSV. Tissue specimens were also inoculated in a number of cell lines to look for any other potential viral agents. Animal inoculation studies were conducted using original specimens and cell culture positive extracts to determine the PRRSV virulence and to identify other possible pathogenic agents present in the original tissues not readily cultured by standard methods.

Virus isolation

Several Tissues were prepared as 10% w/v homogenates in Hank's minimal essential media with antibiotics. Confluent 25 cm^2 cell culture flasks were inoculated with 500 ul of the sample. Alternatively, 50 ul/well was used to inoculate 96-well plates. The following tissue culture cells were used: secondary lamb kidney (LK), Instituto Biologico Rim

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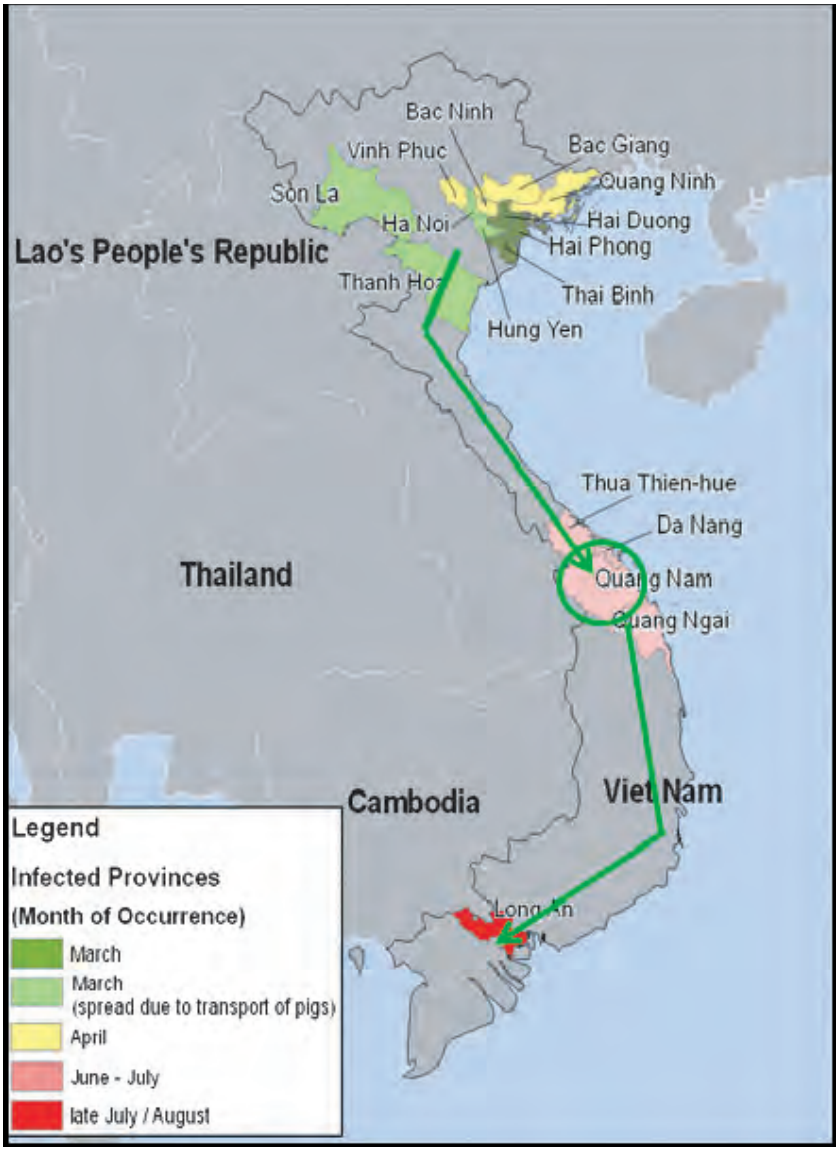

Fig. 1. Vietnam map. Outbreaks spreading from north to south (arrow) in 2007. Disease outbreaks first reported in the northern province of Vietnam and spread to central and southern provinces (arrows). Specimens submitted for testing were collected from Hai Duong district in April and from Qaung Nam district in June 2007.

Suino-2 swine kidney (IBRS-2), Vero, swine kidney-6 (SK-6), primary swine peripheral blood mononuclear (PBMC) and MARC-145. Monolayers were monitored for cytopathic effect (CPE) for two consecutive blind passages; LK and IBRS-2 3 days per passage, and Vero, SK-6, MARC-145 and PBMC 7 days per passage. For CSF virus detection, SK-6 cells were inoculated and stained 72 h post-inoculation for the presence of CSF antigen using an immunohistochemistry assay (ABC- avidin biotin complex assay) utilizing V3 monoclonal antibody. An additional set of PBMC were inoculated and monitored for CPE and rosette formation (hemagglutination) as evidence of ASF virus (ASFV) infection. MARC-145 cells were inoculated and monitored for CPE in attempt to isolate PRRSV.

Serology and bacteriology

The presence of PRRSV antibodies was tested using the PRRS HerdChek 2XR ELISA kit from IDEXX Laboratories (Westbrook, ME, USA). The test was performed on samples collected from the animal inoculation study as explained later. The sample to positive (S/P) ratio was calculated according to manufacturer's specifications. Standard methods of bacteriology were used for analysis of the spleen, lung and lymph nodes.

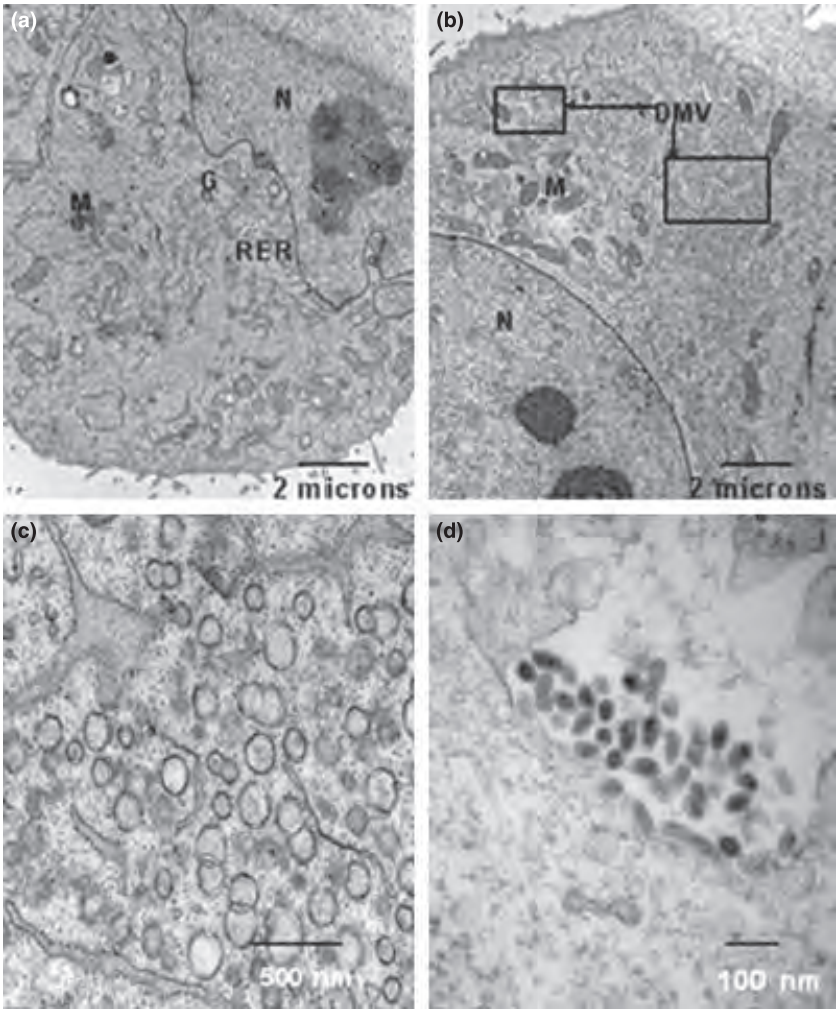

Fig. 2. Electron micrographs of mock and Vietnam isolate infected-MARC-145 cells. (a) Mock infected MARC-145, mitochondria (M), rough endoplasmic reticulum (RER) nucleus (N). (b) Vietnam-isolate infected cells showing changes in mitochondria (M) and nucleus (N). Cytoplasm filled with double membrane vesicles (DMV), a high power view is shown in plates C and D. (c) Clusters of cytoplasmic double-membrane vesicles. (d) Extracellular clumps of mature 40–50 nm electron-dense particles.

Immunofluorescence and Electron Microscopy

MARC-145 cell cultures infected with Vietnam isolates showing >50% CPE were prepared for immunofluorescence assay (IFA) and transmission EM. For IFA, the cells were fixed with 100% acetone at −20°C for 10 min, rinsed in phosphate-buffered saline (PBS), blocked with 10% foetal calf serum in PBS (FCS-PBS), incubated with a 1 : 100 dilution of anti-PRRSV Mab, SDOW17 (Nelson et al., 1993), in FCS-PBS for 1 h, washed and incubated with anti-mouse antibody conjugated to Alexa Fluor® 488 Dye (Molecular probes, Eugene, OR, USA).

For EM examination, the cells were fixed with a solution containing 2% glutaraldehyde, 2 mM calcium carbonate and 0.1 M HEPES buffer (pH 7.4). The cells were then osmicated, stained *en bloc* with uranyl acetate and embedded in Spurr's Resin via acetone dehydration. Tonsil, lymph node, liver, spleen and lung from experimental pigs inoculated with tissue homogenate or PRRSV were harvested at necropsy and processed for EM as noted earlier. For tissue orientation, 1-μm-thick resin tissue sections were stained with 1% Toluidine Blue and examined with a Zeiss microscope. Ultrathin sections of cells and tissues were picked up on Formvar and carbon-coated slot grids, stained with uranyl acetate and lead citrate and examined using a Hitachi T-7600 electron microscope operating at 80 kV. Electron microscope images were captured with an advanced microscopy techniques (AMT) digital camera and contrast adjusted using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA).

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Polymerase chain reaction

For reverse transcriptase PCR tests (RT-PCR), RNA was extracted from 140 μl of tissue homogenate or cell culture supernatant using RNeasy extraction kit (Qiagen, Stanford, CA, USA), following the manufacturer specifications. RNA was eluted in 40 μl of RNase-free water and stored at −70°C. Similarly, for PCR tests, DNA was extracted from 200 μl of tissue homogenate or cell culture supernatant using DNeasy extraction kit (Qiagen), following the manufacturer specifications. DNA was eluted in 100 μl of RNase-free water and stored at −70°C. Original specimens and virus culture passages with CPE were tested for the presence of nucleic acid of PCV-2, porcine enterovirus (Zell et al., 2000, Krumbholz et al., 2003), CSF virus (CSFV) (Risatti et al., 2003), ASFV (Zsak et al., 2005), FMD virus (FMDV) (Callahan et al., 2002) SVD (Callens and De Clercq, 1999) and PRRSV (G.R. Risatti, personal communication) as described in their respective published references.

Sequence analysis

Nucleic acid sequences for non-structural protein 2 (nsp2), open reading frame 5 (ORF5) and ORF6 of the original and cultured PRRSV from Vietnam were obtained. The original homogenate (lymph node and lung) of pig 07-HN and a pool of macrophage virus culture/passage 1 of the following pigs: 07-TB, 07-BG, 07-HY, 07-HN, 07-QN-1, 07-QN-2 and 07-QN-3 were used. Sequences were obtained using the following primers: 14682-F: 5′-CTTGCTAGGCCGCAAGTACAT and 15396-R: 5′-TAATTTCGGCCGCATGGTT, which amplified a 714-bp product containing ORF-6; primers in positions 13562-F: 5′-TGCTTCYGAGATGAGTGAAA and 14525-R: 5′-CAAAAGGTGCAGAAGCCCTA, resulted in an amplification product of 964 bp from ORF5, and 2492-F: 5′-GRACTTCCTCARCTTCTTGC and 3160-R: 5′-TCGACGAGCTTAAAGACCAGA yielded an amplification product of 669 bp from the nsp2 genomic region. Amplified products were column purified (Qiagen) and cloned in TOPO-TA cloning system (Invitrogen, Carlsbad, CA, USA). After transforming into bacteria, at least three colonies of each construct were selected and grown in 3 ml of Carbenicillin-LB media (20 mg/l). Plasmid DNA was purified using S.N.A.P.™ MiniPrep Kit (Invitrogen) and sequenced in ABI 3900 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences were assembled using the Sequencher® program (Gene Codes Corporation, Ann Arbor, MI, USA) and submitted to GenBank® database Basic Local Alignment Search Tool (BLASTn) for analysis (Benson et al., 2008).

For phylogenetic studies, sequences were aligned to public (GenBank) and an internal PRRSV database containing over 8600 viruses detected in North America, using the Megaline® software package (DNASTAR, Inc, Madison, WI, USA).

Animal inoculation study

Twelve 4- to 6-week-old Yorkshire pigs were purchased from a PRRS-free herd and were pre-tested negative for antibodies to PRRSV, PCV-2, pseudorabies virus, transmissible gastroenteritis virus and swine influenza virus (H1N1 and H3N2). Pigs were acclimated at Plum Island Animal Disease Center (PIADC) biocontainment Biosafety Level 3 Agriculture facility for 10 days before the start of the experiment, observed daily for clinical signs, and body temperature was recorded throughout the experiment starting 3 days prior to inoculation.

Animals were divided into three groups. Group one consisted of three pigs that received intranasal (IN), intramuscular (IM) and intraperitoneal (IP) inoculation of 10% unfiltered, original tissue homogenate obtained from sick pigs from Vietnam (spleen and lung), 1 ml per route. Two days post-inoculation (dpi), two naïve pigs were introduced and comingled with the inoculated pigs. Group two consisted of three pigs that received one ml per route IN, IM and IP of pooled PRRSV/PBMC passage one composite of individually inoculated lymph nodes, lung and spleen. Inoculum contained $10^{4.5}$ $TCID_{50}$/ml of PRRSV titrated on PBMC, corresponding to $10^{3.7}$ $TCID_{50}$/ml when titrated on MARC-145 cells. At 2 dpi, two naïve pigs were introduced and comingled with the inoculated pigs. Group three consisted of two sham-inoculated control pigs; one pig was inoculated with EMEM media used for making the tissue homogenate, and the other pig was inoculated with the negative control PBMC culture supernatant with the same lot used for virus propagation. Pigs were monitored daily for fever and clinical signs. Serum and heparinized blood were collected at 0, 2, 5, 7, 9, 12, 15, 22, and 28 dpi or day post-contact (dpc), with day 0 representing the day of inoculation or first day of contact. White blood cell (WBC) counts were performed on heparinized blood using a Beckman Coulter Counter (Beckman Coulter, Inc., Miami, FL 33196, USA), and sera were used for PRRSV isolation and detection by reverse transcriptase PCR (RT-PCR). Animals were necropsied at the end of the experiment or when died or euthanized because of severe illness. At the time of necropsy, a complete set of fresh tissues was collected for bacteriology, virology and PCR analysis, and a set of tissues was fixed in 10% neutral-buffered formalin for histopathology. The animal experiment was performed in compliance with an Animal Use Protocol and all the regulations of the Institutional Animal Care and Use Committee (IACUC).

Results

Diagnostic submission

Tissue homogenates of lung and lymph nodes, and tissue samples of spleen, lung and lymph node from seven affected pigs from five districts in the northern and central provinces of Vietnam tested negative for CSFV, FMDV and SVD virus by both virus isolation and RT-PCR, negative for ASFV by both virus isolation and PCR, and negative for PTV (formerly porcine enterovirus serotype 1) by RT-PCR. PBMC cell cultures inoculated with spleen and/or lung samples from the seven pigs showed CPE with no rosette formation. RT-PCR confirmed the presence of PRRSV nucleic acid and the absence of CSFV, and PCR the absence of ASFV on CPE-positive PBMC; however, MARC-145 did not support the growth of PRRSV from samples collected from QN province. Samples from the three pigs from the QN province were positive for PCV-2 by PCR, while samples from the provinces of TB, BG, HY and HN were negative (Table 1).

Escherichia coli and *Streptococcus equi* subsp. *zooepidemicus* were cultured from lungs and spleens, and *Streptococcus suis* from one spleen sample from the QN province (Table 1).

PBMC-inoculated cells revealed intense cytoplasmic staining by IFA with the anti-PRRS MAb SDOW17, which reacts to an epitope on the 15 kDA nucleocapsid protein of the PRRSV but not with the p54 anti-ASFV MAb (data not shown). Electron microscopy of ultrathin sections of infected swine PBMC revealed cytoplasmic proliferations of single (35–40 nm) and double (70–85 nm) membrane structures. Extracellular clumps of 50 nm particles were seen in cells infected with the Vietnamese isolates as well as with PRRSV reference strain (Fig. 2).

All samples except for the lung of pig 07QN-2 were positive for PRRSV nucleic acid by RT-PCR (Table 1). Results of PRRS RT-PCR were confirmed through sequencing of the ORF6 amplification product. The four samples from the QN province were PCR positive for PCV-2. The PCV-2 PCR results were confirmed by restriction enzymatic digestion of the PCR product with NcoI that digests the PCV PCR product in two easily distinguishable fragments of 168 and 75 bp each (Fenaux et al., 2000).

Sequences and phylogenetic analysis

Sequence analysis of targeted genomic regions amplified from the original 10% lung and lymph node homogenate of pig 07HN, swine PBMC infected with 07HN homogenate, and a pool PBMC culture/passage of pigs, 07TB, 07BG, 07HY, 07HN, 07QN-1, 07QN-2 and 07QN-3 (Table 1), confirmed that the Vietnam 2007 PRRSV isolates belong to the North American PRRSV genotype (Type 2) and have a close identity with the recently reported Chinese strains with the identical deletions in the nsp2 region. The BLAST search of the Vietnamese ORF6 sequence against the whole nucleotide GenBank database confirmed 99% homology with the following Chinese PRRSV strains: HUN4, JX0612, JXA1, HUB2, HUB1, HuN and HEB 1. All these strains had been identified during the recent high virulent PRRSV epidemic in China (Tian et al., 2007a; Tong et al., 2007; An et al., 2010).

Sequence alignments of the 2006 Chinese strain HUN4 and the 2007 Vietnamese sequences generated from the highly variable genomic region ORF4-6 showed 99.4–99.7% nucleotide identity, whereas identity of the Vietnam isolates to the North American prototype strain VR-2332 (Type 2) and to the highly virulent North American strain MN184 was approximately 90% and 86–88%, respectively. Similarly, nsp2 region nucleotide comparison showed that the Vietnamese isolates have

Table 1. Diagnostic results on samples from a swine disease outbreak in Vietnam. Samples analysed using PRRSV conventional polymerase chain reaction (PCR), virus isolation (VI) on swine peripheral blood macrophage cell (PBMC) and MARC-145 cells

		Sample		PCR		VI		Bacteriology	
Province	Pig	ID	Type	PRRS	PCV-2	PBMC	MARC-145	*S. zooep*[a]	*E. coli*[b]
Quang Nam	1	07QN-1	Sp[c] & Lg	+	+	+	−	+	+
	2	07QN-2	Lg	−	+	−	−	+	+
			Sp	+	+	+	−	+	+
	3	07QN-3	Sp & Lg	+	+	+	−	+	+
Thai Binh	4	07TB	LN & Lg	+	−	+	+	NT	NT
Bac Giang	5	07BG	LN & Lg	+	−	+	+	NT	NT
Hung Yen	6	07HY	LN & Lg	+	−	+	+	NT	NT
Ha Noi	7	07HN	LN & Lg	+	−	+	+	NT	NT

Samples positive for [a]*Streptococcus equi subspecies zooepidemicus,* [b]non-hemolytic *Escherichia coli* and [c]*Streptococcus suis*. Samples not tested (NT). Sample types are defined as Sp, spleen; Lg, lung; LN, lymph node; PCV, porcine circovirus; PRRSV, porcine reproductive and respiratory syndrome virus.

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98.6–99.6% identity to Chinese strain HUN4, in contrast to their low percentage of identity to VR-2332 (77%) and MN184 (71%).

Comparison of predicted amino acid (aa) sequence in the nsp2 region revealed a range of 95.7–99.4% identity between the Vietnamese isolates and HUN4. This wide range of identity could be attributed to the variations within the Vietnamese PRRSV isolates. The nsp2 amino acid identity with North American strains was even lower, only 68–69% and 58–59% identity to VR-2332 and MN184, respectively. For the GP5 protein, all Vietnamese isolates were 99.5–100% identical to the Chinese strain HUN4, whereas all isolates were 88–89% identical in amino acid sequence to the prototype North American (Type 2) strain VR-2332 and 86–87% identical to strain MN184. Phylogenetic analyses of nsp2 and ORF-5 was

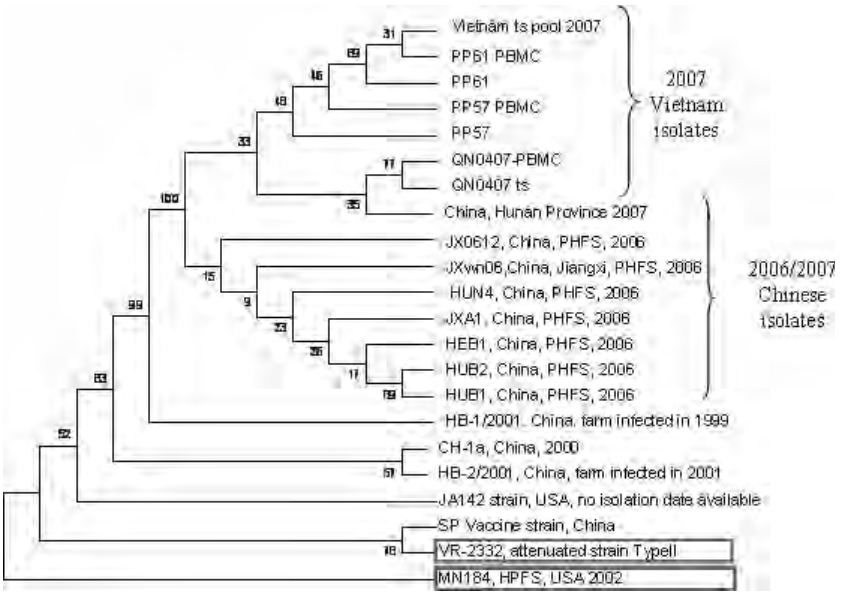

Fig. 3. PRRSV phylogeny of ORF-5 nucleotide sequences. ORF-5 unrooted tree by neighbour-joining method with bootstrapping. ts: sequence obtained from original tissue samples; PP: Pig Passage; PBMC/p1: sequence obtained from same tissue samples after 1 passage in PBMCs; Boxes: indicate the prototype PRRSV (VR-2332) and highly virulent (MN184).

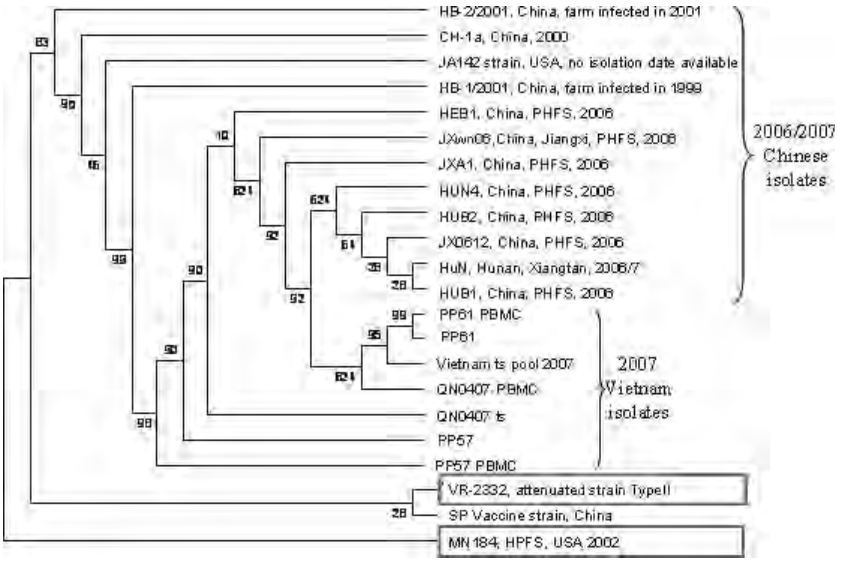

Fig. 4. PRRSV phylogeny of nsp2 nucleotide sequences Nsp2 un-rooted tree neighbour-joining method with bootstrapping. ts: sequence obtained from original tissue samples; PP: Pig Passage; PBMC/p1: sequence obtained from same tissue samples after 1 passage in PBMCs; Boxes: indicate the prototype PRRSV, attenuated (VR-2332) and highly virulent (MN184).

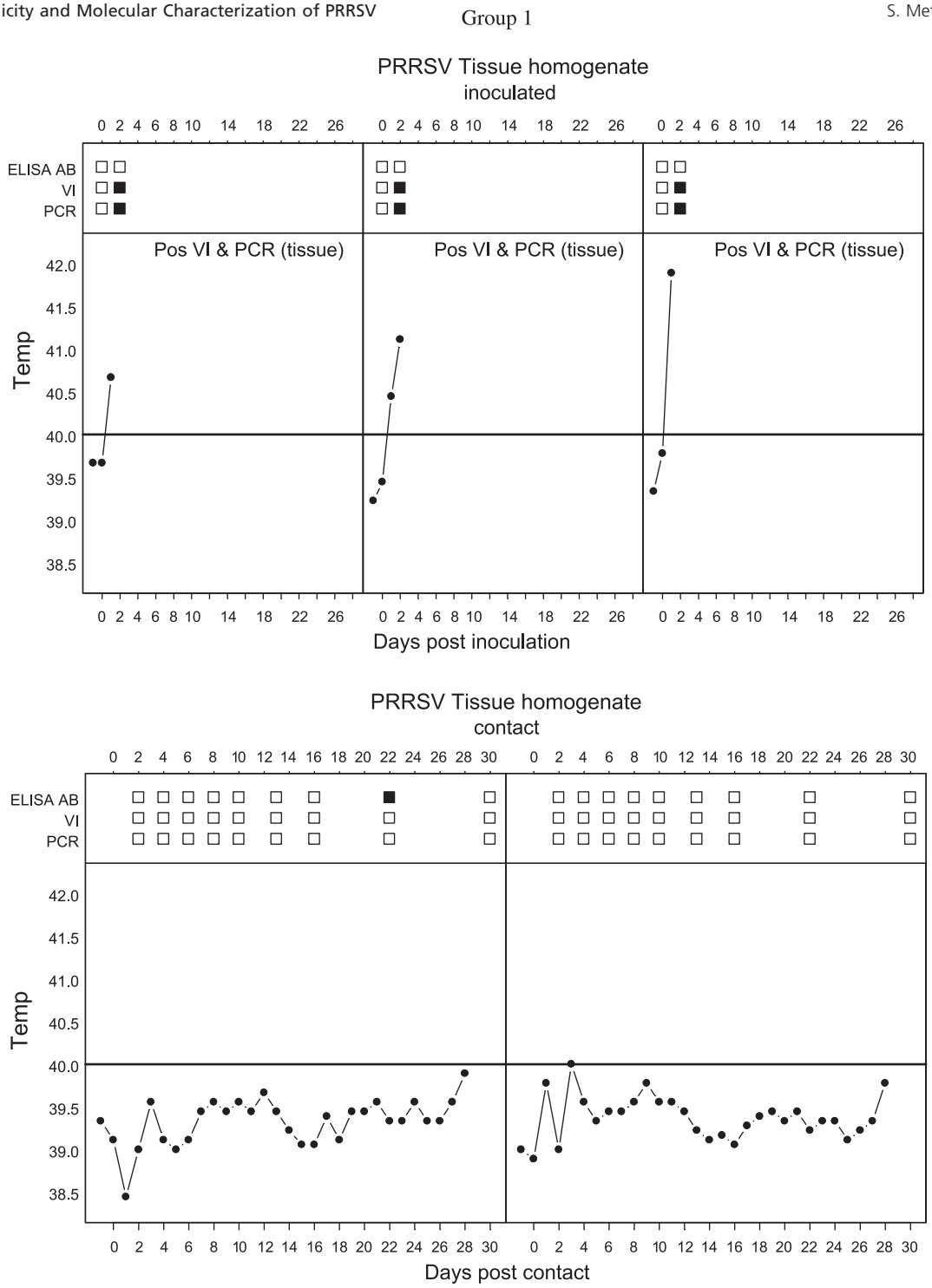

Fig. 5. Body temperature, virus isolation, PCR, and serology results on pigs inoculated with or contact exposed to either tissue homogenate (Group 1) or cultured porcine reproductive and respiratory syndrome virus (PRRSV) (Group 2). Each box represents a different animal. Three inoculated and two contact pigs were used each for PRRSV tissue homogenate group (group 1) and tissue culture (group 2). Days post-inoculation or post contact are represented on the horizontal *X*-axis for each animal. Body temperature was measured in Centigrade degrees shown on the *Y*-axis. The bold horizontal line at 40.0 C represents the highest temperature considered nonfebrile, after which is considered febrile. Antibody ELISA (ELISA AB) positive serum is represented by a filled square and negative serum is represented by an open square opposite the AB ELISA designation. Virus isolation (VI) and RT-PCR (PCR) were performed on serum. An open box represents negative results and a filled box represents positive for VI or PCR results opposite the VI or PCR designations. Spleen, lung, and lymph nodes collected at necropsy that were positive for PRRSV by PCR and VI are indicated as positive by a text note in each box corresponding to a positive animal.

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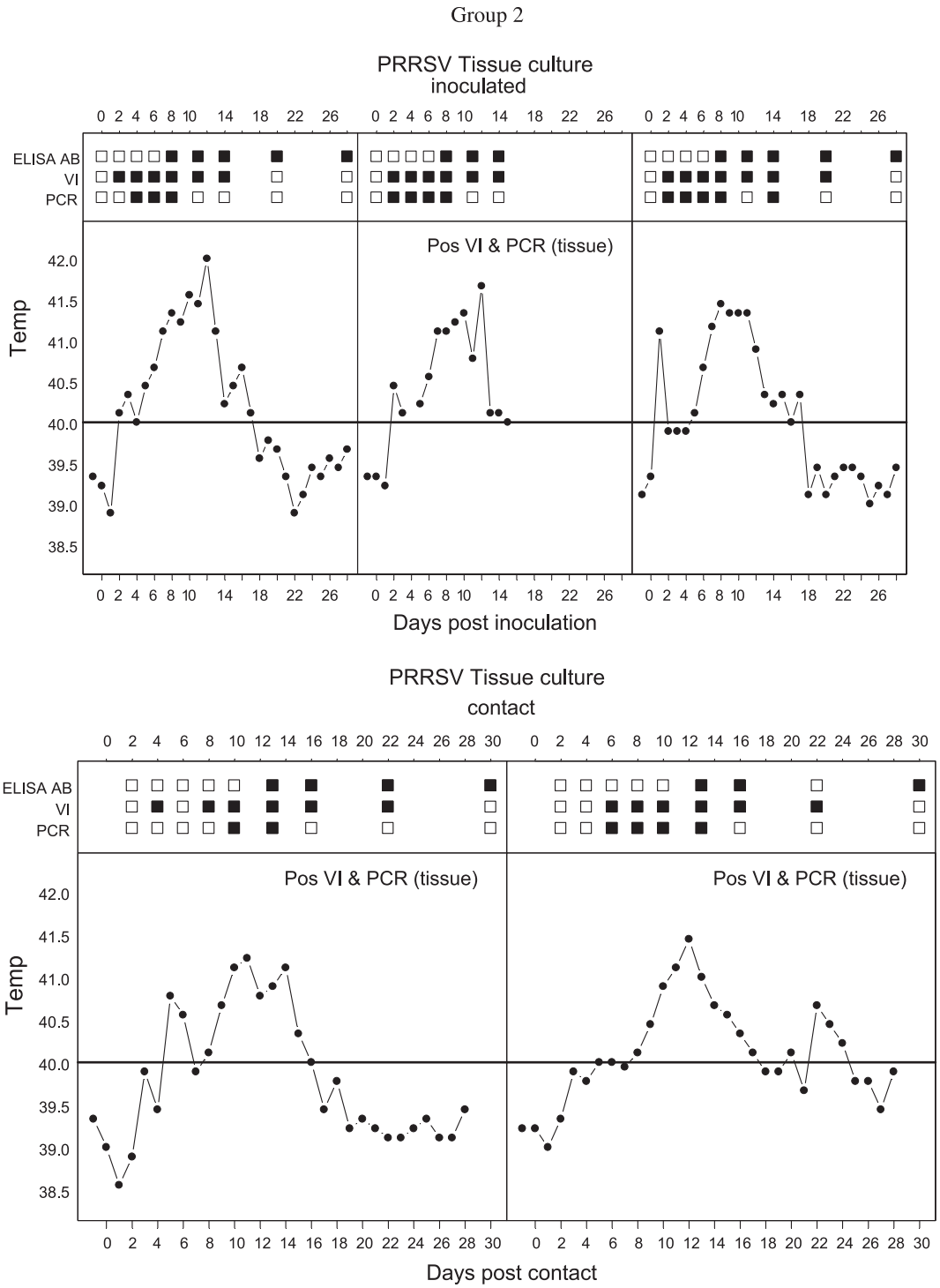

Fig. 5. (Continued).

completed using both Neighbour-Joining and UPGMA algorithms, each with percent identity or BLOSUM62, to confirm the relationships between the different viruses analysed (Figs 3 and 4).

Animal inoculation

The three pigs that received the tissue homogenate (group one) developed high fever (40.6–41.7°C) within 24–72 h,

septicaemia and acute death within 48–72 h after inoculation (Fig. 5). Gross necropsy findings were similar in all three animals and consisted mainly of severe fibrinous peritonitis, pleuritis and pleural effusions. Histopathology showed severe fibrinosuppurative pleuritis. Additionally, necrosuppurative lymphadenitis was observed in two pigs. *Streptococcus equi* subspecies *zooepidemicus* was cultured, and PPRSV was reisolated, detected by RT-PCR, and sequenced from lungs and spleens. The two contact pigs showed no clinical signs throughout the experiment as their exposure to the inoculated pig dying acutely was <24 h. Necropsy findings of the contact pigs were not significant, and histopathology showed mild lymphoid follicular hyperplasia of the mandibular and thoracic lymph nodes. PRRSV was only isolated and detected by RT-PCR from lung and spleen of three inoculated pigs.

The three pigs that received the PRRSV in PBMC culture (group two) had fever starting 2 dpi and their two contact pigs at 5–8 dpc. Body temperature peaked at 7–10 dpi/dpc (40.6–41.1°C) and persisted for 1 week before starting to decline (Fig. 5). Pigs were depressed, had low appetite, and a rough hair coat. One of the tissue culture inoculated pigs in group two was euthanized (15 dpi) because of swollen joints and loss of mobility. Upon necropsy, this pig had bronchopneumonia, fibrinous pericarditis with pericardial effusions and polyarthritis. Histopathology showed myocarditis, perisplenitis, suppurative lymphadenitis, suppurative pneumonia with intralesional bacteria and severe meningoencephalitis. The remaining pigs in group two were euthanized at 28 dpi/26 dpc, and the gross necropsy findings were variable. All animals had fibrinous peritonitis ranging from mild to severe with peritoneal effusions. In addition, inoculated and contact pigs from group two had severe, chronic pericarditis, fibrinous pleuritis and polyarthritis (Fig. 6). Histopathological examination confirmed gross findings of multifocal interstitial pneumonia, lymphoid hyperplasia in some animals (Fig. 6), and additionally pericarditis, peritonitis and meningoencephalitis (Fig. 6). PRRSV was

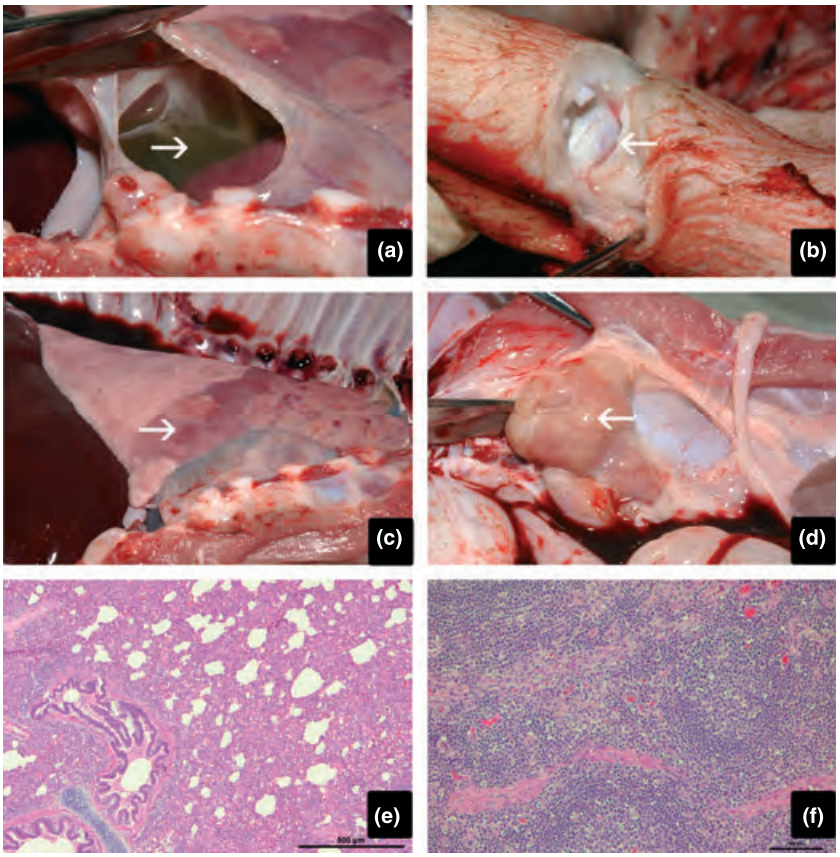

Fig. 6. Gross and histopathological lesions in pigs inoculated with tissue culture PRRSV (group 2). (a) Fibrinous pericarditis and pericardial effusions (arrow), (b) Arthritis (arrow), (c) Interstitial pneumonia (arrow) and (d) lymphoid hyperplasia (arrow). Photomicrographs of interstitial pneumonia showing thickening of the alveolar walls and type II cell hyperplasia (e), and marked follicular lymphoid hyperplasia of the lymph node (f). Pericarditis and arthritis are likely due to secondary bacterial infection.

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isolated from sera of inoculated and contact pigs starting at 2 dpi/2–6 dpc, and PRRS antibody was first detected at 8 dpi/12 dpc (Fig. 5). PRRSV was isolated and detected by RT-PCR from lung and spleen of one of the inoculated pigs and the two contact pigs. The sham-inoculated control pigs (group 3) showed no clinical signs throughout the experiment and tested negative for PRRSV and antibody (data not shown).

Discussion

In this study, a diagnostic investigation was conducted on disease outbreaks causing respiratory distress, which affected thousands of swine in Vietnam in 2007. Similar epidemics of severe swine disease, referred to as Porcine High Fever Disease (PHFD), were reported in Southeast Asia in 2006 (Tian et al., 2007a; Zhou et al., 2008). Investigations into these cases consistently identified PRRSV, and thus this virus has been implicated as the primary cause of these epidemics. Here we present data of PRRSV isolated from specimens submitted from northern, central and southern provinces of Vietnam with sequence analysis similar to the Chinese isolates from 2006 including the identical deletion of nsp2 region. Deletions in the nsp-2 region, and the 5′ and 3′ UTR of the PRRSV sequence have been correlated with the high pathogenicity of these infections (Feng et al., 2008; An et al., 2010). Co-infection with *E. coli*, *Streptococcus equi* subspecies *zooepidemicus, Streptococcus suis* and/or PCV-2 was confirmed in a number of samples from the same or different pigs. As strains of PRRSV are known to vary in pathogenicity (Drew, 2000), an animal study was conducted on the Vietnamese PRRSV strain to determine its degree of pathogenicity in the absence of other agents. The inoculated pigs (except for the ones which died acutely) had persistent high fever for 2 weeks, mild to severe respiratory distress, seroconverted within 8 dpi, and the virus was reisolated from serum, lungs and spleen in most animals The duration of viraemia and the persistence of the virus in tissues past the viremic stage were similar to those reported by Christopher-Hennings et al., 2001. Our findings differ from the previous reports (Tian et al., 2007a; Tong et al., 2007) where the Chinese PRRSV strain was characterized as a highly virulent variant causing high fever and up to 100% mortality within 6–10 days post-exposure; however, the breed of pigs used in those studies was not specified and may have played a role in the susceptibility to infection.

Most pigs in this study showed evidence of secondary bacterial infection manifested by peritonitis, pleuritis and pericarditis, which may indicate increased susceptibility or immunosuppression. Immunosuppresion was often associated with PRRS, and several studies were undertaken to study the mechanism of immunosuppression in PRRS (Drew, 2000). While there is some field evidence of immunosuppression of pigs following PRRSV infection, the experimental evidence is somewhat ambiguous. It is likely that any effects are because of a transient deterioration of local lung cellular defence where the virus tropism is for alveolar macrophages, which is difficult to reproduce experimentally (Pol and Wagenaar, 1992, Voicu et al., 1994). It has also been reported (Gomez-Laguna et al., 2010) that PRRSV modulate the immune response by upregulation of IL-10, which may in turn reduce the expression of cytokines involved in viral clearance. It is important to note that pigs inoculated with tissue homogenates from the diagnostic submission samples died acutely with septicaemia with no evidence of toxicity as determined by histopathological examination of tissues. Additionally, when filtered tissue homogenate was inoculated into two naïve pigs, no clinical signs of toxicity were noted (data not shown).

In this study, pigs did not show significant decrease in total WBC count throughout the experiment (data not shown). A recent report (Shi et al., 2008) showed that the total WBC count was significantly lower in pigs inoculated with HB-2 strain than the control group at 7 dpi but became significantly higher than the control at 0, 14 and 28 dpi. To reproduce similar disease as reported in the outbreaks, experimental group one was inoculated with the original tissue homogenates neither pre-treated with antibiotics nor filtered, so that any agent present might have the opportunity to exacerbate disease. Inoculated pigs died acutely within 72 h with a septicaemia.

PRRSV and bacterial infection were detected in infected animals confirming earlier observations that PRRSV and other agents were involved in these epidemics. It is likely *Streptococcus suis*, and *E. coli*, or other agents may have played a role in increasing the severity of the outbreak in Vietnam, as previously reported in other PRRSV infections (Thanawongnuwech et al., 2000a; Feng et al., 2001; Rovira et al., 2002).

In 2001, many virulent isolates of PRRSV from Minnesota were genetically closely related to the Canadian isolates of the 1990s (Mardassi et al., 1994) and Minnesota isolates of 1998 (Wesley et al., 1998). Isolates with similar ORF-5 genomic patterns to the MN184 have been reported in Iowa, Wisconsin, South Dakota, Kansas, Missouri, Illinois, Nebraska, Kentucky, Oklahoma and Wyoming but showed variable levels of pathogenicity suggesting no direct correlation between severity of disease and genetic makeup (Han et al., 2006b). In our experiment, partial genome sequencing of the PRRSV reisolated from blood samples and tissues of the inoculated pigs confirmed the presence of the exact same GP5 mutations and nsp2 deletions found in the field samples. Transmission EM examination of tissue collected from inoculated

pigs and in cell culture passage showed typical single and double membrane profiles described previously (Snijder, 1998; Tian et al., 2007a) with the extracellular accumulation of 70-nm particles found with EM analysis of PRRSV exposed cells *in vitro* and *in vivo* (Dea et al., 1995; Balasuriya et al., 2004). Therefore, our results suggest that the PRRSV emerging in Vietnam 2007, despite the presence of the characteristic nsp2 deletions previously described as a marker of virulence and high pathogenicity for pigs, is not solely responsible for the high death rate that characterizes these epidemics.

Phylogenetic analysis revealed that the Vietnamese isolates belong to the North American genotype (Type 2) PRRSV and are closely related to Chinese strains from 2006 to 2007 (HUB1, HuN, JX0612 and others) with two nsp2 deletions when compared to the prototype strain VR-2332. The nsp2 of PRRSV is a multidomain protein that has been shown to undergo remarkable genetic variation. From its three major domains (den Boon et al., 1995; Han et al., 2006a, 2007), nsp2 is the most divergent protein between PRRSV types 1 and 2 (Allende et al., 1999; Nelsen et al., 1999) and also between the pathogenic PRRSV 16244B and an attenuated vaccine and its parental strain VR2332 (Allende et al., 1999, 2000; Nelsen et al., 1999). A large amount of data supports the theory that the middle section of the nsp2 protein is highly susceptible to mutation and tolerant to insertions and deletions, regardless of the pathogenic phenotype of the isolates (Allende et al., 1999, 2000; Nelsen et al., 1999; Shen et al., 2000; Fang et al., 2004, 2006; Gao et al., 2004; Ropp et al., 2004; Diao et al., 2005; Han et al., 2006b, 2007; Kim et al., 2007; Ran et al., 2008). In fact, reverse genetics experiments have mapped several non-essential viral replication regions in nsp2, including a large deletion of 402 nucleotides in the middle region of the gene (Han et al., 2007). These reports and our data contrast with the suggestion that the nsp2 deletions found in the atypical PRRS outbreak in 2006 in China could be correlated with a highly virulent phenotype (Li et al., 2007, 2008; Tian et al., 2007a,b; Zhou et al., 2008).

Co-circulation of other pig disease causing agents with PRRSV has been noted by other authors. PCV-2 co-infection with PRRSV was detected in some of these samples, and CSFV was detected in addition to PRRSV from samples received in a subsequent diagnostic submission to FADDL from other locations in Vietnam. As final evidence, in agreement with our conclusions, a recently published reverse genetics experiment has demonstrated that there was no direct correlation between the 30 aa deletion in nsp2 of the Chinese PRRSV and virulence (Zhou et al., 2009). Additional studies are needed to determine and compare the pathogenicity of the Vietnamese PRRS virus to prototype PRRSV type 1 strains.

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Research Article

In-Depth Global Analysis of Transcript Abundance Levels in Porcine Alveolar Macrophages Following Infection with Porcine Reproductive and Respiratory Syndrome Virus

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a major pathogen of swine worldwide and causes considerable economic loss. Identifying specific cell signaling or activation pathways that associate with variation in PRRSV replication and macrophage function may lead to identification of novel gene targets for the control of PRRSV infection. Serial Analysis of Gene Expression (SAGE) was used to create and survey the transcriptome of *in vitro* mock-infected and PRRSV strain VR-2332-infected porcine alveolar macrophages (PAM) at 0, 6, 12, 16, and 24 hours after infection. The transcriptome data indicated changes in transcript abundance occurring in PRRSV-infected PAMs over time after infection with more than 590 unique tags with significantly altered transcript abundance levels identified ($P < .01$). Strikingly, innate immune genes (whose transcript abundances are typically altered in response to other pathogens or insults including IL-8, CCL4, and IL-1β) showed no or very little change at any time point following infection.

1. Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), the causative agent of porcine reproductive and respiratory syndrome (PRRS) in swine, is a member of the *Arteriviridae* family in the order *Nidovirales*. PRRSV causes significant losses to the swine industry worldwide [1] as a result of both reproductive failure (late-term abortions and stillbirths) in pregnant sows and respiratory disease (pneumonia) in nursery and grower/finishing pigs [2]. Infection with PRRSV also predisposes pigs to infection by bacterial pathogens as well as other viral pathogens [3–7]. Clinical disease caused by PRRSV is highly variable, ranging from mild, subclinical infections to acute deaths of swine of any age [8]. Differences in virulence have been attributed to numerous factors including host genetics, management practices, and virus strain heterogeneity [9–16].

Relatively little is known about the interactions of PRRSV and host cells. The primary cellular target of PRRSV is the porcine alveolar macrophage (PAM) [17, 18]. PRRSV has been shown to replicate to varying degrees in peritoneal macrophages, pulmonary intravascular macrophages, type II pneumocytes, testicular germ cells, and PAMs [19–22]. MARC-145 cells, a monkey kidney cell line, are used to propagate this fastidious virus in culture. A putative cell surface receptor has been identified that may contribute to the propensity of PRRSV to readily infect these cells. The CD163 protein, sialoadhesin, and heparan sulphate have been reported to play significant role in helping PRRSV attach and be internalized into cells [23].

A primary function of the PAM is to combat bacterial insults within terminal airways of the lung, in part, by regulating the local host immune response in the alveoli. Reports have shown pigs infected with PRRSV have a higher rate of concurrent or secondary bacterial infections [23, 24]. This has led investigators to examine the effect of PRRSV infection on bacterial killing by PAMs [25–28]. Studies have reported PRRSV infection significantly decreases production of superoxide anion and hypohalous acid, both of which contribute to the oxidative ability of PAMs to kill bacteria [25]. Another report [29] demonstrated that PRRSV infection resulted in cytotoxicity to PAMs that led to a 40% reduction in phagocytic uptake of *Escherichia coli*. The specific mechanism(s) by which PRRSV infection alters PAM function is unknown. It has been reported PRRSV infection of PAMs results in lowered transcript abundance of proinflammatory response cytokines including TNF-α, IL-1α, and MIP-1β [30]. However, other studies showed that TNF-α, IL-8, IFN-α, and IL-1β transcripts are not significantly altered by PRRSV infection [31–35]. Thus, it remains unclear which macrophage genes PRRSV affects upon infection.

One study attempting to better understand the altered transcript abundance of PAMs upon infection by PRRSV used differential display reverse-transcription PCR to identify host cell gene responses to PRRSV infection of PAMs over a 24 hour period [35]. Four transcripts were identified that specifically responded to PRRSV infection and were induced *in vivo* in tissues where PRRSV persistently resides. Of the four transcripts identified, three of these came from identified genes and the fourth remains a novel expressed sequence tag (EST). The three genes identified are Mx1 (myxovirus resistance), UBP (ubiquitin protease), and RHIV-1 (RNA helicase). Presumably there are more, yet to be identified, genes that differentially respond to PRRSV infection.

Various research techniques provide the potential to look at cellular processes and response to infection in a comprehensive and unbiased manner [36]. Combined with targeted approaches such as gene knockouts, transgenics, targeted overexpression and other methods, it is now possible to dissect pathways and networks of genes, proteins and small molecules that define cellular functions. Technologies have been developed that permit high throughput quantification of transcript abundance. Most prominent of these are various forms of solid phase microarray hybridization [37] and serial analysis of gene expression (SAGE; [38, 39]). Information derived from these methods can be exploited in a number of ways including development of diagnostic assays, understanding molecular mechanism(s) behind disease states and formulating intervention regimens to inhibit or minimize infections and negative outcomes. SAGE has been used extensively to evaluate changes in transcript abundance in a number of experimental systems. The availability of nearly 600,000 swine and over 1 million bovine ESTs in the NCBI dbEST greatly increases the utility of SAGE for gene expression studies in these species. SAGE has also been used for gene discovery and enabled identification of genes not previously known to be expressed in granulocytes [40], and in identifying potential new cell surface diagnostic markers in astrocytomas [41].

Table 1: Summary statistics of SAGE libraries.

Library (hours PI)	Noninfected	Infected			
	0	6	12	16	24
Tags sequenced	111,214	96,968	103,662	134,990	196,421
Unique tags	24,367	30,709	33,601	33,255	37,942
PRRSV tag (CGGCCGAAAT)	0	255	9500	6902	3632

It is our hypothesis that PRRSV infection of PAMs alters their normal transcriptome in a manner that enables virus replication and dysregulates the normal host immune response. Here we report a comprehensive evaluation of transcript abundance levels in noninfected and PRRSV-infected PAMs as an initial step towards a more comprehensive understanding of PRRSV pathogenesis. Although a comprehensive understanding of differential posttranscriptional and posttranslational responses in PAMs remains to be determined, detection of altered transcriptome patterns may identify PRRSV virulence mechanisms that contribute to a delayed or lack of a protective immune response and viral persistence.

2. Results

2.1. Serial Analysis of Gene Expression. Total cellular RNA was prepared from *in vitro* PRRSV-infected PAMs at 0, 6, 12, 16 and 24 hours after infection, and mock-infected PAMs at 0 and 24 hours. Five SAGE libraries were constructed from the 0 hour mock-infected and the 6, 12, 16 and 24 hours PRRSV-infected cells. The libraries were subsequently sequenced to obtain approximately 100,000 tags each, with the exception of the 24 hour infected library, which was sequenced to nearly 200,000 tags (Table 1). Five SAGE libraries yielded 643,255 sequenced tags that were used to populate a modified Identitag database. Examination of the SAGE data indicated that there were major changes in transcript abundance occurring in the PRRSV-infected PAMs based on more than 590 unique tags with significantly altered transcript abundance ($P < .001$ with Bonferroni correction). Table 1 summarizes the general statistics of these libraries. Tags with a frequency of 1 were not considered for quantitative purposes, because they could represent artifacts of sequencing or of the SAGE procedure [42].

2.2. Functional Classification of Transcripts with Changes in Abundance. To obtain a greater understanding of cellular responses to PRRSV, the identified transcripts were further categorized with biological processes, defined by the Gene Ontology Consortium (http://www.geneontology.org/), according to the Ingenuity Pathway Analysis (IPA) database (Ingenuity Systems, http://www.ingenuity.com/). Fischer's exact test was used to calculate a P-value determining the probability that each biological function assigned to that data set is due to chance alone. Transcriptional

abundance differences between high-level function groups observed during the first 24 hours of PRRSV infection were compared. Cellular movement, which describes the cellular functions associated with movement and localization of cells, was significantly upregulated among differentially expressed transcripts in the first 6 h p.i. Cell-to-cell signalling and interaction, which describes functions involved in intercellular interactions and includes functions associated with specific cellular components that are involved in signalling and interaction, an important aspect of macrophage function, included the most significantly down-regulated, differentially expressed transcripts. Our interest was in those expression changes that affect PAM function; particularly in regard to innate immunity, antigen presentation, and intra- and extra-cellular signalling.

2.3. Detection of Viral RNA Transcripts. Analysis of the data revealed the presence of the tags derived from transcripts produced by the infecting PRRSV strain; thus confirming the virus infected the PAMs as intended. PRRSV produces 3′ co-terminal subgenomic RNAs, thus all viral RNAs had the same tag [43]. The viral tag, not detected in the mock-infected library, was first present at 6-hours PI and increased in number to its highest point at 12 hours after which it declined (Table 1). These data were validated by real-time PCR (data not shown). There was a high level of viral RNAs present in the infected cells at 12 hours after infection, where viral RNAs accounted for almost 10% of all polyadenylated RNA in these cells. The amount of viral RNA declined by 24 h hours after infection perhaps because of a decline in RNA replication/transcription, degradation of viral RNAs, release of virus from the cells or a combination of all three.

2.4. Real-Time PCR Validation of Transcript Abundance Levels. Changes in transcript abundance of genes of interest were validated using real-time reverse transcriptase (RT)-PCR (Figure 1). In this study, β2-microglobulin was found to have stable transcript levels across all times tested (Figure 1(a)) and was chosen as the internal control in all real-time RT-PCR assays.

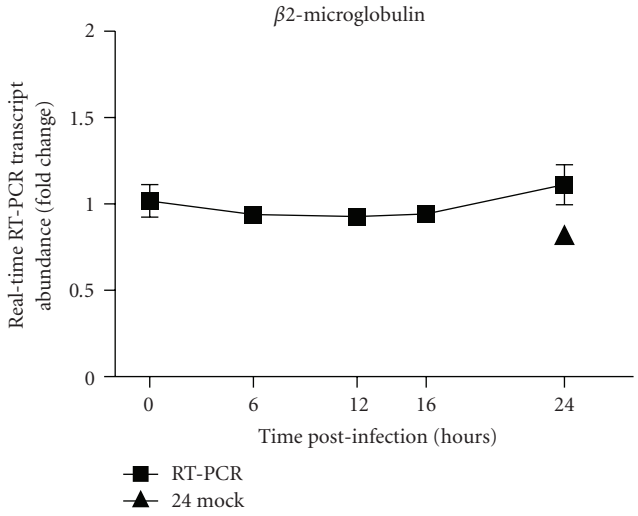
Real-time RT-PCR amplification of cellular transcripts previously shown to have altered transcript abundance levels following PRRSV infection was done to confirm the infection had proceeded as expected (Figure 1(b)). As expected, transcripts encoding the proteins Mx1 and rHIV (a RNA helicase) showed 6.4- and 4.8-fold increases at 24 hours PI, respectively (Figure 1(b)), and were induced between 0 and 12 h postPRRSV-infection [35]. The transcripts encoding the proinflammatory proteins IL-1α and CCL4 (macrophage inflammatory protein, MIP-1β) declined in abundance as previously demonstrated [44]. The tags for the Mx1 transcript were identified in the 16 and 24 hour infected SAGE libraries but at very low levels and were therefore insignificant following normalization of the SAGE data.

Overall, the real-time RT-PCR validation described above, in addition to that which was not shown, had a high degree of correlation to the SAGE data, thus providing confidence that the tag counts for each library was an accurate representation of transcript abundance levels in these PAMs. This demonstrated that additional validation of the SAGE libraries by real-time PCR was not necessary.

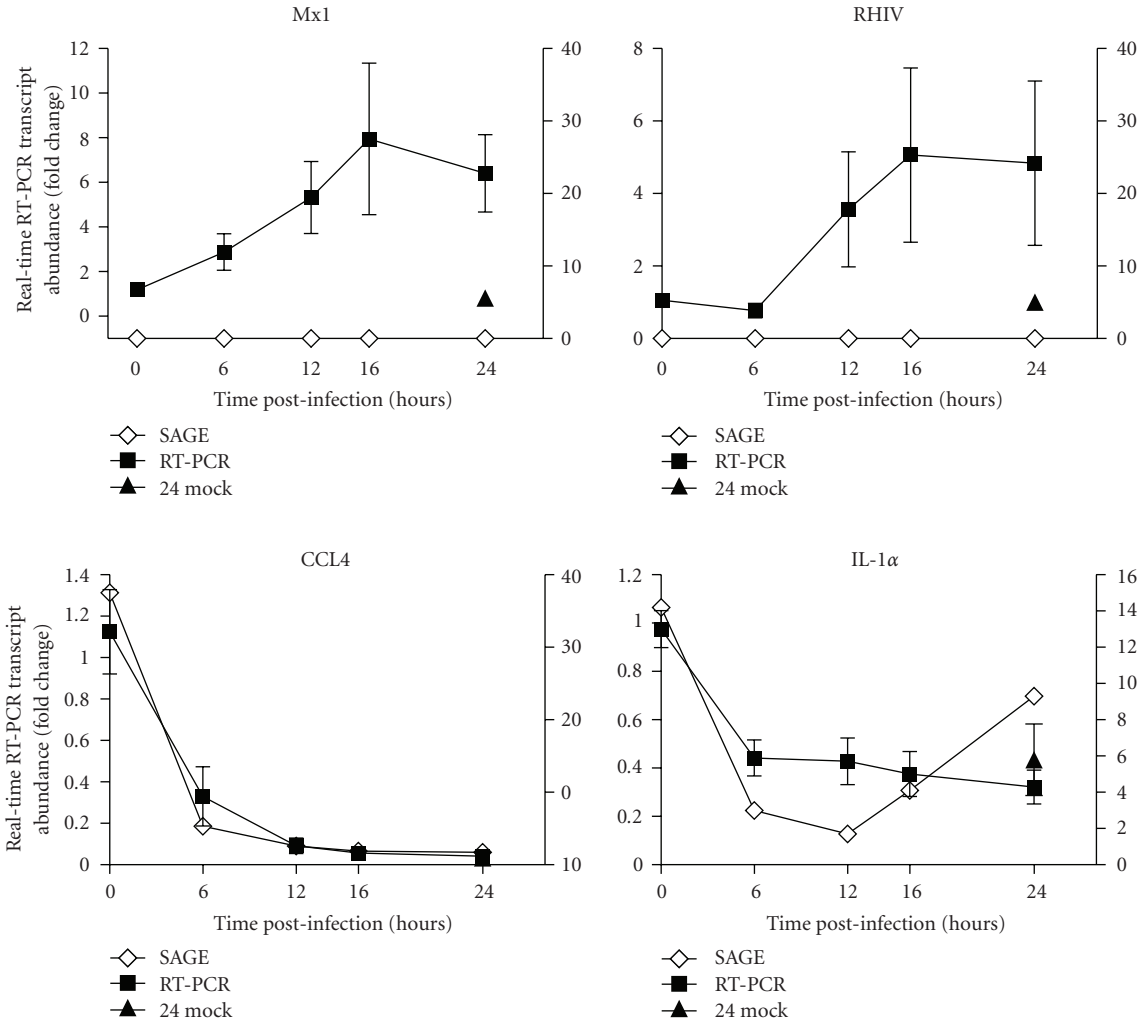
2.5. Transcripts Encoding Immune Response Proteins. There was a general decline in numbers of the cytokine and chemokine transcripts, indicating that there was no induction of expression that would be expected in an innate immune response to a viral infection. SAGE revealed decreased transcript abundance IL-1α and IL-1β (Tables 2(a) and 2(b)). RANTES, MIF, MCP3 and CXCL5, showed increased transcription during the infection (Table 2(a)). MCP3 and CXCL5 showed increases only late in the infection process, while CXCL8 (IL-8) showed increased transcription early in the infection then decayed to noninfected levels (Table 2(a)). Interestingly, there were 2 tags identified for CXCL8 with both showing similar patterns of expression. Proinflammatory cytokines CXCL8 and CXCL5 are chemoattractants for neutophils, IL-1α and IL-1β are important proinflammatory cytokines and would be expected to be increased early in infection. IL-1α and IL-1β promote infiltration of various leukocytes by inducing chemokine production and increasing expression of various adhesion molecules on mesenchymal cells and postcapillary venule endothelial cells.

The increase in transcript numbers of cytochrome p450 3A29, BNIP3 (a proapoptotic protein), and GRP78 (an endoplasmic reticulum chaperone protein) were also confirmed (Figure 1(c)). Changes in levels of transcripts encoding proteins involved in defense and the innate immune response were also validated (Figure 1(c), Table 2). Changes in levels of transcripts encoding CCL3 (MIP1-α), CXCL8 (swine IL-8), and IL-1β were found to be very close to that indicated by SAGE with all declining as the infection progressed. An additional transcript of interest, that encoding IL-6, was not found represented in the SAGE database. Interestingly, the transcript encoding IL-6 does not encode a tag (no *Nla* III restriction site in the transcript), therefore, it was not detected by SAGE. The only information on IL-6 transcript abundance levels in these cells was obtained by real-time RT-PCR. This revealed a sharp decline in IL-6 transcripts during the first 24 hours following infection by PRRSV (Figure 1(c)).

2.6. Additional Pathways and Functions. Data was evaluated for indications of activation of intracellular signaling or other pathways in PRRSV-infected PAMs. Tags derived from transcripts encoding major proteins making up the AP-1 immediate-early transcriptional complex were identified (Table 2(c)). Tags corresponding to the *c-jun*, *junB, junD* and *c-fos* transcripts showed decreased abundance levels, dropping to the lowest levels at 12 hours and showing some rebound by 24 hours PI. These data indicated that there was no overt induction of the AP-1 signaling pathway following PRRSV-infection. Similarly, activation of NF-κB was not observed (Table 2(c)). A20, a negative regulator of NF-κB as well as a gene that is transcriptionally activated by NF-κB,

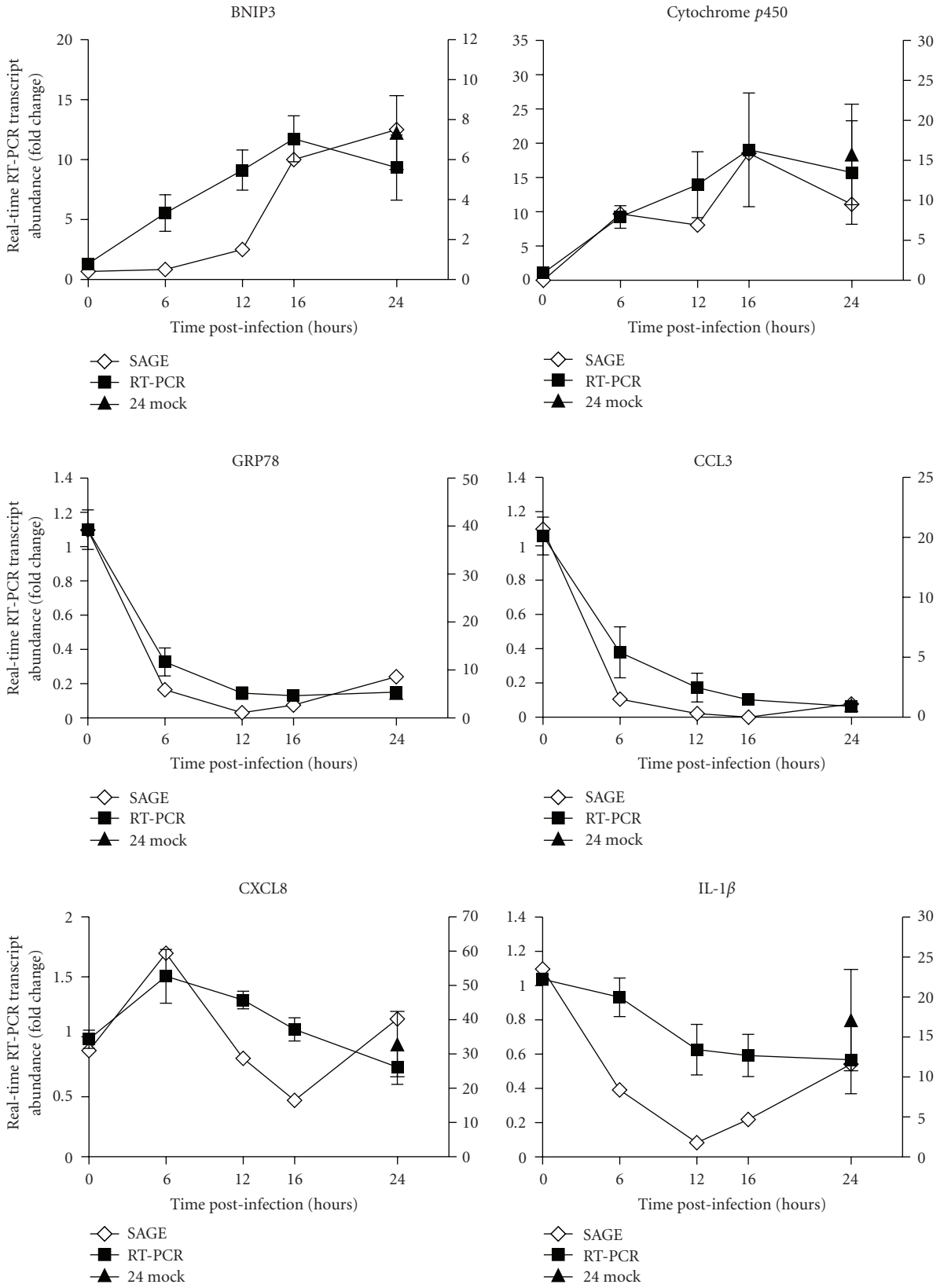


(a)



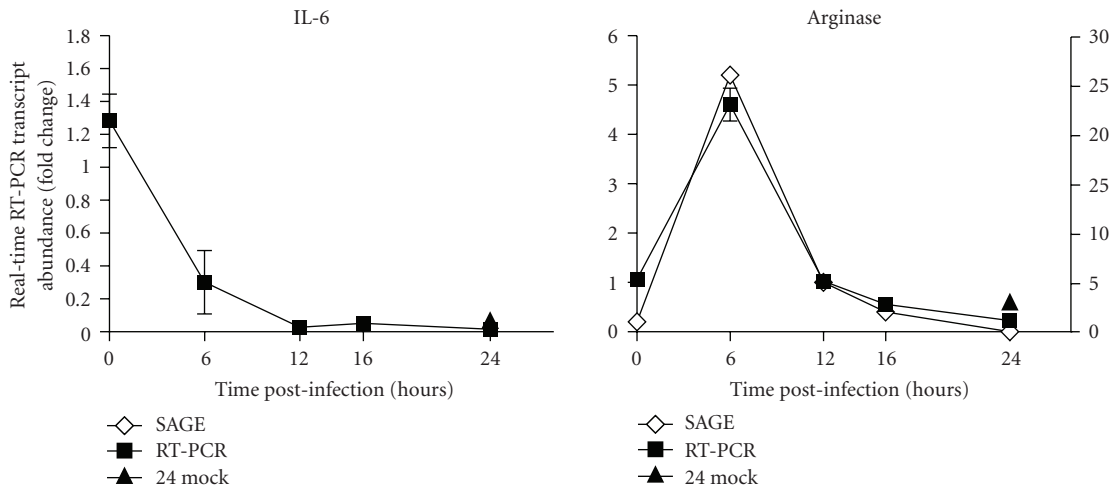
(b)

Figure 1: Continued.



(c)

Figure 1: Continued.



(c)

Figure 1: Real-time RT-PCR validation of SAGE results. Validation by real-time RT-PCR on RNA from the *in vitro* PRRSV-infected PAMs samples used in the SAGE analysis of selected transcripts showing differential transcript abundance. Real-time RT-PCR transcript abundance results (filled squares; left y-axis) expressed as the mean fold increase ± SEM in gene expression ($n = 3$) relative to mock-infected cells and SAGE tag counts normalized to total tags per library (open diamonds; right y-axis) are shown for transcripts. The "24 mock" result shows the real-time RT-PCR fold change in transcript abundance as a result of being in cell culture.

showed no significant changes in the SAGE data. Interestingly, IκBα showed a steady decline in transcript abundance out to 24 hours PI while IκKα showed no significant changes over the course of the experiment. Activation of toll-like receptor 4 (TLR4), a cell surface receptor that recognizes bacterial cell membrane lipopolysaccharides, results in the downstream activation of NF-κB. The transcript encoding TLR4 showed a steady decline during the course of the PRRSV infection (Table 2(c)).

An interesting finding was the sharp increase in arginase transcript levels at 6 hours PI, with the RT-PCR and SAGE data closely mimicking each other (Figure 1(c)). In addition, a total of four distinct tags, presumably representing different mRNA species, were found that were derived from arginase-encoding transcripts; all showed increased transcript abundance at 6 hours PI (Table 2(c)). The primer set used to validate the arginase transcript levels amplified sequences found within the coding sequences of the transcript, thus, the real-time PCR results were from amplification of sequences from all four transcripts. The 6 hour PI time point examined here represents only a "snap-shot" of the cells at that time, so the full pattern of expression in not known.

3. Discussion

The derived catalog of expressed genes reported here represents a first attempt to generate comprehensive analyses of PRRSV-infected PAMs transcript abundance profiles at different time points after infection. The wealth of information obtained allows detection of altered transcription of genes involved in normal porcine alveolar macrophage physiology, as well as genes whose transcript abundance is altered by PRRSV infection.

The ability of an animal to respond to specific foreign characteristics or molecular patterns of pathogens is an important aspect of innate immunity. This is the first, generally rapid step in the response to invasion by a pathogen and the beginning of a protective immune response. It is important that this response begin quickly and in sufficient fashion to stop the spread of the invader and terminate the infection. In many cases, the pathogen possesses the ability to inhibit or thwart the immediate innate immune response, thus giving it an early advantage. Deciphering the mechanisms of disruption of the innate immune response is the focus of considerable research and is beginning to provide answers into the myriad of different means pathogens employ to achieve this. The focus here was to discern how PRRSV inhibits the innate immune response in infected PAMs. This study produced transcriptional profiles of noninfected and PRRSV-infected PAMs that provided insight into the suppression by the virus on host transcript abundance levels necessary for a strong immune response. This work has resulted in the characterization of macrophage transcript abundance in normal cells as well as transcript abundance changes that occurred with progressive PRRSV replication. Virus-specific transcript abundance changes that were found provided intriguing clues to possible mechanisms behind immune suppression and the lack of a strong innate and adaptive immune responses. Functional genomic analyses of pure populations of PAMs freshly obtained from healthy pigs revealed that PRRS virus fails to elicit a significant (>2-fold) increase in the transcription of immune-related genes. The character of the innate immune response to a virus is thought to dictate the quality of the adaptive immune response that ensues. Given the key roles that the cytokines produced by cells of the innate immune system, play in the development of

Table 2

(a) Changes in chemokine transcript abundance in PRRSV-infected PAMs

Transcript	Function	RT-PCR	Normalized SAGE tag counts (per million tags)				
			Mock	6	12	16	24
CCL3 (MIP1α)	Chemotaxis	↓	191	21	3	0	21
CCL4 (MIP1β)	Chemotaxis	↓	419	68	25	19	16
CCL5 (RANTES)	Chemotaxis	nd	7	7	17	23	13
MIF	Inhibits macrophage migration	nd	8	1	25	35	32
CCL20 (MIP3α)	Chemotaxis	nd	196	78	14	19	11
CCL2 (MCP-1)	Chemotaxis	nd	1	1	1	4	31
CCL7 (MCP-3)	Chemotaxis	nd	0	0	4	1	22
CXCL2 (GROβ)	Chemotaxis	↓	1596	659	335	215	155
CXCL8 (AMCF-1/IL-8)*	Chemotaxis	6 hr ↑	353 8	807 172	370 121	202 55	449 17
CXCL5 (AMCF-2)	Chemotaxis	6 hr ↑	0	88	31	13	80

(b) Changes in cytokine transcript abundance in PRRSV-infected PAMs

Transcript	Function	RT-PCR	Normalized SAGE tag counts (per million tags)				
			Mock	6	12	16	24
IL-6	Acute phase response, T- and B-cell growth & differentiation	↓	No tag in transcript				
GM-CSF	Dendritic cell growth & differentiation	nd	4	3	1	0	4
TNF-α	Local inflammation, endothelial activation	nd	49	3	1	0	0
IL-1α	T-cell activation, macrophage activation	↓	152	31	23	16	40
Il-1β	T-cell activation, macrophage activation	↓	283	103	31	52	163

(c) Changes in immediate early-response transcript abundance in PRRSV-infected PAMs

Transcript	Function	RT-PCR	Normalized SAGE tag counts (per million tags)				
			Mock	6	12	16	24
AP-1	Transcription factor	nd	1	0	0	0	0
C-jun	Transcription factor for AP-1	nd	8	3	0	1	5
C-fos	Transactivating regulator of gene expression	nd	134	9	11	18	9
JunB	Transcription factor for AP-1	nd	101	6	11	17	33
JunD	Transcription factor for AP-1	nd	13	1	0	4	9
NFκB	Transcription factor	nd	2	1	3	0	0
IκBα	NFκB inhibitor	nd	205	72	42	11	26
IκKα	Dissociates inhibitor from NFκB	nd	4	7	3	2	1
A20	Inhibits activation of transcription factors	nd	11	12	6	1	4
TLR4	Activation of transcription factor NFκB	nd	24	22	14	4	15
Arginase*	Regulator of nitric oxide synthesis	6 hr ↑	227 37 1 14	915 131 88 84	169 21 17 31	141 18 4 5	103 24 1 3

* Alternative spliced transcripts.

adaptive immunity, clarification of the pathways responsible for modulating their generation during the initial innate immune response to PRRSV could have important implications in the development of effective vaccines against this major pathogen of swine. The results obtained in this project helped us understand that the unique character of the innate immune response to PRRS virus is likely to be influencing the quality of adaptive (acquired) immune response to this virus. Thus, the knowledge derived from this study will contribute to the elucidation of the molecular mechanisms controlling the development of adaptive immunity in swine to PRRS virus and will allow for the rational development of effective vaccines against this pathogen.

Previous work has demonstrated transcript abundance differences in 13 genes between noninfected and PRRSV-infected PAMs [33, 35, 45, 46]. More recently, Genini et al.

Table 3: Real-time PCR primer sequences.

Transcript	Sequence [1]
β2-microglobulin	GCAGTCAGACCTGTCTTTCAGCAA ATCTCTGTGATGCCGGTTAGTGGT
β-actin	AGATGTGGATCAGCAAGCAGGAGT AGCTAACAGTCCGCCTAGAAGCAT
A20	GATGCTGCCTTTGATGCTGGTCTT AAAGCACACAGAGACACTGCAAGC
Arginase	AAGAACGGAAGGACCAGCCTTGTA TCGTGGTTGTCAGTGGAGTGTTGA
BNIP3	TTCGCGTCTCCTGAATCACCTGTA AGTGCCTAACTCAAGGCTGCAGAT
CCL3 (MIP-1α)	AAACAGCCACTCTCTGGGACTCAT AAAGGTGTCTTCGGACCTCTTGGA
CCL4 (MIP-1β)	AAGCTTCCTCGCAACTTCGTGACT TCAGAGCAGCTCAGTTCAGTTCCA
CD163	TCTGTTGGCCTGTCTCATCGCATT TCAGGCAAGAATTCATCTCCCGGT
CXCL2	GACCGTGCAAGGAATTCACCTCAA CAGTTGGCACTGCTCTTGTTTAGC
CXCL5(AMCF2)	AGCCACCCTGAAGAATGGAAAGGA CTTCTGCTGAAGAACTGGGCGATT
CXCL8 (IL-8/AMCF1)	GCAGAACTTCGATGCCAGTGCATA TCTGTACAACCTTCTGCACCCACT
Cytochrome P450 3A29	TACCTACGATGGTCTGGCGCAAAT CGAACACGCCATGGATTTCCACAT
GRP78[2]	TGGCATTCTTCGAGTGACTGCTGA GTGTCAATGCGCTCCTTGAGCTTT
IL-1α	TTCAAATCAGCCGCCCATCCAAAG TGGTACATACGGCCTGTCAACACT
IL-1β	GAAATGGGAGCATCCAGCTGCAAA TTGCACGTTTCAAGGATGATGGGC
IL-6	ATGCTCTTCACCTCTCCGGACAAA TTCTGCCAGTACCTCCTTGCTGTT
MIF	TACTACGACATGAACGCGGCCAAT GCGCCATCTCCACACCGTTTATTT
Mx1	TTCGCACATCCTCCTGTGGTTAGT GCGTGCTTATCACAGCTTCTTGCT
PRRSV	CAACGGCAAGCAGCAGAAGAGAAA TGATCTTACCCAGCATCTGGCACA
RHIV	TTTGGACTCTGTTCTCAGGCAGGT AGACTTAAACCCGAGCCTCAGCAA

[1] First line of sequence is plus sense and second line is minus sense.
[2] Glucose responsive protein 78.

[47] profiled gene expression of PAMs infected in vitro with the European Lelystad strain of PRRSV, with the biological similarities but distinct serological properties from the North American VR-2332 isolate [48], over 12 h p.i. by utilizing an Affymetrix 24 K Porcine Chip array. In Genini's study, statistical analysis of variance (ANOVA) showed differential expression of 1409 genes. After applying a cut-off threshold based on a fold-change of 1.5 between infected and control PAMs, 148 genes were differentially expressed compared with the controls [47]. In our study, greater than 590 significant ($P < .01$) changes in transcript abundance levels were identified.

Analysis of this transcriptomics data in the context of gene ontology allows us to ascribe biological function to the differentiated transcript abundance dataset. The P-values and scores calculated in IPA act as starting points for further investigation and act as rough guides for identification of significant processes or pathways affected in the experiment. Note, however, that functions whose significance values exhibit little or no change from one time-point to another may be changing.

However, an important aspect of this study was not in what was altered, but rather what was not. Of particular interest was the apparent lack of any overt innate immune response in the PRRSV-infected PAMs. This was borne out by the lack of increased transcription of chemokine and cytokine genes that are commonly observed to increase in infection with other pathogens. These included CCL3, CCL4, TNFα, type 1 interferons, and a number of proinflammatory chemokines and cytokines.

In the initial stage of a PRRSV infection, the primary target cells of the virus are PAMs. It is as yet unknown what signals are necessary to call the immune system into action. The most likely candidates are cytokines and particularly those that initiate migration and activation of leukocytes. Sprenger et al. [49] have shown that influenza A virus selectively induces mononuclear leukocyte-attracting chemokines MIP-1, MCP-1, and RANTES and suppresses neutrophil-attracting chemokines IL-8 and GRO-α. In this study, we have shown that PPRSV does not activate an alveolar macrophage proinflammatory response while suppressing type 1 IFN production and apoptotic pathways. No activation of the PAMs was indicated by a decrease in IL-1α, IL-6, or IL-8. Knoetig et al. [50] has shown that IL-1 is released from CSFV-infected macrophages. IL-8 is, for example, an important chemo-attractant for immune cells, while IL-1 and IL-6 prime B- and T-cell responses against infected cells. The chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), RANTES, MCP1, MCP3, and MIP3 are important chemo-attractants and mediators of virus-induced inflammation *in vivo* [51]. Cytokine mRNAs have a short half-life after synthesis and the rapid reduction in mRNA levels a few hours after addition of virus suggests rapid intracellular cytokine mRNA degradation or suppressed transcription. Virus replication and protein synthesis has been shown to commence 10–15 h PI [52] (although SAGE showed an increase in viral RNAs at 6 hours PI), suggesting that any increase in cytokine mRNA levels after 15 h PI was due to the presence of replicating virus. Similarly, we showed no activation of NF-κB, indicating a lack of transcriptional activation of these genes. The transcription factor NF-κB is a central regulator of the transcript abundance of these proinflammatory genes and many viruses manipulate the NF-κB pathway, resulting in suppression of antiviral responses or prevention of apoptosis [53].

The finding of a sharp spike in transcription of the arginase gene at 6 hours PI may give a clue to the early events following infection that inhibit an innate immune response. Arginase competes with nitric oxide synthases (NOS) for the substrate arginine for the production of nitric oxide (NO). NO is an important early signal in many pathophysiologic

processes. Early inhibition of its production would have an impact on downstream events. Increased transcript abundance of arginase has been shown to modulate NO production in macrophages and impact the downstream immune response [54–56].

4. Conclusions

It is well established that many pathogens cause changes in expression of specific genes that act to protect the host and clear the infection. This type of response was not seen in these PRRSV-infected PAMs. Of particular interest was the minimal expression of genes that are involved in attracting other immune cells to the area of the infection. Additionally, there was no response by genes that cause inflammation. This is the first comprehensive study to show the inhibition of an immune response in PAMs by PRRSV. However, the results have also given us tantalizing clues to the mechanism(s) behind this inhibition. There are specific cellular proteins that control the expression of the protective genes and future studies of the genes, their transcript abundance, protein level, and protein function will enhance our understanding of the interaction of PRRSV with the porcine macrophage. Possible outcomes may include identification of virulence mechanisms, development of diagnostic assays and rational vaccine design to more effectively limit viral replication and spread.

5. Methods

5.1. Cells and Virus. Primary PAMs were isolated, cultured, and infected, as previously described [57]. Briefly, PAMs were harvested from three clinically healthy, PRRS-negative gilts 6–8 weeks of age. Animals were humanely euthanized, following animal care and use protocols, and PAMs were harvested under aseptic conditions. PAMs were tested by PCR for porcine circovirus and *Mycoplasma* spp [58, 59] and found to be free of both. Aliquots of PAMs were frozen and stored in liquid nitrogen. Typical yields were 10^8–10^9 PAMs with >95% viability. Immediately prior to use, PAMs were thawed and viability of PAMs was determined to be 85% to 90% by trypan blue dye exclusion. Viable PAMs were cultured at 37°C, 5% CO_2 in Dulbecco's Modified Eagles Media with 5% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA) and 1% antibiotic/antimyotic (Gibco-Invitrogen) for 2 hours. PRRSV strain VR-2332 [60] stock was propagated in MARC-145 cells [61] and stored frozen at −80°C until use.

5.2. Infection and RNA Isolation. PAMs isolated from three pigs were maintained separately. All three sets of PAMs were treated identically. After establishing PAMs in culture, cells were infected with PRRSV strain VR-2332. To achieve a near synchronous infection, flasks containing adherent PAMs were infected at a multiplicity of infection (MOI) of 10 in chilled media and incubated at 4°C for 1 hour to allow for virus binding, but not entry into the cell. Pre-warmed media was added and the cells placed at 37°C, 5% CO_2 until collected for RNA isolation. Total cellular RNA was prepared using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA), according to manufacturers instructions, from each PRRSV-infected PAMs flask at 0, 6, 12, 16 or 24 hours after infection. Total cellular RNA from mock-infected PAMs was collected at 0 and 24 hours.

5.3. SAGE Library Construction. SAGE libraries were constructed as described previously [62] using *Nla* III as the anchoring enzyme. Each library was made from pooled equimolar amounts of total RNA from each pig at each time point. The SAGE libraries provided the population means of the transcript abundance levels for each time point. SAGE clones were amplified and sequenced using a high-throughput sequencing pipeline with an ABI 3730 automated sequencer and ABI chemistry (Applied Biosystems Inc., Foster City, CA). The SAGE libraries with tag counts were submitted to GenBank GEO and have the accession number GSE10346.

The database of tags derived from the raw sequence data was analyzed to identity transcripts from which tags were derived as well as their relative abundance. Tag sequences were corrected for sequencing errors using R and sagenhaft [63]. The libraries were normalized to total tags. Relative abundance was calculated based upon the number of times a tag was represented in a given SAGE library [64]. Tags were mapped to transcripts and genes by exact regular expression matching to sequences in GenBank, Harvard Gene Index, and the Pig Expression Database (Japan) databases and parsed into a modified Identitag database [65]. Multidimensional statistical tests: Audic and Claverie pairwise test; the Fisher's exact test; Greller and Tobin test; the R test and pairwise and general Chi-square tests [66] were applied to determine which changes in tag abundance were significant. The Fisher's exact test was adequate for detecting differences in gene expression when dealing with pairwise comparisons. SAGE libraries were compared with each other to identify common or differential patterns of transcript abundance. Attention was given to those transcripts where transcript abundance changes may affect PAM function; particularly in regard to innate immunity, antigen presentation, and intra- and extracellular signaling.

5.4. Real-Time RT-PCR Validation. Validation of the results and corroboration of the altered transcript abundance levels were analyzed by real-time reverse transcription-PCR (RT-PCR) on the individual sample of 100 ng total RNA from each pig at each time point. Real-time RT-PCR was done in 25 μL reaction volumes using the SuperScript III Platinum SYBR green One Step qRT-PCR kit (Invitrogen, Carlsbad, CA) according to the supplier's specifications. The primer sets used for this analysis are shown in Table 1. All primers were used at 200 nM. PCR cycling conditions were 95°C for 15 minutes followed by 40 cycles of 94°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds using an Opticon 2 fluorescent thermocycler (BioRad, Inc., Hercules, CA). Final analysis of amplification products was done by melt curve where the PCR reactions were heated from 50 to

94°C at a rate of 0.5°C /second. Equal amplification kinetics of the target and the reference genes (β2-microglobulin) were confirmed by serial dilutions as described [67]. Quantification of levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method, which expresses mRNA in treated cells relative to mock infected cells after normalizing to β2-microglobulin (β2m) [68]. For real-time PCR and SAGE tag count comparisons, β2-microglobulin (β2m) served as the internal control where the number of SAGE tags for β2m derived from this library and the amplification curve from real-time PCR were considered equal.

Authors' Contributions

L. C. Miller: study conception, data collection and analysis, research design, manuscript writing. J. D. Neill: study conception, research design, SAGE library construction, manuscript production. G. P. Harhay: research design, SAGE database construction, data analysis, manuscript production. K. M. Lager: virus stocks, data collection, manuscript production. W. W. Laegreid: study conception, manuscript production. M. E. Kehrli: study conception, manuscript production. All authors read and approved the final manuscript.

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Cytokine Protein Expression Levels in Tracheobronchial Lymph Node Homogenates of Pigs Infected with Pseudorabies Virus[▽]

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Pseudorabies virus (PRV) is a neurotropic alphaherpesvirus that produces fatal encephalitis in newborn pigs, respiratory disorders in fattening pigs, and reproductive failure in sows. Following primary infection of the respiratory tract, PRV can develop into a systemic infection with dispersion of the virus via the lymphatic system that involves mononuclear cells in tracheobronchial lymph nodes (TBLNs). The objectives of the present study were to evaluate the pathogenesis and to determine the early immune cytokine profiles in TBLNs following experimental infection with a feral swine PRV isolate at 1, 3, 6, and 14 days postinfection (dpi). Forty healthy pigs were purchased from a PRV-negative herd. Twenty pigs received the Florida strain isolate (FS268) of feral swine PRV intranasally, and 20 uninfected controls received a sham inoculum. Compared to the levels in the controls, the levels of alpha interferon (IFN-α), interleukin-1β (IL-1β), IL-12, and IFN-γ were increased in TBLN homogenates from PRV-infected pigs at 1 dpi, whereas the IL-18 levels were decreased from 3 to 6 dpi. The protein levels of IL-4 and IL-10 did not differ between the controls and the PRV-infected pigs at any time point. Flow cytometric analysis of TBLN homogenates of PRV-infected pigs and the controls revealed increases in the percentages of B cells at 6 dpi, $CD4^+$ cells at 14 dpi, and CD25 expression in TBLN homogenates (in the total mononuclear fraction and on B cells) in the PRV-infected pigs. Collectively, these findings demonstrate that a feral PRV in commercial swine can modulate the host's early immune response to allow the virus to establish an infection.

Pseudorabies virus (PRV) is a neurotropic alphaherpesvirus that produces fatal encephalitis in newborn pigs, respiratory disorders in fattening pigs, and reproductive failure in sows. There are three classes of swine in the United States, as defined in the USDA Veterinary Services Pseudorabies Virus Program Standards: feral swine that are wild and free roaming, transitional swine that are considered owned by someone and that may enter the food chain, and commercial swine that are raised in an environment in which there is no contact with feral swine. Studies of feral swine populations in the United States have demonstrated that PRV is indigenous (7), and it is believed to spread primarily through a venereal route rather than the respiratory route typical in domestic herds (34, 36). In contrast to the trigeminal ganglion latency of PRV in domestic swine, feral swine typically show latent infection of the sacral ganglia (35). Viral strain differences may explain the difference in tropism, as a comparison study of virus strains originating from either domestic or feral pigs found the feral strains to be markedly attenuated (18). Since the eradication of PRV from the U.S. commercial swine herd, there have been rare case reports of transitional swine becoming infected with feral swine PRV isolates (1a, 1b). The continued expansion of the feral swine population in North America presents an emerging threat to the PRV-free status of the U.S. commercial swine herd.

In general, the recognition and the subsequent elimination of herpesvirus-infected cells involve three major immune effector mechanisms: antibody-dependent lysis, major histocompatibility complex (MHC) class I-restricted cytolytic T lymphocyte (CTL)-mediated lysis, and natural killer (NK) cell-mediated lysis. PRV virions contain several envelope glycoproteins important for interactions between the virion and the host cell and constitute major targets for the immune response of the infected host (reviewed in references 27 and 28). Glycoprotein D (gD) is required for entry of the virus into host cells and is probably the most potent inducer of neutralizing antibodies (25). gC is the major attachment protein of PRV and a target of neutralizing antibodies (2), CTLs (31), and effector/memory $CD4^+$ $CD8^+$ T cells (21, 48). gB is required for entry and cell-to-cell spread and is highly conserved among herpesviruses. It represents a major constituent of the viral envelope and has been shown to induce complement-dependent and -independent neutralizing antibodies and helper T cells (21). gE is important for the neurovirulence of PRV and plays a role in transneuronal spread, although it is nonessential for viral replication in cell culture (29).

After acute infection, pigs develop specific antibody and T-cell responses against PRV (7, 8, 21, 46). Besides the induction of type I interferons (IFNs), gamma interferon (IFN-γ) production by peripheral blood mononuclear cells (PBMCs) is an indicator of antigen-specific cell-mediated immunity in swine with PRV and other viral infections (26, 40, 46, 47). IFNs not only play an important role as antiviral agents but also are interconnected regulators in the innate and adaptive immune responses (4). Administration of potent IFN-γ-eliciting cytokines, such as interleukin-12 (IL-12) and IL-18, with PRV antigens promotes the antigen-specific induction of IFN-γ and

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IL-12 yet suppresses PRV-specific antibody production and increases the susceptibility of pigs to subsequent challenge with virulent PRV (44).

In vitro studies have demonstrated that porcine IFN-α, IFN-β, and IFN-γ act synergistically to inhibit the replication of PRV (43). PRV avoids innate defenses via the active suppression of type I IFNs and downstream IFN-β-stimulated genes (6). Numerous studies have been performed to evaluate the protective efficacy of PRV vaccines (27, 28), and the association of vaccine efficacy with balanced IFN-γ and IL-4 memory responses has been described (3, 12, 17). Using the murine model, Bianchi et al. reported that both IFN-γ- and $CD4^+$-producing T cells play important roles in conferring protection against a lethal PRV infection (3). Studies evaluating the kinetics of cytokine production by leukocytes in the lung-draining lymph nodes of PRV-infected pigs, however, have not yet been reported, especially for feral PRV isolates. Accordingly, the goals of the present study were 2-fold: first, to evaluate the pathogenic effects of a feral swine PRV isolate and, second, to characterize the production of cytokines by cells of the tracheobronchial lymph node (TBLN) that drains infected lungs, one of the target organs in PRV-infected pigs.

MATERIALS AND METHODS

Virus, animals, and experimental design. Forty conventionally raised 4- to 5-week-old pigs, free of clinical disease, were purchased and tested negative for porcine circovirus 2, swine influenza virus, porcine respiratory and reproductive virus, and PRV. The pigs were allotted to two equal treatment groups, and each group was housed in an isolation room for about 1 week prior to the beginning of the experiment. On 0 days postinoculation (dpi), the pigs received an intranasal challenge with 3 ml of a sham inoculum ($n = 20$), prepared from swine testicle (ST) cell culture, or an inoculum ($n = 20$) of the Florida strain isolate (FS268) of feral swine PRV (34) at 1×10^6 50% cell culture infectious doses ($CCID_{50}$s) per pig. Five pigs from each group were euthanized and necropsied on 1, 3, 6, and 14 dpi.

Clinical signs, gross pathology, and pulmonary histopathology. Pigs were monitored daily for clinical signs, including rectal temperature and a clinical respiratory score, as described previously (19). Blood samples were collected on 0, 1, 3, 6, and 14 dpi. The rectal temperatures of pigs intended for necropsy on 14 dpi were recorded daily. Pig weights were recorded on 0 dpi and at necropsy. During the postmortem examination, gross lung lesions were evaluated by visual inspection and each lung lobe was scored to reflect the approximate volume or percentage of the lung tissue affected (19). Bronchoalveolar lavage fluid (BALF) and TBLNs were collected. The BALF was cultured for the presence of bacterial pathogens. Two milliliters of BALF was frozen at −80°C. A section of the TBLN was homogenized with a pellet pestle for flow cytometry analysis. Another section of TBLN was homogenized in tissue lysis buffer for cytokine analysis (16).

Antibody detection. For the PRV group, sera collected at 0 and 14 dpi were tested for antibody to the gB antigen of PRV by using the commercial HerdChek PRV gB blocking enzyme-linked immunosorbent assay (ELISA; IDEXX, Westbrook, ME), according to the manufacturer's directions. Test values were reported as the sample optical density value as a ratio of the positive control provided in the kit (*S*/*N* ratio). The cutoff *S*/*N* values for the PRV gB assay were as follows: negative, greater than 0.70; positive, less than or equal to 0.60; and suspect, greater than 0.60 and less than or equal to 0.70.

Virus detection. On 0, 1, 3, 6, and 14 dpi, all serum and BALF samples were tested for virus. Real-time PCR assays for the specific detection of the gB and gE genes of PRV were performed by the use of TaqMan chemistry, as described previously (24). The analytical sensitivity of the assays was approximately 0.1 PFU per reaction (24).

Virus isolation for the detection of PRV in the samples collected was performed with an ST cell line, as described previously (32). Briefly, each well of 24-well plates with 80 to 90% confluent monolayers of ST cells was inoculated with 100 μl of diluted medium in which the nasal swab specimen, BALF, or tissue homogenate had been placed. The inoculated cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 h and monitored daily for the development of cytopathic effects.

Cytokine assays. After removal, approximately 1 g of TBLN was homogenized in 750 μl of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl, and 1 mM MgCl, pH 7.40, with a tissue homogenizer (Biospec Products, Bartlesville, OK). The homogenates were incubated on ice for 30 min and then centrifuged at 2,500 rpm for 10 min (16). The supernatants were collected, passed through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, MI), and then stored at −20°C prior to the assessment of cytokine levels.

The total protein concentration in the supernatants was measured by use of a commercially available bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's directions, on a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Tumor necrosis factor alpha (TNF-α), IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12 protein levels were measured by use of the respective porcine DuoSet ELISA development kits (R&D Systems Inc., Minneapolis, MN), according to the manufacturer's protocol. IL-18 and IFN-γ protein levels were measured by use of the respective porcine ELISA kits (Biosource International, Inc., Camarillo, CA).

IFN-α protein was measured by a porcine IFN-α-specific ELISA with monoclonal antibody (MAb) F17 and MAb K9 (R&D Systems Inc.), as described previously (10). MAb K9 was conjugated with horseradish peroxidase (HRP) by use of a peroxidase labeling kit (Roche Molecular Biochemical, Indianapolis, IN). Immulon 2 flat-bottomed 96-well plates (Fisher Scientific, Houston, TX) were coated overnight at 4°C with MAb F17 at a concentration of 0.3 μg/well in coating buffer (100 mM carbonate buffer, pH 9.6; Sigma Inc., St. Louis, MO). After the wells were blocked with 1% nonfat dried milk and 0.05% Tween 20 in phosphate-buffered saline (PBS) for 1 h at 37°C, the plates were washed five times with 0.05% Tween 20 in PBS. Samples (50 μl) containing 50 μl of 1% nonfat dried milk and 0.05% Tween 20 in PBS were added to each well, and the plates were incubated for 2 h at 37°C. Following five washes, 100 μl of peroxidase-conjugated MAb K9 was added to each well. After 1 h of incubation at 37°C and five washes, 100 μl of tetramethylbenzidine substrate solution (KPL Inc., Gaithersburg, MD) was added to each well. After 30 min, the reaction was stopped with tetramethylbenzidine stop solution (KPL Inc.) and the optical density at 450 nm was measured with an ELISA plate reader. Quantified recombinant porcine IFN-α (rIFN-α; R&D Systems Inc.) was used as a standard, and the IFN-α concentrations were calculated on the basis of the concentrations on a standard curve. One unit per milliliter of rIFN-α was equivalent to 26 pg/ml.

Flow cytometry analysis. Cells from the TBLNs were phenotyped by flow cytometry, as described previously (38). Commercially available antibodies to the following markers were used: CD3 (2B3C), CD25 (PGBL25A), MHC class II (TH16B), B cell (BB6-11C9), CD4 (PT90A), CD8 (76-2-11), and γ/δ T-cell (PGBL22A) (VMRD Inc., Pullman, WA). Secondary antibodies (VMRD) targeted to murine primary antibodies included IgG1-fluorescein isothiocyanate (FITC), IgG2a-FITC, IgG2b-phycoerythrin (PE), IgG1-PE, IgG2a-PE, and IgM-PE. Data were acquired with CellQuest Pro software (BD Biosciences, San Jose, CA) on an LSR II flow cytometer (Becton Dickinson) and were analyzed with FlowJo software (TreeStar, Ashland, OR).

Statistical analysis. Differences in virus titers, body temperatures, and respiratory scores between the study groups were evaluated by analysis of variance (ANOVA). Two-way ANOVA and posttests were used for comparison of the cytokine titers. Spearman rank correlation tests were used to compare the individual virus titers, cytokine titers, body temperatures, and respiratory scores with the other parameters.

RESULTS

Clinical evaluation and gross pathology. PRV-inoculated pigs developed a mild clinical disease typical of PRV infection, as reflected by fever (Fig. 1A), anorexia, sneezing, increased respiration rates, and dyspnea that began at 2 to 3 dpi and that resolved by 14 dpi. Control animals did not display clinical signs or gross lung lesions consistent with PRV infection. Pigs inoculated with PRV had an increased ($P < 0.05$) rectal temperature from 1 to 6 dpi (Fig. 1A). No significant differences ($P > 0.05$) in daily weight gain (Fig. 1B) were found between pigs inoculated with PRV (average, 0.6 kg/day) and control pigs (average, 0.6 kg/day). Gross lung lesions in PRV-inoculated pigs mainly consisted of hilar multifocal areas of purple to red areas of consolidation. The lesions were most pronounced at 3 and 6 dpi (Fig. 1C). Bacteria typically associated

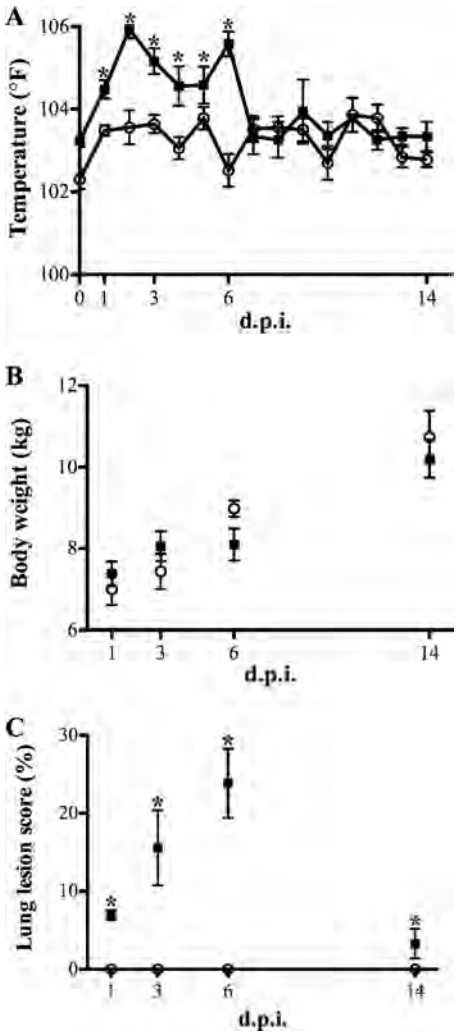

FIG. 1. Clinical evaluation and gross pathology. (A) Rectal temperature for control (○) and PRV-inoculated (■) pigs measured each day out to 14 dpi; (B) body weight measured prior to necropsy for control (○) and PRV-inoculated (■) pigs; (C) macroscopic lesion score for lungs at necropsy for control (○) and PRV-inoculated (■) pigs. Data are expressed as means ± standard errors ($n = 5$). *, $P < 0.05$.

with porcine respiratory disease were not isolated from the BALF of any of the pigs. Pig serum was negative for PRV gB antibody at 0 dpi (mean *S/N* ratio, 1.22 ± 0.03) and positive at 14 dpi (mean *S/N* ratio, 0.26 ± 0.2).

Quantitative PCR for virus nucleic acid and virus isolation. Table 1 summarizes the results of the virus detection assays. After intranasal infection, PRV was detected in the tonsil, lungs, BALF, and nasal swab of the inoculated pigs from 1 to 6 dpi; however, PRV was not detected in the brain, spleen, or sacral ganglia.

Cytokine assays. Cytokine protein level measurements were normalized to the total amount of protein per gram of TBLN. The ex vivo protein levels of the proinflammatory cytokines IFN-α and IL-1β were increased ($P = 0.06$ and $P < 0.05$, respectively) in the TBLN homogenates of PRV-inoculated pigs compared with those in the control pigs at 1 dpi (Fig. 2A and B), whereas the IL-6, IL-8, and TNF-α levels did not differ ($P > 0.05$) between the treatment groups at any time point. As with IFN-α and IL-1β, at 1 dpi the levels of the Th1 cytokines IL-12 and IFN-γ were increased ($P < 0.05$) in the PRV-inoculated pigs compared with the levels in the control pigs (Fig. 2C and D). In contrast, at 3 and 6 dpi the IL-18 level in the PRV-inoculated pigs was decreased ($P < 0.05$) compared with that in the control pigs (Fig. 2E). At 6 dpi, the IL-12 level in the PRV-inoculated pigs was also decreased ($P < 0.05$) compared with the level in the control pigs (Fig. 2C). Protein levels of the Th2/regulatory T cell cytokines IL-4 and IL-10 did not differ between the treatment groups at any time point (Fig. 2F and G).

Flow cytometry. Flow cytometric analysis of TBLN homogenates revealed increases ($P < 0.05$) in B-cell percentages (Fig. 3A) and a concomitant decrease in $CD4^+$ cells at 6 dpi in PRV-infected pigs compared with the results for the control pigs (Fig. 3B). By 14 dpi, the percentages of $CD4^+$ cells were increased ($P < 0.05$) in PRV-infected pigs compared with the percentages in the control pigs (33.4% ± 1.2% and 26.0% ± 2.5%, respectively). The mean fluorescence intensity of CD25 was increased ($P < 0.05$) both in total TBLN homogenates (Fig. 3C) and on B cells in TBLN homogenates (Fig. 3D) at 14 dpi in PRV-infected pigs compared with the mean fluorescence intensity for the control pigs. Differences ($P > 0.05$) between treatment groups in the numbers of B cells and $CD4^+$ cells were not detected at 1 and 3 dpi. Differences ($P > 0.05$) between treatment groups in the numbers of $CD4^+$ $CD8^+$, γδ T-cell receptor-positive, $CD8^+$, $CD3^+$, or MHC class II-positive cells were not detected.

DISCUSSION

Feral swine PRV isolate FS268 was pathogenic in this study, inducing fever, anorexia, listlessness, and dyspnea following

TABLE 1. Distribution of PRV in tissues of intranasally inoculated pigs[a]

Tissue sampled	No. of pigs positive for PRV by the indicated assay/total no. of pigs tested on the following dpi:							
	1		3		6		14	
	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI
Brain	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Sacral ganglion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonsil	3/5	4/5	4/5	5/5	3/5	2/5	0/5	0/5
Lung	5/5	5/5	4/5	4/5	4/5	1/5	1/5	0/5
BALF	5/5	5/5	5/5	4/5	4/5	0/5	2/5	0/5
Nasal swab	4/5	5/5	5/5	5/5	5/5	5/5	0/5	0/5

[a] No PRV was detected in the tissue from control animals (data not shown). RT-PCR, reverse transcription-PCR; VI, virus isolation assay.

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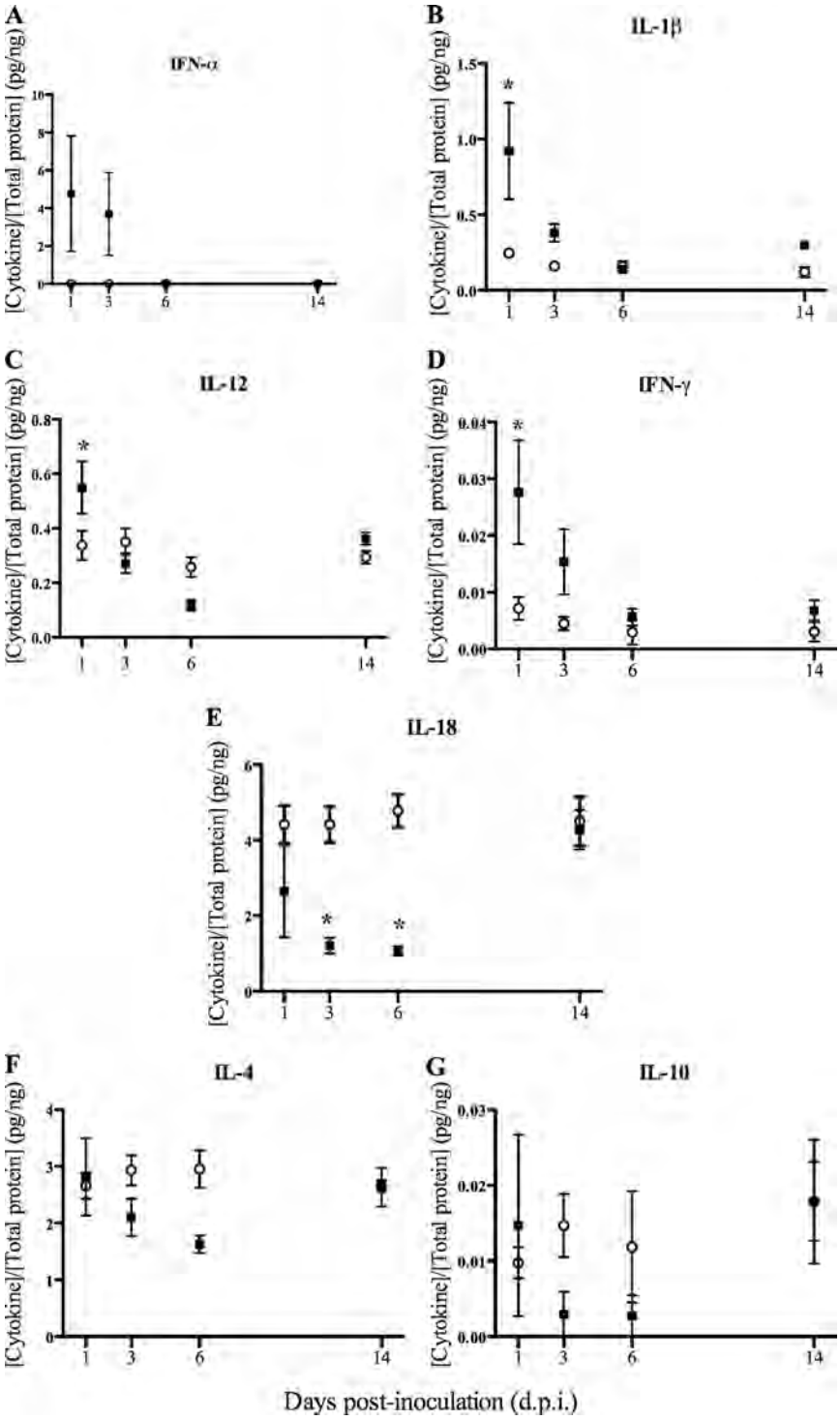

FIG. 2. Cytokine concentration in homogenates of TBLNs from pigs inoculated with PRV (■) or controls (○) normalized to the total protein concentration per gram of TBLN. Due to differences in the concentrations of the cytokines, the graphs use different scales. Means and standard errors of the means ($n = 5$) are shown. *, significantly different value than that for the control ($P < 0.05$).

experimental infection of young pigs. The presence of clinical signs was related to the detection of PRV in tissues associated with the respiratory tract. Likewise, the lack of central nervous system (CNS) clinical signs may be related to the absence of detectable virus in CNS tissues. The results of this study indicate that while isolate FS268 may induce moderate to severe pneumonia, it may have a limited effect on the CNS, supporting the assumptions of others that feral swine PRV isolates may have a phenotype different from that of isolates from commercial swine. Few studies evaluating the kinetics of cyto-

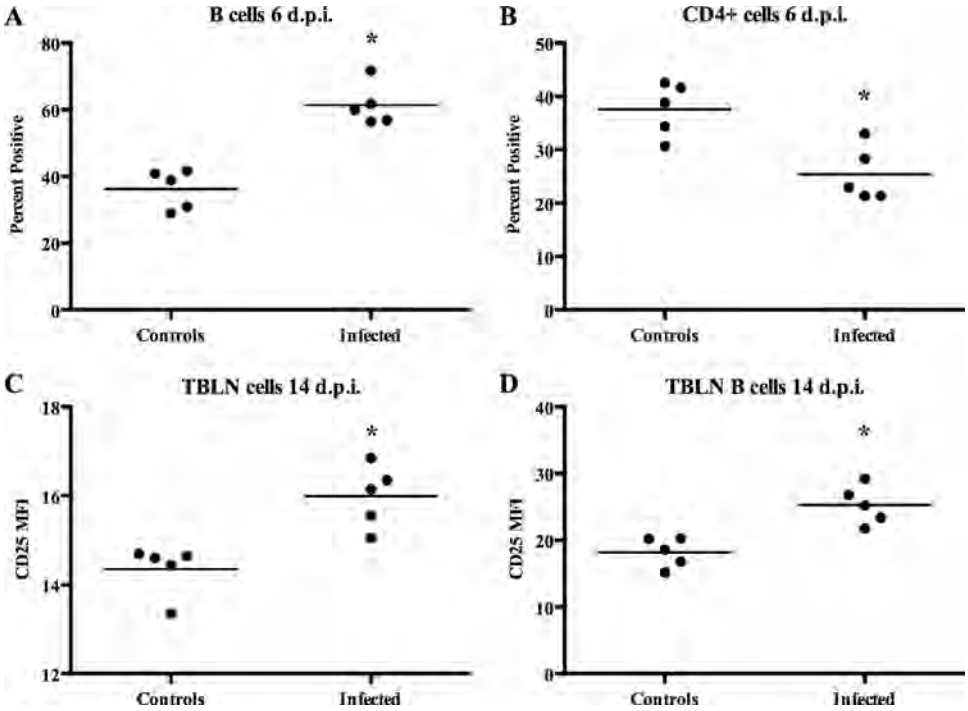

FIG. 3. Flow cytometric analysis of homogenates of TBLNs from pigs inoculated with PRV or controls. (A) B-cell percentages; (B) $CD4^+$-cell percentages; (C) mean fluorescence intensity (MFI) of CD25 for total TBLN homogenates; (D) mean fluorescence intensity of B cells in TBLN homogenates. *, significant difference ($P < 0.05$) from the values for the controls.

kine production in PRV-infected pigs have been reported (5, 7, 14, 21, 33, 42, 48). Previous studies used PBMCs *in vitro* or from pigs infected with attenuated (gE- or thymidine kinase-deleted) laboratory or wild-type PRV strains.

During the 14-day course of this study, the infected pigs began to recover clinically, and this was reflected in a variety of cellular and cytokine changes in the TBLNs. Overall, an early increase in proinflammatory cytokine levels was elicited by PRV in TBLNs, leading to an increase in the Th1 (IL-12 and IFN-γ) responses concurrently with increased percentages of B cells at 6 dpi and unchanged Th2 (IL-4 and IL-10) responses. It has previously been observed in PRV (domestic strain)-primed pigs that upon PRV reexposure of PBMCs *in vitro*, there is a significantly enhanced transcription of Th1-type cytokines (IL-2 and IFN-γ) but not of Th2-type cytokines (IL-4 and IL-10), but in naïve pigs there was no increased transcription of cytokines (14). The activation of B cells, as evidenced by the upregulation of CD25 (IL-2 receptor α chain marker), and their proliferation and expansion (likely with the support of Th cells) were observed at 14 dpi in our study. A significant decrease in the IL-18 response early following PRV infection could be a virulence mechanism and could be related to the persistence and latency of PRV, given that the IL-18-interacting Th1 cytokine (IL-12 and IFN-γ) responses were not sustained. IL-18 expression can be activated by Toll-like receptor signaling (1) or can be induced by IFN-γ via interferon consensus sequence-binding protein and activator protein 1 pathways (20), by inflammasome activation (39), or by autocrine signaling through the NF-κB pathway (15). In the presence of IL-12, IL-18 induces strong Th1-type immune responses (9, 23, 30, 41), primarily through the induction of IFN-γ expression by T cells and natural killer cells (11, 45). Impairment of IL-18 expression, which is involved in the induction of antiviral cytokines, could represent a strategy that the virus uses to evade the immune response of the host.

A B-cell response is induced after PRV inoculation but alone is not a protective response (7). The proliferation of T cells after restimulation with PRV suggests that T cells are involved in protective immunity (21). These cells may produce IFN-γ and TNF-α (13). IFN-γ can be involved in the prevention of PRV replication either by a direct local antiviral effect or, indirectly, by the induction of proinflammatory cytokines, or by a combination of both mechanisms (17, 37). IFN-γ and TNF-α can also activate macrophages and natural killer cells, which are involved in the clearance of PRV (17). It has previously been demonstrated that $CD8^+$ T cells lysed PRV-infected cells and that $CD2^+$ $CD4^-$ and $CD8^-$ or $CD8^{dull+}$ T lymphocytes (i.e., NK cells) lysed PRV-infected target cells (22, 48). More cytolytic cells may be found at specific sites in tissues involved in the infection process, such as mucosal tissues, tonsils, and draining lymph nodes (7). Lymphocytes that proliferate after pigs are inoculated with PRV probably migrate to the site of infection. This possibility is supported by the observation of a very localized influx of various T-lymphocyte subsets to the site of PRV infection, which was restricted to specific areas in the draining lymphoid tissues (5).

A change of lymphocyte subset frequency in TBLNs can be caused by an expansion or depletion of specific subsets after the inoculation of PRV. Our results showed that the proportion of various lymphocyte subsets in TBLNs changed after inoculation (Fig. 3C and D). This finding is in accordance with the observations of leukopenia after challenge with PRV and

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lower percentages of total T and B lymphocytes (33, 42) and is in contrast to the finding that the lymphocyte subset proportion did not change after inoculation of an avirulent strain of PRV that induced protective immunity (7).

In conclusion, the TBLNs from PRV-infected pigs presented an altered cytokine protein expression profile. It was evident that the key cytokines responsible for driving the immune responses (IFN-α, IL-1β, IL-12, IL-18, and IFN-γ) showed changes in their levels of protein expression in the TBLNs of diseased pigs. These changes suggest a lack of induction of the IL-4 and IL-10 responses and a decrease in the IL-18 response. To our knowledge, this is the first *in vivo* study of the cytokine response to a feral pseudorabies virus strain in the TBLNs of pigs. Moreover, the feral swine strain of PRV used in this study caused mild clinical disease during the acute phase that included fever and lung lesions, which are associated with an inflammatory cytokine response. These resolved by 14 dpi, and there was an increase in the B-cell percentages in association with the induction of immune cytokines in the TBLNs. The presence of PRV-specific antibodies in serum at 14 dpi was consistent with the observation of increased B-cell percentages in TBLN homogenates.

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General Interest

Response to Questions Posed by the Food Safety and Inspection Service Regarding Determination of the Most Appropriate Technologies for the Food Safety and Inspection Service To Adopt in Performing Routine and Baseline Microbiological Analyses†‡

ADOPTED 20 MARCH 2009, WASHINGTON, DC
NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS

NACMCF Executive Secretariat, U.S. Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, Room 333 Aerospace Center, 1400 Independence Avenue S.W., Washington, DC 20250-3700, USA*

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EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF or Committee) reviewed available and developing detection technologies that the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) could evaluate for use in routine and baseline microbiological analyses. The NACMCF determined that the recommendation of any new technology for use by the FSIS must be presented in an appropriate context to have applicable meaning and utility. The context agreed upon was the application of a new technology as a fully validated microbiological testing method ready for implementation. The method, in turn, must be rooted in the broader public health goals of the FSIS, and further defined by the microbiological testing objectives as applied to an FSIS program activity. The NACMCF provided background information on the role of testing in the protection of the food supply, particularly by the Federal regulatory system. General considerations for the application of various microbiological testing methods to food safety were reviewed, followed by a description of new and emerging technologies, including a discussion on critical performance criteria when selecting, evaluating, and validating new methods that incorporate these technologies. The advantages and disadvantages of potential emerging methods that employ new technologies are presented in a manner that is relevant to the regulatory ''gold standard'' of culture-based testing. Finally, an outline of a systematic process to identify and evaluate new methods was developed for the FSIS to consider when adopting a new method. The Committee then identified barriers and research gaps which should be addressed as the FSIS adopts new methods to enhance public health. Major recommendations to address barriers and research gaps included: continued articulation of public health goals and testing objectives; sharing of methods and promotion of harmonization across Federal and state agencies; assessing methods development needs and providing a structure and process for method evaluation and implementation; strengthening of the FSIS's method development capabilities; increase efforts to integrate pre-analytical sample processing with advanced detection

technologies; review the requirement of a viable microbial isolate; give priority to enumeration of pathogens by using real-time methods; consider charging the NACMCF to examine statistical considerations relating to microbiological sampling and testing; and consider charging the NACMCF to review new genotyping and subtyping technologies.

1. INTRODUCTION: STATEMENT OF CHARGE TO NACMCF AND THE RATIONALE FOR THE APPROACH TO ADDRESS THE CHARGE

1.1. Charge to the Committee

Determination of the Most Appropriate Technologies for the FSIS to Adopt in Performing Routine and Baseline Microbiological Analyses

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) should provide guidance to assist with the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS or Agency) goal of moving into the next generation of microbiological testing methods. To do so, NACMCF should review the current status of molecular methods, including genotyping assays, nanotechnology, and other available or evolving technologies for potential applicability to the FSIS's microbial analysis and explore their roles for incorporation into the FSIS's microbiological testing programs at both the laboratory and in-plant level.

The Agency suggested that the charge might best be approached by NACMCF in two stages. The first would focus on **laboratory methods** for pathogen detection, and the second on **in-plant testing** to reliably assess process control. Analyses for use in the FSIS laboratories versus within plants are likely to require different technologies. Analyses carried out in the FSIS laboratories will be used for baseline monitoring of national microbial trends and regulatory sampling. In-plant sampling may primarily help in assessing process control and real-time monitoring of plant performance.

The FSIS requested the NACMCF to examine the merits of available technologies for application to the FSIS's microbial testing with a focus on:

- Selectivity and sensitivity
- Adaptability to various matrices (including foods, the processing environment, and human clinical samples)
- Scope of analyses (including species identification, serotype equivalence, antibiotic resistance, pulsed-field gel electrophoresis (PFGE) equivalence, and additional indicators of microbial hazards, such as virulence factors)
- Enumeration
- Data acquisition and transfer
- Speed
- Ability to be effectively incorporated into the FSIS methods
- Cost and resource efficiency

Charge Questions:

1. What are the most appropriate technologies the FSIS should consider for improved microbiological analyses? What are the most promising methods that could replace or complement those currently used at the FSIS? What are the important parameters to be considered in determining the suitability of a method for a particular application (such as laboratory analyses for pathogens versus in-plant testing for process control, or routine versus baseline testing, and enumeration of pathogens and indicators)?
2. What are the advantages and disadvantages of these newer technologies/methods? When selecting newer technologies/methods consider the FSIS approach of reliance on culture-confirmed positives for target organisms in the context of method correlation, substitution, and degree of confidence. For instance, if the technology does not measure or correlate with viable cell presence, can reasonable decisions be made about the safety of the product?
3. When adopting new technologies and testing platforms, what considerations must be made regarding sampling protocols? How does sampling (size, site, rinse, excision) impact assay sensitivity, specificity, and limit of detection (LOD)? Are there any practical ways (concentration technologies, etc.) that could be adopted to compensate for potential loss in specificity, sensitivity, and detection limit requirements for microbiological targets?
4. Consider specifically the accuracy, applicability, and validation of an assay capable of detecting thousands of single-nucleotide polymorphisms (SNPs) in a single reaction. Would such an assay be timely, cost-effective, and capable of screening specimens to monitor process control? Would it be capable of differentiating multiple microbial species in a single sample? Could it have application for differentiating bacterial subspecies (particularly relevant for salmonellae, which are currently characterized by serotype), or detecting antibiotic resistance genes and virulence factors? Determine the suitability of incorporating SNPs in meeting the current and future testing needs of the FSIS.
5. When selecting a new technology, what factors should be considered, such that the data generated would be useful in an expanded manner to include attribution/risk profiles and models for human illnesses?
6. What issues will need to be considered to make newer and promising technologies a reality in the FSIS's future testing for pathogens and indicator organisms? For technologies that may be useful in the future, identify research gaps that need to be addressed prior to implementation.

1.2. Public Health Focus

Foodborne infections cause an estimated 76 million acute illnesses and 5,000 deaths each year in the United States *(84)*. These infections are the result of the contamination of food with a variety of disease-causing bacteria, viruses, and parasites that can occur as food moves from the farm to the consumer. The USDA's Economic Research Service (ERS) estimates that illnesses caused by Shiga toxin–producing *Escherichia coli* (*E. coli*), *Salmo-*

TABLE 1. *Healthy People 2010 objectives*[a] *(152)*

Pathogen	1997 baseline infections	2010 target
Campylobacter	24.6	12.3
Escherichia coli O157:H7	2.1	1.0
Listeria monocytogenes	0.5	0.24[b]
Salmonella	13.7	6.8

[a] Laboratory-confirmed cases per 100,000 humans (Food Net).
[b] Changed to year 2005 by Executive Order (President Clinton).

nella, *Campylobacter,* and *Listeria monocytogenes* (*L. monocytogenes*) result in $6.9 billion in medical costs and lost productivity each year in the United States *(26)*. The Department of Health and Human Services, Food and Drug Administration (DHHS-FDA) further estimates that 2% to 3% of foodborne illnesses result in secondary long-term health consequences *(71)*. With the globalization of the food supply and emerging foodborne pathogens, foodborne illness is clearly a serious public health issue that requires continued attention.

Recognizing this threat to the public health, the U.S. regulatory agencies charged with the oversight of food safety have evolved from a command-and-control (and largely visual) to an increasingly science-based, data-driven inspection approach that shifts significant responsibility for ensuring the safety of domestic and imported food products to the food industry. In 1996, the FSIS adopted hazard analysis and critical control points (HACCP), a proactive, preventive system of process control *(144)*. Microbiological testing plays a critical role in enhancing and verifying HACCP systems. For example, during food production and processing, microbiological testing can be used to continually improve HACCP systems, reducing the likelihood of pathogen contamination, and in so doing, enhance public health. Although end-product testing cannot ensure the safety of food products, microbiological testing data are also pivotal in making policy decisions, guiding compliance and enforcement actions, and developing risk assessments. This is not to say that microbiological testing methods are perfect; in fact, to assure appropriate use, microbiological testing must be accompanied by appropriate sampling techniques which are statistically valid. Taken together, microbiological detection methods employed by regulatory agencies must be robust, dependable, and defensible.

Traditionally, the FSIS has set public health-based performance goals to assure that the products under their regulatory jurisdiction have a minimal impact on the overall burden of foodborne illness. These goals are based on the Healthy People 2010 (HP 2010) objectives in Table 1 *(152)* and estimates by the DHHS Centers for Disease Control and Prevention (CDC) *(84)*. Specifically, the FSIS used the HP 2010 goals to establish public health-based performance goals for three pathogen/product pairs: *E. coli* O157:H7 in not-ready-to-eat (NRTE) ground beef products, *L. monocytogenes* in ready-to-eat (RTE) meat and poultry products, and *Salmonella* in NRTE broiler carcasses. Regarding *Campylobacter*, the FSIS is in the process of analyzing the results of a year-long baseline study for broiler carcasses, and recently initiated a similar study for turkey carcasses. The FSIS expects to establish a quantitative standard for these species in the near future.

The impact of the FSIS regulatory activities on the HP 2010 goals cannot be measured directly, in large part because of the absence of reliable and detailed foodborne illness attribution data (data which are used to allocate the burden of foodborne illnesses to specific commodities). In recent years FoodNet data have demonstrated that the incidence of reported laboratory-confirmed foodborne illnesses has remained relatively unchanged. Furthermore, the Office of the Inspector General *(148)* has stated that the FSIS must develop goals, objectives, and methods in support of an effective microbial testing program. Therefore, NACMCF recommends that as a first step, the FSIS clearly articulate measurable public health goals and demonstrate how those goals advance the Agency's public health mission to reduce the burden of foodborne illness attributable to the FSIS-regulated food products. Once the FSIS has articulated its public health goals, the FSIS must clearly define its microbiological testing objectives and how they address these goals. Microbiological methods that employ any new technology must then fulfill the necessary test criteria that clearly support the FSIS testing objectives and public health goals.

1.3. Committee's Approach to Answering the Charge

Upon reviewing the language in the charge (the title, the preamble, and the charge questions), the NACMCF determined the need to establish a context for the use of the terms ''technology or new technology'' and ''microbiological method or testing or analysis'' and then to maintain this context throughout the document for clarity. At this early deliberative juncture, the Committee also believed strongly that the FSIS must adopt a longer-term vision which includes development of a process for applying appropriate new microbiological technologies as part of a broad food safety and public health strategy. Thus, the Committee's approach for addressing the charge, that both delineates the terms mentioned above and puts a public health focus front and center, emerged as:

> The recommendation of any new technology for use by the FSIS must be presented in an appropriate context to have applicable meaning and utility. The context agreed upon was the application of a new technology as a fully validated microbiological testing method ready for implementation. The method must be rooted in the broader public health goals of the FSIS, and further defined by the microbiological testing objectives as applied to an FSIS program activity.

The NACMCF's full charge and the explicit explanation of the need for public health to be the main driver for how NACMCF addressed the charge (and, in turn, how NACMCF recommended that the FSIS should develop microbiological testing as part of a food safety strategic plan) is presented above in Sections 1.1 and 1.2, respectively. This Section (1.3) continues below with a

description of how the document is further structured to address the charge.

In reviewing the charge questions, the NACMCF determined that there was substantial overlap and oftentimes the questions were too prescriptive, making it difficult to address the longer term vision of the Agency. Therefore, the Committee chose to address the charge in a holistic manner, rather than answering the specific charge questions independently. The need for this approach became more apparent as the Committee began its deliberations and recognized that applying new technologies to improve microbiological methods is a dynamic process. Moreover, new technologies emerge at a rapid rate and certain ones may not be practical for use in a food safety testing laboratory, because of expense, operator training needs, ability to transfer into a high throughput testing format, and sample preparation and matrix interference concerns.

Because of these issues, the Committee determined that the best way to structure the document was first to provide a ''Background'' that discussed the role of testing in the protection of the safety of the food supply (Section 2), particularly in the context of the Federal regulatory system. Next, a review of the general considerations for the application of various microbiological testing methods to food safety is provided (Section 3). This is followed by a description of new and emerging technologies, including a discussion of the critical performance criteria which need to be considered when selecting, evaluating, and validating new methods that incorporate these technologies (Section 4). A discussion of the advantages and disadvantages of potential emerging methods that employ new technologies follows, which covers multiple issues (e.g., rapid, on-site analysis; discrimination between viable and non-viable cells; the need for an isolate; qualitative versus quantitative results; and multianalyte considerations) in a manner that is relevant to the regulatory ''gold standard'' of culture-based testing (Section 5). Finally, NACMCF described the critical elements that need to be considered as the FSIS seeks to apply a new method that takes advantage of new technologies for an intended food safety and public health-related programmatic purpose (Section 6).

One caveat to the NACMCF approach to address the charge is that the discussion on SNP technology (Question 4) was limited largely to addressing the focus given in the charge preamble ''on **laboratory methods** for pathogen detection'' and ''on **in-plant testing** to reliably assess process control.'' Therefore, while the Background (Section 2) describes the present status of several methods, including those based on SNP technology, the discussion is confined to the detection function and does not address the use of these technologies in genotyping and subtyping applications. This is not to say that their use in genotyping is not promising, but rather that the Committee believed that this topic was worthy of a wholly separate charge to NACMCF. The FSIS did brief the Committee on an extensively researched internal ''white paper'' on new subtyping technologies that could supplement and/or potentially replace PFGE, the current gold standard typing method employed in the CDC-managed PulseNet program. Both the FSIS and the FDA fiscally co-support PulseNet with CDC by Interagency Agreements.

In summary, NACMCF chose a public health thrust to drive its response to the charge, gathered and described background information on the current and future detection technologies which could be applicable to a regulatory setting, and used this foundation as the basis upon which to address the broad charge in a holistic manner. The Committee identified, both in the Table of Contents and the Introduction to each section, the location of discussions addressing the specific charge questions. Some questions are addressed in more than one section. It is the opinion of the Committee that the charge has been adequately addressed in this document.

2. BACKGROUND: TESTING AND METHODS DEVELOPMENT PROGRAMS OF FEDERAL FOOD SAFETY AGENCIES

In the U.S., a number of Federal and state agencies have complementary roles in ensuring the safety of a myriad of domestic and imported food products. The two major Federal regulatory agencies responsible for the safety of the food supply are the USDA-FSIS and the DHHS-FDA. The DHHS CDC conducts human disease surveillance for foodborne and other illnesses of public health importance. In addition, the Environmental Protection Agency (EPA) sets limits on the amount of pesticide residues permitted in food, and the National Marine Fisheries Service (NMFS) within the Department of Commerce (DOC) provides fee-for-service inspections of seafood safety and quality. In the Department of Defense, the U.S. Army Veterinary Service is the Executive Agency responsible for food safety and defense. The Veterinary Service audits food processors and monitors food safety and quality throughout the supply chain, which is critically important during deployments.

During information gathering for this background section from the FSIS, the NACMCF learned of the restrictions on method development by the FSIS, which apparently occurs because this activity is perceived as research and hence outside the purview of USDA-FSIS. This information prompted the Committee to explore in greater depth the method development activities of other agencies relative to their food safety responsibilities.

2.1. Roles and Responsibilities of Food Safety Agencies

The USDA's FSIS is the public health agency responsible for ensuring that the nation's commercial supplies of meat, poultry, and processed egg products are safe, wholesome, and correctly labeled and packaged *(145)*. The FSIS monitors domestic and imported meat, poultry, and processed egg products for bacterial contamination, residues of pesticides, drugs, and other chemicals through implementation of HACCP and verification testing. The FSIS is actively involved in recalls and trace-back or -forward activities for products that may be adulterated and/or related to foodborne disease outbreaks. The FSIS has a

pre-market approval process for all labeling applied to meat, poultry, and processed egg products. In addition, by statute, the FSIS is required to conduct inspection in all regulated facilities each day. In the case of slaughter and processed egg inspection, the FSIS personnel must be continually present during the entire operation. The FSIS regulated products are regularly tested for foodborne pathogens such as *Salmonella, L. monocytogenes,* and *E. coli* O157:H7, to verify and ensure that process controls are effective. For the meat, poultry, and egg products regulated by the FSIS, the pathogens with the greatest impact on the public health are the bacterial agents Shiga toxin–producing *E. coli* (such as *E. coli* O157:H7), *Salmonella, Campylobacter,* and *L. monocytogenes*, while viral agents such as norovirus, and parasitic agents, such as *Toxoplasma gondii* are also of concern. In addition, zoonotic pathogens such as *Mycobacterium bovis* and *Brucella abortus,* which are now largely controlled as foodborne problems in this country, still occur in food animals and in wildlife animal reservoirs.

The FDA Foods Program consists principally of activities of the Center for Food Safety and Applied Nutrition (CFSAN) and field programs of the Office of Regulatory Affairs (ORA). The Center for Veterinary Medicine (CVM) has a role in animal feed and veterinary drug safety for animals, including those destined for human consumption. The FDA's Foods Program mission is to promote and protect the public health and economic interest by ensuring that the food and feed supply is microbiologically, chemically, nutritionally, and toxicologically safe and wholesome and cosmetics are safe; and that food and cosmetic products are honestly and accurately labeled. The FDA's Foods Program is unique relative to the FSIS (and FDA's own drug, medical device, and biologics centers) because the predominant focus for ensuring food safety relies mostly on post-market activities which require the documentation of risk. To fully appreciate the significance of this food protection mission, however, it must be understood that the underlying assumption of the laws the FDA enforces is that foods are safe. Thus, with the exception of certain pre-market food and feed additive and labeling requirements, the FDA must rely on post-market surveillance and scientific evidence to prove that a product is a threat to public health to take action against it.

In contrast to the FSIS and the FDA, the CDC is nonregulatory. In collaboration with local and state public health departments, the CDC conducts surveillance for human illness, investigates disease outbreaks, estimates the burden of illness caused by specific agents, and monitors longer term trends as prevention efforts are implemented. Public health surveillance depends on reports from clinical laboratories of the isolation of clinically meaningful microbes from sick persons. Active sentinel site surveillance through the FoodNet platform provides reliable information on the incidence of diagnosed foodborne infections and the trends over time, that are integral to setting and tracking progress towards national disease reduction goals *(23).* For some microorganisms such as *Salmonella,* this surveillance is strengthened by sending the strains isolated from patients to public health laboratories for further testing to characterize and subtype them. This subtyping enhances the capacity of the public health system to detect and investigate outbreaks. Traditional subtyping has depended on tests for microbe characteristics such as serotype and toxin production. In recent years, the public health laboratories have used molecular subtyping methods (or ''fingerprinting'') for the same purposes. The National Network for Molecular Subtyping of Foodborne Bacteria, PulseNet, connects all 50 states with the database and methods development hub at the CDC, as well as the laboratories of the FSIS and the FDA. PulseNet makes it possible to detect widespread and dispersed outbreaks that would likely have been missed in the past and improves the precision of epidemiological investigations *(136).* Most outbreaks are investigated by local and state public health authorities. The CDC scientists are consulted on many of these, and coordinate or lead investigations of outbreaks that are particularly severe, unusual or widespread. In outbreak investigations, diagnostic and subtyping tests have been critical to define which illnesses are likely to be part of an outbreak, and which are not, and to link isolates from suspected or implicated foods to the clinical cases, as well as to potential upstream or environmental sources of contamination.

Interagency coordination occurs through numerous formal and informal collaborations. The CDC, FSIS, and FDA are all connected to PulseNet and participate in FoodNet, as well as other surveillance networks. Interagency liaisons foster communication and coordination. If methods are standardized across the agencies, then sharing microbiological data across the agencies can answer additional questions. This is important to monitoring antimicrobial resistance in foodborne pathogens in people, animals, and foods through the National Antimicrobial Resistance Monitoring System (NARMS) *(150).* Comparing the organisms identified by regulatory product testing with those coming from clinical, environmental, and animal sources can help to allocate the disease burden of a pathogen across a variety of potential food sources.

The National Oceanic and Atmospheric Administration (NOAA), through its Seafood Safety Research and Monitoring Program (SSRMP) and the Seafood Inspection Program (SIP), plays an important role in food safety. The SSRMP represents NOAA Fisheries' foundation to proactively and rapidly respond to seafood safety and aquatic animal health issues and episodic events. This program has provided NOAA the capability to respond quickly to environmental disasters and episodic seafood processing malpractices. As part of the SSRMP, the SIP is a voluntary, fee-for-service program for inspection and certification of fishery products for quality and safety. The mission of the SIP is to assist industry and consumers in improving the overall quality and marketability of seafood and ensuring that all processing firms are compliant with the FDA and DOC regulations. The SIP supports the FDA's mission by enforcing regulatory requirements and referring non-compliant seafood and processing firms to the FDA. A variety of services, including in-plant inspections, product evaluation and grading, HACCP services, and consultation for regulatory compliance, are offered to the industry.

2.2. Current Microbiological Testing Programs

USDA's regulatory (FSIS and Animal and Plant Health Inspection Service [APHIS]) and research (Agricultural Research Service [ARS]) agencies are empowered with diverse missions. As a result, these sister agencies differ in resources and capacity to develop and validate detection and subtyping methods. In addition to USDA, other government entities with microbiological testing programs are also discussed below.

2.2.1. The FSIS Microbiological Testing Programs and Objectives

The FSIS currently has two microbiological testing programs: the Baseline Microbiological Surveys and the Verification Testing Programs. Data from these microbiological testing programs are used to (i) establish microbiological performance standards and testing objectives for specific meat and poultry products, (ii) verify process control, (iii) improve risk assessments, (iv) provide epidemiological information, (v) assess the effectiveness of the FSIS inspection programs, and (vi) measure the Agency's progress toward meeting its public health goals.

The FSIS Microbiological Baseline Surveys were started in the 1990's to provide data as a prelude to the promulgation of the HACCP Final Rule and serve as the basis for the microbial performance standards used in the HACCP Verification Testing Program. These baseline studies sample the FSIS-regulated products from federally inspected establishments to determine the presence and levels of specific pathogens and indicator organisms. The intent was to estimate (i) the prevalence of specific foodborne pathogens in selected meat and poultry products and (ii) the likelihood of exposure of the public to foodborne pathogens of public health concern in meat and poultry products. The number and frequency of samples are driven by statistical considerations as well as the establishment's production volume and within the constraints of existing agency inspection, laboratory, and financial resources. Recently, the National Academies of Science (NAS) *(93)* reported that the original baseline studies were flawed by significant sampling deficiencies and recommended that the FSIS conduct new baseline studies on a periodic basis that are representative and statistically valid. Furthermore, the NAS stressed the need for increased transparency in the development of food safety criteria, noting difficulties in reviewing and assessing the validity of the data and assumptions used to create the microbial performance standards. Since then, a number of new baseline studies have been conducted which attempt to address the deficiencies in the original studies. However, only one of the original baseline studies (broilers) has been repeated, and baselines for turkey and hog carcasses have been initiated. Since 2001, the NACMCF has provided guidance to the FSIS on the design of five baseline studies as they relate to establishing performance standards *(91)*.

The HACCP Verification Testing Program is a regulatory program that was designed to verify process control (i.e., effectiveness of in-plant HACCP programs) in federally regulated establishments over a specific interval of time. This includes sample sets of meat and poultry tested for *Salmonella,* and sampling of selected meat and poultry products for *E. coli* O157:H7 and *L. monocytogenes* (see Tables B-1, B-2, and B-3). To verify process control and prioritize future inspection activities, the FSIS collects verification samples of products during production and, depending on the purpose of the testing program, conducts microbial testing to detect the presence of *Salmonella, E. coli* O157:H7, or *L. monocytogenes*. The number of verification samples collected by the FSIS is pre-determined each year for each pathogen-product pair based on the constraints of existing agency inspection, laboratory, and financial resources. Different establishments may be tested from year to year and the frequency of sampling is dependent upon a number of factors (e.g., the establishment's production volume, degree of process control, and prior FSIS testing history). As pointed out by the Office of the Inspector General and the FSIS *(148, 149),* the HACCP Verification Testing Program was not designed to provide estimates of nationwide prevalence of foodborne pathogens and should not be used to measure the overall effectiveness of HACCP in an establishment or nationally, or to make year to year comparisons. Even so, the FSIS tracks the percent positive rate in verification samples quarterly and regularly reports these results to the public as a measurement of its progress toward meeting public health goals. In an attempt to improve its ability to estimate population exposure to pathogens, the FSIS calculates the volume-adjusted percent positive rate and has established a new data integration and food protection program *(146)*. The FSIS should ensure that the Agency analyzes and reports data in a coordinated, efficient, and statistically valid manner.

2.2.2. Overview of the FSIS Testing Methods

For regulatory food safety testing, the consuming public and the regulated industry expect the FSIS test results to be above reproach. Therefore, the FSIS uses generally accepted biochemical, serological, and genetic criteria for pathogen identification methods that have been historically accepted by the public health and microbiological scientific communities.

For every microbiological testing method, there is a functional limit to the amount of product (sample) that can be accommodated by an analysis. This may be called the ''test portion'' or ''analytical portion.'' Standard protocols specify the portions of submitted samples that are tested for each type of product and each type of agent. The test portion provides a theoretical limit for detecting a pathogen. The typical test portion specified by most pathogen testing protocols is 25 g but larger test portions are sometimes used, as these can enhance the detection of low levels of the contaminant or facilitate detection when the contaminant is distributed unevenly throughout the food product.

Pathogen testing methods currently in use by the FSIS typically employ a one- or two-stage broth enrichment step followed by a rapid screening test, typically based on detection of an antigen (i.e., immunoassay) or genetic determinants (i.e., polymerase chain reaction [PCR]). The use of screening tests expedites identification of samples

that are negative and enables the FSIS to determine potentially contaminated product more quickly. This allows the FSIS laboratories to utilize their limited testing resources more efficiently, and industry to expedite disposition of held product.

2.2.3. Methods Development and Validation Capabilities of USDA

The USDA's non-fee for service regulatory (FSIS and APHIS) and research (ARS) agencies are empowered with diverse missions. As a result, these sister agencies differ in resources and capacity to develop, optimize, and validate detection and subtyping protocols.

2.2.3.1. The FSIS. According to information provided to this Committee, FSIS has no in-house laboratory capabilities at any of their locations to specifically address microbiological methods development. Thus, this regulatory arm of the USDA is reliant upon other sectors (USDA-ARS, academia, and industry) to develop candidate microbiological methods.

2.2.3.2. The ARS. The USDA-ARS National Program 108 (NP-108), ''Food Safety, (Animal and Plant Products)'' *(141)* conducts both pre- and post-harvest food safety research, including methods development. ARS provides scientific information and technology to producers, manufacturers, regulatory agencies (APHIS, FDA, FSIS), and consumers to support their efforts to provide a secure, affordable, and safe supply of food, fiber, and industrial products. Included in this mission is the development and validation of methodologies that have regulatory, industry, and research use.

To foster interagency collaboration, a formal FSIS-ARS liaison, similar to the FSIS-CDC liaison, and the APHIS-CDC liaison, is in place. The FSIS-ARS liaison meets with the National Program Leader (NPL) for food safety quarterly and annually for the planning of joint FSIS-ARS research projects. Currently, at the national level, the FSIS priorities are shared with the ARS NPLs who may assign specific methods development and/or validation projects to a suitable ARS research scientist(s) as the need arises. The ideal time for this to occur is during the drafting of the 5-year research project plan. Ideally, the FSIS counterpart should participate as a stakeholder in the planning of such projects.

In general, since the ARS research is outlined in the 5-year project plan, short-term needs tend to fall by the wayside unless they are addressed within the scope of the broadly-written project plans. Less formal collaborations are realized when the ARS and FSIS personnel interact with one another at various venues. Again, these collaborations usually fall within the purview of the ARS project plans. Nonetheless, successful projects resulting from ARS-FSIS collaboration have been showcased at annual ARS-FSIS Research Planning Workshops, in the ARS NP-108 annual report, in peer-reviewed journal articles, and by awards to the ARS and FSIS staff.

2.2.3.3. The APHIS. Although not a food safety agency per se, APHIS, which is the animal health regulatory arm of USDA, has agency-sponsored facilities to support in-house methods development and to evaluate published methods or commercially available systems. In general, APHIS performs its own validation before adopting a method or protocol. In-house developmental projects conducted by APHIS personnel address the Agency's immediate diagnostic needs and yield publishable data. APHIS proactively seeks technical support from ARS investigators, as evidenced by publications resulting from these collaborations. In addition, APHIS enlists the cooperation of government and university partners, updates stakeholders at national meetings, and solicits extramural support. APHIS conducts microbiological testing in response to either disease outbreaks or producers' needs. For example, the National Animal Health Monitoring System (NAHMS) *(143),* an APHIS-based initiative, enlists state and Federal veterinarians to distribute questionnaires and collect field samples (livestock feces), which are then distributed to collaborating laboratories for analysis. Originally, NAHMS samples were processed for *Salmonella* and *E. coli* isolation at the National Veterinary Services Laboratories, Ames, Iowa; recently, testing has been expanded to include other pathogens (*Campylobacter, Yersinia,* protozoa, helminths, and viruses) with isolations performed in collaborating ARS and academic laboratories, funded in part by extramural initiatives. The U.S. Animal Health Association (USAHA) is a major forum to address the needs of stakeholders and to garner their support and to facilitate ARS-APHIS collaboration *(142).* APHIS is a major contributor to USAHA working committees as evidenced by the annual update summarizing *Salmonella* serotyping and phagetyping results. The APHIS also provides support to the FSIS in the form of the serotyping of *Salmonella* field isolates and is a participant in studies to evaluate the CDC's molecular-based alternatives to traditional serotyping. Finally, the APHIS is an active participant in extramurally funded research projects with academic and ARS partners.

2.2.4. The FDA

The FDA conducts inspections of production, processing, and storage facilities for the food products it regulates. Sampling and testing will occur when violations in good manufacturing practices (GMPs), sanitation, and where applicable, deviations from HACCP programs are cited. Additionally, CFSAN issues targeted surveillance assignments to ORA for high risk foods, high risk situations (e.g., food service for high profile national events such as political conventions), and certain emergency response situations (e.g., outbreaks) to obtain a short term assessment of pathogen prevalence.

Two relevant research programmatic thrusts for the CFSAN include:

- Development of methods for sampling, detecting, and confirming the identity of pathogens in a variety of food types so that the FDA can unequivocally establish evidentiary support to its regulatory actions.
- Identification of virulence factors, epidemiological markers, and other determinants that influence the ability

of pathogens to use foods as a vehicle for disease transmission, thereby providing enhanced epidemiological investigation, earlier interventions, and more accurate product trace-back.

In addressing each of these needs, the FDA has also relied heavily on the basic work of the DHHS's National Institutes of Health (NIH), USDA's ARS and Cooperative State Research, Education, and Extension Service (CSREES), commercial entities (e.g., platform technologies), and academia. However, without the ability to augment those studies with the unique capabilities, expertise, and focus of the FDA researchers, this scientific knowledge could not have been translated into the FDA relevant programs. Although the charge to the NACMCF is focused on the FSIS regulatory model and mission, the FDA can clearly benefit from the analysis and recommendations cited here, and the NACMCF membership considered this in their deliberations.

2.2.5. The DOC-NOAA Fisheries

Within NOAA Fisheries, the seafood safety activities are primarily carried out by the SSRMP. Activities include working with the CDC, NIH, and others to advance the understanding of mercury issues in fish, providing scientific oversight to the SIP, identifying and characterizing marine pathogens, and improving detection and forecasting of harmful algal blooms.

The SIP conducts inspections of seafood establishments including vessels, processing plants, and retail facilities. Validation and audit inspection are conducted in order to assure adherence to all sanitation, HACCP, and other regulatory requirements. Inspections often include surveillance and compliance sampling of high risk products which are sent to the National Seafood Inspection Laboratory (NSIL) for microbiological and chemical analysis. The NSIL conducts laboratory analyses using screening methods as well as methods approved by other Federal and international bodies. Samples analyzed at the NSIL include surveillance and compliance samples in support of the SIP, compliance samples from industry and other Federal agencies, and research samples. The laboratory has methods development and validation capabilities.

In addition to NOAA Fisheries activities, NOAA's National Oceanic Service conducts seafood safety related activities at its Center for Coastal Environmental Health and Biomolecular Research (CCEHBR). The CCEHBR conducts interdisciplinary research to resolve issues related to coastal ecosystem health, environmental quality, and related public health impacts. Chemical, biomolecular, microbiological, and histological research is done to describe, evaluate, and predict the significant factors and outcomes of natural and human influences on marine and estuarine habitats. Chemical, biomolecular, microbiological, ecological, toxicological, and histological methods are developed and used in both laboratory and field studies.

2.2.6. The CDC

The CDC conducts routine testing to support the network of state and local public health laboratories. This testing function includes identifying problematic organisms, providing specialized diagnostic testing for rare infections (e.g., botulism), and testing clinical specimens (and occasionally, environmental and food samples from outbreak settings), as well as supporting specialized surveillance and research activities. The reference laboratories also develop and validate new methods for diagnosis and subtyping for use in the public health system. In general the CDC develops and/or validates its own methods in-house and performs multi-laboratory comparisons with other public health laboratories before adopting a method for surveillance purposes. Specialized protocols for biothreat agents are developed and distributed through the Laboratory Response Network (LRN).

2.2.7. Food Emergency Response Network (FERN)

The FERN is a USDA-FDA led activity that comprises over 150 collaborating laboratories. Its mission is to integrate the nation's food testing laboratories for the detection of pathogens and select agents in foods at the local, state, and national level. FERN laboratories use validated methods that have been developed by the FDA, USDA, CDC, or by the military. Laboratories in the FERN also develop and validate methods for targeted analytes that are a priority for the network.

2.3. Current Methodological Approaches

There are a number of methodological approaches applied to the detection and further characterization of microorganisms in foods *(22, 32)*. Some of the most commonly used technologies are summarized in Table 2 *(36)*.

The following sections describe the three basic categories of microbiological methods as applied to food microbiology, i.e., enumeration of microbiological indicators, detection of foodborne pathogens, and methods for further strain characterization.

2.3.1. Detection of Foodborne Pathogens

There are many widely used culture-based methods for the detection of common enteric pathogens in clinical specimens. Clinical specimens usually have large numbers of the target organism and the sample matrices (urine, blood, feces, etc.) are relatively consistent from sample to sample. Adapting such methods to the detection of the same pathogens in foods can be challenging, but over the last 50 years, food microbiologists have developed well validated and robust culture-based methods. These methods are designed to address several issues unique to the detection of pathogens in foods:

- The ability to detect as little as 1 target cell per sample, with sample sizes ranging from 25 to 325 g;
- The recovery of pathogens sublethally injured as a consequence of previous treatments applied for food processing and/or preservation;
- A high degree of assay specificity to reduce the likelihood of a false-negative result.

Standard cultural procedures for foodborne pathogen isolation and detection encompass the sequential steps of (i)

TABLE 2. *Existing technologies for the detection and identification of bacterial pathogens, toxins, and indicator organisms in foods*[a]

Technology	Format	Selected targets	Limitations
Bioluminescence	ATP	Viable bacteria	Cannot determine species, total count only
Chromogenic and fluorogenic dyes	Media	*Campylobacter,* coliforms, *Cronobacter sakazakii, E. coli* O157:H7, *Listeria, Salmonella, Staphylococcus aureus, Vibrio*	Selective plating media; need incubation; presumptive data; need confirmation
	Assay	*E. coli,* coliforms	Automated enumeration; instrument cost
Manual identification	Biochemical	Most bacteria	Pure cultures required
Auto identification	Biochemical	Most bacteria	Pure cultures required
	Fatty acid C oxidation		
Nucleic acids	DNA probe	Bacterial communities	Limited database
		Campylobacter, E. coli O157:H7, *Salmonella, Listeria, Yersinia*	Need culture enrichment; detects nucleic acid sequences but not gene expression; cannot determine cell viability; confirmation required
	PCR	*Campylobacter, Clostridium, C. sakazakii, E. coli* O157:H7, *Listeria, Salmonella, S. aureus, Shigella, Yersinia*	Need some enrichment; detects gene sequences but not gene expression; cannot determine cell viability; many inhibitors in foods; need confirmation
Antibodies	Latex agglutination	Many pathogens and serotypes, some toxins	Pure culture required; good for serotyping; not sensitive for detection
	Lateral flow	Most pathogens, some toxins	Culture enrichment and confirmation required
	Magnetic bead	Most pathogens	May not yield pure culture; matrix-dependent efficiency; not stand alone
	ELISA	Most pathogens and toxins	Culture enrichment and confirmation required

[a] Most assays provide presumptive data and will need confirmation for definitive results *(35).*

cultural enrichment, (ii) selective and differential plating, (iii) confirmation, and (iv) subtyping. Each individual step in this process takes a minimum of 18 to 48 h. Sometimes this first phase of testing is referred to as ''screening.'' Based on standard cultural procedures, the screening process is completed after incubation of selective and differential plating media. In this case, two different outcomes are possible. If a characteristic colony is not present after the selective and differential plating steps, there is no need to continue the test and the result is reported as confirmed negative. It typically takes 3 to 4 days to obtain a confirmed negative test result. On the other hand, if suspicious colonies are identified on selective-differential agar, confirmatory testing is necessary, and the sample is designated as a presumptive positive. These samples typically require further testing to characterize the phenotypic properties of the organism that may include: the ability to metabolize specific compounds, antigenic properties associated with the organism which distinguish it by serotype, and biochemical characteristics such as the presence of specific proteins or fatty acids. Confirmatory testing assures that the isolate(s) is the target pathogen; not all presumptively positive isolates are actually confirmed as the pathogen. Depending on additional subtyping needs (described below), complete characterization of a confirmed positive isolate may require a few days to a few weeks.

Over the last two decades, the time to result in screening foods for pathogens has improved with the introduction of detection platforms such as enzyme-linked immunosorbent assay (ELISA) and PCR. These are the approaches most commonly used by the FSIS for initial pathogen screening. These methods allow the analyst to bypass selective plating by replacing it with a step that takes only a few minutes to hours to complete. Cultural enrichment is still necessary to bring the target organism to detectable limits (usually $>10^3$ CFU/ml of enrichment broth). With this approach, a confirmed negative test result can be obtained in 1 to 2 days. However, a presumptively positive sample must be further processed by selective plating, isolation of suspect colonies, and subsequent confirmation steps. The process is therefore faster for a negative test result but does not result in more rapid results for those samples screened as presumptively positive.

Cultural enrichment techniques typically provide qualitative presence-absence data but no quantitative estimates of the number of target pathogens that are present in the sample. In recent years, enumerative methods have emerged for some pathogens and indicator organisms. For example, for detecting *Campylobacter* in baseline studies, the FSIS uses an enumerative selective plating method which does not require prior cultural enrichment *(90).* The *FDA Bacteriological Analytical Manual* (BAM) *(3)* describes a colony lift hybridization method for the enumeration of *Vibrio parahaemolyticus* and *V. vulnificus* in molluscan shellfish. Theoretically, any enrichment-based pathogen detection approach can be adapted for quantitative analysis by converting it to the most probable number (MPN) format; however, MPN enrichment is cumbersome and resource intensive. There may be opportunities in the future to combine MPN enrichment with PCR, thereby streamlining the process *(98).* For indicator organisms, a commercial system for automated MPN determinations for coliforms, *E. coli* and *Enterobacteriaceae* claims to provide quantitative

results (including confirmation) in 22 h versus the 3 to 4 days needed for traditional MPN *(106, 107)*.

2.3.2. Non–Culture-Based Approaches

For detection of organisms of public health importance that are not easily cultured or are difficult to detect, diagnostic methods are almost always based on the detection of nucleic acids specific to the target pathogen. For example, the NACMCF report entitled ''Assessment of Food as a Source of Exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP)'' details several PCR-based methods to detect *Mycobacterium avium* subsp. *paratuberculosis,* a fastidious pathogen which is often recalcitrant to growth in culture *(91)*.

Oligonucleotide fingerprinting of rRNA genes (OFRG) has identified microbial communities in soil by employing DNA probes in a microarray *(16, 154)*. OFRG, which does not rely on isolation of fastidious microbes, correlated shifts in the microbiota of the turkey intestine with *Campylobacte*r colonization *(120)*. Using a similar approach, *Salmonella* colonization status of cattle has been correlated with the fecal microbiota *(105)*. Sequence-based approaches to characterize entire microbial communities are supplementing culture-based methods and in some instances replacing them. For example, the power of rapid pyrosquencing technology can be applied to entire microbial communities, many of which cannot be cultured *(79)*. Pyrosequencing of entire microbial communities may have applications for detection of population shifts in abused, low quality, or pathogen-contaminated products and ultimately may be more sensitive than screening for indicator organisms or specific pathogens. Theoretically, approaches such as these could be used in detection but as is described in Section 4.1, there are a number of hurdles that must be overcome before their routine use in pathogen screening and confirmation can be adopted.

2.3.3. Methods for Strain Subtyping (Question 4)

The process of strain typing at the subspecies level is often referred to as subtyping. There are four major applications of subtyping: taxonomy, phylogeny, outbreak detection-investigation, and risk assessment including attribution. Once definitively isolated and identified, bacterial isolates can be further subtyped based on phenotypic and/or genotypic characteristics of the organism. The automated Phenotype Microarray Technology™ offers the potential to characterize an isolate by measuring the expression of thousands of genes during growth *in vitro* and *in vivo (14)*. Traditional phenotypic subtyping (including serotyping and antibiotic resistance profiling, among others) is still performed for many organisms, but recent improvements in molecular subtyping have replaced some of these methods *(9, 25, 54)*. Of particular interest is PFGE, which is the current ''gold standard'' method used in the CDC's PulseNet Program. Much of the developmental work and subsequent implementation of these types of molecular methods has been done by the CDC. For example, the CDC developed a molecular equivalent for *Salmonella* serotyping which is based on detection of the genes that encode serotype-specific antigens. This assay is now in final evaluation at state public health laboratories and appears to be faster, easier, and more reliable than traditional serotyping *(38)*. Other promising technologies include multiple-locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), SNPs, microarrays, and mass spectrometry, which are described later in this document. MLST, a method based on sequence comparison of the sequences of 5 to 10 genes, is particularly useful for subtyping of many foodborne pathogens *(24, 54)*.

Development and future prospects of subtyping foodborne bacterial pathogens are beyond the scope of this document and have been reviewed elsewhere *(54)*. Of particular relevance is the recent FSIS document entitled *Analysis of Molecular Subtyping Methods for FSIS Regulatory Testing: The Present and Future of FSIS Regulatory Subtyping*. This internal ''white paper'' offers recommendations for future molecular subtyping to be undertaken by the FSIS *(33)*. Consult this document for further details about potential molecular typing methods which could be applied by the Agency but are currently beyond the scope of this document.

3. PURPOSES OF MICROBIOLOGICAL TESTING (QUESTIONS 1, 3, AND 5)

Multiple factors must be taken into account when considering new testing methodologies for regulatory laboratories. Not only should the method's appropriateness for meeting a particular public health goal be a factor, but method reliability and validation are critical issues for results that may become legal evidence in court. This section reviews a few of the criteria that must be taken into consideration. The limitation of using pathogen indicator organisms is mentioned in the Philadelphia report *(34)*.

3.1. Microbiological Testing for Public Health

Microbiological testing is an essential tool for protecting consumers from contaminated food. The various roles for microbiological testing are described below.

3.1.1. Surveillance and Investigation

Public health surveillance is the routine reporting of health events in a defined population. Surveillance data are used to estimate the burden of a disease *(83)* to set public health objectives (e.g., Healthy People 2010 goals), to detect and investigate outbreaks, and to track trends over time.

Public health surveillance depends on standard diagnostic testing in clinical laboratories to identify cases of reportable infectious diseases. This is supplemented by routine characterization and subtyping of those organisms for public health purposes, which is conducted largely by the local and state public health laboratories. The use of standardized subtyping methods for foodborne pathogens, and the linking of the results through the PulseNet database has enhanced the ability of the public health network to detect, investigate, and control outbreaks. For example, outbreaks can be identified sooner when there is a cluster or

an increase in the number of cases caused by one particular subtype. Epidemiological investigation can be targeted to those clusters, and to those instances in which matching clinical and environmental or food isolates have been obtained, improving the ability to identify vehicles of transmission and ultimately, the source(s) of contamination.

When a foodborne illness outbreak is identified, it is likely that more than one Federal agency, as well as state and/or local authorities, will be involved in the investigation. Analytical methods applied by multiple agencies tend to reflect the perspective and mission of each of the agencies. While this can be very useful, it is important that there be coordination and communication between agencies with respect to methodological issues.

3.1.2. Estimating Prevalence

Estimating the prevalence of pathogens in the food supply is critical to understanding and addressing the public health risk of foodborne disease in the United States. Prevalence estimates can provide (i) a mechanism for measuring performance against public health goals, (ii) data for risk assessment, and (iii) the basis for regulatory performance standards. Currently, the Baseline Microbiological Surveys are used to estimate the prevalence of pathogens in specific meat and poultry products and serve as the basis for the FSIS microbiological performance standards. However, a number of parameters including method sensitivity and limit of detection (LOD) significantly affect the reliability of the results and must be taken into consideration when evaluating analytical data.

3.1.3. In-Plant Process Control

Microbiological testing can be used to assess in-plant process control. The key to success in process control is implementing a cost-effective, real-time, on-site testing platform that can be used to rapidly identify a process deviation trend relative to the established acceptable limits. Rapid testing allows for swift correction of process deviations, reducing the likelihood of contaminated finished product. For ease and reduced expense, testing for microbial indicators is often chosen as an alternative to pathogen testing. It is likely that a number of emerging technologies might be applicable to monitoring and verifying process control.

3.1.4. Providing Data for Risk Assessment and Attribution

Quantitative risk assessment is a prelude to the promulgation of food safety regulations and relies on valid microbiological data to support risk estimates. One component of risk assessment, i.e., exposure assessment, requires data on the prevalence and levels (numbers) of a select pathogen in the food in question. Quantitative data obtained from microbiological testing provide this type of information which can then be used to populate risk models, increasing their scientific rigor and relevance *(76, 77)*. The introduction of enumerative methods for pathogen detection, which might be associated with some of the emerging technologies, would provide much needed quantitative data for risk assessment.

Food attribution, or the ability to attribute the proportion of specific foodborne diseases associated with specific food commodities, is of great interest as food safety agencies move toward risk-based management approaches. Current epidemiological and microbiological data that are used to inform attribution estimates are limited and subject to uncertainty; microbial data are currently limited to *Salmonella* strains from meat, poultry, and some egg products. Application of standardized subtyping methods to build libraries of isolate data from diverse sources including foods, environments, animals, and humans, would improve attribution estimates.

3.2. Indicators versus Pathogens

Direct testing for specific pathogens is not always practical when considering technical requirements, cost and the low prevalence of pathogen contamination in many food products. While indicator methods are rapid, inexpensive, and often enumerative, the most important question is whether the chosen indicator is a valid representative of the conditions conducive to the presence of the pathogen of concern *(91)*.

Detection of one or more microbiological indicators may be applied in place of specific pathogen detection. Although not a direct measure of pathogen contamination, indicators have historically been used as part of process control systems, to assess the hygienic status of processing operations, and to monitor the efficacy of antimicrobial interventions at critical control points in production and processing of foods. Indicators have also been used to evaluate the overall microbiological quality of finished products and to estimate product shelf life. Typical indicator systems include aerobic plate counts, coliform counts (CC), and *E. coli* biotype I counts (ECC). *E. coli,* fecal coliforms, and *Enterococcus* spp. of fecal origin have been used extensively as indicators for the potential presence of enteric pathogens *(39, 88)*.

3.3. The Concept of "Zero Tolerance"

Some microorganisms are considered so hazardous to the public health that they are not allowed in certain foods at any detectable level. This principle has led to the concept of ''zero tolerance,'' which can be defined as the inability to detect the target organism in a certain number of samples of a specified size. Both statistical sampling and microbiological methodology play key roles in the practical application of the concept of ''zero tolerance.'' As only a small number of samples are likely to be contaminated, and pathogens in contaminated foods tend to be distributed in a non-homogeneous manner, the sampling design and method will influence the likelihood of collecting a pathogen in any given sample, if present. While technological advances have resulted in microbiological methods with improved (lower) limits of detection, the performance of these methods in detecting low-prevalence pathogens is inherently impacted by sampling. Therefore, ''zero tolerance'' provides some protection of public health but cannot guarantee that the product in question is completely free of the pathogen of concern. It is clear that microbial testing alone cannot ensure food safety. Negative and positive pathogen test results do not necessarily indicate

absence or presence, respectively, of the target in the sample due to the possibility of false reactions.

3.4. Sampling and Statistical Considerations in Microbiological Testing

As stated above, the ability of microbiological methods to detect foodborne pathogens is intimately dependent upon sampling. The term ''sampling'' refers both to the statistical methods used to determine which and how many samples to test in order to represent a larger amount of product, and to the technical methods used to collect, preserve, and process that sample for microbiological testing. Although the charge to the Committee explicitly focused on the non-statistical issues of sampling, the Committee nonetheless recognizes that the questions in the charge raise statistical issues related to sampling.

According to the International Commission on Microbiological Specifications for Food (ICMSF), ''the purpose of sampling a food is to collect a representative sample to obtain information on its microbiological status'' *(55)*. Sampling plans, when designed properly using sound statistical concepts, provide a systematic means for assessing the microbiological status of food with a high degree of confidence *(92)*. A sound sampling plan should specify the number of samples collected; the methods used to select and collect them; the laboratory testing methods; and criteria for interpreting the results. All of these factors depend on the purpose for the microbiological testing.

1. Sampling can provide an estimate for the parameter of interest, however it does introduce uncertainty. To reduce uncertainty, the sampling must be representative of the population of interest and the sample size must be sufficient to provide a high degree of confidence that the sampling results correctly characterize the parameter of interest. For example, in routine microbiological testing, there is a risk that a lot will be misclassified: lots with acceptable levels of pathogens are rejected (producer risk) and lots with unacceptable levels of pathogens are accepted (consumer risk). While it is not possible to eliminate these risks, the probability that misclassification occurs can be minimized. The extent to which these risks can be minimized depends on a number of factors, including sample size and representativeness as well as the sensitivity, specificity, repeatability, and reproducibility of the laboratory methods *(93)*. An appropriate statistical sampling plan will address these issues and minimize producer and consumer risk. From a public health perspective, it is more important to minimize consumer risk.
2. Obtaining representative samples is crucial to the interpretability and generalizability of the sampling results. A representative sample should reflect the composition of the population of interest, which will affect the number of samples taken as well as the sampling methods. For example, a 1-pound sample from a production lot of 10,000 pounds may be representative if pathogens were distributed uniformly (i.e., homogeneous population). However, it is well established that microorganisms and pathogens are unevenly distributed in food *(93, 149)*. With a heterogeneous population, there is increased risk that sampling results will not accurately characterize the parameter of interest. Increasing the sample size and using appropriate sampling methods, such as stratified sampling, will minimize this risk and provide a greater degree of confidence in the sampling results. There are mechanisms for determining the appropriate number of samples needed for maintaining an acceptable level of risk. An appropriate statistical sampling plan will address heterogeneity within the population to be sampled.

In short, the entire testing spectrum from sampling through laboratory analysis must be considered when determining the most appropriate technologies for performing routine and baseline microbiological analyses. Sampling plans should include an explicit description of the trade-offs in sample size and statistical power that were considered during design and implementation.

3.5. Performance Criteria for Methods Selection and Evaluation

The basic assumption of microbiological testing is that it will result in some protection to public health. The purpose for testing will influence the criteria used in method selection. For example, for assays intended to provide presumptive identification (screening), the foremost characteristics are sensitivity, reliability, cost, and speed. For confirmatory tests, sensitivity and specificity must be considered to minimize false-negative and false-positive results. For assays that are used to subtype isolates, it is necessary to demonstrate that, in addition to being a practical and reliable assay, the method reliably separates outbreak-related strains from the background of sporadic infections, and provides data that are epidemiologically meaningful. Validity, reliability, feasibility, effectiveness, and validation, all of which are important considerations in choosing and evaluating candidate methods, are described briefly below.

3.5.1. Validity

Validity is a measure of the ability of the test to do what it is intended to do under specific conditions of use, i.e., to detect the organism(s) of interest if it is present, and to not detect it if it is absent. The components of validity are described below.

3.5.1.1. Sensitivity. Imprecise use of the term sensitivity causes confusion in the interpretation of microbiological test results. The reason for this confusion is that there are two distinct types of sensitivity, analytical sensitivity and diagnostic sensitivity *(116)*. Analytical sensitivity, also known as the LOD, represents the smallest amount of an analyte in a sample that can be accurately measured by a platform or assay. Therefore, analytical sensitivity relates only to the detection platform or assay. In contrast, diagnostic sensitivity is the probability of detecting an analytical target (i.e., pathogen, toxin) in a sample from a

population of samples (i.e., a production lot) which is contaminated. Therefore, diagnostic sensitivity measures the ability to detect (''diagnose'') contamination in environments and foods. Significant progress has been made in enhancing the analytical sensitivity of various cultural and molecular detection platforms and assays. For example, some cultural methods can detect one viable cell in a 25-g sample and PCR can theoretically detect one molecule of target DNA in small PCR tubes holding microliter volumes. Unfortunately, little progress has been made in improving diagnostic sensitivity, which remains a major barrier to the detection of pathogens in foods and thus represents a major research gap (see Number 2 in Section 7).

3.5.1.2. Specificity. Specificity is a performance characteristic that judges the ability of a laboratory test method to exclude non-target analytes in chosen matrices, and it is therefore a reflection of ''false-positive'' rate. As with sensitivity, there are also two distinct types of specificity: analytical and diagnostic *(116).* Analytical specificity is defined as the ability of an assay to exclusively identify a target rather than other similar analytes in a sample. Diagnostic specificity is defined as the probability that the sample tests negative when the pathogen is absent from the sampled population. Therefore, a highly specific test will rarely be positive in the absence of the contaminant. Specificity is highly influenced by test method and sample matrix, as well as by the presence of closely related species. Like sensitivity, specificity is often established under controlled laboratory conditions and this may not adequately represent the real analytical challenges to the method. For efficient sample processing and use of laboratory resources, methods should minimize the generation of false-positive results that require additional laboratory work.

3.5.1.3. Predictive value. Sensitivity and specificity define the operating characteristics of an assay, but it is the predictive value (positive or negative) of the assay that is of most importance to the FSIS and public health. Positive predictive value is the probability that a sample whose test result for a specific pathogen is positive truly contains that viable pathogen, which can be calculated as one minus the false-negative rate. Negative predictive value is the probability that a sample whose test result is negative does not contain the viable pathogen, which can be calculated as one minus the false-positive rate. It is important to apply the concepts of positive and negative predictive value to the entire lot of food being produced, not just to the sample being tested (see Section 7, especially Number 2). High diagnostic sensitivity improves negative predictive values and high diagnostic specificity improves positive predictive values, regardless of analytical sensitivity or analytical specificity, and vice versa. Therefore, it is important to realize that assays having very high analytical sensitivity and specificity, but low diagnostic sensitivity and specificity have poor predictive value. Many of the available pathogen tests fit this description, and thus this represents a major barrier and research gap (see Number 2 in Section 7).

While the ideal test method will be both highly sensitive and highly specific, there is an inherent trade-off between these two. In order to protect public health, the false-negative rate should be minimized. However, a low false-negative rate results in a corresponding higher false-positive rate which can create unnecessary follow-up testing and consume laboratory resources. Clearly, altering the criteria for positivity will influence both the sensitivity and specificity of the test. Therefore, any decision regarding specific criteria for acceptable levels of sensitivity and specificity must be made by weighing the consequences of both false-negative and false-positive results. This also needs to be considered when trying to achieve very low (1 CFU/sample) limits of detection.

3.5.1.4. Gold standard. When evaluating the validity of a new assay, it is necessary to compare it to a reference method, which is often referred to as the ''gold standard.'' For foodborne pathogen detection assays, the reference method is almost always the culture-based method, i.e., cultural enrichment followed by selective-differential plating and confirmation. Complications can arise when the new assay outperforms the reference method. In this case, the new assay might classify a higher proportion of the samples as positive, but the reference method will identify these as false positives because of its poorer sensitivity. This presents a difficult situation for validation because samples containing low numbers of pathogens cannot necessarily be ''confirmed'' as positive. In addition, because of the possibility of greater sensitivity and specificity of non–culture-based molecular assays, a more ideal method (a ''platinum standard'') might be considered in the future.

3.5.2. Ruggedness and Credibility

Method durability (ruggedness) is required for reliability in a high throughput testing program. As most tests are performed in several laboratories which are using different personnel and different equipment, it is critical that results obtained under varied environments be comparable. Although laboratory conditions should be consistent, they are rarely identical. Methods should be tolerant of minor variations and must be validated by varying critical test parameters. Methods used by the FSIS should have the highest levels of credibility since the results of laboratory tests can have considerable regulatory (and economic) implications. It is critical that official laboratory test methods have extensive, well-designed validation to achieve defensibility in scientific and legal proceedings.

3.5.3. Workflow: Throughput, Speed, Turnaround

Methods used in a national testing program have specific requirements in terms of the number of sample analyses that need to be performed simultaneously and within a defined timeframe. While related to throughput, the timing of sample processing has important logistical considerations. As many samples are shipped by overnight carrier, assay start times are dictated by the time of sample arrival. To efficiently schedule personnel, the various steps undertaken to complete an assay should fit within reason-

able time parameters while also providing results in a timely fashion. As many of the producers operate on a hold-and-test basis, laboratory test turnaround times can have important economic consequences. Perhaps most critical is the time required to obtain a negative test result so the particular lots of product can be released into commerce in a timely manner. In this case, improving the speed of screening methods may have substantial positive impact.

3.5.4. Validation

Validation encompasses the entire process by which it is demonstrated that a method meets claimed performance characteristics. Methods that are selected by the FSIS for validation must have significant potential to meet the Agency's regulatory need for analytical capacity and should be compatible with Agency laboratory resource demands. Because the FSIS laboratories analyze a variety of diverse products types with different microbial loads and compositions, the Agency conducts extensive validations prior to implementing new methods. The FSIS laboratories are also accredited to perform within the ISO (International Organization for Standardization) 17025 standard and therefore are required to use validated methods that are fit for purpose.

4. EMERGING MICROBIOLOGICAL TECHNOLOGIES (QUESTIONS 1, 2, 3, AND 4)

A variety of technologies are available for incorporation into microbiological testing of foods. Some of these technologies could be used to supplement current FSIS methods with only minor modifications; others would require a completely new way of interpreting positive test results. The Committee reviewed several technologies for potential consideration by the FSIS in sampling (pre-analytical sampling and sample processing), microbial detection, and identification. In so doing, the Committee developed performance criteria for evaluation of these technologies.

4.1. Overview of Emerging Technologies

Culture-based methods have been by default the gold standard given their ease of use, low cost, established sensitivity, and ability to be standardized. In addition, a tremendous amount of historical data exists from the use of culture methods. A major drawback is the time it takes to enrich, screen for, and confirm the presence of pathogens of interest (e.g., 24 to 48 h of cultural enrichment followed by rapid detection using ELISA or PCR, with the potential of another 5 to 8 days for confirmation using conventional biochemical and serological assays).

The most appealing promise of emerging technologies is reducing time to detection without compromising assay validity. In fact, with initial usage of PCR, food microbiologists recognized the theoretical potential to replace cultural enrichment with specific nucleic acid enrichment, which could reduce detection time to a matter of hours rather than days. In more recent years, interest has focused on nucleic acid-based assays that can provide rapid detection of DNA sequences (including antibiotic resistance, virulence factors, etc.). Nanotechnology-based methods have the potential for real-time microbiological detection for process control and could be used to detect pathogen harborage in relatively inaccessible sites in processing environments. Portable technologies are particularly appealing because of their potential application to on-site testing.

A comprehensive review of emerging technologies is available and briefly summarized below with representative applications given in Table 3 *(32, 36)*. A caveat for these methods is that the analytical sensitivity and specificity realized for pure cultures will likely be better than those observed when applied to the detection of the target analyte in a food matrix. Because many of these technologies and methods are still in development and few have been applied to detection of pathogens or indicator organisms in foods, it is premature to assess all their ''advantages'' and ''disadvantages,'' as requested in the original charge.

Real-time PCR (RT-PCR) technology. RT-PCR combines traditional nucleic acid amplification (PCR) with DNA hybridization which occurs while the reaction is progressing. This is accomplished by including a fluorescently-labeled probe in the PCR amplification reactions. In most cases, the probe's fluorescence is quenched in its normal stochiometric conformation. However, if the target DNA is amplified by PCR, the probe will bind specifically during the annealing phase, resulting in a change in conformation which results in the loss of quenching and the occurrence of fluorescence, which is recorded during amplification using a thermocycler with fluorescent detection capabilities. The RT-PCR consists of the sample, primers specific to the target to be amplified, nucleotides, and a polymerase enzyme, which adds nucleotides complementary to the single DNA strand to yield the PCR product or amplicon, and a probe to detect the formation of the PCR product. The method is referred to as ''real time'' since PCR detection and confirmation of amplicon identity occur at the same time, in ''real time.'' The probe may be either non-specific (e.g., SYBR Green I) or a fluorescently labeled sequence-specific probe (e.g., TaqMan, Molecular Beacon, fluorescence resonance electron transfer [FRET]). When the latter is used, the strength of the fluorescent signal is directly proportional to the initial copy number of the target DNA sequence. RT-PCR assays have the potential to simultaneously identify and quantify the DNA target in a single reaction vial (i.e., closed system).

Although faster and more specific than culture-based methods, PCR platforms require (i) primers specific for the target sequence of interest, (ii) stringent amplification conditions, and (iii) optimized DNA extraction to remove PCR inhibitors in foods while simultaneously isolating DNA. Multiple pathogens can be simultaneously detected in a single PCR (multiplex PCR), as detailed in Section 5.5. Multianalyte detection for real-time platforms is possible but restricted to no more than four targets due to the limited commercial availability of non-overlapping fluorophores. Finally, incorporating an internal amplification control (IAC) is important in assuring the absence of reaction

inhibitors, which may result in false-negative results, and as a measure of an analytical method's capacity to remain unaffected by small but deliberate variations in method parameters. Therefore, inclusion of an IAC is an indication of assay reliability. RT-PCR (and real-time reverse transcriptase PCR) as well as portable real-time thermal cyclers for on-site analysis are commercially available. The fundamentals and application of PCR to food matrices have been reviewed elsewhere *(36, 83)*.

DNA microarrays and SNP technologies. In contrast to PCR assays which identify one or a limited number of genes, microarrays are used to simultaneously screen for hundreds or even thousands of genes in a high throughput format *(62)*. Often referred to as ''lab-on-a-chip'' technology, probes, including oligonucleotides (<100 bp) or PCR amplicons (100 to 1,000 bp), based on highly specific nucleic acid sequences which may differ by only a single nucleotide (SNP), are attached or printed to a solid support (e.g., polymer, membrane, glass) in a spatially pre-determined order for simultaneous analysis of many different DNA sequences. Theoretically, microarrays can be designed to rapidly detect multiple pathogens, virulence factors, antimicrobial resistance genes and/or any number of targets useful for detection. Nonetheless, while pre-printed oligonucleotide microarrays are commercially available for a limited number of foodborne pathogens (e.g., Affymetrix GeneChip), the technology is not currently ready for routine use as applied to the detection of pathogens in foods. Of particular importance is the fact that successful hybridization requires $\geq 10^5$ gene copies which means that some type of amplification (cultural enrichment or PCR) must precede microarray detection. Hence, microarray or SNP analysis must be inherently linked to both pre-analytical sample processing and amplification, and is therefore subject to the same considerations required for these methods. In addition, microarray detection requires expensive and sophisticated equipment, and interpretation is tied to complex computer algorithms, neither of which is currently amenable for routine use in pathogen detection in foods or environmental samples.

Spectroscopy technology: matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectroscopy (MS). Whereas genomics identifies genes, proteomics measures the level of proteins. Proteomics is defined as ''use of quantitative protein-level measurements of genes expression'' *(62)*. Analysis utilizes two-dimensional polyacrylamide gel electrophoresis to separate proteins in the first dimension by their isoelectric point and in the second dimension by their molecular weight. The resultant spot is then excised from the gel, digested into peptides, and analyzed by mass spectroscopy. MALDI-TOF MS ''simplifies'' the analysis and generates a characteristic spectrum or fingerprint for either proteins or nucleic acids. For protein analysis, the starting material ranges from a single colony or liquid culture to a single peptide generated by 2D gel electrophoresis *(133)*. Analysis is robust and reproducible (e.g., the acquired profile spectra are comparable between different MALDI-TOF instruments). Assays are rapid with minutes needed for sample drying, loading the instrument, and spectra acquisition. Computer software analyzes and compares results against a growing database of ~40,000 protein spectra. MALDI-TOF offers high throughput analysis, and the potential to detect multiple analytes simultaneously. This technology has identified SNPs of *E. coli (119)*, can distinguish species of *Campylobacter*, and is being applied to serotype *Salmonella*.

Biosensor technologies. A biosensor uses biological recognition molecules (i.e., antibodies) to detect and identify a target with high selectivity and sensitivity. The high affinity and avidity of antibodies to their target antigen underlies the specificity of immunosensors. Binding of the antigen to the antibody or cells is measured by light scattering, fiber-optic biosensors (FOBS), evanescent wave biosensors, surface plasmon resonance (SPR), and piezoelectric-excited millimeter-sized cantilever (PEMC) sensors. Living cells may also be used to detect the presence of specific pathogens; collagen encapsulated hybridoma cells (Ped2E9) lyse and release alkaline phosphatase, which is colorimetrically detected in pure cultures of *L. monocytogenes* but not *L. innocua (7)*.

Light scattering directs a laser beam on bacterial colonies which scatter light forward into a camera. Unique bacterial by-products (e.g., extracellular polysaccharides or toxic proteins) generate distinctive images (concentric rings, spokes, and bright central spots), which are analyzed with a computer algorithm. Colonies are viable for further analysis including confirmatory assays. A prototype portable unit facilitates on-site testing.

Optical biosensors achieve detection through optical transduction mechanisms, such as changes in refractive index, absorption, fluorescence, and SPR. Because methods are predominantly antibody-based they are subject to variable sensitivity, especially low level detection, antibody production limitations, and inhibition by high background. Early versions were tested only with pure bacterial cultures but current focus is bacterial detection directly from food.

FOBS use fiber-optic cable with covalently attached antibodies. Target antigen binds to the antibody, which is detected by a secondary antibody conjugated to molecules that, when stimulated, emit fluorescent light measured by a laser detector. Fluorescence is quantitatively related to amount of antigen immobilized on the fiber surface. The RAPTOR™ is a commercially available example used to detect *Salmonella (36)*.

SPR sensors use antibodies (or other receptors) immobilized on gold electrode sensing surfaces. Binding of antigens alters resonance frequency generating a signal. Although results generated are in real time (few seconds to minutes) interpretation is difficult in the absence of a strong signal. BIAcore, a commercially available SPR sensor, detected 10^5 *L. monocytogenes* in less than 30 min *(36)*.

Piezoelectric biosensors measure resonance frequency changes when the mass of quartz crystals changes in

TABLE 3. *Representative applications of emerging technologies in food pathogen detection*

Assay name	Target pathogen (matrix, detection levels reported)	Selected references
Real-time PCR (RT-PCR) format		
SYBR Green I	*E. coli* O157:H7, *L. monocytogenes, Salmonella* (fresh produce; 1–10, 1,000, 1–10 cells/ml, respectively); *Salmonella* (fresh vegetable rinse water, 1–10 cells/ml); *S. aureus* (beef samples, 10 cells)	*1, 12*
SYBR Green I + immunomagnetic separation (IMS)	*Salmonella* (milk, ground beef, alfalfa sprouts; 1 CFU/ml, 25 CFU/25 g, 1.5 CFU/25 g, respectively)	*85*
5′ nuclease (hydrolysis probes)	*E. coli* O157:H7 (pure culture, milk, apple juice, beef, and beef enrichment; 10^3–10^9, 10^4–10^9, 10^5–10^9, 10^0–10^3 CFU/ml, respectively); *S. aureus* (beef samples, 100 cells)	*1, 52*
5′ nuclease (hydrolysis probes) + IMS	*E. coli* O157:H7 (buffer solution and ground beef; $<5 \times 10^2$ cells/ml and 1.3×10^4 cells/g, respectively); norovirus (strawberries, 3–7 RT-PCR units)	*41, 103*
Molecular beacon	*E. coli* O157:H7 (skim milk, 10^3–10^9 CFU/ml); *Salmonella* (cantaloupe, mixed salad, cilantro, and alfalfa sprouts; as few as 4 CFU/25 g with enrichment)	*69, 82*
Fluorescence resonance electron transfer (FRET)	*L. monocytogenes* (reconstituted nonfat dry milk, 10^3–10^4 CFU/25 ml); *E. coli* O157:H7 (25 g of raw ground beef and 375 g of raw boneless beef, 10 cells)	*31, 64*
Reverse transcriptase PCR	Detects *Listeria* spp. in 8 h (includes 4-h enrichment) from stainless steel surfaces. Sensitivity ~10 CFU/ml for all *Listeria* except *L. grayi,* which is ~30 CFU/ml	*29*
Biosensor		
Fiber-optic biosensors (FOBS) (evanescent-wave biosensors)	*E. coli* O157:H7 and Shiga-like toxins (SLTs) (pure SLTs, ~0.5 μg/ml; ground beef, 10^5 cells with SLTs); *L. monocytogenes* (frankfurter sample, 5.4×10^7 CFU/ml); *E. coli* O157:H7 (pure culture, 10^3 CFU/ml; ground beef, 1 CFU/ml after 4 h of enrichment); *S. enterica* serovar Typhimurium (spent sprout irrigation water, 50 CFU/g); *L. monocytogenes* (hot dog and bologna after enrichment, 10–1,000 CFU/g); FRET-based *Salmonella* Typhimurium (homogenized pork, 10^5 CFU/g; *E. coli* O157:H7 (buffer solution, 6.5×10^5 CFU/ml); staphylococcal enterotoxin A (hot dogs, potato salad, milk, and mushrooms, 10–100 ng/g)	*13, 43, 44, 60, 61, 63, 65, 110, 126, 139*
Surface plasmon resonance (SPR)	*Salmonella* Typhimurium (10^2–10^9 CFU/ml); *E. coli* O157:H7, *Salmonella* Typhimurium, *Y. enterocolitica, L. monocytogenes* (10^5 cells/ml); *Salmonella* Enteritidis and *E. coli* (skim milk, ~25 CFU/ml); *E. coli* O157:H7 (milk, apple juice, ground beef, 10^2–10^3 CFU/ml); *L. monocytogenes* (whole cell, 2×10^6 CFU/ml)	*68, 89, 100, 101, 162, 163*
Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors	*E. coli* O157:H7 (buffer solution, 1 cell/ml); *E. coli* O157:H7 (broth and ground beef, 50–100 cells/ml); *E. coli* O157:H7 (10^3–10^8 CFU/ml); *E. coli* O157:H7 (ground beef, ~10 cells/ml)	*20, 21, 78, 132*
Cell-based sensors (B cell and cytotoxicity assays)	*Listeria* spp. (pure cultures); *L. monocytogenes* and *Bacillus cereus* (bacteria culture); with immunoseparation *L. monocytogenes* (hot dogs, bologna, raw beef, chicken, and pork samples; enriched food samples)	*8, 48, 124*
Optical scattering	*Listeria* spp. (1.2–1.5 mm colony size, approximately 10^{12}–10^{13} individual bacteria)	*6, 10, 109, 134*
Multi-analyte array biosensor	*Salmonella* Typhimurium and *L. monocytogenes* (''complex samples''); *E. coli* O157:H7 (pure culture and liquid food samples 10^4–10^7 CFU/ml)	
Proteomic biosensor (reflective interferometry)	Label-free detection of enteropathogenic *E. coli* (EPEC) in cell cultures	*51*
Microarray		
Oligonucleotide/amplicon arrays	*Salmonella, E. coli* (screened for 25 virulence and 23 antimicrobial resistance genes); with IMS *E. coli* O157:H7 (chicken rinsate without enrichment, 55 CFU/ml); *Campylobacter* spp., *S. aureus,* enterotoxin genes, *Listeria* spp., and *Clostridium perfringens* toxin genes	*18, 19, 24, 123*

TABLE 3. *Continued*

Assay name	Target pathogen (matrix, detection levels reported)	Selected references
Suspension microarray (Luminex/xMAP)	*L. monocytogenes* (broth cultures)	*16*
Spectroscopy		
Surface-enhanced Raman scattering	*E. coli* (aqueous suspensions, 10^3 CFU/ml); *Listeria* spp. (discrimination between six species)	*47, 49, 121*
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)	16S rRNA PCR amplified; various bacteria colonies; *E. coli* and *Bacillus cereus* (bacteria mixture)	*58, 74, 156*
Intact cell MALDI-TOF MS	*E. coli* (single colony); *E. coli* O157:H7 (bacterial cells)	*17, 81*
Fourier transform infrared (FTIR)	Various (bacterial cocktail of three different species, 10^9 CFU/ml); *E. coli* O157:H7, *B. cereus, Listeria innocua* (apple juice, 10^9 CFU/ml)	*2, 167*
Others		
BEADS (biodetection enabling analyte delivery system)	With integrated IMS/multiplex conventional PCR, *E. coli* O157:H7, *Salmonella, Shigella* spp. (aqueous solution, 100 cells/organism)	*131*
Flow cytometry	*E. coli* O157:H7 (with IMS + enrichment, ground beef, 4 cells/g); *E. coli* O157:H7 (milk, apple juice, ground beef; 10^3 cells/ml of milk or apple juice, 10^3 cells/g of ground beef); *L. monocytogenes* (with IMS, 10^2–10^8 CFU/ml)	*50, 122, 164*
Immunomagnetic bead–immunoliposome (IMB-IL) fluorescence assay	*E. coli* O157:H7 (aqueous matrices: water, apple juice, and cider, <1 CFU/ml)	*28*
Phage	*E. coli,* 1–10^8 CFU/ml in 1.5–10.3 h (pure culture). In lettuce leaf washings, 130–10^8 CFU/ml in 2.6–22.4 h	*111*
PCR + MS	Distinguished 10 bacterial species. LOD: 0.5 genome equivalents/PCR. Human adenovirus screen: 500 samples/day at sensitivity of 100 genomes/reaction. Automated system: 1,500 PCRs/day	*15, 80, 118*
Electrochemiluminescence (ECL)/luminometer	ECL-IMS detection (~1 h) of *E. coli* O157:H7 and *Salmonella*: 10^2–10^3 cells/ml in buffer; 10^3 cells/ml in foods (milk, juices, ground beef, and minced chicken and fish)	*168*
	Detection of *C. botulinum* toxins A, B, E, and F in foods (milk, apple juice, ground beef, pastry, and raw eggs). LOD: 50–100 pg/ml	*112*
Quantum dots	IMS–quantum dot analysis for *Salmonella* in chicken carcass wash water, sensitivity of 10^3–10^7 CFU/ml. *Salmonella* and *E. coli* O157:H7, 10^4 CFU/ml in 2 h (buffer). Immunostaining of *L. monocytogenes*	*140, 165, 166*

response to the binding of analytes to antibodies immobilized on the crystal surface.

Cell-based sensors use interdigitated microsensor electrodes to measure changes in conductivity seen in cell membranes when eukaryotic cells interact with pathogens. Live bacteria or active cytotoxins that affect the integrity of the membrane alter the conductivity and provide a measurable signal (i.e., impedance of the cells). In the commercial CANARY™ (''cellular analysis and notification of antigen risk and yields'') system, antibodies bound to B lymphocytes are engineered to express aquorin, a bioluminescent protein, which emits a light signal in the presence of a specific antigen *(36)*.

4.2. Evaluation of Emerging Technologies Based on Performance Criteria

In response to the FSIS request that NACMCF examine the merits of emerging technologies, the Committee evaluated each of the assays listed in Table 4, using the criteria specified in the charge. A few additional criteria, which the Committee believed were important, were added and also considered in the assessment.

The Committee would like to clarify how several criteria were used to assess new and emerging technologies. The charge requested an assessment of technologies that can be used for enumerating indicator organisms. The Committee decided that the criterion of ''scope'' of analysis, should include the flexibility of a technology to detect indicators and/or pathogens. The ability to enumerate indicators is addressed under the criterion ''quantify.'' The charge also requested an assessment of the adaptability of the assay to different sample matrices and/or testing situations; i.e., food, environmental, clinical, etc. The Committee found it difficult to score this criterion, as few assays can be applied to the direct detection of the agent in the sample matrix without some sort of pre-analytical sample preparation. Therefore, the assay by itself should not be regarded as the

TABLE 4. *Evaluation of emerging technologies for analysis of pathogens in foods*[a]

Format	Assay name	Speci[b]	Sensi[c]	Scope[d]	Adapt[e]	Quantify[f]	Viability[g]	Data/Tran[h]	Speed[i]	xFSIS[j]	Target[k]	Afford[l]	Through[m]	Maturity[n]
RT-PCR	SYBR Green I	+	++	−	NA	+	−	+	+	++	−	+	++	++
	SYBR Green–IMS	+	++	−	NA	+	+/−	+	+	++	−	+	++	++
	5′ nuclease	++	++	−	NA	+	−	+	+	++	+	+	++	++
	5′ nuclease–IMS	++	++	−	NA	+	+/−	+	+	++	+	+	++	++
	Molecular beacon	++	++	−	NA	+	−	+	+	++	+	+	++	++
	FRET	++	++	−	NA	+	−	+	+	++	+	+	++	++
	Reverse transcriptase PCR	++	+	−	NA	+	+	+	+	++	+	+	++	+
Biosensors	Fiber optic	++	++	+	NA	+	+	+	++	+	++	+	++	+
	SPR	++	++	+	NA	+	+	+	++	++	++	+	++	+
	PEMC	++	++	0	NA	+	+	+	++	+	0/+	+	++	0
	Cell-based sensor	+	+	−/0	NA	+	+	+	++	+	−/0	++	+	+
	Optical scattering	0/+	+	−	NA	+/−	++	+	+/−	+	0	++	−	0
	MAAB	++	++	++	NA	+	+	+	+	+	++	+	+	+
	Proteomic	++	+	0	NA	0	+	+	0	−	−	0	0	0
Microarray	Amplicon arrays	++	++	++	NA	−	−	0	+	−	++	−	−	0
	Oligo arrays	++	++	++	NA	−	−	0	+	−	++	−	−	+
	Suspension array	++	++	++	NA	−	−	0	+	−	++	−	−	0
Spectroscopy	Surface-enhanced Raman	++	0	0	NA	0/+	−	0	++	−	++	−	−	0
	MALDI-TOF MS	0/+	0	NA	NA	−	−	+	+	−	−	−	−	0
	FTIR	0	0	−/0	NA	−	−	+	+	−	−	−	−	0
Other	Flow cytometry	0/+	+	+	NA	++	+/−	+	+	+	+	−	−	+
	Immunoliposomes	++	+	+	NA	0	+	+	+	+	−	++	0	0
	Phage	+	+	−	NA	+	+	0	+	+	−	0	+	0
	PCR + MS	++	++	0	NA	0	+	+	++	+	++	0	++	+
	Luminometer/ECL	++	++	++	NA	0	+	+	+	+	++	++	+	++
	Quantum dots IMS	+	+	+	NA	+	++	+	+	+	+	++	+	+

[a] Scoring system: −, poor; 0, unknown or neutral; +, good; ++, better; NA, not applicable.
[b] Speci, specificity; scored as the ability of the assay to detect the target specified.
[c] Sensi, sensitivity; scored as limit of detection (LOD). However, because the LOD for an assay can vary greatly depending on food matrix, it was scored here based on pure cultures only.
[d] Scope, scope of analyses; capability of the assays to expand to include more targets, which in addition to those mentioned in the charge could also include viruses, SNPs, and indicator organisms. In accordance with our interpretation of the charge, existing RT-PCR assays, which already can simultaneously detect multiple (3–4) targets, but yet scored poor (−) due to the limited availability of fluorophores to enable adding more targets. In contrast, DNA microarrays that have the capability to test thousands of targets score very well for scope. Assays such as MALDI-TOF or others that require pure cultures for analysis were scored as NA.
[e] Adapt, adaptability to other matrices; ability of the assay to adapt to various matrices and testing situations, i.e., food, environmental, and clinical samples.
[f] Quantify, enumeration; capability of an assay to enumerate the number of bacteria present in the sample or to quantify the target. For many assays, a standard curve using known target number versus signal strength can be established from which, the target levels in the sample can be quantified based on the signal detected.
[g] Viability; capability to determine whether the target is viable or non-viable. Because some of these assays will detect the target regardless of the organism's viability, this criterion is important to assess the public health significance of the data.
[h] Data/Tran, data acquisition and transfer; ease with which the analytical data are collected and whether they can be disseminated electronically.

TABLE 4. *Continued*

[i] Speed; scored on whether the assays are faster to perform than conventional microbiological methods. The scoring was based solely on the performance of the assay itself and did not consider the time required for culture enrichment or sample preparation. However, we did take into account procedures that are part of the assay. For instance, DNA microarray requires PCR prior to analysis, so PCR is part of the method and requires additional time. Hence, arrays were scored as only good (+). Biosensors, which require little or no additional procedures prior to testing, were scored as better (++).

[j] xFSIS, incorporation into the FSIS methods. The three pathogens currently tested by the FSIS are *Salmonella*, *Listeria monocytogenes*, and *E. coli* O157:H7, so we evaluated how easily the assays can be incorporated into existing procedures. All the RT-PCR scored better (++) because the FSIS is already using some of these assays, and it will be easy to change to another test. The Committee also felt that biosensors can be easily incorporated into existing methods and were scored as good (+). However, implementation of arrays and other assays may be complex logistically, hence they scored as poor (−).

[k] Target, simultaneous testing of multiple targets; criterion added to assess the assay's capability to detect various targets simultaneously. For example, RT-PCR that uses SYBR Green scored as poor (−) because it is based on intercalation of dye to double-stranded DNA without differentiation. However, other RT-PCR that use specific probes scored as good (+). Some biosensors and certainly DNA arrays can accommodate multiple targets and hence scored as better (++).

[l] Afford, cost and resource efficiency; scored not on a per test basis but rather as overall cost of the test, including capital equipment, maintenance contracts, training needs, and assay costs.

[m] Through, throughput; criterion added to assess whether the assay can be used to screen large numbers of samples. For example, many RT-PCR and biosensors can accommodate multiple samples, so they scored as better (++), but arrays, which can test for multiple targets within one sample but not multiple samples, scored as poor (−).

[n] Maturity; assay's commercial availability. Unlike the rest of Table 4, a score of good (+) was given when the assay was commercially available and better (++) was given when the assay had been evaluated.

sole component in the testing protocol. In fact, the efficiency and performance of any assay is strictly dependent on whether the sample was adequately prepared prior to analysis. For example, an assay used to screen a blood sample for microbial contamination may not be directly applicable to foods unless the food has been previously subjected to a short culture enrichment period to suppress competitive microflora, resuscitate stress-injured pathogens, dilute potential assay inhibitors, and/or increase the numbers of the target analyte. Similarly, some assays require that the toxin or the DNA content of the target organism in the sample be extracted prior to analysis. Once properly extracted, the target DNA or toxin can be screened using a variety of assays, regardless of whether the original sample was a food, a swab or blood. Because of considerations such as these, the Committee scored the ''adaptability'' criterion as not applicable (NA) for all assays.

Table 4 scored each assay based on the criteria specified in the charge. The scoring system used was: −, poor; 0, unknown or neutral; +, good; ++, better. The following are descriptions of criteria used specifically in Table 4, with the abbreviations used in the table in parentheses.

Specificity (Speci). The ability of the assay to detect the target specified.

Sensitivity (Sensi). The analytical sensitivity (LOD) of the assay based on pure culture.

Scope of analyses (Scope). The capability of the assay to expand to include more targets, which in addition to those mentioned in the charge could also include viruses, SNPs, and indicator organisms. In accordance with the Committee's interpretation of the charge, existing RT-PCR assays, which already can simultaneously detect multiple (3–4) targets, scored ''poor'' (−), due to the limited availability of fluorophores to enable adding more targets. In contrast, DNA microarrays that have the capability to test thousands of targets score very well on scope. Also, assays such as MALDI-TOF or others that require pure cultures for analysis were scored ''NA.''

Adaptability to other matrices (Adapt). The ability of the assay to adapt to various matrices and testing situations, i.e., food, environmental, and clinical samples.

Enumeration (Quantify). The capability of an assay to enumerate the number of bacteria present in the sample or to quantify the target. For many assays, a standard curve using known target number versus signal strength can be established from which the target levels in the sample can be quantified based on the signal detected.

Data acquisition and transfer (Data/Tran). The ease with which the analytical data are collected and whether they can be disseminated electronically.

Speed. The assays were scored on whether they are faster to perform than conventional microbiological methods. The scoring was based solely on the performance of the assay itself and did not consider the time required for culture enrichment or sample preparation. However, procedures inherent to the assay were considered in the scoring. For instance, DNA microarray requires PCR prior to analysis, so PCR is part of the method and requires additional time, hence arrays only scored ''good'' (+). Biosensors require little or no additional procedures prior to testing and hence were scored ''better'' (++).

Incorporation into the FSIS methods (xFSIS). The ease with which the assay can be incorporated into existing procedures for *Salmonella, L. monocytogenes* and *E. coli* O157:H7. All the RT-PCR assays were scored ''better'' (++) because the FSIS is already using some of these assays and it should be easy to change to another test. Biosensors were thought to be easily incorporated into existing methods and so were scored ''good'' (+). Implementation of arrays and other assays whose implementation would be complex logistically were scored ''poor'' (−).

Cost and resource efficiency (Afford). The overall cost of the test including capital equipment, maintenance contracts, training needs, and assay costs.

Criteria not included in the charge but which the Committee decided were worthy of consideration are:

Viability. The capability to determine whether the target is viable or non-viable. As some of these assays will detect the target regardless of the organism's viability, this criterion is important to assess the public health significance of the data.

Simultaneous testing of multiple targets (Target). This criterion was added to assess the assay's capability to detect various targets simultaneously. For example, RT-PCR that uses SYBR Green scored ''poor'' (−), as it is based on non-specific intercalation of the dye to double stranded DNA, but other RT-PCR tests that use specific probes scored ''good'' (+). Some biosensors and certainly DNA arrays can accommodate multiple targets and hence scored ''better'' (++).

Throughput (Through). This criterion was added to assess whether the assay can be used to screen large numbers of samples. For example, many RT-PCR assays and biosensors can accommodate multiple samples and so scored ''better'' (++), but arrays which can test for multiple targets within 1 sample but not multiple samples scored ''poor'' (−).

Maturity. This criterion evaluated the assay's commercial availability. Unlike the rest of Table 4, a score of ''good'' (+) is used if the assay is commercially available and ''better'' (++) if the assay has been evaluated and validated for use in food testing.

4.3. Sampling and Pre-Analytical Sample Processing Technologies

A variety of techniques are available for sampling and pre-analytical processing of foods and environmental samples. Integration of these techniques with new technologies is essential for enhancing the FSIS's analytical capabilities.

4.3.1. Sampling and Pre-Analytical Sampling Considerations

Optimal strategies for collecting, transporting, and preparing test specimens are critical to the quality and interpretation of pathogen detection results. At a very basic level, sampling may be categorized as either ''destructive'' or ''non-destructive.'' In destructive sampling, such as excision sampling of carcasses, a specific weight of product is collected and tested by the laboratory as a sample test portion measured in grams. The destructive sampling-testing approach offers the advantage of near 100% recovery of the target pathogen from the sample as well as the potential for detection of the pathogen if internalized within the product. Excision is generally considered to be the sampling method that yields the highest recovery of pathogenic and indicator bacteria *(102)*. However, comparisons between swab and excision sampling showed no significant differences *(45)*.

Non-destructive sampling, such as the whole-bird rinse technique used for chicken carcass sampling or the sponge technique used to collect samples from turkey, cattle, and hog carcasses, employs an indirect means of collecting the pathogen from the surface of the product to be tested. A non-destructive sampling approach is warranted where the focus is detection of contaminants which do not penetrate below the surface of the product. Such an approach may be advantageous when it is desirable to sample a large surface area of the product (e.g., to increase sensitivity and/or potential detection of heterogeneously distributed contamination), or where the entire product or sampled surface cannot be submitted to the laboratory due to its size. For any indirect sampling approach, recovery from the product and, in some cases, the test portion may be significantly less than 100%.

The appropriate sampling method depends on the purpose of the test. For example, the optimal sampling location and method might differ if one were trying to determine the prevalence of an organism in live animals (e.g., rectal, fecal, cecal, hide, feather, pen samples) versus its prevalence in market samples (e.g., whole birds or cuts of meat) versus evaluation of the efficacy of a candidate control strategy (e.g., in-process sampling of carcasses or equipment). The sampling location and method may also be influenced by the type of information desired. For example, there may be specific locations on a carcass where

contaminants are concentrated. Carcass mapping studies have predicted the areas with concentrated contamination levels *(45, 46, 127, 128).* Another consideration in choosing the sampling location and method is minimizing the degree of disruption to the production process and the cost of product lost to sampling. These considerations have led to comparisons of the efficacy of excision and sponge sampling *(46, 53, 102, 160).* Recovered pathogen subtypes may vary with the sampling location and protocol *(125).*

Finally, suitability of the sample for the specific detection method being used (''fit for purpose'') must be considered. For example, if sampling previously cleaned or disinfected surfaces, there is a need to neutralize or remove residual antimicrobials or compounds that may interfere with the detection system. There may also be a need to dislodge attached microorganisms from the sampled portion or site, for example, as might occur during optimization of the recovery of *Salmonella* and *Campylobacter* imbedded in feather follicles or *E. coli* O157:H7 encapsulated in beef fat.

4.3.2. Novel or Emerging Sample Collection Methods

Much more research has been conducted on detection technologies than on sampling methods. Some of the relatively few examples of novel or emerging sampling technologies include the Microbial-Vac system *(87),* the sampling of beef trim combo purge *(30),* and thin surface sampling of trim *(59).* The package rinse method for *L. monocytogenes (72)* has been evaluated and was found to be superior to several other product sampling methods. Tissue paper wipes have been found to be a good alternative to sponges or swabs for environmental monitoring *(157).* Other novel or emerging sampling ideas include sampling of rinsate from spray cabinets in slaughter facilities and turkey wing tip sampling.

4.3.3. Pre-Analytical Sample Processing

There are factors aside from assay validity that can also impact the performance of a test method for pathogens. One of these is volume considerations. For example, while most nucleic acid amplification methods and biosensor approaches are theoretically able to detect a single target molecule (or cell) per sample the volume amplified utilized in these assays is very small (<10 μl). Clearly, it is not feasible to screen the entire sample volume in such a test method, so if intermittent and/or low levels of contamination are present, they are likely to be missed. An additional consideration is the fact that food samples frequently contain relatively high levels of non-pathogenic bacterial flora and/or food components which can inhibit the assay or otherwise raise the lower LOD. Furthermore, most rapid detection methods require the sample as a liquid but most foods are not liquid. These and other important issues that might otherwise influence assay performance are described in detail by Feng *(35).* These also provide the basis for the recent increased interest for the use of novel pre-analytical sample preparation technologies, most of which are intended to reduce sample volumes, remove matrix-associated inhibitors, yet simultaneously result in recovery of most (if not all) of the target pathogen.

4.3.4. Novel Approaches to Sample Preparation

Cultural enrichment could be considered the first form of pre-analytical sample processing in that this process is intended to suppress the growth of competitive microflora, dilute food-associated inhibitors, and increase the numbers of the target organism. Recent studies have focused on the refinement of enrichment media resulting in faster multiplication of the target pathogen. For example, enrichment in non-selective broth can be done with the addition of bacteriophages which eliminate certain competitive or interfering microflora *(129).* Enrichment times have also been shortened by enriching in a non-selective broth followed by immunomagnetic separation, which will provide both amplification and concentration in a single test protocol. This is the current approach being used in some *E. coli* O157:H7 testing protocols as applied to foods *(5).*

Theoretically, improvements in how samples are collected and shipped to the testing laboratory could enhance the speed, sensitivity, and selectivity of a pathogen assay. One option might be to prepare and place the sample into the enrichment medium immediately after sample collection, then ship the inoculated medium to the detection laboratory in a temperature-controlled incubation chamber, i.e., enriching the sample en route. At this time, there do not appear to be any practical methods to achieve this, at least using U.S. commercial overnight carriers. Another option might be to lyse the bacterial cells and stabilize the nucleic acids in a transport medium prior to shipping, preventing the laboratory from having to undertake time-consuming nucleic acid extraction steps. A commercially available method for preparing vaginal swabs or urine samples for the detection of *Chlamydia trachomatis* is based on this principle *(151).* Similar systems for foodborne pathogens could be developed.

Over the last decade, there has been recognition of the need for pre-analytical sample processing prior to the application of rapid and emerging test methods. This is based on the supposition that ultimately the LOD for a test could be improved if the pathogen(s) were separated and concentrated from the matrix prior to detection. The general principles applied to pathogen concentration have been reviewed elsewhere *(42, 130, 138)* and some of these approaches are detailed in Table 5.

To date, almost all of the methods outlined in Table 5 have only been applied after a prior cultural enrichment step. None of the sample preparation approaches described in Table 5 are ideal and the choice of method depends on the purpose of the analysis. For example, some sample preparation methods will concentrate and purify the entire bacterial population, while others are specific for one or more pathogens; some will result in recovery of viable cells, others will kill the target cell but maintain the integrity of the target molecule. No pre-analytical sample processing method recovers 100% of the target from a complex sample matrix, and the efficiency of concentration and purification can be matrix dependent. Further, not all methods are applicable to all types of food products. Many of the sample preparation methods are cumbersome, require specialized equipment or training, and are not adaptable to the routine processing of

TABLE 5. *Partial listing of microbiological sample preparation approaches*[a]

Method	Principle/application	Advantages/efficacy	Comments	References
Ion exchange resins	Cationic exchange resins bind bacteria by ion exchange; release of bacteria from resin accomplished by pH manipulation	Rapid; relatively inexpensive; broadly inclusive	Not practical for large sample numbers; sample pre-treatment to remove debris recommended; pH manipulations needed for desorption; destroys cell viability	*57*
Metal hydroxides	Hydroxides of zirconium, titanium, or hydroxyapatite adsorb and ''flocculate'' bacteria; used in conjunction with centrifugation	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	Not practical for large sample numbers; sample pre-treatment to remove debris required; appears to work best on less complex sample matrices	*11, 27, 73*
Aqueous two-phase partitioning	Cells partition in one of two immiscible liquid phases (polyethylene glycol and dextrans) based on charge	Rapid; inexpensive; simple; broadly inclusive	Not practical for large sample numbers; partitioning frequently incomplete; composition of the phases may impact cell viability; fat interferes with separation	*67, 75*
Affinity separation	Immobilization of molecules (lectins) with high affinity for bacteria to a solid support such as agarose beads, affinity columns, or magnetic particles	Rapid; simple; specificity unknown	Not practical for large sample numbers; expensive; sample pre-treatment to remove debris recommended; release of bound cells may be inefficient; best applied to small sample volumes	*104, 108*
Simple centrifugation	Low speed ($<1{,}000 \times g$) sediments debris; high speed ($>8{,}000 \times g$) sediments bacteria; used with or without coagulation or flocculation	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	Not practical for large sample numbers; bacteria adhere to and sediment with matrix components; best if preceded by an elution step	*158*
Differential centrifugation	Low speed centrifugation followed by high speed centrifugation; used with or without coagulation or flocculation	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	Not practical for large sample numbers; bacteria adhere to and sediment with matrix components; few products available to promote desorption without destroying cell viability	*86, 94*
Density gradient centrifugation	Cell separation by centrifugation within a density gradient; requires use of chemical additives to establish a gradient	Can be designed to separate very distinct species from one another	Not practical for large sample numbers; expensive; difficult to perform; osmotic strength of gradient destroys cell viability; fat entraps bacteria at interfaces	*70*
Crude filtration	Cheesecloth; filter paper; filter homogenizer bags	Rapid; inexpensive; simple; broadly inclusive; amenable to large sample sizes	May not be practical for large sample numbers; highly particulate foods clog filters; bacterial cells can absorb to the filter or retentate	*37, 153*
Electropositive and -negative filtration	Bacteria tend to have a net negative charge, so electropositive filters often used; sample pre-filtration to remove debris frequently required	Rapid; inexpensive; simple; broadly inclusive	Not practical for large volumes and sample numbers; filters clog rapidly even if samples are pre-filtered; desorption of bacteria from filters frequently inefficient	*137*
Immunoseparation	Immobilization of antibodies to a solid support such as polystyrene beads or magnetic particles	Rapid; simple; highly specific; standard method for some foods	Not practical for large sample numbers; expensive; sample pre-treatment to remove debris recommended; many formats available; best applied to small sample volumes although recirculating IMS is available for larger volumes	*41, 56, 99, 153, 161*
Nucleic acid extraction	Purification of DNA or RNA template	Removes matrix-associated inhibitors and concentrates template; matrix- and method-dependent efficacy	Not practical for large volumes or sample numbers; many commercial kits available, some with matrix specificity; automation available but expensive; destroys cell viability	*117*

TABLE 5. *Continued*

Method	Principle/application	Advantages/efficacy	Comments	References
Novel methods	Phage based, synthetic phage ligand to capture target bacteria; magnetic nanoparticles, ultra small magnetic particles to which target-specific ligands are conjugated	Less susceptible to cross-reactivity; reagent stability; nanoparticles have higher capture efficiency than microbeads	Very new technologies with limited history of performance	*66, 155*

[a] Adapted from Stevens and Jaykus, 2004 *(130).*

large numbers of samples. The volume that can be processed in sample preparation is also method dependent. Sometimes the complexity of matrices requires the use of multiple sample preparation methods in sequence. Taken together, it is clear that the field of pre-analytical sample preparation is fertile ground for future research that is needed to maximize the potential benefits of emerging methods.

5. CONSIDERATIONS WHEN CHOOSING EMERGING TECHNOLOGIES AND METHODS (QUESTIONS 1 AND 2)

Some of the advantages of emerging technologies are a reduced time to detection, a high degree of sensitivity and specificity, and a low LOD. If robust and dependable pre-analytical sample preparation methods were available, one could even envision completely bypassing cultural enrichment. While this is theoretically possible, there are many other considerations which must be taken into account before adopting emerging technologies, whether preceded by cultural enrichment or not. These are discussed below.

5.1. Potential for Rapid, On-Site Analysis

Rapid, or ideally real-time, screening methods that might be suitable for on-site and in-plant use (e.g., biosensors) would be particularly valuable. Such methods offer the opportunity to screen samples prior to shipment to the laboratory, thus saving resources and decreasing the time a product needs to be held while being tested. However, these methods must be held to high performance standards and accountability to minimize false-positive and -negative results, and they must be appropriately validated before use.

Cultural enrichment is the universal starting point for most pathogen detection assays. The manipulation of cultures enriched for pathogens within or even adjacent to a food processing facility requires strict precautions to prevent cross-contamination. For on-site analysis to become widely practical, either enriched pathogen cultures would need to be self-contained, leak proof, and disposable, or cultural enrichment steps would need to be eliminated. Self-contained pathogen assays are currently available, but only for a few applications, e.g., an assay for the detection of *Listeria* spp. *(4).*

5.2. Discrimination between Viable and Non-Viable Cells

An inherent advantage of culture-based methods is the detection of viable cells capable of causing illness. Culture-based methods are considered to be the ''gold standard'' and are critical in helping the FSIS to meet its mandate of assuring the safety of meat, poultry, and egg products. However, many of the newer tests target the pathogen's nucleic acids, which may be detected long after cell death (days to weeks). This means that nucleic acid amplification methods cannot always differentiate living from dead cells. For foods, this is especially important due to commonly used food processing or preservation methods which are intended to inhibit or inactivate pathogens.

The use of nucleic acid amplification methods in pathogen screening is easily defensible if followed by culture-based confirmation. However, if the elimination of cultural enrichment is an eventual goal, the ''live-dead'' dilemma will need to be resolved. Recently, the DNA intercalating agents ethidium monoazide and propidium monoazide have been used in conjunction with quantitative PCR for the selective detection of live cells of foodborne pathogens *(95–98, 114, 115, 159).* At the time of this writing, none of these methods have been commercialized and it is still unclear as to whether the approach will be suitable for widespread application for viability discrimination for the detection of pathogens in foods.

Though not a viability issue per se, a positive result with a toxin gene-specific PCR assay indicates that those gene sequences are present in the target organism, and that the cells are potentially toxigenic. It does not, however, assure that the gene is actually expressed or that the toxin, if produced, is functional *(36).*

5.3. The Need for a Viable Isolate

Related to the viability issue is the need for a live culture in order to further characterize the strain by phenotypic and/or genotypic methods (see Section 2). Many of the newer detection platforms are based on the detection of one or more genes or antigens that are present in the microbial target. Such molecular targets might be species or serotype specific, associated with virulence located on plasmids, cell surface components, or associated with biochemical abilities. Because of the sensitivity and discriminatory power of some of these methods, especially the genetically-based ones, it is no longer essential that viable isolates be generated for testing purposes. However, when a pure culture is not available for further testing, subsequent confirmation or subtyping cannot be performed. Even though this situation may not be an important factor for tests that target a single gene, consider cases in which

tests rely on results from multiple genetic targets. The interpretation of these results can have serious shortcomings because the result might indicate a positive test for all the required markers that would ordinarily identify the designated pathogen. However, in a non-clonal culture, the individual positive test results might have been generated by genes present in different cells, with no one cell having the required genotype to give a confirmed positive result. In this case, further analyses on purified strains would fail to confirm the presence of the pathogen. While such a test might be appropriate for screening purposes, especially when time is of the essence, one may anticipate a higher level of false positives under these circumstances.

Bacterial strain isolates can be readily archived and stored for years. Although nucleic acid extracts also can be archived, the stability of the material is questionable. Since the material would undoubtedly consist of a mixture of nucleic acid moieties, differential degradation would increase uncertainty that the identical material is being tested upon subsequent analysis. An additional difficulty is that the same material (that is, a pure culture of an isolated pathogen) would not be available in a legal dispute. Such inconsistencies, understandable from a scientific standpoint, could lead to substantial difficulties in a legal context.

With newer technologies often come faster, more specific and sensitive assays, but the complexities of testing foods remain. Cross-contamination with positive controls or other sources and the potential for antibody cross-reactivity or non-specific binding linger as issues to be addressed. Furthermore, matrix-associated inhibitors can impact assay performance. In short, having an isolate for confirmation remains the definitive proof of contamination.

5.4. Qualitative versus Quantitative Results

Most foodborne pathogen detection methods are qualitative and yield positive or negative results (see Section 3). However, determining the number of pathogenic cells in a sample can provide important information for process control, risk assessment, and support of regulatory decision-making. With the introduction of quantitative real-time PCR (qPCR) techniques, direct estimation of pathogen load is becoming practical. The basis for such quantification is that the fluorescent signal generated by the amplification reaction is proportional to the concentration of DNA in the sample. Hence, by incorporating standards in RT-PCR assays, it is possible to estimate the absolute or relative amounts of nucleic acid target, which indirectly estimates the number of microorganisms present in the sample *(113, 135)*. While qPCR has promise, issues related to viability, the requirement for enrichment, the effects of matrix-associated inhibition and subsequent target recovery continue to affect accuracy.

5.5. Multianalyte Considerations

Within a testing program designed to screen foods for the presence of specific pathogens, single-target assays meet a critical need. However, in surveillance situations, the process control setting, and outbreak investigations, multianalyte analysis (sometimes called multiplexing), in which two or more targets are measured simultaneously in a single assay, offers an increase in test throughput, work simplification (i.e., fewer assay tubes, fewer pipeting operations, etc.), and possibly a reduction in the overall cost per test. Many of the newer technologies, e.g., RT-PCR, biosensors, genotype and phenotype microarrays, offer the potential to detect several genes, species, or toxins simultaneously.

Obstacles do exist that might preclude the routine implementation of multianalyte assays. These include the possibility of cross-reactions and difficulties in optimizing the assay as applied to the individual analytes or the wide variety of sample matrices. Also, with the addition of multiple targets comes the possibility of quality control failure for one analyte that could jeopardize the validity of an entire run. If an assay needs to be repeated, any savings of cost or analyst time could be lost. The difficulties with non-clonal cultures have been discussed above (Section 5.3).

5.6. Fit for Purpose

The selection of new methods must always be made with the consideration that they must be appropriate for intended use. Presently, it appears that the emerging pathogen detection methods under development will be most appropriate for screening purposes. Due to complications described above, methods used for regulatory decision-making will most likely need to remain based on standard cultural procedures, at least in the near term.

6. REVIEW OF TECHNOLOGIES AND METHODOLOGIES TO MEET PUBLIC HEALTH GOALS (QUESTION 6)

As stated in the rationale for addressing the charge (Section 1), the NACMCF determined during its deliberations that the recommendation of any new technology for use by the FSIS must be presented in an appropriate context to have applicable meaning and utility. Microbiological testing objectives and resulting test criteria of any proposed new technology or method should clearly support the FSIS testing objectives outlined in the FSIS Strategic Plan *(146)*. The broad elements of testing itself must be addressed in the submitted proposal, including statistical and sampling requirements, sample collection and transportation, laboratory analysis and reporting, database generation, and statistical analyses. The proposal should also address the degree of validation required for adoption and use of the method within the agency (e.g., interim, no validation or emergency use only, single lab validation, full collaborative validation). This section describes a process for the FSIS to consider before adopting a new method for an intended programmatic purpose, within the context of the public health focus. It was the intent of the Committee to describe the process in broad, rather than prescriptive, terms to allow the FSIS flexibility to develop their own policy and protocols.

6.1. Overview of the Proposed System for Evaluating New Technologies and Methods

Because laboratory methodologies for regulatory use do not exist in an analytical vacuum, it is necessary to consider

external factors when evaluating the appropriateness of technologies. A model for method evaluation could consist of a holistic approach such as:

1. Indicate which FSIS public health strategic goal or objective the method attempts to address
2. Describe what sampling plans can be implemented and resulting statistical consequences
3. Analyze the performance and capabilities of candidate method(s)
4. Establish reporting requirements

The development of microbiological methods for the analysis of food to detect and enumerate bacterial pathogens is a complex and costly process. Presently, the FSIS does not have the mandate or the resources to conduct methods research in-house and therefore must rely on a variety of resources from outside the Agency. This leveraging could include other governmental agencies (such as the ARS, FDA and CDC), companies that carry out methods development research (especially methods for industry use), and academic researchers who may have innovative ideas needing further development before they can be adopted for regulatory use. For methods developed by these diverse groups to receive a fair, timely and technically appropriate review and evaluation, the FSIS should consider adopting a comprehensive system for ongoing evaluation, selection, optimization, validation, and implementation of new microbiological testing technologies/methodologies to meet public health goals. This recommended ''idealized'' system should include the staff, facilities, and organizational structure necessary for successful implementation of appropriate new technologies that will allow the agency to meet its public health goals. Descriptive text and a schematic diagram (Fig. 1) of the proposed system for evaluating new technologies and methods follow.

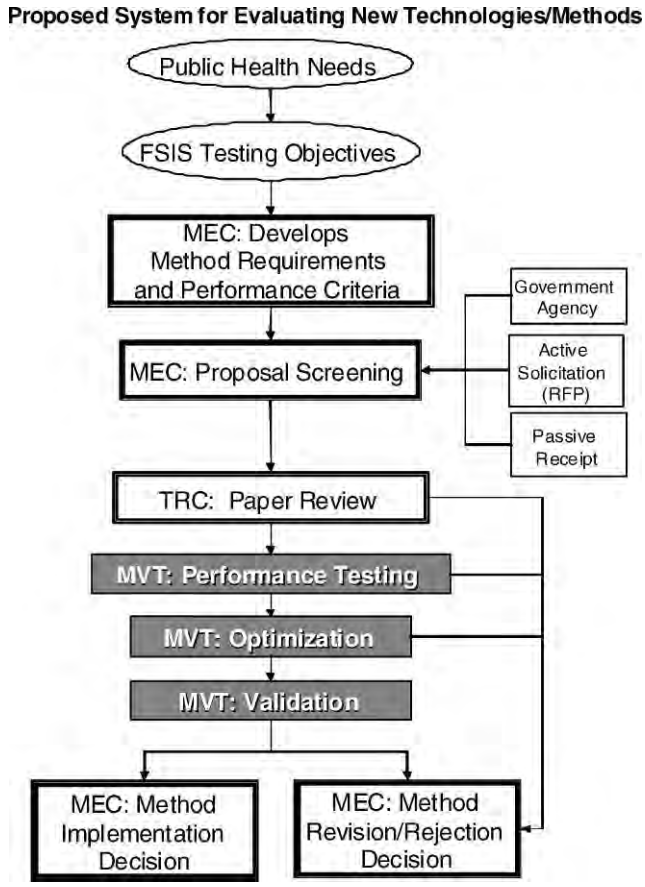

FIGURE 1. *Proposed system for evaluating new technologies/methods. The proposed system includes a Method Evaluation Committee (MEC), a Technical Review Committee (TRC), and a Method Validation Team (MVT). The MEC is a standing committee composed of subject matter experts that identify, define, and develop testing objectives/needs, proposal requirements, and performance criteria. The TRC is an* ad hoc *committee of technical experts that conducts technical reviews of proposals for new technologies/methods referred by the MEC. The MVT is a committee of laboratory and other experts responsible for performance testing, optimization, and validation of those methods that have been selected by the TRC for further testing.*

6.2. Method Evaluation Committee (MEC)

The Methods Evaluation Committee (MEC) is envisioned as a standing committee that will organize and coordinate the solicitation, receipt, and initial categorization and screening of proposed methods. The MEC should be composed of subject matter experts from multiple program areas within the FSIS with input from academia, industry, and other stakeholders as needed. The MEC should include experts who have responsibility for policy development, data analysis, public health, and contracting.

To ensure that the FSIS has the opportunity to consider and evaluate all appropriate new methods and technologies for use in its laboratories, the MEC should serve as the point of contact for method submissions coming into the FSIS. The MEC would receive method descriptions and other testing proposals and review them for their applicability in supporting the Agency's public health objectives. If these methods appear to be able to fulfill an FSIS testing need, they will be forwarded to a specially constituted Technical Review Committee (TRC).

The MEC will also work closely with senior management to identify, define, and develop the FSIS's testing objectives and needs and develop proposal requirements and performance criteria, including checklists and guidelines to determine if proposed methods should proceed to the technical review stage. If at any point in the process, the proposal fails to meet the established criteria the MEC may choose to generate a report detailing the method's failings and notify the submitter.

6.2.1. Develop Proposal Requirements and Performance Criteria

A standardized evaluation protocol should be developed and applied to any proposal for new or revised technologies and methods for use in the FSIS laboratories. Once the testing objectives have been defined by the FSIS, the MEC should construct checklists for (i) proposal format requirements and (ii) method performance criteria that an analytical method must possess to be considered for use in the FSIS laboratories.

6.2.1.1. Proposal format requirements. For a technology or method to be considered by the FSIS, a formal

written proposal must be submitted to the FSIS that meets the proposal format requirements. The written submission must be organized for easy review, with all logically related materials sorted into appropriate sections and all pages numbered. The FSIS should develop a standardized form and make it available on its web site.

At a minimum, the written report must include the following information *(40)*:

- *Contact information.* All technologies and methods submitted to the FSIS for consideration must include an address, phone number, and e-mail address for the point of contact (POC). The POC should be able to answer detailed questions concerning the development and application of the submitted technology or method.
- *Date submitted.*
- *Background.* A summary of the test principle and the target agent must be included, as well as the matrices to which the test system or method can be applied. The nature of the method, either qualitative or quantitative, should be identified. The background should also include justification and reasons for either an initial submission or substitution of the method for an existing FSIS method.
- *Safety precautions.* A description of any biological, chemical, or radiological hazards associated with the method must be included along with any special instructions for disposal of hazardous materials.
- *Sample collection.* Instructions for the collection, handling, and storage of the test samples, including criteria for sample rejection, must be included.
- *Sample preparation.* A description of the special procedures that are used to prepare a sample for analysis must be submitted.
- *Reagents.* Critical reagents required to complete the submitted test or method must be identified, including the source (commercial or governmental), storage requirements, and any regulatory stipulations for purchase and utilization. Suitable reagent substitutions should be provided, as applicable.
- *Reagent preparation.* Procedures for the preparation of the submitted test or method reagents must be clearly delineated.
- *Equipment, supplies, and analytical instrumentation.* Sources (commercial or governmental) and regulatory requirements for instrumentation and supplies needed to complete the test or method must be identified. Suitable equipment substitutions should be provided, as applicable.
- *Equipment operation.* Instructions for operation of equipment necessary to complete the submitted test or method must be included. These may include manufacturer instructions, identification of variable parameters, etc.
- *Laboratory protocol.* Clear and concise step-by-step instructions of the test method must be given for rapid implementation in another laboratory.
- *Data analysis.* Raw data, statistical methods, and a summary of data analysis must be included. Results from multiple laboratories should be included, if available.
- *Quality assurance.* Procedures and controls for reagents and instrumentation must be included.
- *Method performance.* Reportable range, sensitivity, specificity, accuracy, precision, linearity, throughput, and sample process time must be determined on test matrices as well as standards.
- *Limitations and interferences.* Concerns related to analytes and matrices.
- *References.* Documentation used to support the development, testing and validation of the submitted test or method must be included.

These generic proposal format requirements may be modified by the MEC as needed to address specific needs within the FSIS.

6.2.1.2. Method performance criteria. To evaluate new technologies and methods, the FSIS should develop specific method performance criteria based on practical considerations for the intended use. Information must be provided in order to evaluate the degree to which the method has been optimized or validated and to determine if the method will be suitable for its intended use by the FSIS. These validations may be done by the submitter with appropriate data submitted for review by the TRC or may be done internally by the FSIS.

Administration information and data needed to evaluate method performance should include:

1. **Need for Method:**
   - Has the need for a new method been clearly defined?
   - Does the method address a specific FSIS public health objective?
2. **Method Background:**
   - Was there sufficient summary of the test principle and the target agent?
   - Was there inclusion of matrices to which the test or method can be applied?
   - Was the qualitative or quantitative nature of the method identified?
3. **Safety Precautions:**
   - Was a description of any biological, chemical, or radiological hazards associated with the method included?
   - Were there instructions for the disposal of hazardous materials included?
4. **Sample Collection and Sample Prep:**
   - Were there instructions for the collection, handling, and storage of test samples, including criteria for sample rejection included?
   - Was there a description of the special procedures that are used to prepare a sample for analysis?
5. **Reagents:**
   - Were any critical reagents required to complete the submitted test or method identified?
   - Did the submitter identify sources, storage requirements, and any regulatory stipulations for purchase, utilization and disposal of reagents?
   - Were suitable reagent substitutions provided, if applicable?
   - Were procedures for the preparation of reagents clearly delineated?

6. **Equipment, Supplies, and Instrumentation:**
   - Did the submitter identify sources and regulatory requirements for instrumentation and supplies needed to complete the submitted method?
   - Were suitable equipment substitutions provided, if applicable?
   - Were sufficient instructions for operation of equipment provided?
7. **Quality Assurance Procedures:**
   - Were quality assurance procedures and controls for reagents and instrumentation included?
8. **Method Performance:**
   - Was information provided on method performance, including methods used to determine the following parameters?
     Sensitivity
     Specificity
     Accuracy
     Precision (includes repeatability and reproducibility)
     Linearity
     Measurement Uncertainty
     Ruggedness
     Matrix Effects
     Throughput
     Sample Process Time
   - Were known limitations and interferences reported?
   - Were all step-by-step procedures for the method provided?
9. **Biosafety and Biosecurity:**
   - Was information given on the level of required laboratory biosafety or biosecurity?
10. **Clarity:**
    - Was the submitted method sufficiently understandable or clear for rapid assimilation and use in another laboratory?
11. **Laboratory Validation and Optimization:**
    - Were multiple strains of the target organism used (inclusivity)?
    - Were strains of non-target organisms used (exclusivity)?
    - Were a number of foods and/or food types used?
    - What was the analyte level and matrix (inoculated and uninoculated)?
    - Were appropriate replicates per food at each level tested?
    - Were samples inoculated prior to testing?
    - Were additional competitor strains present?
    - Was the method compared to the FSIS recognized method(s)?
    - Was a multiple laboratory collaborative study conducted?
12. **Final Review Recommendations by the MEC:**

After the TRC review, the MEC may recommend:

- Approved or Accepted for the FSIS implementation as submitted (sufficient laboratory review and validation done by submitter)
- Not appropriate for current FSIS stated objectives, but recommend the FSIS use this information to inform future objectives
- Not approved (provide a brief summary of deficiencies that need correction before acceptance or resubmission)

The FSIS will determine the specific acceptable numbers based upon the intended use of the method within the program.

Any other supporting documents and/or publications needed for a review and understanding of the new technology or method should also be included. All raw data should be available for review if necessary. These may include:

1. Worksheets and notebooks.
2. Identification of all matrices and analytes tested.
3. A unique identifier for all standards, controls, or test portions analyzed.
4. Organism inoculation levels and protocols.
5. Test portion weights, volumes, etc.
6. Identification of all critical standards, reagents, and instrumentation used during analysis.
7. Instrumental readouts.

These criteria may be modified by the USDA-FSIS depending upon the specific objectives and need for the new method.

6.2.1.3. Receipt of proposals: active, passive, government. Once proposal requirements have been developed, a request for proposals may be issued to advertise the FSIS's requirements and generate interest. New technologies and methods can be submitted to the USDA-FSIS as either a direct response to a call for proposals by the USDA-FSIS (active) or by another government agency, academia, or industry submitting a new or revised method to the USDA-FSIS without a formal request for proposals (passive).

Regardless of how the technology or method is submitted, the MEC will then conduct a non-technical review of the proposals and determine which ones generally meet the testing objectives defined by the FSIS. At this point, the MEC will pass onto the TRC those proposals that appear to satisfy the overall testing objectives.

A general scheme for submitting method proposals:

1. USDA-FSIS will put out a formal call for proposals in the *Federal Register* (active only).
2. The FSIS will collect all proposals and submit to the MEC for consideration (active or passive).
3. The FSIS will determine if USDA-FSIS has a need for the proposed technology or method and if there is merit for a full evaluation of the method (passive only).
4. The MEC will review proposals, obtain appropriate documentation and prioritize the submitted proposals (active or passive).
5. The MEC in consultation with appropriate USDA-FSIS personnel will identify potential technical reviewers for the proposals (active or passive).
6. Methods will be evaluated by the TRC using established criteria and recommendations will be made to the MEC (active or passive).

6.3. Technical Review Committee (TRC)

The TRC is an *ad hoc* committee constituted to conduct technical reviews of proposals for new technologies or methods referred by the MEC. The TRC will be composed of technical experts from within and outside the FSIS and will be constituted under the direction of the MEC to assure that the committee's composition contains the expertise required to perform the technical review. Thus, the TRC membership is not constant but changes to accommodate changes in technical expertise needs. Where disparate proposals are being considered more than one TRC may be required at any given time.

The TRC will undertake a technical proposal review of the method, its claims, and supporting data. The method will be evaluated and rated with respect to, but not limited by, the following parameters: sampling requirements, method sensitivity (CFU/sample), pure culture requirement, false-positive and -negative rates, ruggedness, throughput, workflow, turnaround time, credibility, cost, flexibility, data integration, quantitative and qualitative capabilities, and portability.

The TRC's review will objectively evaluate the proposals against the checklist criteria that were constructed to assure that methods would allow the FSIS to meet its testing objectives. If any of the proposed methods appear to be more appropriate for an alternative testing objective, they will be referred back to the MEC to determine if the testing objectives should be redefined.

6.3.1. Proposal Review

Following an initial proposal screening by the MEC, a TRC will be established to conduct a review of the documentation submitted for the proposed technology or method. The make-up of the TRC will be dependent upon the intended use of the method, the type of method, and the degree to which it has previously been validated. The TRC will consist of reviewers from the FSIS, other Federal agencies such as the CDC and FDA, academia, and any other expert reviewers called in by the FSIS as needed. As long as the data are submitted according to the submission requirements provided previously, this review will be conducted by a process similar to a journal review. Each panel member will review the submitted documents based upon the generic criteria established by the MEC using the checklist provided as well as any additional criteria specific to that method (to be supplied by the FSIS). Following the individual reviews, the panel will discuss the overall review by a teleconference or a face-to-face meeting in order to provide the FSIS with a consensus technical review recommendation. The proposed method can be accepted for immediate use; accepted to proceed to the next step; rejected; or recommendations made for revisions to the submitted documentation. This technical proposal review will be completed in a timely manner, within no more than one month from the time of submittal.

Following the TRC technical paper review, the proposal will be referred to the method validation team (MVT) with any necessary comments sent to the MEC. Once the MVT has completed laboratory evaluation, a complete report with recommendations will be sent to the MEC.

6.3.2. Laboratory Data Review

Submitted data will be reviewed by the TRC. The performance of top-rated methods under close-to-real-world conditions will be assessed by the TRC. As a rule, the data should be sufficient to evaluate the performance of methods. If the data are determined to be insufficient, then additional laboratory validation may be requested. If multiple promising new technologies or methods have been identified, the FSIS may invite the various method proponents to test a panel of coded samples, similar to the AOAC review process *(4)*. In emergency situations where rapid response is necessary, the FSIS may use alternate mechanisms to select new technologies or methodologies that are transparent and defensible. The TRC would statistically evaluate the test results and determine overall method performance. Those methods meeting the minimum requirements may be selected by the FSIS for further evaluation in its own laboratories with actual samples. The FSIS should have mechanisms for recovering the costs of method evaluation.

6.4. Method Validation Team (MVT)

The MVT is charged with conducting (i) performance testing, (ii) optimization, and (iii) validation of the proposed method(s). Here, the FSIS has considerable discretion in how these tasks will be conducted. Where disparate proposals are being considered more than one MVT may be required at any given time.

Following the technical proposal review of a method, the FSIS may determine that, based upon the submission of the data outlined in Section 5.4, enough data are provided to validate the method for its intended use without further laboratory review. However, if insufficient data are provided by the submitter, the FSIS may request additional data and/or conduct an internal laboratory review. In-house testing of the method using appropriate matrices and organisms would determine if the technology or method is repeatable and meets the needs of the FSIS. Specific criteria for the laboratory review will need to be developed by the FSIS based upon the nature of the technology or method and its intended use. At this level of the review process, the submitter may be asked to provide necessary training, test kits, reagents, and labor required to evaluate the technology or method in an FSIS laboratory. If sufficient multiple laboratory testing and method optimization or validation has already been done, the method may be recommended for acceptance and implementation as submitted.

Methods that have successfully passed the review process conducted by the TRC are those that show considerable promise for meeting the FSIS needs as defined in the method requirements and performance criteria. However, as it is quite unusual for laboratory testing methods to be ''off-the-shelf'' ready for use in a regulatory laboratory, the FSIS must collect data as to actual method performance under ''real-world'' conditions.

6.4.1. Performance Testing

Method performance testing should be conducted under controlled circumstances to prevent undue outside influence on the test results. The MVT would supervise performance

testing and analyze data to determine if any or all of the methods meet the FSIS performance goals for the testing requirements. If multiple methods are to be considered, a number of approaches to method comparison might be taken, both within the FSIS's own laboratories and externally. One scenario might include a parallel comparison of methods conducted under the actual conditions used by the FSIS. Appropriate blinded samples representative of actual FSIS samples should be provided. The details of the actual testing protocol depend on the FSIS's goals for the method but the testing design could resemble that used by the AOAC for performance tested methods.

6.4.2. Method Optimization and Validation

The evaluation of new technologies can be divided into three phases: (i) selection, (ii) optimization, and (iii) validation. These phases become increasingly more expensive as a method moves from selection to optimization to validation. In addition, adaptation of inappropriate methods would be both expensive and potentially harmful to public health. Therefore, it is critical that appropriate *objective* processes be put in place to ensure that only optimized and validated methods that meet the performance and convenience criteria set by the FSIS and that maximize public health go forward and are adopted.

Prior to adoption of a new microbiological testing technology, the FSIS should first subject all potential new methods to Phase 1, selection. This phase includes reviewing: inputs from the MEC and TRC; Public Health Goals; the FSIS Microbiological Testing Objectives; the Criteria Checklist; and relevant paper and laboratory reviews. The two general types of criteria to consider when reviewing and evaluating new microbiological testing technologies are: (i) performance (efficacy) and (ii) convenience (efficiency). These criteria should be evaluated in the context of the FSIS's regulatory and public health objectives. To help in selection of new methods, the FSIS should first prioritize and weight performance and convenience criteria using an objective mathematical formula developed and updated as needed by the MEC and TRC.

6.4.2.1. Optimization. New methods that show the most promise of meeting the performance and convenience criteria and contributing to public health should proceed to Phase 2, optimization. After a new technology has been reviewed and selected by the TRC, it should be handed over to the MVT for Phase 2, optimization. Optimization is defined as the procedure or procedures used to make a system or design as effective or functional as possible. While a method's performance might be satisfactory for the FSIS's applications, it might not be totally suitable for implementation into the regulatory environment of the FSIS's own testing laboratories. The MVT will make appropriate adjustments to the method so that it will be compatible with normal laboratory operations. Such variables that might be considered may be sample volumes, incubation durations and incubation temperatures.

Given the many and often competing criteria that must be considered before adopting a new technology (accuracy, precision, sensitivity, specificity, reproducibility, speed, cost, etc.) it will typically not be feasible to achieve maximum values for each criterion. The Committee recommends that the MEC take advantage of ''optimization'' computer software to aid in the optimization process. Prioritization, weighting and use of computer software will help ensure that the selected method will be truly optimized for its intended purpose. The optimization phase can be conducted at either the FSIS or ARS laboratories.

6.4.2.2. Validation. If and when a new method has been optimized it is then necessary to subject it to Phase 3, validation. Method validation is defined as the process of verifying that a method is fit for purpose. The process of validation ensures that a new method meets the defined performance and convenience criteria when analyzing multiple samples of every type that the FSIS analyzes. Once the method has been shown to perform adequately under the FSIS regulatory laboratory conditions, a final method validation will be conducted by the MVT to assure that regulatory results will be supported by the appropriate scientific testing underpinnings. Thus the methods will be appropriate for regulatory use and supportable in legal proceedings.

In order for this critical phase to be performed correctly the Committee strongly recommends that these validation studies be conducted at the FSIS laboratories by scientists that are familiar with the FSIS's samples and testing needs and are specifically dedicated to new method validation. Personnel working in method validation at the FSIS should include experts in microbiology, molecular biology, and statistics. If such personnel are not currently available at the FSIS for this purpose, then the Committee strongly encourages the FSIS to recruit such personnel and organize them into an effective MVT under appropriate leadership within the FSIS.

Following the optimization and validation of the technology or method, the MVT will make specific recommendations on the acceptability and appropriateness of the method for use by the FSIS to the MEC. Based upon these recommendations and those of the TRC, the MEC will provide a report to the proposal submitter.

7. BARRIERS AND RESEARCH GAPS (QUESTION 6)

The Committee identified barriers and research gaps which should be addressed as FSIS adopts new technologies to enhance public health.

1. There are three major barriers that need to be addressed as part of making newer and promising technologies an effective reality: (i) inadequate in-house methods development and validation capabilities at the FSIS; (ii) insufficient application and transparency of statistically-based sampling and analysis plans; and (iii) limited data and methods harmonization and sharing across Federal agencies.
2. As the Committee evaluated technologies applicable for laboratory testing, it became apparent that portable user-friendly instrumentation for in-plant testing offered the

potential for ''real-time'' monitoring of process control and pathogen detection. Although advanced on the spot detection methods are not ready for implementation, reduction in cultural enrichment time could be pursued now. At a minimum, research should be pursued to incorporate enrichment or DNA extraction of samples during transport and to develop shortened enrichment protocols to reduce analysis time.

3. The major barrier to the implementation of real-time detection methods is the need for pre-analytical sample preparation to compensate for (i) matrix-associated residual compounds which impact assay sensitivity, specificity, and LOD; and (ii) the need to test large sample sizes to account for uneven distribution and low levels of pathogen contamination. Methods to concentrate and purify the target agent(s) from the matrix prior to detection are critical for achieving representative recovery and true real-time detection. This problem is not unique to food and environmental samples and continues to be a major impediment for the application of biotechnological methods in general.
4. An enrichment-related problem is the biased selection of strains that flourish in conventional media. The strain that predominates in current enrichment methods may not be the strain that is predominant in the natural setting. This barrier results in the potential for over-representation of one or more strains which may or may not be of public health importance. For example, research is needed to understand the competitive dynamics between *Salmonella* serotypes in various enrichment environments.
5. A barrier to the regulatory adoption of enrichment-independent or non–culture-based detection methods is the need to confirm that the agent is viable and/or infectious. Although there are candidate methods (e.g., reverse transcriptase, fluorescent activated cell sorting) that can detect organisms without growth or enrichment, none of these methods has been validated to unequivocally confirm viability, as well as to provide other important public health information, e.g., strain subtyping and virulence. For regulatory action, however, it is beneficial to have a physical isolate to compare different isolates as well as demonstrate that an adulterant was indeed present. Development of a non–culture-based technology to reliably differentiate viable and non-viable agents is a research gap.
6. There are alternatives for molecular subtyping which may perform better than PFGE. To implement these technologies, they must be thoroughly evaluated and standardized. Development of alternative molecular subtyping methods is a research gap. A barrier to implementation is the necessary protocol standardization across agencies. Only then can such data be meaningfully interpreted for epidemiological purposes.
7. Every new detection method has its own set of strengths and weaknesses (see Table 4). The ''ideal'' method might include the following characteristics: rapid or real-time detection at a high degree of sensitivity and specificity; low LOD; simplicity and ease of use; cost efficacy; high throughput and reliability; the ability for multianalyte detection; adaptability to a wide variety of sample matrices; discrimination between viable and inactivated cells; production of enumerative data; portability; and simultaneous isolate characterization and subtyping. The absence of ideal methods that adequately fulfill all of these criteria is a formidable research gap.

8. RECOMMENDATIONS

1. The NACMCF recommends that the FSIS continue to clearly articulate measurable public health goals and microbiological testing objectives and integrate new technologies to achieve these goals and objectives.
2. To meet public health goals and the FSIS's microbiological testing objectives, appropriate statistically-based sampling and analysis plans must be developed. The plans should address the required sample size to achieve statistical power, the frequency and process of sample collection in the field and in laboratories, microbiological criteria, and the final statistical analysis. Given the importance of statistical considerations and the fact that this Committee was specifically directed to not address statistical issues, the Committee recommends that the NACMCF be charged to look at the statistical considerations as they relate to microbiological testing.
3. Diverse methods are used to collect data by multiple agencies. There is a need to harmonize methodologies and share data among agencies and other partners (industry, academic) in the interest of improving public health. The Committee recommends continued collaboration between the USDA, FDA, CDC, Federal agencies, state health departments, and relevant national and international entities. In addition, representatives from the scientific community (public health and epidemiology, veterinary and human medicine, agriculture and food science, among others) can help bring technologies to fruition in a timely manner.
4. The Committee is concerned that the FSIS has no clearly defined mandate and limited infrastructure for method development and validation activities to support its public health regulatory program. This Committee is also concerned with the current interpretation that methods development constitutes a research activity and therefore falls outside the FSIS mandate. Consequently, this Committee recommends that the FSIS assess the needs to conduct methods development and validation and seek resources for this effort, including in-house staff, facilities, equipment, and organizational structure necessary for successful implementation of appropriate technologies that will allow the Agency to meet its public health goals.
5. The creation of new testing methods that apply new technologies is a multi-disciplinary and resource intensive process. Stringent prerequisites must be met to take full advantage of state-of-the-art advancements in science and technology and the translation to the testing laboratory. To introduce, enhance, and maintain scientific expertise in methods development and

implementation and/or to develop methods that address public health goals and microbiological testing objectives, the Committee recommends that the FSIS devote resources to strengthen its laboratory research capabilities. For example, the FSIS could:

- initiate formal inter-governmental personnel agreements (IPA);
- expand the FSIS Fellows program;
- promote further collaboration with academia and the private and Federal sectors, through the USDA/ARS-FSIS liaison;
- contract directly with appropriate private companies and academia through the Federal government's open and competitive process;
- award cooperative agreement-type grants, administered through CSREES, either to principal investigators or Centers of Excellence (e.g., academic or academic/industry consortia); and
- develop cooperative research and development agreements and other agreements between the FSIS and commercial method developers.

6. The Committee recommends that the FSIS adopt a systematic process to identify and evaluate new technologies that address the FSIS's public health goals and microbiological testing objectives as discussed in Section 6. All methods should be evaluated against a set of previously established performance and efficiency criteria.
7. Safety cannot be tested into a food product, but must be built into prerequisite programs and HACCP systems by the food industry. Food processors can utilize new technologies or methods to enhance their food safety systems. Therefore, the Committee recommends that the FSIS establish a mechanism for sharing new detection technologies with the food industry as they are validated and adopted by the FSIS. The Committee further recommends the reciprocal exchange of data and ideas between industry and regulators, which can lead to the application of improved methods that can enhance public health.
8. Some current and emerging detection platforms are quite promising, provided the test analyte is stable, free of inhibitors, and present in adequate concentration in a sample of low volume. This situation is seldom the case for food and environmental samples (Research Gap No. 2) and in the opinion of the Committee, this is the ultimate limitation to the practical application of emerging technologies. Therefore the NACMCF recommends broad-based multi-disciplinary research efforts that integrate pre-analytical sample processing technologies with advanced detection technologies to yield new methods that are adaptable to a wide variety of sample matrices. This recommendation could be achieved through the referral to the Executive Office of the President's Office of Science Technology and Policy to appoint a working group with broad expertise to plan and implement a ''collection-to-detection'' initiative to:
   - Engage all relevant constituencies (e.g., food, water, environmental, biological, and chemical preparedness);
   - Identify high priority agents and/or matrices;
   - Identify relevant disciplines and experts for participation in the initiative (e.g., microbiologists, food technologists, chemists, engineers, physicists, statisticians);
   - Develop a coordinated Federally funded initiative in pre-analytical sample processing with direct linkage to emerging detection platforms (e.g., perhaps a centrally managed industry-academic-government consortium may be the ideal mechanism); and
   - Work within the mission of the initiative (or consortium) to develop relatively simple, inexpensive, and rapid pre-analytical sample processing methods that can be commercialized in the near term (3 to 5 years) and in an environment flexible enough to respond rapidly to both known and unknown agents or unexpected events.
9. Under certain circumstances, the FSIS should consider accepting results based on stringently validated new technologies in the absence of cultured isolates. For agents that cannot be cultured, the agency should lay the groundwork to allow decision-making to occur in the absence of a viable isolate, with the ultimate goal of acceptance of these new detection and typing methods as equivalent to cultural methods. The Committee recommends that these issues and their ramifications be carefully considered before adoption of new technologies.
10. Enumeration of foodborne pathogens and indicator organisms using real-time molecular methods would accelerate the evaluation of control strategies and provide quantitative data to support risk assessment. Therefore, the Committee recommends that such new technologies be given priority for adoption by the FSIS.
11. Microarray and/or SNP analyses, while promising for genotyping and subtyping applications, are not yet practical for detection although they are relevant for molecular epidemiological purposes. The Committee recognizes the importance of this issue and therefore recommends that evaluating new genotyping and subtyping technologies should be a potential future charge to the NACMCF.

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10. APPENDICES

10.1. Glossary of Terms

Accuracy	The closeness of agreement between a measured value and the accepted ''true'' or reference value.
Adaptability	The applicability of an assay to various matrices and testing situations, i.e., food, environmental, and clinical samples.
Amplification	A step or procedure that either increases the quantity of the analyte or enhances the signal resulting from the analyte's presence.
Analyte	The specific organism or chemical substance sought or determined in a sample.
Assay	The specific analytical component of a method that is used to detect a specific analyte.
Clone	A strain or group of strains descended asexually from a single ancestral cell (source strain) that has identical or similar phenotypes or genotypes as identified by a specific strain typing method.
Confirmation	The unambiguous substantiation of an analyte's presence by comparison to a standard or reference culture.
Detection	The act of discovering or determining the presence of a specific microorganism in a sample. Note that this may apply to the detection of nonviable cells by a non–culture-based method.
Epidemic	One or more outbreaks caused by an epidemic clone that survives and spreads over a long period of time.
False negative	A test result that wrongly determines that an analyte is absent.
False-negative rate	The ratio of false negatives found divided by true positives present, expressed as a percentage.
False positive	A test result that wrongly determines that an analyte is present.
False-positive rate	The ratio of false positives found divided by the number of true negatives present, expressed as a percentage.
Fluorophore	A tag or marker that generates a fluorescent signal.
Format	The material form or layout of a platform.
Generalizability	The ability to apply inferences drawn from a sample to the population from which the sample is drawn.

Genotyping — Testing to determine the complete genetic constitution of an organism or group, as determined by the specific combination and location of the genes on the chromosomes.

Gold standard — A reference method, to which candidate procedures are compared.

Identification — The process of determining that a viable microbial isolate belongs to one of the established, named taxa.

Indicator organism — A non-pathogenic microorganism that may be naturally present in food or water, which is used to indicate a state or condition suggesting the presence of a pathogenic microorganism.

Isolate — A population of microbial cells in pure culture derived from a single colony on an isolation plate.

Limit of detection — The lowest amount of analyte that can be reliably observed or found in the sample matrix by the method used. Limit of detection is matrix and analyte dependent.

Matrix — The substrate of a test sample.

Method — A body of pre-analytical and analytical procedures and techniques for performing an activity (e.g., sampling, analysis, quantification), systematically presented in the order they are to be executed.

Nanotechnology — A field that focuses on control of matter on an atomic and molecular scale.

New technology — A technology that has not existed previously, or that is being applied in a novel way.

Outbreak — An acute appearance of a cluster of an illness that occurs in numbers in excess of what is expected for that time and place. In the case of a foodborne outbreak, the source is often a specific food vehicle that contains one specific outbreak clone.

Platform — The physical surface or structure to which a technology or technologies is/are applied.

Precision — The closeness of agreement between independent test results obtained under stipulated conditions.

PR-HACCP — Pathogen reduction–hazard analysis critical control point (PR-HACCP) is an adaptation of HACCP intended to achieve reduction of the incidence of a particular pathogen in food. FSIS implemented the PR-HACCP rule in 1996.

Pyrosequencing — A DNA sequencing technique in which complementary strands are synthesized and nucleotide sequences are determined by the pyrophospate released during the addition of the nucleotide base.

Quality assurance — Those systematic activities, defined by management, that are done outside of the actual analysis to provide confidence that the analysis will satisfy given requirements for quality. Examples of these activities include training, audit, and review.

Quality control — Those activities that are performed during the analysis to fulfill the requirements for assuring quality. Examples include control charting, blank determinations, spiked samples, repeat determinations, and blind samples.

Recovery — The amount of analyte quantified by the analytical method, expressed as a percentage of the amount known to be present in the sample.

Repeatability — The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Reproducibility — The measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

Ruggedness — The ability of an analytical procedure to resist changes in results when subjected to minor changes in environmental and procedural variables, laboratories, personnel, etc.

Sample — Any material brought into the laboratory for analysis.

Sample preparation or processing — The process of obtaining a representative test portion from the sample which includes selecting a sub-sample(s) and in-laboratory processing (e.g., mixing, reducing, coring, quartering, blending, and grinding).

Sampling — A procedure whereby a part of a substance, material or product is taken to be used for testing or calibration as a representative sample of the whole. In some cases, such as forensic analysis, the sample may not be representative but is determined by availability. The term refers both to the statistical methods used to determine which and how many samples to test in order to represent a larger amount of product, and to the technical methods used to collect, preserve and process that sample for microbiological testing.

Screening method — A method designed to detect the presence of an analyte in a sample at or above some specified concentration (target level). Screening method results are usually reported as yes or no values.

Selectivity — The extent to which the analytical method can determine a particular analyte(s) in a complex mixture without interference from the other components in the mixture. The probability that the method will classify a test sample as negative, given that a test sample is a known negative. A method that is perfectly selective for an analyte or a group of analytes is said to be specific.

Sensitivity — The probability that the method will classify a test sample as positive given that a test sample is a known positive. Analytical sensitivity, also known as limit of detection (LOD), represents the smallest amount of an analyte in a sample that can be accurately measured by a platform or assay. Diagnostic sensitivity is the

probability of detecting an analytical target (i.e., pathogen, toxin) in a sample from a population of samples (i.e., a production lot) which is contaminated.

Specificity — A performance characteristic that judges the ability of a laboratory test method to exclude non-target analytes in chosen matrices, whereby the method will classify a test sample as negative, given that the test sample is a known negative. Analytical specificity is defined as the ability of an assay to exclusively identify a target rather than other similar analytes in a sample. Diagnostic specificity is defined as the probability that the sample tests negative when the pathogen is absent from the sampled population.

Strain — An isolate or group of isolates exhibiting phenotypic and/or genotypic traits that are distinctive from those of other isolates.

Subtype — A specific pattern, or set of marker scores, displayed by a strain upon application of a particular typing system.

Technology — A capability given by the practical application of knowledge, specifically, the method, and material used to attain a microbiological testing objective.

Test — A technical operation that consists of the determination of one or more characteristics or the performance of a given product, material, equipment, organism, physical phenomenon, process, or service according to a specified procedure.

Test method — Specified technical procedure for performing a test.

Test portion — The actual material weighed or measured for the analysis.

Test Sample — Material prepared from the laboratory sample and from which test portions will be taken.

Throughput — The volume of samples that an assay can process.

Validation — Establishment, by systematic laboratory studies, that the performance characteristics of the method meet the specifications related to the intended use of the analytical results.

Validity — Validity is a measure of the ability of the test to do what it is intended to do under specific conditions of use, i.e., to detect the organism(s) of interest if it is present, and not to detect it if it is absent. The two major measures of validity are sensitivity and specificity.

Verification — Confirmation, through the provision of objective evidence, that specified requirements have been fulfilled.

Viability — Ability of an organism to multiply in culture or in a matrix.

10.2. List of Acronyms

AFLP — Amplified fragment length polymorphism
AOAC — Association of Official Analytical Chemists
APC — Aerobic plate count
APHIS — Animal and Plant Health Inspection Service, USDA
ARS — Agricultural Research Service
CC — Coliform count
CCEHBR — Center for Coastal Environmental Health and Biomolecular Research of NOAA's National Ocean Service
CDC — Centers for Disease Control and Prevention, DHHS
CFSAN — Center for Food Safety and Applied Nutrition, FDA
CFU — Colony-forming units
CRADA — Cooperative Research and Development Agreement
CSREES — Cooperative State Research, Education, and Extension Service (now IFA), USDA
CVM — Center for Veterinary Medicine, FDA
DHHS — Department of Health and Human Services
DOC — Department of Commerce
ECC — *E. coli* biotype I count
ECL — Electrochemiluminescence
ELISA — Enzyme-linked immunosorbent assay
EPA — Environmental Protection Agency
EPEC — Enteropathogenic *E. coli*
FAO — Food and Agriculture Organization of the United Nations
FDA — Food and Drug Administration, DHHS
FERN — Food Emergency Response Network
FOBS — Fiber-optic biosensors
FRET — Fluorescence resonance electron transfer
FSIS — Food Safety and Inspection Service, USDA
FTIR — Fourier transform infrared
HACCP — Hazard analysis and critical control points
HCV — Harmonized collaborative validation
HP — Healthy People
IAC — Internal amplification control
IAEA — International Atomic Energy Agency of the United Nations
ICMSF — International Commission on Microbiological Specifications for Food
IFA — Institute for Food and Agriculture (formerly CSREES), USDA
IMS — Immunomagnetic separation
IPA — Inter-governmental personnel agreements
ISO — International Organization for Standardization
LOD — Limit of detection
LRN — Laboratory Response Network
MAAB — Multi-analyte array biosensor
MALDI — Matrix-assisted laser desorption/ionization
MAP — *Mycobacterium avium* subspecies *paratuberculosis*
MEC — Method Evaluation Committee
MLG — *Microbiology Laboratory Guidebook*
MLST — Multilocus sequence typing
MLVA — Multiple-locus variable number tandem repeat analysis
MPN — Most probable number
MS — Mass spectroscopy
MVT — Method Validation Team
NA — Not applicable
NACMCF — National Advisory Committee on Microbiological Criteria for Foods
NAHMS — National Animal Health Monitoring System, USDA-APHIS

NARMS	National Antimicrobial Resistance Monitoring System
NAS	National Academies of Science
NIH	National Institutes of Health, DHHS
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
NPL	National Program Leader
NRTE	Not ready-to-eat
NSIL	National Seafood Inspection Laboratory, DOC-NOAA Fisheries
OFRG	Oligonucleotide fingerprinting of rRNA genes
ORA	Office of Regulatory Affairs, FDA Foods Program
PCR	Polymerase chain reaction
PEMC	Piezoelectric-excited millimeter-sized cantilever
PFGE	Pulsed-field gel electrophoresis
PR-HACCP	Pathogen Reduction–HACCP
PVM	Peer-Verified Methods^{SM}, AOAC
qPCR	Quantitative real-time PCR
RFP	Request for proposal
RTE	Ready-to-eat
RT-PCR	Real-time PCR
SIP	Seafood Inspection Program, NOAA
SLTs	Shiga-like toxins
SLV	Single laboratory validation
SNP	Single-nucleotide polymorphism
SPR	Surface plasmon resonance
SSRMP	Seafood Safety Research and Monitoring Program, NOAA
TOF	Time of flight
TRC	Technical Review Committee
USAHA	U.S. Animal Health Association
USDA	U.S. Department of Agriculture
xMAP	Suspension microarray

10.3. Details about the FSIS's Testing Protocols

The FSIS method protocols currently report foodborne pathogens using the following criteria in the FSIS *Microbiology Laboratory Guidebook* (MLG) *(147)*:

Salmonella

- Non-Typhi/Paratyphi *Salmonella* strains are not necessarily detected (i.e., the MLG 4.04 method does not provide sensitive detection of *Salmonella* strains that are not typically harbored by food animals or non–*S. enterica* species that are not implicated in human foodborne illness).
- Atypical hydrogen sulfide-negative strains are detected and identified.
- Traditional biochemical and serological definitions are applied.
- Genetic criteria are currently not applied.

Listeria monocytogenes

- β-Hemolytic *L. monocytogenes* strains are detected (i.e., non-hemolytic strains are not detected but are rare and generally regarded as having attenuated virulence potential).
- Genetic criteria, serology and virulence capability testing is currently not applied in the FSIS methodology.

E. coli O157:H7

- Isolates that are biochemically confirmed as ''*E. coli,*'' serologically or genetically positive for ''O157,'' and positive for either of the following criteria are reported by the FSIS as ''*E. coli* O157:H7'':
  - genetically confirmed as ''H7'' or
  - serologically confirmed for Shiga toxin production or harbor a gene sequence associated with Shiga toxin capability.

TABLE B-1. *PR-HACCP* Salmonella *carcass testing conducted by the FSIS laboratories (146, 147)*

Carcass	Sampling method and test portion	Maximum *Salmonella*$^+$ samples allowed	
		Category 1	Category 2
Heifer/steer	3-site sponge, 300 cm^2 total or 60 cm^2 excision	0 of 82	1 of 82
Cow/bull	3-site sponge, 300 cm^2 total or 60 cm^2 excision	1 of 58	2 of 58
Market hog	3-site sponge, 300 cm^2 total or 60 cm^2 excision	3 of 55	6 of 55
Chicken	Whole carcass, 400 ml rinse with 30 ml tested	6 of 51	12 of 51
Young turkey[a]	2-site sponge, 100 cm^2 total	7 of 56	13 of 56
Goose[a]	2-site sponge, 100 cm^2 total	5 of 54	9 of 54

[a] New for 2006.

TABLE B-2. *PR-HACCP raw ground product* Salmonella *testing (147)*

Commodity	Test portion	Maximum *Salmonella*$^+$ samples allowed	
		Category 1	Category 2
Raw ground chicken	25 g	13 of 53	26 of 53
Raw ground turkey	25 g	15 of 53	29 of 53
Raw ground beef	25 g	3 of 53	5 of 53

TABLE B-3. *''Zero tolerance'' verification testing conducted by the FSIS laboratories for domestic and imported products (147)*

Commodity	Pathogen	Test portion
Raw ground beef	*E. coli* O157:H7	Five individually analyzed 65-g portions (325 g total)
Raw ground beef components	*E. coli* O157:H7	Five individually analyzed 65-g portions (325 g total)
RTE products (except commercially sterile products)[a]	*L. monocytogenes*	25 g
	Salmonella	325 g
Certain RTE products (i.e., dried or semidried fermented sausages and cooked meat patties)	*E. coli* O157:H7	Five individually analyzed 65-g portions (325 g total)
Food contact surfaces in RTE establishments	*L. monocytogenes*	Sponge sample representing surface area of various sizes

[a] RTE, ready-to-eat.

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Review

Assessment of Food as a Source of Exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP)†‡

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NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS

NACMCF Executive Secretariat, U.S. Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, Room 333 Aerospace Center, 1400 Independence Avenue S.W., Washington, DC 20250-3700, USA*

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ABSTRACT

The National Advisory Committee on Microbiological Criteria for Foods assessed the importance of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is the causative agent of Johne's disease, which affects primarily the small intestine of all ruminants. The significance of MAP as a human pathogen is unknown and is being investigated by several research groups. This document also reviews the efficacy of current detection methods, processing interventions, and MAP inactivation. Research needs related to MAP are provided. The Committee reached the following conclusions: current methods for detection of MAP have significant limitations, and a standard method for the detection of viable MAP cells is needed. Aside from MAP-infected domestic ruminant animals, the organism is found infrequently. If MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated. Milk, particularly raw milk, may be a likely food source for human exposure to MAP. Given the prevalence of MAP in U.S. cattle herds, ground beef may be a potential source of MAP. Although humans may be exposed to MAP through a variety of routes, including food and the environment, the frequency and amount of exposure will require additional research.

EXECUTIVE SUMMARY

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF or Committee) was asked to assess the importance of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease, which affects primarily the small intestine of all ruminants. The significance of MAP as a human pathogen is unknown and is being investigated by several research groups. The Committee was directed not to consider whether MAP is a human pathogen, and therefore did not evaluate the public health relevance of foodborne and other exposures to MAP. In the United States, dairy cattle represent the largest population of MAP-infected animals (Johne's disease–positive herds) and therefore are the most likely source of direct or indirect exposure to humans. There are several possible modes of MAP transmission to humans, including exposure to a contaminated environment, person-to-person transmission, direct contact with infected animals, as well as exposure to contaminated foods.

After examining the scientific literature, the Committee reached the following conclusions:

- Current methods for detection of MAP have significant limitations.
- A standard method for the detection of viable MAP cells needs to be developed and adopted by researchers in order to accurately determine the presence and numbers of MAP in foods and other potential sources of exposure.
- Aside from MAP-infected domestic ruminant animals, the organism is found infrequently. This may be a function of low prevalence and/or a consequence of the absence of reliable detection methods.

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§ Note to the reader: On 20 March 2009 the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) reviewed current scientific literature on *Mycobacterium avium* subspecies *paratuberculosis* relevant to this report. The NACMCF agreed to update this report with additional references as appropriate. The reference section of this report reflects the 20 March 2009 updates agreed upon by the NACMCF. The addition of references to this report and supporting text where appropriate leave the conclusions of the report adopted on 28 September 2007 unchanged.

- If MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated.
- Milk, particularly raw milk, may be a likely food source for human exposure to MAP.
- Thermal processes that deliver a 4- to 7-log reduction in the number of MAP cells should be adequate to inactivate the numbers of MAP estimated to be present in raw milk.
- A small percentage (<3%) of commercially pasteurized milk may contain small numbers of viable MAP cells.
- Although the data are limited, cheese made from pasteurized milk is probably not a significant source of exposure to MAP, but the potential for exposure to MAP from milk products made from raw milk is unknown.
- Given the prevalence of MAP in U.S. cattle herds, ground beef may be a potential source of MAP.
- MAP survives in cattle feces, water, and soil and is found in many wild animals; therefore, farm runoff may potentially contaminate irrigation water, which can come in contact with fruits and vegetables and result in human exposure.
- Although there is no information to indicate that municipal drinking water is a source of human exposure to MAP, further study is needed.
- Although humans may be exposed to MAP through a variety of routes, including food and the environment, determination of the frequency and amount of exposure will require additional research.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease in dairy cows and other ruminants. According to the National Animal Health Monitoring System (NAHMS) 1996 Dairy Study, this transmissible disease has been estimated to affect approximately 21.6% of U.S. dairy herds *(137)*. MAP, which is excreted in feces and milk, is reportedly not inactivated by thermal treatments as easily as other bacteria of public health and animal health concern *(129)*. The Committee was charged with assessing the role of food in exposure of humans to MAP by answering six specific questions. The Committee was directed not to consider whether MAP is a human pathogen and therefore did not evaluate the public health relevance of foodborne and other exposures to MAP.

There are several potential modes of transmission of MAP to humans. These include exposure to a contaminated environment (soil, water), person-to-person horizontal transmission, direct contact with infected animals, and consumption of contaminated foods, including produce and food products originating primarily from dairy cattle but also from beef cattle, sheep, and goats. In fulfilling its charge, the Committee reviewed the pertinent available literature, with an emphasis on the most recent publications since these reflected more current methodologies and results.

WORK CHARGE AND BACKGROUND

Paratuberculosis, or Johne's disease, is an infectious bacterial disease in animals that is caused by MAP. Johne's disease has been spreading slowly through domestic livestock populations for nearly a century and has become endemic in many countries. MAP, the etiologic agent of Johne's disease, is being investigated as a pathogen of animals that may be naturally transmitted to humans. In the United States, dairy cattle represent the largest population of MAP-infected animals (Johne's disease–positive herds) and therefore the most likely source of direct or indirect exposure to humans.

MAP, like other members of the *M. avium* complex (MAC), is an opportunistic pathogen in immunocompromised persons. The question remains whether generally immunocompetent individuals can be infected with MAP and whether this leads to disease. Leaving aside whether or not MAP is a pathogen to humans, there are several possible modes of transmission, including exposure to a contaminated environment (soil, water), person-to-person horizontal transmission, direct contact with infected animals, and preharvest and postharvest contamination of foods, including produce and food products originating primarily from dairy cattle but also from beef cattle, sheep, and goats. Specifically, the presence of MAP in raw milk has raised concerns about whether MAP has potential public health significance.

Charge to the Committee. The Committee was asked to limit their deliberations to the consideration of a very specific set of questions. The Committee was not being asked to consider the question of whether or not MAP is a human pathogen.

The Committee was asked to consider the following questions during their deliberations:

1. What food, water, or environmental sources are of most concern with respect to exposure of humans to MAP?
2. What are the frequencies and levels of MAP contamination found in the above referenced sources?
3. What is the efficacy of the current methods of detection for MAP?
4. What processing interventions are available for the foods of concern to eliminate or reduce the levels of MAP contamination to an acceptable level or to ensure that MAP does not enter the food supply?
5. What are the research needs to determine:
   a. additional sources of MAP;
   b. the frequencies and levels of MAP contamination in specific sources of concern;
   c. potential processing interventions to eliminate or reduce the levels of MAP contamination; and
   d. potential processing interventions to prevent MAP from entering the food supply?
6. Additional research needs?

RESPONSE TO QUESTIONS IN THE CHARGE

The Committee agreed to change the order of the questions in order to allow for a more logical progression as follows below. The Committee chose to address detection methods for MAP first.

1. What is the efficacy of the current methods of detection for MAP?

The ability to culture MAP and detect the organism in various relevant sample matrices, including veterinary clinical samples (feces, tissues), milk, water, and other samples, has been limited by significant challenges. The Committee reviewed the pertinent current literature describing the detection of MAP in these sample types (summarized in Tables 1 through 5). A review compared the various methods, including their specificities and sensitivities, costs, and average time to obtain test results that can be used to screen, diagnose, and confirm MAP infection in animals *(19)*. Four general categories of methods for MAP detection were available: immunological, cultural, molecular, and histopathological methods, the last of which was restricted to veterinary diagnostic settings. A brief discussion of the other three categories is given below.

Immunological methods. Commercially available immunological methods detect antibodies against the organism that are present in blood and milk and hence are most often used in clinical screening or diagnosis of infection of livestock. The commercial assays generally have high specificity, although that varies with the particular matrix being screened. These tests are most accurate when diagnosing animals that have seroconverted in the final stages of the disease when antibody responses are well developed *(59)*. They have low sensitivity for detection of serum antibodies to MAP, especially in subclinical infections *(4, 59),* detecting only about 30% of infected animals when they are in the preclinical disease stage *(4),* and likewise perform poorly for detection of antibodies to MAP in milk *(19, 59, 83, 126)*. However, Nielsen *(94)* concluded that MAP antibodies were generally detected prior to bacterial shedding based on a 3-year study involving analysis of over 24,000 milk and 10,000 fecal samples. An evaluation of four commercial serum enzyme-linked immunosorbent assays (ELISAs) for detecting MAP infection in dairy cows in herds with a history of clinical paratuberculosis indicated that none of the ELISAs were effective for early detection of infection *(28)*. Nonetheless, immunological methods are widely used on-farm because they are rapid and inexpensive. New ELISAs show promise for greater sensitivity. For example, Shin et al. *(118)* developed a JTC-ELISA (a novel ELISA based on the MAP strain JTC303) using antigens secreted by early to mid–log phase culture of MAP strain JTC303 that had a significantly higher sensitivity and equivalent specificity compared to those of five commercial ELISA kits. The sensitivity of detection of low-level fecal shedders was 40% compared to 20% for the commercial kits.

Culture methods. There is no ''gold standard'' culture method that can be considered the definitive approach for identifying viable cells of MAP. All of the cultural methods available are cumbersome and time-consuming, requiring at best up to 16 weeks of incubation, which is often further extended (to 8 or more months) to ensure detection of injured cells that might occur due to heat or other treatments. The general steps for isolation of MAP include concentration, decontamination, culturing (solid or liquid media), and confirmation.

Concentration. Because MAP may be present in low numbers and grows slowly, especially in subclinically infected animals and food or environmental samples, a cell concentration step often precedes detection. This step also reduces total sample volume and may remove some matrix-associated inhibitors. In some protocols, sonication may be used to aid in the disintegration of clumps of cells. Sonication may, however, have a negative impact on assay detection limits *(44)*. The most common concentration methods are sedimentation, centrifugation, and immunomagnetic separation (IMS); filtration has been used but with limited success. Many protocols combine two or more of these approaches. Data indicate that MAP may segregate differentially during centrifugation, particularly in milk fat, which can cause losses during the concentration steps that precede detection *(87)*.

Decontamination. Due to the prolonged incubation required for propagation of MAP and the nature of most specimens that are tested (e.g., feces), rigorous sample decontamination methods are typically applied before transfer into growth media to reduce the number of unwanted organisms. Typical decontamination protocols expose the sample to antimicrobial agents such as hexadecylpyridinium chloride (HPC), amphotericin B, vancomycin, naladixic acid, carbenicillin, trimethoprim, sodium hydroxide, and zepharin, among others. Some of these compounds also may be added in the media to suppress competitive microbial growth during the incubation period. Use of decontaminants at high concentrations or for extended time periods may be bactericidal, and it is important to balance reduction of the growth potential of competitors with optimal recovery of MAP *(33)*. The true extent of such inhibition depends on the agent, time and temperature of treatment, and other factors *(42)*. Investigators have attempted to diminish this effect by optimizing combinations of the agents and/or their concentrations *(69),* which may improve the sensitivity of cultural methods.

Culturing. Currently there are three solid media commonly used to culture MAP using standard plating methods. These media are Herrold's egg yolk agar (HEYA), Lowenstein-Jensen (LJ) agar, and Middlebrook 7H11 agar. MAP lacks the ability to produce the compound mycobactin J, an iron chelator required for growth; therefore, media used to grow MAP must be supplemented with mycobactin J. These media have been shown to support the growth of some but not all MAP isolates *(25)*. To avoid culture bias, investigators have begun using multiple media for isolation of MAP. For example, de Juan et al. *(25)* reported that some strains could be isolated using LJ or Middlebrook 7H11, but the use of these two agars in conjunction with HEYA was necessary to isolate all MAP strains. Better results were obtained when HEYA was supplemented with sodium

TABLE 1. Mycobacterium avium *subsp.* paratuberculosis *detection methods in water*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop an RT-PCR method to detect MAP, incorporating IAC with appropriate pre-PCR processing for application to drinking water and milk *(109)*.	Purified, seeded drinking water	Varying quantities of MAP were added to 20 ml of drinking water and centrifuged in sequence: 2,000 × *g* for 10 min, recover and suspend precipitate in 1 ml of PBS, 13,000 × *g* for 5 min, recover pellet by resuspending in 100 μl of PBS, DNA extract (Wizard kit), PCR. TaqMan probes for RT-PCR targeting IS*900* with independently amplified internal control; primers designed by investigators.	60 strains total: 18/18 (100%) MAP strains positive; 17/17 (100%) non-MAP *Mycobacterium* and 25/25 (100%) other bacterial species were negative. 100 CFU/20 ml, high degree of relative accuracy in drinking water.	Relative accuracy: percentage of the number of cells calculated by the Q-PCR assay versus the number of cells calculated by hemocytometer.
Develop a sensitive detection method for MAP in water by modifying and optimizing an existing IMS-PCR method *(143)*.	Sterile distilled water, spiked	50 ml of water was centrifuged (2,500 × *g*, 20 min) and the resulting pellet was resuspended in 1 ml of PBS with Tween 80. This was followed by IMS (polyclonal antibody–coated beads) and SDS–proteinase K DNA extraction method with ribolysing and IS*900* PCR. This was optimized by the addition of Tween 80 (0.4% being optimal). IMS-PCR targeting IS*900* with primers previously described *(68, 89)*.	Two strains of MAP used. Detection limit of 10 MAP/ml water in original 50 ml of water sample using 0.4% Tween 80.	The attempted filtration method for sample pretreatment was not successful.

[a] LOD, level of detection; RT-PCR, real-time PCR; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; IAC, internal amplification control; PBS, phosphate-buffered saline; Q-PCR, quantitative PCR; IMS-PCR, immunomagnetic separation PCR; SDS, sodium dodecyl sulfate.

pyruvate. All studies with solid media have used the incubation temperature of 37°C for lengths of time ranging from 6 weeks to 40 months under aerobic conditions.

Automated liquid culture systems (including BACTEC, MGIT, TREK ESP II) use selective media in conjunction with indicator systems to detect growth. TREK (TREK Diagnostics, Cleveland, OH) uses a computer controlled incubation cabinet to continuously monitor changes in head space pressure within each individual sample vial. As bacteria grow, nutrients from the media are metabolized and the pressure inside the sample vial changes. This change in pressure is used as an indicator of bacterial growth. Although samples can be called suspect as soon as a change in head space pressure is detected, in order for a sample to be called negative or ''none detected'' it must remain in the incubation chamber for a minimum of 6 weeks.

The BACTEC (Becton Dickinson [B-D], Sparks, MD) system detects ^{14}C-labeled carbon dioxide as an indicator of bacterial growth. As bacteria metabolize ^{14}C-labeled palmitic acid in the media, they produce $^{14}CO_2$. The BACTEC is one of the most sensitive culture methods. However, due to its reliance on radioactive media, its use in laboratories is steadily declining. In response to these concerns, B-D has developed the MGIT system. This method uses the reduction or consumption of O_2 as an indicator of growth. Each sample tube is continuously monitored in a computer-controlled incubation chamber. As O_2 is depleted from the tube, an indicator substance in the bottom of the tube fluoresces when the tube is exposed to UV light. Both the BACTEC and MGIT systems also require lengthy incubation periods.

All three liquid culture systems use selective media to inhibit competing organisms. However, no medium is 100% selective. All three use time to detection to estimate cell number, based on the assumption that the higher the MAP load, the more rapid the detection endpoint. Liquid culture (BACTEC 12B) was found to be more sensitive than a solid medium (Middlebrook 7H10 agar with mycobactin J) for isolation of sheep strains from fecal and tissue samples and may be more sensitive for cattle strains as well *(147)*. Addition of ampicillin to BACTEC medium can reduce contamination with organisms other than MAP *(147)*. Shin et al. *(117)* developed an immunoassay to screen liquid mycobacterial cultures for evidence of MAC. This MAC-ELISA test was effective in determining cultures that warranted the resources to identify the organism by PCR.

Confirmation. A sample that gives a presumptive positive result using any of the culture systems must undergo further testing. Growth with and without mycobactin J, combined with acid-fast staining and/or PCR, are confirmatory tests for MAP. Although culture methods are cumbersome, they are the only ones available that result in isolation of a viable bacterial culture, which can be further characterized if so desired.

Molecular methods. As is the case for cultural methods, molecular amplification methods must also be preceded by sample concentration. An additional consideration is the need for nucleic acid extraction prior to detection. There is no single method of choice for this extraction, but as MAP cells are extremely difficult to disrupt, sonication or disruption with glass beads is usually recommended before DNA extraction.

Improvements in nucleic acid detection by PCR have accelerated rapid detection of MAP DNA, theoretically reducing the detection time to 1 to 3 days. The majority of these assays have targeted the IS*900* DNA base sequence (there are approximately 17 copies of this insertion sequence per cell) *(7, 9, 11, 37, 46, 66, 68, 76, 96, 98, 108, 109, 122, 126, 139, 143)*, which provides the potential for a higher level of sensitivity relative to assays based on detection of single copy genes. Single-copy gene targets include *hspX (5, 7, 11, 36, 37, 132, 139)* and F57 *(85, 119, 132)*. The recent completion of the MAP genome sequencing project has identified the ISMAP*02* sequence, which is present in six copies, as an alternative target *(122)*.

Limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, matrix tested, and PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, and real-time PCR). The real-time PCR format eliminates the subjective interpretation of gels, providing an objective determination of the presence or absence of MAP DNA and theoretically can be adapted to produce quantitative estimates of MAP concentration *(60, 109, 119)*. Incorporation of an internal amplification control to each reaction minimizes the likelihood of false-negative results in both conventional and real-time PCR formats *(7, 11, 98, 109, 132)*. Investigators have attempted nested amplification with little improvement in overall detection limits *(122, 139)*. PCR assays have a detection limit of 1 to 10 MAP per reaction. When applied to artificially contaminated sample matrices, detection limits are approximately 10^2 to 10^3 CFU per sample. Some, but not all, of the assays have been evaluated using naturally contaminated samples. A major concern about PCR-based diagnostics is that DNA-based methods do not discriminate between viable and nonviable MAP cells. The application of reverse transcriptase PCR may address this issue. Interestingly, a recent publication described a phage-PCR method for the differentiation of viable from nonviable MAP as applied to naturally contaminated raw milk samples *(127)*. This may be more practical than previous vital staining methods for demonstration of viable cells in clumps *(57)*. Application of robotic technology for both DNA and RNA extraction coupled with real-time PCR formats offers the possibility of high throughput.

Interpretation of results. Negative test results should be interpreted with caution, given the sensitivity and specificity of the test methods *(19)*. The cultural methods could be considered a ''reference'' standard, but they are not ideal. For example, decontamination and declumping steps may affect the accuracy of detection. After thorough review of the literature, it appears that the molecular methods hold the most promise, as this approach is rapid and sensitive when targeting multicopy genes (e.g., IS*900*). PCR is an excellent method for confirmation of cultures

TABLE 2. Mycobacterium avium *subsp.* paratuberculosis *detection methods in milk and dairy products*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop an RT-PCR method to detect MAP, incorporating IAC with appropriate pre-PCR processing for application to drinking water and milk *(109)*.	Semiskim milk, seeded	Varying quantities of MAP were added to 20 ml of drinking water and centrifuged in sequence: 2,000 × *g* for 10 min, recover and suspend precipitate in 1 ml of PBS, 13,000 × *g* for 5 min, recover pellet by resuspending in 100 μl of PBS, DNA extract (Wizard kit), PCR.	60 strains total: 18/18 (100%) MAP strains positive; 17/17 (100%) non-MAP *Mycobacterium* and 25/25 (100%) other bacterial species were negative.	Relative accuracy defined as percentage of the number of cells calculated by the Q-PCR assay versus the number of cells calculated by hemocytometer.
		TaqMan probes for RT-PCR targeting IS*900* with independently amplified internal control; primers designed by investigators.	Detection limit in artificially contaminated semiskim milk was 100 CFU/20 ml with low relative accuracy.	
Develop quantitative RT-PCR assay for detection and quantitation of MAP in milk *(96)*.	Whole, pasteurized milk, seeded	24-ml samples of milk were seeded with 10^4 MAP cells, incubated, precentrifuged for MAP concentration followed by DNA extraction using seven approaches: (1) proteinase K and lysis; (2) lysozyme and proteinase K; (3) chloroform-methanol; (4) basis lysis; (5) alternative buffer; (6) GITC lysis; (7) clarifying solution.	Specificity not reported. Lower detection limit of 40 CFU/ml in seeded milk.	No IAC.
		FRET-based RT-PCR targeting IS*900,* primers designed by investigators.		
Comparison of four decontamination methods for recovery of MAP on HEYM *(33)*.	Raw milk spiked with 10^6–10^7 CFU/ml and dilutions of decontaminated milk samples were used.	Three different isolates were grown in Middlebrook broth with OADC enrichment and mycobactin J (7–8 wk), pelleted and resuspended in PBS to yield 10^6–10^7 MAP CFU/ml. 0.5 ml of suspension was added to 40 ml of raw milk. Samples were centrifuged (2,500 × *g,* 15 min) and the pellet was decontaminated by four different methods: (1) pellet resuspended in 0.75% HPC; (2) overnight incubation in BHI and 0.75% HPC, then addition of an antibiotic mixture; (3) method 2 but with 0.9% HPC; (4) use of CB-18. Samples inoculated onto HEYM, incubated (18 wk), then quantified.	Minimum detection limits per 40 ml of raw milk for four decontamination methods evaluated: (1) 30; (2) 10^5; (3) not determined; (4) 10^3. Recoveries from methods 2 and 3 were 2–3 log lower than for method 1.	Decontaminants are bacteriostatic at low concentrations and bacteriocidal at higher concentrations; therefore, a balance must be struck between efficient inactivation of undesirable microbes at the lowest possible toxicity for MAP. Even most effective decontamination method recovered a fraction (28.7%) of MAP present in milk. Extra centrifugation steps may also yield lower recoveries.
To develop rabbit polyclonal antibody–coated Dynabeads to selectively isolate MAP from milk samples to enable end-point detection by another method (e.g., IS*900* PCR or ELISA) *(46)*.	Spiked raw cow's milk from a ''healthy'' cow or pasteurized whole cow's milk; authors did not distinguish which was used in each individual test.	Sample prepared for analysis (centrifuged at 2,500 × *g* and resuspended in PBS); coated Dynabeads added to sample for IMS; inoculated onto HEYM (12 wk); also tested by IS*900* PCR.	Polyclonal IgG gave weak agglutination in the presence of *Hafnia* when undiluted; for *Pseudomonas* sp. and *Staphylococcus* sp. up to 1:10 dilution; for *M. avium* and *M. intracellulare* up to 1:1,000 dilution. There were moderate cross-reactions with three other *Mycobacterium* spp. and weak cross-reactions with other bacterial	Under the conditions used the beads had a maximum binding capacity of 10^4–10^5 CFU.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
			isolates from raw milk. Detection limit of 10 CFU/ml without centrifugation. Detection limit increased to 10 CFU per original sample volume with centrifugation.	
Evaluate the efficacy of various media to suppress the growth of cheese starter cultures and other interfering bacteria to enable enumeration of MAP *(31)*.	Cheddar cheese manufactured from pasteurized milk (and one unpasteurized sample) and various starter cultures and MAP strain NCTC 8578.	Cheddar cheese suspensions and dilutions of starter cultures were decontaminated (0.75% HPC, 5 h), centrifuged (2,500 × *g*, 15 min) then inoculated onto (1) HEYM, (2) HEYM + VAN, (3) Middlebrook 7H10 agar + PANTA supplemented with OADC and PANTA PLUS, or (4) BACTEC 12B radiometric medium supplemented with PANTA PLUS and incubated at 37°C (98.6°F) or up to 12 wk. Parallel samples not decontaminated prior to inoculation. MAP strain NCTC 8578 was added to pasteurized milk, mixed with starter cultures, and inoculated as above.	Without decontamination, starter cultures grew on HEYM at initial levels $>10^8$ CFU/ml but not on HEYM +VAN or HEYM + PANTA. BACTEC gave false-positive results in the presence of two starter cultures. Decontamination required for cheeses to prevent overgrowth of competitors on culture media. PANTA was significantly better than BACTEC 12B at suppressing growth of cheese microflora. PANTA allowed growth of ''contaminants'' once (identified as *Lactobacillus rhamnosus*). Growth was observed on all media from cheeses made from unpasteurized milk.	Study illustrates the critical need to suppress non-MAP microbes in complex samples bearing high numbers of other microbes. HEYM + PANTA appeared most effective for starter cultures and cheeses; BACTEC 12B was the least effective.
Evaluate sensitivity, specificity, and predictive value of IS*900* PCR assay for detection of MAP in raw cow's milk *(68)*.	Raw, bulk tank and pooled quarter milk samples, naturally contaminated.	50-ml milk samples, centrifugation (1,950 × *g*, 30 min), pellets were washed twice in PBS, decontaminated overnight at RT in 0.75% HPC, recentrifuged (1,600 × *g*, 10 min), resuspended in 0.75% HPC. Culture: inoculated into slants of HEYM supplemented with mycobactin J and incubated at 37°C (98.6°F) for 16 wk. Typical MAP colonies counted; confirmed by Ziehl-Neelsen staining. PCR: DNA extraction by Instagene DNA purification matrix with additional lysis by sequential incubation (56°C [132.8°F] for 30 min, 100°C [212°F] for 15 min), centrifugation (20,800 × *g*) and recovery of DNA-containing supernatant. PCR detection targeting IS*900*; primers of Moss et al. *(89)* yields a 229-bp fragment for sequence confirmation.	By PCR and compared to fecal culture, positive pooled-quarter milk samples tested by PCR displayed sensitivity, specificity, and predictive value of 0.87, 0.95, and 0.71, respectively. This is juxtaposed to 0.17, 0.98, and 0.67, respectively, for pooled-quarter milk samples tested by cultural methods.	Fecal cultures done by double incubation and centrifugation technique (Cornell method); fecal culture considered gold standard. True positives: culture-positive individual cows. Improved sensitivity, specificity, and detection limits when using individual cow samples compared to bulk tank milk samples.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To determine if MAP is present in retail supplies of whole pasteurized cow's milk obtained over 19 mo in England and Whales *(87)*.	Milk, raw and pasteurized, seeded and naturally contaminated	For seeded samples, 15-ml milk samples were centrifuged (41,000 × *g*, 1 h at 4°C). Each fraction (whey, cream, and pellet) was recovered individually. 500-μl aliquots were boiled (20 min), centrifuged, and the supernatant was used in PCR amplifications. For naturally contaminated samples: culture confirmation was done on aliquots (whey, pellet, and cream) decontaminated with 0.1% BAC or 0.75% CPC (overnight at RT). After centrifugation and washing, samples were inoculated in Dubos broth supplemented with mycobactin J and incubated at 37°C (98.6°F) for 13–40 mo. Microscopic evaluation by Ziehl-Neelsen staining; aliquot of the culture supernatant processed for DNA extraction (GITC and heat) and tested by IS*900* PCR. PCR detection using IS*900* primers not previously referenced. Amplicons confirmed by internal probe hybridization (radiolabeled) produced by PCR product internal to the amplicon (226 bp).	Spiking trials: sensitivity of detection in milk was 200–300 MAP cells/ml. In naturally contaminated samples, 0–25% (total 22/312 or 7.05% positive) of cartons of retail milk were positive by IS*900*; difficult to confirm by culture due to overgrowth; IS*900* PCR on pellets of cultures were positive in 9/18 (50%) samples and negative in 6/36 (16%) samples tested.	MAP partitioned in fat and pellet fractions, not whey.
Improve procedure for isolating MAP from bulk tank raw milk. Specifically interested in (1) effects of different decontamination agents and procedures on recovery of MAP and (2) partition of MAP in milk fractions *(42)*.	Bulk tank (raw) milk and pasteurized milk, seeded	Seeded milk centrifuged (3,100 × *g*, 30 min); decontaminate pellet with 0.75% HPC or 0.75% HPC + half-strength BHI for 2–48 h with shaking. Samples centrifuged (1,000 × *g*, 15 min at RT), and pellet resuspended in sterile antibiotic brew (amphotericin B 50 mg/ml; vancomycin 100 mg/ml; nalidixic acid 100 mg/ml, BHI 18.5 mg/ml) and held at RT or 37°C (98.6°F) for up to 96 h. Plated on HEYM slants and LB plates. Incubated 1, 2, 4, 8, and 16 wk at 37°C (98.6°F).	Not reported.	Freshness of milk is critical; should be kept at 4°C for no more than 3 days. 0.75% HPC was more effective than HPC with half-strength BHI; optimal time of disinfection was 2–5 h at RT. Extensive incubation during decontamination reduced the number of culture-positive samples in both raw and pasteurized milk. High levels of MAP were recovered in the cream fraction; best to pool cream and pellet. 16.6% of input MAP recovered culturally using the optimized sample prep method.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Use of solid phase cytometry for rapid detection of MAP in milk *(27)*.	Retail pasteurized milk, seeded	50 ml of seeded milk treated as follows: 0.5% ammonia, 5% diethylether, 12% petroleum ether, and 0.1% SDS. Centrifuge (9,500 × *g*, 15 min), wash the pellet in Ringer solution, decontaminate with 0.75% CPC. After double washing, pellet resuspended in Ringer solution, incubated (RT, 1 h), homogenized, digested with 1 ml of 4% trypsin (37°C [98.6°F], 1 h), filtered through a polycarbonate membrane filter (0.4 μm). Bacteria eluted in 10 ml of Ringer solution, centrifuged (9,500 × *g*, 15 min). Pellet resuspended in 2 ml of 0.5 M NaCl, subjected to solid phase extraction in loose C_8 sorbent, washed with 2 ml of 0.5 M NaCl, and MAP eluted with 2 ml of 100% n-propanol and collected in 18 ml of physiological saline. Aliquots were filtered through a polyester membrane filter (0.4 μm), counterstained with 1 ml of Chemunex, stained with ChemChrome V6, and incubated at 30°C (86°F) for 45 min to label viable cells. The membrane filter was then subjected to automated laser scanning using a ChemScan.	Method combined, when applied to milk spiked at 10^2 and 10^4 CFU/50 ml; recovered 70 and 11%, respectively, for *M. smegmatis,* whereas the recovery for MAP (10^2 cells/50 ml) was 73%.	Most of the developmental work was done with *M. smegmatis,* not MAP. The test organism segregated in the pellet and not cream. About 0.1% of the inoculum was lost in the cream fraction. Total 5 min time in 0.75% CPC killed all seeded representative gram-positive and gram-negative bacteria but not *M. smegmatis.* Trypsin improved filterability of the milk pellet. 100% retention-adsorption of MAP on C8 hydrophobic beads. Feasibility of dual labeling approach using red and green fluorescent antibodies and fluorescent viability substrate (for cellular identity and viability) was demonstrated but needs additional optimization.
Compare detection capabilities of the nonradiometric MGIT and radiometric BACTEC 460TB culture systems for recovering MAP from milk *(51)*.	UHT milk, spiked	UHT milk seeded with 10^1–10^7 cells/ml was inoculated into the two systems with and without prior chemical decontamination in 0.75% CPC for 5 h. Time to detection of growth was measured in days from each system; in addition, counts were calculated from BACTEC readings using a published formula.	Both systems detected 10–100 MAP cells/ml in UHT milk in 30–40 days with no prior decontamination treatment. Only 10^2–10^3 cells/ml were detected with chemical decontamination. Correlation between the two methods was 0.6983.	The nonradioactive MGIT systems could be substituted for the radiometric BACTEC system without loss of detection sensitivity. Chemical decontamination before culture caused a significant reduction in the numbers of viable MAP. Detection time for the two systems was not significantly different.
Develop an RT-PCR assay for detection of MAP in milk that targets a more specific genetic marker (F57 gene) *(132)*.	Raw milk, spiked	10-ml samples of raw milk seeded with 10^6, 10^2, or 0 MAP cells/ml were treated with 100 μl of Triton X-100 and centrifuged twice (30 min at 4,500 rpm and 10 min at 14,000 rpm) with recovery of pellets. Pellets were stored at 20°C (68°F) until testing was performed. Three different DNA extraction approaches were applied: (1) precipitation	On purified DNA, assay could detect equivalent to two to three gene copies per reaction. Specific for MAP (10 of 10 [100%] MAP cultures positive), no cross-reactivity with 24 non-MAP *Mycobacterium* cultures or 9 representative gram-positive and gram-negative enterics.	Assay performance was not impacted by high levels of background DNA. Best DNA extraction was achieved with precipitation or column-based methods. Assay successfully detected MAP in raw milk obtained from two clinically confirmed cases. Assay was also applied to 80 individual cow's milk

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
		(two commercial methods); (2) column based (two commercial methods); and (3) DNA binding beads (two commercial methods). Primers targeted the F57 region (254-bp amplicon), not previously reported, in RT-PCR method and IAC (400-bp amplicon, additional set of primers).	Detection limits in spiked raw milk: 10^3 and 10^2 MAP cells/ml (10/10 [100%] positive); 10 MAP cells/ml (7/10 [70%] positive); <10 MAP cells/ml (0/10 [0%] positive).	samples (pooled to 16 samples total) obtained from a farm with a history of MAP; 2 of 16 pooled samples were positive for MAP.
Rapid enumeration of viable MAP in milk *(27)*.	Milk	50-ml retail pasteurized milk samples were treated with 0.5% ammonia, 5% diethyl ether, 12% petroleum ether, and 0.1% SDS and centrifuged at 9,500 × *g* for 15 min. The resulting pellet was decontaminated with 0.75% CPC for 10 min. The pellet was double washed by homogenizing by aspiration through a syringe and needle. This procedure was followed by digestion with trypsin. Bacteria were collected by filtration, centrifuged, and resuspended in 2 ml of 0.5 M NaCl. The suspension was transferred to a column for solid phase extraction with Bio-Rad C_8 sorbent. The extraction was followed by a wash step with 0.5 M NaCl. MAP was eluted with 100% n-propanol. Rapid detection of viable cells was performed using solid phase cytometry.	Recovery for 10^2 cells/50 ml of milk was 73%.	MAP detected in a single working day. Investigation of dual labeling as a means of detection the presence of viable MAP, but optimization is needed, as well as testing on field samples.
To compare commercially available DNA extraction–IS*900* PCR assays and three culture-based methods (MGIT, HEYM, and BACTEC 12B radiometric medium) for detection of MAP in milk in multiple laboratories *(30)*.	Raw milk inoculated with naturally infected feces	For PCR assays, DNA was extracted by three methods (using combinations of magnetic beads [IMS and other], mechanical disruption [Ribolyser], phenol-chloroform extraction, and commercial extraction kits). Various decontamination methods were used, including centrifugation and HPC treatment; diethyl ether–petroleum–SDS–NH_3 followed by centrifugation; or sodium citrate clarification, centrifugation, and HPC treatment.	Commercial PCR method gave overall detection rates of 100, 90, 85, and 25%, respectively, for MAP concentrations of 300, 30, 3, and 0.3 copies of IS*900*/ml in raw milk. The Adiavent PCR method yielded 100, 88, 65, and 11% for the same concentrations of IS*900*. At 20 MAP cells/ml in raw milk, 100% of samples tested by MGIT were positive; 10/12 were positive by HEYM, and 0% were positive using BACTEC. Of the three cultural methods, the MGIT method showed the best performance, with consistent positive detection in samples inoculated with approx 20 MAP cells/ml.	RT-PCR had increased sensitivity over conventional endpoint PCR. PCR methods were more sensitive than cultural methods. Interlaboratory variability was surprisingly small.

TABLE 2. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Use of Pourquier ELISA for detection of antibodies against MAP in individual and bulk milk samples *(140)*.	Milk	ELISA	99.8% specificity for individual milk samples and 100% for bulk milk samples; 87–96% sensitivity depending on the level of shedding by the cows tested.	Method for detection of antibodies in milk from infected cows.
Evaluate phage-PCR method for live-dead differentiation and molecular characterization of MAP *(127)*.	Pure culture then applied to naturally contaminated raw milk samples	Bacteria in milk pelleted by low speed centrifugation, with the pellet subsequently used in the phage amplification assay (FASTPlaque TB, Biotech Laboratories, UK). Presumptive colonies tested by IS*900*-based PCR to confirm MAP.	10/14 milk samples from 14 animals with evidence of clinical infection were presumptively positive by phage amplification assay; all 10 confirmed as MAP by IS*900* PCR.	The phage amplification assay PCR method allows rapid and specific detection of viable MAP in milk in as little as 48 h.

[a] LOD, level of detection; RT-PCR, real-time PCR; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; IAC, internal amplification control; PBS, phosphate-buffered saline; Q-PCR, quantitative PCR; GITC, guanidinium isothiocyanate; FRET, frequency resonance energy transfer; HEYM, Herrold's egg yolk medium; OADC, oleic acid, dextrose, catalase; HPC, hexadecylpyridinium chloride; BHI, brain heart infusion; CB-18, C_{18}-carboxypropylbetaine; ELISA, enzyme-linked immunosorbent assay; IMS, immunomagnetic separation; VAN, vancomycin, amphotericin B, nalidixic acid; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; PANTA PLUS, polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and 0.5% (vol/vol) glycerol; RT, room temperature; BAC, benzalkonium chloride; CPC, cetylpyridinium chloride; LB, Luria-Bertani; SDS, sodium dodecyl sulfate; MGIT, mycobacteria growth indicator tube; UHT, ultrahigh temperature.

thought to be MAP. However, the sensitivity of molecular-based methods as applied to complex samples is limited by matrix and sampling effects, which can result in false-negative results if internal controls are not used in the assay. DNA-based molecular methods are hampered by an inability to discriminate viable from nonviable MAP. This continues to pose concerns regarding the potential for false-positive detection. Reliance on molecular methods alone means that an isolate is not obtained for further characterization.

Pinedo et al. *(105)* analyzed feces, blood, and milk from 328 cows in four infected dairy herds and determined that the combined use of ELISA and fecal PCR has the potential to increase the overall sensitivity for detection of MAP infection. Currently, neither cultural nor molecular methods are able to provide accurate quantitative information about the numbers of MAP present in the sample. Finally, lack of method standardization complicates comparison among studies.

To avoid duplication, the Committee determined that the two questions below could best be answered concurrently:

2. **What food, water, or environmental sources are of most concern with respect to exposure of humans to MAP?**
3. **What are the frequencies and levels of MAP contamination found in the above referenced sources?**

MAP, which is shed in the feces of infected animals, can potentially enter the food supply through several routes, including fecal contamination of raw food products, contaminated water due to runoff containing feces, and mammary gland secretions. Therefore, potential sources of MAP include domestic livestock, wildlife, insects, protozoa, environmental contamination, water, milk (raw and pasteurized), milk products, meat, fish and shellfish, and produce. Since MAP is an extremely fastidious pathogen requiring mycobactin J for in vitro culture, it is unlikely to grow in the environment or food. However, it has been shown to persist in both.

Prevalence and Numbers of MAP in the Agricultural Environment

Domestic livestock. Cattle are the most significant source of MAP that could impact the U.S. food supply. There are several studies examining the prevalence of MAP in cattle, with variable results *(10, 23, 68, 82, 95, 111, 112, 134, 137, 138)*. Several of these studies indicate there are regional and seasonal differences in prevalence; however, seropositive animals are widely distributed geographically. The most comprehensive multistate, multiherd studies are those conducted by U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service for NAHMS. According to the 1997 NAHMS Beef Cattle Study, it was estimated that 7.9% of the U.S. beef herds tested ($n = 380$) had one or more ELISA-positive animals, and 0.4% of all animals tested ($n = 10{,}371$) were positive

TABLE 3. Mycobacterium avium *subsp.* paratuberculosis *detection methods in pure culture*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To improve specificity of PCR assays for MAP detection by multiple targets and nested approaches *(139)*.	190 pure mycobacterial cultures: 74 MAP, 116 non-MAP	DNA was extracted by GITC-lysis-alcohol precipitation. The researchers refined IS*900* primers and developed nested PCR as well as developed F57 region primers and nested PCR.	Detection limit of 1 CFU/PCR for nested reactions for both assays. 74/74 (100%) MAP strains were correctly identified by either assay; 116/116 (100%) non-MAP strains were negative by either assay.	Both assays performed equivalently.
Describes cloning and sequencing of a single-copy gene unique to MAP and application of an efficient nucleic acid diagnostic test that identifies mycobacterial infection, specifically MAP *(36)*.	Pure cultures 14 MAP and 27 non-MAP isolates	DNA was extracted from all isolates using a lipase digestion followed by lysozyme digestion and proteinase K plus SDS and purified with repeated phenol–chloroform–isoamyl alcohol extractions. MAP DNA was digested with *Pst*1, size fractionated by agarose gel electrophoresis, and screened using eight 15-bp DNA probes that contained in one open reading frame the nucleotide sequence encoding the RGD peptide adhesion motif.	Screening using degenerated RGD probes identified a unique sequence encoding 144 amino acid residues for a putative heat shock protein called *hspX*. Southern blot analysis using the sequence for *hspX* positively identified 100% of the MAP isolates and negatively identified 100% of non-MAP isolates.	*hspX* is a single-copy gene unique to MAP.
To detect viable MAP using plasmid and phage-based luciferase reporter constructs *(115)*.	FSL S2-02, 04, and 16 for phage study; eight MAP isolates for plasmid study	Grow pure culture, sonicate, infect with phage.	The lowest MAP numbers detected by luminescence were 10^3 CFU from whole milk or saline, 10^2 CFU from skim milk.	Luciferase reporter phage shows promise for rapid and sensitive detection of viable MAP cells in milk.
Compare Accu-Probe, serotyping, and single IS*900*, IS*901*, IS*1245*, and IS*901* flanking region PCR with internal standards *(7)*.	4,729 mycobacterial isolates	Isolates were grown on HEYM or LJ medium. A loopful of a single colony was resuspended in 50 ml of distilled water, boiled for 20 min, centrifuged for 5 min at 14,000 × *g*; 2 μl of supernatant was used for PCR amplification.	Sensitivity was 1×10^1 CFU/reaction for IS*900*, 5×10^3 CFU/reaction for IS*901*, 1×10^2 CFU/reaction for IS*1245*, and 1×10^3 CFU/reaction for ISFR*300*. Percent inhibition during PCR analysis ranged from 0 to 50% depending on the strain being tested.	PCR was completely inhibited by more than 50 ng of chromosomal DNA per reaction.
Detection of MAP by RT-PCR and differentiation from other mycobacteria using SYBR Green and TaqMan assays *(108)*.	6 MAP, 25 non-MAP strains	The template DNA used for real-time detection of target sequences was extracted with InstaGene matrix according to the manufacturer's protocol.	The TaqMan assay detects as low as 4 fg of MAP-specific DNA per assay (equivalent to 0.8 cells) and has the potential to detect even smaller quantities by increasing the amplification cycles. The SYBR Green assay yielded a minimum detectable limit of 0.07 cells (equivalent to 0.34 fg of DNA).	Multiple primer sets may be needed to detect all MAP.

TABLE 3. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop nested PCR assay using ISMAP*02* *(122)*.	Fecal samples from cows naturally infected with MAP and negative control samples from noninfected cows	A previously published methodology was used that applied double-centrifugation, double-decontamination *(120)*.	Only MAP DNA was detectable after amplification with the ISMAP*02* primers. The sensitivity of detection for the ISMAP*02* element in conventional PCR or RT-PCR was <100 fg or 10^2 CFU/ml.	RT-PCR and conventional PCR were equivalent. Maximum recovery was obtained with IS*900* conventional PCR and RT-PCR.
Develop an internal control plasmid for MAP detection by RT-PCR *(11)*.	3 MAP, 13 non-MAP strains	The methodology for the extraction of DNA from the 16 bacterial strains was not provided.	The optimal plasmid amounts are 10 fg/reaction for *hspX* detection and 1 fg/reaction for IS*900* detection. Positive results were only observed for the MAP strains.	Internal control amplification was detected by fluorescence at 705 nm on the LightCycler. Amplification of IS*900* was detected by fluorescence at 640 nm on the LightCycler.

[a] LOD, level of detection; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; GITC, guanidinium isothiocyanate; SDS, sodium dodecyl sulfate; HEYM, Herrold's egg yolk medium; LJ, Lowenstein-Jensen; RT-PCR, real-time PCR.

(23). The 1996 NAHMS Dairy Cattle Study *(137)* found a 21.6% apparent herd prevalence based upon two or more positive tests or one positive test with at least 5% of cull animals with clinical signs of disease. Serological testing may have underestimated the true incidence of positive cows in each herd, since seroconversion occurs at the later stages of the disease. Also, factors such as within-herd prevalence, sample size, test sensitivity and specificity, and the definition of a positive herd cause uncertainty in estimating herd prevalence. The 2002 NAHMS Dairy Study *(138)* was done to study positive herds ($n = 62$) and examine the prevalence of MAP within these herds. This study found 8.6% of the animals ($n = 7{,}272$) in positive herds tested positive for MAP. Because the study focused on positive herds, the 2002 NAHMS Dairy Study cannot be used to accurately estimate the true prevalence of positive herds or animals in the United States. A 2007 NAHMS study yet to be published may provide more accurate information on herd and animal prevalence of MAP.

Wildlife. Understanding the ecology of the host species can provide valuable information about disease risk to susceptible livestock. Multiple studies have demonstrated the presence of MAP in the feces and tissues of wild nonruminant animals, including feral cats, mice, foxes, stoats, weasels, crows, rooks, jackdaws, rats, wood mice, rabbits, hares, and badgers *(8, 21, 22, 54, 70, 99)*.

Two studies have documented histopathological changes in wild animals consistent with MAP infection *(8, 22)*. The finding of viable MAP organisms in the feces of nonruminant animals could indicate a potential for spread of MAP beyond the range of infected ruminant herds *(22)*. Molecular genotyping could not discriminate between rabbit and cattle isolates of MAP *(54)*. MAP has also been isolated from several species of wild ruminants, including red deer, roe deer, fallow deer, moufflons, and North American bison *(12, 101)*. There is evidence of the transmission of MAP between wild ruminant populations and free-range cattle *(101)*.

Insects. Insects may be vectors of MAP, but their significance is unknown *(38–40)*. Fischer et al. *(39, 40)* recovered MAP in the droppings from nymphs of the oriental cockroach and from the abdomen and head of blowflies experimentally infected with MAP. Another study performed in the Czech Republic found MAP in Diptera flies in areas occupied by MAP-infected cattle *(38)*.

Protozoa. Mycobacteria (MAP and non-MAP) have been described in protists *(74, 88, 133)*. In laboratory studies, growth of bovine and human MAP isolates in *Acanthamoeba polyphaga* as well as replication of clinical and environmental strains of *M. avium* (non-MAP) within *Acanthamoeba castellanii* suggest that protozoa may provide a reservoir for mycobacteria *(15, 92)*. Mura et al. *(92)* reported the growth and survival of MAP in *A. polyphaga* for up to 4 years using in situ hybridization and quantitative real-time PCR; however, these authors did not attempt to culture the organism. Others have reported survival of MAP within *Acanthamoeba* and demonstrated

TABLE 4. Mycobacterium avium *subsp.* paratuberculosis *detection methods in fecal matrices*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
To estimate sensitivity, specificity, and predictive value of an ELISA relative to fecal culture for fecal and milk samples *(126)*.	Fecal samples from 712 individual cows within 61 herds and bulk tank samples from 52 of the 61 herds	ELISA protocol was not described. For culture, fecal samples (2 g) were added to 35 ml of sterile deionized water, settled for 30 min; supernatant removed and centrifuged (1,700 × *g*, 20 min); pellet was resuspended in 0.9% HPC-BHI, held overnight at 37°C (98.6°F); centrifuged (1,700 × *g*, 20 min). Pellets were resuspended in 1 ml of antibiotics (naladixic acid, vancomycin, amphotericin B) and incubated overnight at 37°C (98.6°F). Samples inoculated onto HEYM with naladixic acid, vancomycin, and mycobactin J and incubated for 12 wk. Milk was centrifuged (2,000 rpm, 30 min), pellet was resuspended in 0.9% HPC-BHI and incubated overnight at 37°C (98.6°F). Samples were recentrifuged (2,500 rpm, 20 min); pellets were resuspended in 1 ml of antibiotic solution. Samples were inoculated onto HEYM with antibiotics and incubated for up to 12 mo at 37°C (98.6°F). For DNA extraction, 0.5 ml of composite milk sample was centrifuged (15,000 rpm, 5 min). Proteinase K was added to cell pellets, vortexed to resuspend the pellet, and incubated overnight at 50°C (122°F). TEN buffer and NaOH were added, mixed, and placed in a boiling water bath for 30 min, then extracted with phenol–chloroform–isoamyl alcohol, then centrifuged (10,000 rpm, 5 min). DNA was amplified by nested PCR targeting IS*900*. Positive PCR results were seen with spiked samples containing 10^2 or more MAP/ml of milk.	Only 25% of cows that were fecal-culture positive were also positive by fecal ELISA, and 6% of cows that were fecal-culture negative were positive by ELISA (25% sensitivity and 6% false-positive rate). 22% of ELISA-positive cows were fecal-culture positive (22% positive predictive rate). All milk samples cultured negative, but 68% of herds had positive PCR results from bulk tank samples, including 11/21 herds with negative fecal cultures.	Inability to culture MAP from bulk tank samples may reflect insensitivity of culture in conjunction with the very low levels present in the milk. The ELISA was a poor predictor of animal infection in this study.
Evaluate two different methods to recover mycobacterial DNA from bovine fecal specimens for PCR analysis *(98)*.	A total of 130 bovine fecal specimens were used; 70 samples were from MAP-infected farms and the remaining 60 samples were from uninfected farms.	Fecal specimens were processed for culture using conventional techniques. The direct dilution–centrifugation method was used to isolate cells from feces. The use of CB-18 was thought to aid in the isolation of MAP from fecal specimens. DNA was extracted with Qiagen modified method. The PCR target was IS*900* primers: 5′-CACGTGCT GTCCCCATCGGC-3′ and 5′-CTACGTCT TCGTGACCAAAG-3′. IAC targeted a universal plant chloroplast *rpoB* region. Primers were 5′-CCGTGATCAA TCAAATATTGCTAAGTC-3′ and 5′-ACCCATAGCTGATGATAGAACT-3′. Culture decontamination method used HPC (18–24 h), inoculated onto HEYM with mycobactin J.	Centrifugation method followed by PCR detection yielded a sensitivity of 92.6% and a specificity of 83.7%. CB-18 method followed by PCR detection yielded a sensitivity of 100% and a specificity of 53.5%. Sensitivity and specificity of both methods were calculated against culture results.	Low specificity of both methods was due to having a positive PCR result and a negative culture result within the same sample.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Compare two cultivation procedures with PCR confirmation for detection of MAP *(37)*.	Feces (USDA fecal check test kit samples)	Feces were decontaminated with 0.9% HPC in BHI overnight, centrifuged, and incubated with polymyxin B, amphotericin B, carbenicillin, and trimethoprim overnight. 200 μl was inoculated onto HEYM with mycobactin J, 200 μl into TREK ESP II vials, and confirmed with acid-fast staining and PCR. PCR direct extraction of feces with Qiagen QIAmp DNA stool mini kit, and PCR performed targeting IS*900* and *hspX*.	All negative via *hspX* primers. Some false positives with IS*900* primers. TREK ESP II had one false positive.	TREK ESP II missed one sample possibly because of reduced inoculation volume (normally 1 ml). Culture three of four positive. PCR prescreening lacked specificity. Combination of TREK culture and *hspX* PCR was 100% sensitive and specific.
Compare three ELISA methods for MAP using tissue and fecal culture as comparison standard *(83)*.	Fecal samples were collected from 994 cows.	2 g of feces was placed in 25 ml of DHOH, shaken (30 min), and let stand 30 min, and 5 ml was removed and placed into 25 ml of 0.75% HPC (24 h, 37°C [98.6°F]), centrifuged (900 × *g*, 30 min). Pellet was resuspended in half-strength BHI with naladixic acid, vancomycin, and amphotericin B for 12–14 h at 37°C (98.6°F). 1 ml was inoculated into TREK vials for 6 wk then acid-fast stained. If the sample was acid-fast positive it was subcultured to HEYM for 6 wk, then PCR confirmed.	The sensitivity of all three ELISA methods was 13.9–27.8%. The specificity was 90.1–97.1%.	The accuracy of all three ELISAs was very poor and of questionable value for screening food.
To determine if results from ELISAs for bovine milk and serum will correlate with bovine fecal culture samples *(59)*.	Fecal, blood, and milk samples were collected directly from 689 lactating cows.	Serum samples were tested in duplicate with a commercial ELISA at one lab. Milk samples were tested with an unspecified ''in-house'' ELISA at a second lab. Fecal samples were tested by a modified ''double incubation method.'' Feces (2 g) were added to 35 ml of sterile water and allowed to settle for 30 min. The top 5 ml was transferred to 25 ml of 0.9% HPC in BHI and held at 37°C (98.6°F) overnight. The sample was pelleted (900 × *g*, 30 min) and resuspended in 1 ml of an antibiotic solution (vancomycin, nalidixic acid, amphotericin B in BHI) and incubated (overnight at 37°C [98.6°F]). HEYM slants were inoculated and incubated for ~16 wk. Colonies confirmed by IS*900* PCR (primers neither referenced nor specified).	Fecal culture results were used as the ''gold standard.'' ELISA and culture results were compared. Agreement between milk and serum results were statistically ''moderate,'' although the *P* value from the McNemar test indicated significant differences in the proportions of positive results. Serum and fecal results had similar statistical outcomes. Agreement between milk and fecal samples was higher, and proportions of positive results were not different.	In all cases, most reliable results were obtained for cows classified as ''heavy shedders'' by the fecal culture technique.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Compare four culture methods for detection of MAP from cattle feces *(90)*.	240 bovine fecal samples	Sedimentation: 1 g of feces was added to 35 ml of DHOH, shaken for 30 min, and allowed to settle for 30 min. 5 ml was removed and added to 25 ml of 0.9% HPC and incubated overnight at RT. The resulting sediment was used to inoculate two plates of HEYM and one plate of HEYM with mycobactin J and incubated for 16 wk. Double centrifugation: 1 g of feces was added to 35 ml of DHOH, shaken for 30 min, and allowed to settle for 30 min. 25 ml was removed and centrifuged (4,000 × *g*, 20 min), pellet was suspended in 25 ml of 0.9% HPC in 25 ml of half-strength BHI and incubated overnight at 37°C (98.6°F). The mixture was centrifuged again and the pellet was suspended in half-strength BHI with amphotericin B, vancomycin, and naladixic acid and incubated overnight at 37°C (98.6°F). Inoculated onto two plates of HEYM and one plate of HEYM with mycobactin J for 16 wk. Double centrifugation was performed as above and used to inoculate MB/BacT broths. If growth occurred, 500 μl from the vial was inoculated to two plates of HEYM and one plate of HEYM with mycobactin J for 8 wk. Molecular detection: PCR was performed targeting IS*900* (L1 and L9 loci) plus 251-bp unique sequence.	Sensitivity method 1 = 37/240 Method 2 = 53/240 Method 3 = 65/240 Method 4 = 76/240	27 samples positive by all four methods, 76/81 positive samples detected by method 4. All but two of broth enriched–molecular assay positive confirmed by one of three other protocols. Indicated broth-molecular method (method 4) indicates true positives and other methods less sensitive.
Compare culture and RT-PCR for detection of MAP in cattle feces *(9)*.	Feces (bovine), 310 spiked samples	3 g of feces was homogenized in 50 ml of 4% NaOH, shaken 10 min, and centrifuged (3,000 × *g*, 15 min). The pellet was resuspended in 20 ml of 5% oxalic acid, vortexed, and shaken for 15 min. The pellet was resuspended in 4 ml of 0.15 M NaCl. 200 μl was transferred to three slants of LJ medium with mycobactin J and one LJ slant without mycobactin J and incubated 16 wk. RT-PCR performed targeting IS*900*.	Sensitivity 60% Specificity 93.4% LOD 500 CFU/g, 100% LOD 31 CFU/g, 71%	
Development of nested PCR targeting IS*900* and ISMAP*02* for detection of MAP *(122)*.	Feces (bovine naturally infected and spiked feces)	Cultured on HEYM with mycobactin J (double centrifugation, double decontamination procedure; no specifics on decontamination). DNA extracted for PCR targeting IS*900* and ISMAP*02*.	All methods equal in sensitivity; specificity not done; LOD 1–7 CFU.	ISMAP*02* PCR equal to nested conventional or RT-PCR with IS*900* target.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Evaluate DNA isolation method and RT-PCR for detection of MAP in feces *(66)*.	Feces (bovine, spiked)	Evaluated the use of Whatman FTA cards for isolation of DNA from fecal samples. Fecal samples were spiked with known amounts of MAP. PCR was performed using FRET. The target sequence was IS*900*.	Reported LOD in fecal samples was 10^2–10^4 CFU/g. No specificity reported.	Question whether spiking bovine feces would give an accurate estimate for limit of detection. (i.e., how were the feces determined to be MAP free prior to spiking with MAP).
Compare BACTEC 460 and MGIT 960 systems for detection of S strains of MAP in pooled ovine fecal samples. Also evaluate the use of ampicillin for control of contamination *(56)*.	Feces (ovine, naturally infected)	Pure culture of laboratory adapted S strain was used to inoculate both BACTEC 12B radiometric medium and MGIT ParaTB medium vials. Samples from four MAP-free and one MAP-positive animal were pooled together. All pooled fecal samples were decontaminated 24 h in HPC and then in vancomycin, naladixic acid, and amphotericin B for 48–72 h. Sample inoculated into BACTEC and MGIT culture vials. BACTEC vials also inoculated with MAP with and without ampicillin to determine effects of ampicillin on MAP growth. BACTEC vials also inoculated with MAP with and without vancomycin to determine effects of vancomycin on MAP growth.	Growth of pure culture was detected 2 wk earlier in BACTEC compared with MGIT culture vials. Analytical sensitivity was estimated to be 2.3×10^1 and 2.3×10^3 for MGIT and $<2.3 \times 10^1$ for BACTEC. Sensitivity of BACTEC on pooled fecal samples was 21.9%. When data were divided by the pathological lesions, when the positive sample in the pooled sample came from a sheep with multibacillary lesions the sensitivity was 100%, when the positive sample in the pooled sample came from a sheep with paucibacillary lesions the sensitivity was 17.8%. MGIT did not detect any of the pooled samples as positive. Ampicillin had to be added to cultures every 4 wk to prevent overgrowth of contaminants and delayed the growth of MAP 1–2 wk. Addition of vancomycin delayed growth of MAP (initial inoculum $= 2.3 \times 10^4$) 4 wk in BACTEC culture vials.	Animals were diagnosed during necropsy and considered infected with MAP if the terminal ileum had characteristic lesions or if MAP was cultured using BACTEC from the tissue or lymph node associated with the terminal ileum Inhibition experiments were conducted using a laboratory strain of S type MAP.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Develop new PCR for detection in feces *(71)*.	Ovine feces (naturally infected and spiked with laboratory strain of MAP)	DNA isolated using JohnePrep. 0.5 g of feces added to 10 ml of saline and allowed to settle 30 min. 1 ml of supernatant added to zirconia-silica beads, washed in lysis buffer and bead beating for 45 s. DNA precipitation with isopropanol.	Sensitivity determined three ways: plasmid containing portion of IS*900* = one copy; purified DNA from culture = 2 fg (approx 0.2 genomic equivalents); spiked feces = 10 CFU/g. Specificity = 100%.	Sensitivity calculated three ways, using cloned plasmid, purified DNA from culture, and spiked fecal samples. The authors tested 506 unknown samples with both culture and their PCR assay but did not report a sensitivity or specificity using the classical methods of calculation to compare their PCR assay with a ''gold standard.''
Comparison of ELISA testing of milk and serum using a commercial kit with fecal culture on HEYM in goats *(114)*.	Serum, milk, and feces from goats	Serum harvested via centrifugation. Milk centrifuged and sampled below cream layer. Feces decontaminated using ''Cornell method.''	Sensitivity and specificity of ELISA in serum = 74.3 and 98.6%; in milk = 60 and 99.3%.	Classification of low, medium, and high shedders based on culture results was elevated compared to most other studies. The authors categorized animals as low, <50 CFU/g; medium, 50–299 CFU/g; and high, >300 CFU/g.
Use of a radiometric method to detect MAP in pooled feces *(34)*.	Pooled feces from infected and noninfected cows	IS*900* PCR used to detect MAP in pooled fecal samples from infected and uninfected cows after HPC treatment, incubation for 20–26 h at 37°C (98.6°F), centrifugation, addition of VAN antibiotics, reincubation for 70–74 h, aliquot into BACTEC 12B radiometric medium containing egg yolk, mycobactin J, and PANTA antibiotics. Incubation at 37°C (98.6°F) for 12 wk and measured.	High-shedder cow feces could be diluted 1:30 and feces from low shedders 1:10 with feces from uninfected cows and detection still possible.	Detection of an infected cow population by examination of fecal material.
Comparison of RT-PCR with solid and liquid culture media and serum ELISA *(2)*.	Fecal and serum samples from dairy cows	RT-PCR per instructions with Tetracore VetAlert kit. Culture: HEYM and TREK ESP per Stabel et al. *(123)*.	Sensitivity of RT-PCR estimated to be 0.60; specificity 0.97, accuracy 0.90 (compared to 0.91 for solid culture, 0.93 for liquid culture, and 0.82 for ELISA).	Evaluated 143 animals.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Evaluate a triplex RT-PCR *(65)*.	Fecal samples from five calves experimentally exposed	1 g of feces was mixed with 20 ml of 5% SDS and allowed to sediment. The sediment was washed three times with PBS and centrifuged. The pellet was disrupted by boiling and freezing, followed by DNA extraction. PCR was performed with primers for F57, ISMAP*02*, and IS*900*.	LOD 2.5×10^2 MAP/g feces.	Tested 197 fecal samples collected over 3 yr during subclinical phase. Good agreement with culture. Used sequence analysis of IS*900* amplicons from three IS*900*-positive samples to confirm positivity.
Development of a rapid biosensor assay for live MAP *(75)*.	Spiked fecal samples	RNA was extracted using Trizol, treated with DNase and heat (95°C [203°F], 5 min). RNA was amplified using RT-PCR. The assay was performed with a lateral flow biosensor targeting IS*900*. Signal amplification occurred when target sequence hybridized to DNA probe coupled to liposomes encapsulating the dye sulforhodamine B.	Detects 10 viable cells.	No cross-reactivity with other mycobacteria.
Estimate the sensitivity and specificity of direct fecal PCR compared to HEYM; compare abilities of direct fecal PCR and serum ELISA to identify animals shedding MAP *(17)*.	Fecal samples, serum samples	2 g of feces was analyzed as follows. Culture: mixed with 35 ml of water and shaken to disrupt fecal material. After settling for 30 min, 5 ml was removed and decontaminated with 0.9% HPC in half-strength BHI and incubated overnight at 37°C (98.6°F). Material was centrifuged, and the resulting pellet was treated with antibiotics, incubated at 37°C (98.6°F), and cultured on HEYM up to 16 wk. PCR: mixed with 4 ml of water and vortexed. After settling for 60 min, 1.5 ml of liquid was removed to a tube with zirconia-silica beads. The mixture was centrifuged, incubated at 95°C (203°F) for 10 min, and bead beat (45 s, 6,500 rpm). Centrifuge and isolate DNA. PCR analysis with P90 and P91 primers. ELISA: Parachek Johne's Absorbed EIA.	Compared to culture ''gold standard'' Sensitivity: PCR, 70.2% (57.7–80.7) ELISA, 31.3% (20.6–43.8) Specificity: PCR, 85.3% (79.3–90.1) ELISA, 97.8% (94.5–99.4)	Two farms, one with suspected high prevalence and one with low prevalence. Of 250 animals, 26.8% were culturally confirmed to be shedding MAP; PCR detected 29.6% and ELISA 10.0%. A positive ELISA was more likely associated with a diseased animal but a negative ELISA was less likely to predict a nondiseased animal.

TABLE 4. *Continued*

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Describe a rapid effective method for the extraction of MAP DNA for fecal samples for PCR *(123)*.	Fecal samples	Culture using double centrifugation, double decontamination *(120)* and HEYM with mycobactin J and antibiotics. DNA extraction: 1 g of feces diluted with 9 ml of 1 × Tris-EDTA buffer, vortex 5 s, settle 2 min, revortex. Centrifuge. Transfer supernatant and dilute with Tris-EDTA buffer. Centrifuge, resuspend pellet in Tris-EDTA buffer, heat 100°C (212°F) for 10 min. Cool, treat with RNase, then run PCR.	Sensitivity: 81% when >70 CFU/g 45% when <1 CFU/g Reproducibility: 75% when >70 CFU/g 25% when <1 CFU/g	1,000 fecal samples. Sensitivity and reproducibility were highly dependent on the load of bacteria. Method can detect animals that are shedding <1 CFU/g.

[a] LOD, level of detection; ELISA, enzyme-linked immunosorbent assay; HPC, hexadecylpyridinium chloride; BHI, brain heart infusion; HEYM, Herrold's egg yolk medium; TEN buffer, 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), Sigma Chemical Co.; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; CB-18, C_{18}-carboxypropylbetaine; IAC, internal amplification control; DHOH, sterile double-distilled water; RT, room temperature; MB/BacT, a modified Middlebrook 7H9 broth, bioMérieux; RT-PCR, real-time PCR; LJ, Lowenstein-Jensen; FRET, frequency resonance energy transfer; MGIT, mycobacteria growth indicator tube; VAN, vancomycin, amphotericin B, and nalidixic acid; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

that strains sequestered in protozoa were more resistant to chlorination than free-living noningested strains *(144)*. Whether *Mycobacterium* spp. colonize protozoans in their natural habitat is unknown.

Environmental contamination. Cattle manure can serve as a source of contamination for other foods. Runoff from cattle holding areas and improperly composted manure can spread the pathogen in the environment and contaminate water and food products *(78)*. Mycobacteria are known to survive for variable periods in the environment *(149, 150)*. In a study of New York herds ($n = 33$), spreading manure on fields used to grow forage for cattle was identified as a risk factor (odds ratio = 10.3) for on-farm transmission of MAP *(95)*. Grewal et al. *(55)* examined the persistence of MAP during simulated composting, manure packing, and liquid storage of dairy manure. Data indicated that MAP may survive at least 8 weeks in liquid slurry, but MAP was not detected in packed or composted manure beyond day 0.

Raizman et al. *(107)* examined 122 samples from cow alleyways on 66 Minnesota farms and found 77% of these farms were culture positive for MAP. The authors suggested that environmental sampling might be a useful means by which to identify positive herds.

Whittington et al. *(149)* isolated MAP from 25 of 146 soil, creek sediment, and fecal samples on farms with stock having a high prevalence of Johne's disease. Only 1 of the 22 originally positive soil sites was culture positive 5 months after the infected animals were removed from the area, and there were no culture-positive sites after 12 months. The authors noted a decreased recovery of MAP from mixed fecal and soil samples compared to fecal samples alone. They postulated that the reason for the decreased recovery was due to binding of MAP cells to soil particles, which are typically removed from the culture matrix during sample preparation. If soil reduces the recovery of MAP, MAP prevalence in soil may be underestimated.

It has been speculated that MAP may be disseminated via aerosols that land on pastures and are ingested by animals *(148)*. No studies have been reported that evaluate this route of exposure.

Presence of MAP in water. Since cattle and other ruminants are reservoirs of MAP, farm runoff may potentially contaminate water supplies *(78)*. MAP has been isolated from soil and water samples surrounding sheep farms *(149)*, and under experimental conditions MAP has been known to survive for months in both water and water sediments from the environment *(148)*. MAP also was detected in a river in England downstream from cattle- and sheep-grazed grasslands *(103)*. Under experimental conditions, MAP remained culturable in surrounding lake water samples for 632 days *(103)*.

MAP detection by PCR in a river in Wales peaked 5 to 7 days after rainfall, suggesting runoff from up-river cattle and sheep sources *(104)*. In the same study, MAP was not detected immediately after water treatment but was detected by PCR in 1 of 54 posttreatment domestic cold-water storage tanks, indicating the potential for MAP to access

TABLE 5. Mycobacterium avium *subsp.* paratuberculosis *detection methods in tissue matrices*[a]

Purpose	Sample type	Sample pretreatment approach and detection approach	Sensitivity, specificity, LOD	Comments
Test several of the available solid culture media for isolation of both bovine and caprine MAP isolates. Study the differential requirements for isolation depending on MAP type and not host *(25)*.	Tissue—homogenized ileocecal valve, intestine, and mesenteric lymph node	All samples were previously decontaminated with HPC before inoculation onto solid media. Each sample was inoculated onto HEYA with mycobactin J, HEYA with mycobactin J and sodium pyruvate, Middlebrook 7H11 medium with mycobactin J, and LJ with mycobactin J. All media contained polymyxin B, carbenicillin, amphotericin, and trimethoprim. Isolates of MAP classified as either type II or type I/III.	100% of type I/III were isolated from either LJ or Middlebrook 7H11 medium. All four media required to isolate 100% of type II isolates.	Demonstrates that even isolates of the same type may have different requirements for growth.
Describe different diagnostic procedures for detection of caprine paratuberculosis based on clinical signs, pathological lesions, conventional isolation and identification, and nested PCR *(5)*.	Tissue—mesenteric lymph node	Methods were limited to the PCR methods. Nested PCR was conducted targeting IS*1311*. First round PCR primers were 5′-TGAACGGAGCGCAT-CACGAA-3′ and 5′-TGCAGCTGGTGATCTCTGAT-3′. Second round primers were 5′-GCAAAGTCGACGATT-TGGTC-3′ and 5′-AAGTGCTCGGCTTCGACGTC-3′.	Pathology included thickening and corrugation of the mucosal folds of the ileum, jejunum, and colon. Mesenteric lymph node enlarged, pale, and edematous. Acid-fast bacilli found in both the ileum and sections of the mesenteric lymph node.	All tests performed on a single goat. Tissue source and DNA extraction method not described.
Evaluate ability of immunohistochemistry and in situ hybridization to detect MAP in formalin-fixed tissues *(26)*.	Tissue from six cows—ileum, ileocecal, lymph node	Tissues were fixed in 10% formalin and embedded in paraffin, sectioned and stained (hematoxylin and eosin), and tested with anti-MAP antibody. In situ hybridization was conducted with a 284-bp segment of IS*900* and alkaline phosphatate–streptavidin conjugate.	Both techniques were able to detect MAP, but immunohistochemistry produced higher intensity staining.	Four adult animals with clinical signs, two young animals from a disease-free herd. Immunohistochemistry was easier to perform. In situ hybridization was as effective as Ziehl-Neelsen staining.

[a] LOD, level of detection; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; HPC, hexadecylpyridinium chloride; HEYA, Herrold's egg yolk agar; LJ, Lowenstein-Jensen.

domestic outlets *(104)*. A study in Northern Ireland involving 192 samples of untreated source water obtained from nine water treatment facilities found 9 of 192 samples positive by PCR and 8 of 192 positive by cultural methods; the combined prevalence using all methods was 7.8% *(145)*. Surveys of treated and untreated water from treatment facilities in Great Britain failed to detect MAP *(62, 141, 142)*. It is thought that municipal water treatment according to current U.S. Environmental Protection Agency (EPA) requirements will reduce the number of MAP present in water. Studies are needed to determine the extent and adequacy of reduction of MAP by municipal water treatment processes. Representatives of the U.S. EPA advised this Committee that there are no data relative to the prevalence of MAP in the U.S. water supply, but studies to address this issue are being planned.

Prevalence and Numbers of MAP in Food Sources

Milk. The vast majority of the reviewed literature regarding MAP in food dealt with raw (not heat treated) and pasteurized bovine milk. Given the large volume of milk consumed (USDA Economic Research Service estimates the annual per capita consumption of beverage milk to be 21 gal [79 liters]), the potential for exposure to MAP from milk is important to consider. Studies reporting the presence of MAP in milk are summarized in Table 6. Most of these studies only reported the prevalence of MAP in milk and provided no information on the number of cells that may be present. A few studies have provided estimates of the numbers of MAP cells in raw cows' milk using cultural methods; numbers typically were on the order of 1 cell per 10 ml *(49, 80, 106, 131)*. The number of MAP in raw milk may be underestimated due to the effects of clumping and inactivation or loss during decontamination steps used in testing for the presence of MAP *(49, 52)*.

Raw milk. A number of studies have evaluated the prevalence of MAP in raw milk and can be summarized as follows. The percentage of raw bovine milk samples positive for MAP when analyzed by PCR ranged from 7.8 to 32.5% *(6, 20, 49, 68, 97, 119)*. Prevalence of MAP in raw bovine milk samples analyzed by culture ranged from 0.25 to 11.1% *(49, 68, 97, 131)*. One study in Switzerland found that 23% of raw goat milk samples and 23.8% of raw ewe milk samples were MAP positive by PCR *(91)*.

Grant et al. *(49)* found that 19 of 244 (7.8%) 50-ml bulk milk samples from 241 approved dairy processing establishments throughout the United Kingdom were positive for MAP by IS*900* PCR, but only 4 of 244 (1.6%) were positive using culture techniques. In a Pennsylvania study *(41)*, 13.5 and 2.8% of quarter milk samples were positive for MAP by PCR and culture, respectively; 27.5 and 6.8% of bulk milk samples were also positive by PCR and culture, respectively. Slana et al. *(119)* analyzed 342 milk samples by IS*900* and F57 real-time quantitative PCR (qPCR); 13.7 and 18.7% were positive by the F57 and the IS*900* qPCR assay, respectively. No sample, however, was culture positive on HEYM plus mycobactin J.

Regional differences in the prevalence of MAP are known to exist, as indicated in a study by Corti and Stephan *(20)* from Switzerland that found differences in prevalence in bulk tank milk samples ranging from 1.7 to 49.2%, depending on region.

Pasteurized milk. The prevalence of MAP in pasteurized bovine milk samples analyzed using PCR ranged from 2 to 15% *(6, 35, 41, 49, 59, 87)*. Pasteurized bovine milk samples analyzed by culture ranged from 0 to 2.8% positive *(6, 35, 41, 49)*. A study conducted in England and Wales found 9 of 18 PCR-positive retail milk samples as well as 6 of 36 PCR-negative samples were culture positive *(87)*, demonstrating the typical variability in prevalence based on the detection and recovery method(s) used.

O'Reilly et al. *(97)* analyzed 389 raw and 367 pasteurized milk samples in Ireland using immunomagnetic separation–PCR (IMS-PCR) and found that 12.9 and 9.8%, respectively, were positive. Yet, only one positive raw milk sample was identified by bacterial culture. Cream and whole milk samples were more frequently PCR positive than skim milk samples. Positive samples also peaked in June, suggesting possible seasonal variations. Grant et al. *(49)* also found 67 of 567 (11.8%) commercially pasteurized milk samples were MAP positive by PCR, while 10 of 244 (1.8%) were positive by culture.

Another study in Canada found 110 of 710 (15%) of milk samples from retail stores ($n = 535$) and dairy plants ($n = 175$) were positive for MAP by nested IS*900* PCR; however, no MAP was isolated from broth and agar cultures of 44 PCR-positive and 200 PCR-negative retail milk samples *(41)*. Ellingson et al. *(35)*, using two different culture methods, found that 20 of 702 (2.8%) retail pasteurized milk samples from Minnesota, California, and Wisconsin were positive for MAP by one or both methods.

The presence of MAP in pasteurized milk may be due to postprocess contamination, underprocessing, or survival of the process. These issues are discussed further in the response to question number 4.

Milk products. Cheeses may be made from pasteurized, thermized, or unpasteurized milk. The aging process in cheese manufacturing has been shown to reduce pathogens originally present in milk that have not been inactivated by heat treatments. However, pathogens may not be completely eliminated by the aging process *(32, 120, 130)*. If MAP is not inactivated during the aging process, cheese could be a source of exposure to this organism.

A study conducted by Clark et al. *(16)*, in which 98 samples of cheese curds were analyzed for MAP, reported that 23% of the samples were positive by IS*900* PCR, 9% were positive by PCR detection of the *hspX* gene, and 5% of the samples were positive by both methods. No viable MAP could be obtained by culture, although 6 to 11% of rinsates of culture slants were positive for MAP by PCR *(16)*. Depending on brand, 0 to 85.7% and 0 to 14.3% of retail cheeses from Greece and the Czech Republic were positive

by IS*900* PCR and culturing, respectively *(63)*. The study did not report if the milk used to manufacture these cheeses had been heat treated.

A study conducted in Switzerland examined 143 retail raw milk cheeses (9 soft, 133 semihard, and 1 hard) by real-time PCR and BACTEC culture following immunomagnetic capture. The investigators found no positives by culture, but 4.2% of the samples showed evidence of MAP by real-time PCR, suggesting that MAP had been present in the raw material *(128)*.

In addition, there are three studies in which cheeses were manufactured using artificially contaminated milk to determine survival and persistence of MAP during ripening *(32, 120, 130)*. These studies are addressed below in question 4 on processing interventions. Although the numbers of MAP cells decreased during cheese manufacturing, the organism was not completely eliminated at the inoculum levels used and the conditions studied *(32, 120, 130)*. It should be noted that these studies were not conducted with naturally contaminated milk.

Hruska et al. *(61)* tested 51 samples (20-mg portions) of powdered infant formula from 10 manufacturers in seven countries using IS*900* PCR, the fragment F57 real-time PCR method, and culture. A total of 25 (49%) samples were positive for MAP by IS*900* PCR, and 18 (35%) samples were positive by fragment F57 real-time PCR. Cultural confirmation of MAP contamination could not be achieved for any sample. These findings warrant confirmation, particularly in light of the high percentage of PCR-positive samples, the lack of culture confirmation, and the small sample size.

Meat. Since MAP results in a disseminated infection in cattle, including lymphatic tissue and the liver *(64, 110)*, beef should be considered a potential source of exposure for MAP, especially ground beef and organ meats such as liver. The Committee reviewed the limited reports describing the presence of MAP on carcasses, in meat, and in meat products. An abstract provided by Rossiter and Henning *(110)* demonstrated a correlation between animals with disseminated infection (determined by culture of the ileocecal lymph node and feces) and the presence of MAP in the liver and in other lymph nodes (superficial cervical and popliteal lymph nodes) that could be incorporated into ground beef. The authors concluded that the prevalence of MAP in the superficial cervical and popliteal lymph nodes in the total market cow population was very low but should be further investigated. Nelli et al. *(93)*, using a real-time PCR test for IS*900*, detected MAP in the gastrocnemius muscle of cows and sheep.

A survey of ground (minced) beef samples ($n = 113$) collected over 4 months from a single meat processing plant in the Republic of Ireland failed to find viable MAP *(64)*. The absence of a detailed description of the methods used in this study precludes assessment of the suitability of the isolation and/or detection protocols. A Canadian survey of 300 carcasses reported that while 22 to 54% of beef and dairy cow skinned carcasses were IS*900* PCR positive for MAP by a nested PCR assay and 6 to 50% were positive after dressing (including pasteurization), the fractions of IS*900*-positive preparations positive for F57 (specific for MAP) ranged from 5 to 43%. Only two dressed and/or pasteurized carcasses were positive for IS*900* with a quantitative PCR, and none for F57 *(84, 85)*. A survey of retail ground beef samples ($n = 200$) in California failed to detect MAP using an IS*900* PCR assay *(67)*. Meadus et al. *(85)* indicated that the limited data available suggest that contamination of beef carcasses with MAP may not be a major source of human exposure.

Fish and shellfish. Since MAP is present in fecal material from cattle and runoff from fields containing manure may contaminate water sources, there is the potential for fish or molluscan shellfish harvested from waters contaminated with bovine feces to be contaminated. However, there have been no studies published examining fish or shellfish for the presence of MAP.

Produce. In an abstract published by Pavlík et al. *(100)*, MAP was reportedly recovered from lettuce, radishes, and tomatoes cultivated for 4 weeks in soil artificially contaminated with MAP. The Committee did not find any published studies for the presence of MAP on vegetables grown in naturally contaminated soil.

4. What processing interventions are available for the foods of concern to eliminate or reduce the levels of MAP contamination to an acceptable level or to ensure that MAP does not enter the food supply?

At this time the Committee could not determine what frequency and level of MAP in food and water are ''acceptable,'' since the impact of foodborne exposures on human health is unknown; therefore, the Committee is not expressing an opinion on the relative public health importance of different levels of exposure to MAP. The literature reviewed by the Committee suggested that raw milk represents a potential source of human exposure to MAP. Milk pasteurization is an effective mitigation strategy for reducing MAP in milk and dairy products, although the organism has been found in up to 2.8% of pasteurized milk samples tested *(35)*. Studies on the efficacy of pasteurization to inactivate MAP are complicated by a number of factors:

- The tendency of the organism to clump interferes with accurate enumeration.
- The hydrophobic nature of the organism causes cells to congregate at liquid surfaces and in films on the sides of glass tubes, which complicates the preparation of accurate dilutions to be used in enumeration *(43)*.
- Extremely slow growth prolongs the time to results. Because of slow growth (inactivation studies often incubate cultures 2 to 3 months and sometime up to 1 year to recover low numbers of injured survivors), other organisms often overgrow the cultures and may mask the detection of MAP.
- Decontamination of samples reduces interfering organisms but may reduce the efficiency at which MAP is recovered *(52, 80)*.

TABLE 6. *Summary of studies on the prevalence of* Mycobacterium avium *subsp.* paratuberculosis *in milk*[a]

Source	Findings	Method	Comments
Raw milk *(131)*.	11.1% (8/72) cows gave MAP-positive milk; 11.7% (9/77) milk samples MAP positive.	50-ml composite of all four quarters, ice transport to lab. Decontaminated (0.75% HPC, 18 h); HEYM culture (37°C, 16 wk).	Examined cow lymph nodes, antibodies (ELISA), feces.
Whole pasteurized milk; England *(87)*.	7% (22/312) retail samples (cartons and bottles) PCR positive.	PCR IS*900*.	
	82% (18/22) of PCR signal segregated to cream or pellet fraction, consistent with presence of intact mycobacteria (although they may not have been viable).	Culture: decontaminate (overnight at RT with 0.1% benzalkonium chloride or 0.1 or 0.75% HPC in PBS; cultured in Dubos broth with mycobactin J (37°C [98.6°F], 13 to 40 mo).	Seasonal peaks in January–March and September–November when approx 25% of samples positive.
	50% (9/18) PCR-positive samples and 17% (6/36) PCR-negative milk samples culture positive (13–40 mo incubation).	5-μl sample (cream, whey, pellet) screened by PCR.	
	Peaked in January–March and September–November when approx 25% retail pasteurized milk positive.		
	27.7% (15/54) retail samples culture positive; 17% (6/36) PCR-negative samples culture positive. No plating, no isolation.		
Bulk raw and commercially pasteurized milk; UK *(49)*.	814 samples.	IMS-PCR and culture: decontamination with 0.75% HPC (5 h, 21°C [69.8°F]).	March 1999–July 2000.
	Bulk raw: 7.8% (19/244) PCR positive; 1.6% (4/244) culture positive.	Cultured on HEYM and BACTEC 12B radiometirc medium (approx 18 wk).	Commercially pasteurized milk samples included: 228 whole; 179 semiskim; 160 skim.
	Commercially pasteurized: 11.8% (67/567) PCR positive; 1.8% (10/244) culture positive.	The 14 positives were on HEYM; 1 pasteurized milk positive on BACTEC.	
	Ten culture-positive pasteurized milk samples from 8/241 dairy processing establishments.		
	50-ml milk sample for PCR and culture.		
	PCR detection limit approx 1 in 50 ml.		
	Counts based on the number of colonies on HEYM slants, approx 4–20 CFU/50 ml. One sample positive on both HEYM and BACTEC had confluent colony growth on HEYM slants, indicating a higher number of MAP in the sample. Decontamination may underestimate prevalence.		
Bulk milk *(20)*.	19.7% (273/1,394) PCR positive; range 1.7–49.9%, depending on region.	PCR IS*900*. No culturing.	Switzerland: prevalence varied significantly between regions.
Raw and pasteurized cow's milk; Ireland *(97)*.	Raw milk: 12.9% (50/389) IMS-PCR positive; 1 culture positive.	IMS-PCR IS*900*.	Milk from all 27 approved pasteurizing plants:
	Pasteurized milk: 9.8% (35/357) IMS-PCR positive.	Culture: HEYM with mycobactin J and BACTEC 12B radiometric medium (supplemented with egg yolk, mycobactin J, and PANTA antibiotics) after decontamination with 0.75% HPC; incubated 18 wk; confirm by Ziehl-Neelsen, and IS*900* PCR.	Skim milk (0/8); semiskim milk (2/31); whole milk (25/277); cream (8/41). Peak in June; culture may underestimate; subculturing since did not always produce cultures for confirmation.

TABLE 6. *Continued*

Source	Findings	Method	Comments
		50 ml of milk tested by each method; sensitivity approx 1 CFU/50 ml. Centrifuged milk and tested pellet only.	
Raw milk; Canada *(59)*.	1.1% (77/689) milk samples were positive.	Milk samples tested with a commercial (AntelBio Systems) and in-house ELISA for antibodies against MAP; serum tested using IDEXX HerdChek MAP ELISA; feces cultured: decontamination using 0.9% HPC in BHI broth (overnight, 37°C [98.6°F]), centrifuged, pellet resuspended in BHI with antibiotics, incubated (overnight, 37°C [98.6°F]), inoculated onto HEYM (37°C [98.6°F], 15 wk); colonies confirmed as MAP using IS*900* PCR.	Milk samples preserved with bronopol; serum and fecal samples frozen (−20°C [−4°F]).
Raw milk; United States *(68)*.	Quarter milk sample: 2.8% (43/1,493) culture positive; 13.5% (201/1,493) PCR positive. Bulk tank milk: 6.8% (2/29) culture-positive; 27.5% (8/29) PCR positive	Milk samples centrifuged, pellets resuspended 500:1 in PBS; half used for DNA extraction and IS*900* PCR, half decontaminated overnight at RT in 15 ml of 0.75% HPC; centrifuge, resuspend 300:1 in 0.75% HPC, and six drops used to inoculate two plates of HEYM with mycobactin J; incubated (37°C [98.6°F], 16 wk). Colonies confirmed using Ziehl-Neelsen staining.	1,524 lactating cows in 29 herds in Pennsylvania (1,493 complete sets of feces, serum, and quarter milk). 16/29 herds had history of Johne's from July 1999–June 2002.
Raw milk *(119)*.	111/345 milk samples (32.5%) were positive by RT-PCR; none were positive by culture.	72 cows sampled (all four teats; from farm with known history of paratuberculosis) on days 1, 5, 6, and 13. Milk samples from 53 cows taken on day 82. Continuous tank milk samples taken on days 1 and 6, and bulk tank milk samples taken after 11 h of mixing on each day. Samples were centrifuged, pellet was resuspended in 0.75% HPC and decontaminated for 5 h. Pellet was centrifuged, resuspensed in water, and inocuated onto HEYM with mycobactin J, 37°C (98.6°F) for 18–30 wk. RT-PCR for IS*900* and F*57*.	February–April 2007
Commercially pasteurized cow's milk; Czech Republic. Locally pasteurized milk. Raw milk *(6)*.	4/244 bottles and cartons (1.6%) Herd A (low level of MAP infection): 2/100 (2%); herd B (high level of MAP infection): 2/100 (2%); herd C (not infected): 0/100 (0%). From fecal culture-positive animals of herd A: 1/10 (10%); from fecal culture-positive animals of herd B: 13/66 (19.7%).	HEYM (32 wk); confirmed by morphology, Ziehl-Neelsen staining, mycobactin J dependency, and IS*900* PCR.	

TABLE 6. *Continued*

Source	Findings	Method	Comments
Retail pasteurized milk; United States *(35)*.	702 samples. 2.8% (20/702) positive by one or both methods; 7/233 in California; 9/234 in Minnesota; 4/235 in Wisconsin. 12/22 brands yielded at least one MAP-positive sample.	(1) HEYM evaluated at 8 and 16 wk; (2) TREK ESP II Culture System; PCR confirmation of IS*900* and *hspX*.	Seasonal peak July–September.
Retail milk; Canada *(41)*.	15% (110/710) retail milk samples from retail store (535) and dairy plants (175) PCR positive; no MAP isolated from the broth and agar cultures of 44 PCR-positive and 200 PCR-negative retail milk samples.	Nested IS*900*; all PCR-positive and a subset of PCR-negative samples screened for MAP by culture on Middlebrook broth and agar (37°C [98.6°F], 5 to 6 mo), and BACTEC 12B radiometric culture (16 wk).	Southwest Ontario
Goat and ewe's milk; Switzerland *(91)*.	23% (79/344) goat's milk (range, 21.76–32.06%) 23.8% (15/63) ewe's milk (range, 13.98–36.21%)	10 ml; PCR IS*900*; no culture confirmation.	403 farms; milk cooled, tested within 24–48 h; 20 ml frozen (−20°C) before testing. Regional prevalence of IS*900*: 17% north, 34% south. Centrifuged milk and tested pellet only.
Retail cheese; Greece and Czech Republic *(63)*.	84 samples. PCR: 30.9% (26/84; range, 0–85.7%) Culture: 3/84 (3.6%; range, 0–14.3%)	Homogenized; decontaminate (0.75% HPC, 5 h); inoculate HEYM with mycobactin J (approx 8 mo); IS*900* PCR.	Most popular brands. Five brands of feta from Greece; three brands (one each soft, hard, and semihard) from Czech Republic. pH, salt conc, temp during manufacture may inhibit MAP, reducing viability. Decontamination may yield false-negative results on culture when PCR is positive.
Cheese curds; United States *(16)*.	Prescreening: 23% (23/98) positive for IS*900* or P90/P91; 59% (9/98) positive for *hspX*; 5% (5/98) positive by both assays; 0% (0/98) culture positive. PCR confirmatory slant rinse: 11% (11/98) positive for IS*900* or P90/P91; 6% (6/98) positive for *hspX*; 1% (1/98) positive by both assays. Conclusion: unable to detect viable MAP; presence of MAP-specific DNA indicates viable MAP once present in component of cheese curds.	Culture: HEYM (16 wk); confirm by acid-fast staining; PCR: IS*900*, P90, P91, and *hspX*.	Wisconsin and Minnesota; approx five samples from four regions for 6 mo (April–September 2004); P90/P91 are portions of IS*900* detected using LightCycler RT-PCR. 5-g samples tested.

[a] MAP, *Mycobacterium avium* subsp. *paratuberculosis*; HPC, hexadecylpyridinium chloride; HEYM, Herrold's egg yolk medium; ELISA, enzyme-linked immunosorbent assay; RT, room temperature; PBS, phosphate-buffered saline; IMS-PCR, immunomagnetic separation PCR; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; BHI, brain heart infusion; RT-PCR, real-time PCR.

- Populations of MAP include dormant and metabolically active cells, which may vary considerably in their susceptibility to inactivation *(57, 58)*.
- Inactivation studies conducted with laboratory-grown strains may not be representative of natural contamination. Laboratory-adapted strains may have altered heat resistance, growth characteristics, or ability to clump, all of which can lead to over- or underestimation of inactivation *(106)*. For example, Sung and Collins *(129)* indicated that low-passage clinical strains seem more sensitive to killing by heat treatment than high-passage laboratory strains.
- When MAP is present as a natural contaminant, the number of cells is too small for accurate evaluation of inactivation *(106)*. On the other hand, MAP in artificially contaminated milk may not respond the same as naturally occurring cells. By way of illustration, Grant et al. *(50)* found that increasing holding time at 73°C (163.4°F) from 15 to 25 s did not affect inactivation in naturally infected cow's milk, but another study by Grant et al. *(48)* did find a difference in milk inoculated with laboratory cultures heated at 72°C (161.6°F).
- PCR assays detect a sequence of DNA rather than viable cells and thereby may overestimate the number of surviving cells. In addition, culture-positive samples have sometimes shown PCR-negative results *(87)*.

Thermal inactivation studies. A number of studies have examined the effect of heat on the inactivation of MAP. The results have been quite variable, with several studies reporting survival of MAP at many time and temperature combinations. Studies by Pearce et al. *(102)*, Stabel and Lambertz *(124)*, Rademaker et al. *(106)*, and Lynch et al. *(80)* indicate that pasteurization conditions (72 to 74°C [161.6 to 165.2°F] for 15 s) should be adequate to ensure the absence of viable MAP (>4- to 7-log reduction), whereas other studies such as those by Grant et al. *(50, 53)* and Hammer et al. *(58)* indicate that viable MAP cells can be recovered from milk following pasteurization treatments that meet regulatory requirements. Grant et al. *(47)* indicated that high-temperature, short-time (HTST) pasteurization in a laboratory unit was only completely effective at eliminating the organism when ≤10 MAP/ml were present in the raw milk.

Laboratory studies on the heat resistance of MAP have been conducted in a static mode using a batch process *(14, 72)*; however, most commercial milk pasteurization is done in continuous flow processing systems using laminar flow. Klijn et al. *(73)* indicated that the way heat is applied may influence inactivation. Chiodini and Hermon-Taylor *(14)* reported that methods simulating HTST pasteurization were more effective than batch pasteurization, but Gao et al. *(41)* showed the opposite. The degree of turbulence and the distribution of residence times for fastest and slowest milk particles are different for pilot scale and commercial scale pasteurizing equipment. This also complicates study design and interpretation of results *(106)*.

Several studies have calculated thermal *D*-values for MAP in milk *(72, 80, 102, 129)*. Using a capillary tube technique, Keswani and Frank *(72)* calculated $D_{60°C(140°F)}$-values ranging from 8.6 to 11 and 8.2 to 14.1 min for clumped and declumped cells, respectively. $D_{63°C(145.4°F)}$-values ranged from 2.7 to 2.9 and 1.6 to 2.5 min for clumped and declumped cells, respectively. Sung and Collins *(129)* determined *D*-values for human and bovine strains of MAP in milk at 62, 65, 68, and 71°C (143.6, 149, 154.4, and 159.8°F) using sealed vials. The *D*-value for clinical strains of MAP in milk at 71°C (159.8°F) was 11.7 s. Lynch et al. *(80)* obtained a $D_{65°C(149°F)}$-value of 20 s for one strain of MAP (ATCC 19698) in raw milk, while Pearce et al. *(102)* reported a $D_{66°C(150.8°F)}$-value of 5 s for the same strain. Both *D*-values were obtained in pilot plant pasteurizers. Interpretation of *D*-values determined by cultural enumeration of survivors is complicated by methodological considerations described previously. The use of *D*-values to establish a recommended process generally assumes first-order inactivation, which likely is not the case for MAP over a range of temperatures.

An excellent review of a number of heat resistance studies, including some of the reasons for the disparities, is found in Gould et al. *(43)*. A summary of a number of recent studies on the heat resistance of MAP in milk is found in Table 7; these focus on studies using pilot and commercial pasteurizers. The interpretation and comparison of the experiments and results is complicated due in part to the different experimental conditions employed *(106)*. Some key factors that could impact the results include laboratory versus natural contamination, the degree of turbulent flow, presence or absence of a resuscitation step after heating, the use of chemical decontamination after heating, and the volume of milk tested *(50, 53)*.

Although pasteurization appears to achieve at least a 4- to 7-log reduction of MAP, whether pasteurization completely eliminates MAP from the milk supply remains unresolved. Lund et al. *(79)* suggested that more controlled laboratory studies, with particular attention given to time and temperature of the heat treatment and the presence of clumps of cells, are needed to determine whether the heat treatment required for pasteurization ensures destruction of MAP. Studies also point out the risk of postprocess contamination *(35, 79)* as well as the potential for laboratory contamination during sample analysis *(86)*.

The detection of MAP in surveys of retail milk samples *(6, 35, 49)* is consistent with studies suggesting that low levels of MAP may be present after pasteurization at 71.7 to 72°C (161.06 to 161.6°F) for 15 s *(50, 53, 58)*; these findings are also consistent with postprocess contamination. Moreover, studies on heat inactivation are conducted in artificially contaminated products with levels of MAP far exceeding those which might be expected in naturally contaminated raw milk, making it difficult to determine if complete inactivation of the organism would routinely occur in a real-world setting. Since the organism is unlikely to replicate in food once it leaves the host, the number of any potential survivors should not increase.

TABLE 7. *Heat resistance of* Mycobacterium avium *subsp.* paratuberculosis *in milk heated in pilot and commercial pasteurizers*[a]

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Raw milk, including milk from two dairy herds fecally positive for MAP. Pilot plant pasteurizer (580 liters/h; Reynold's no. 10,348), with and without homogenization. 16 trials at 72.5°C (162.5°F), 27 s *(80)*.	10^2–10^5 CFU/ml	Samples stored at 4°C (39.2°F) for 24–48 h prior to assay; no decontamination; pellet and resuspend in PBS; plate on HEYM plus mycobactin J (37°C [98.6°F] up to 6 mo).	No MAP survivors were detected in any of the 16 trials with laboratory strains, fecal material, or naturally contaminated milk, indicating a 4–5-log kill.	No	MAP reference strains, bovine field isolate; two natural isolates from feces and milk. MAP detected in milk from Johne's-positive herds on one of six occasions (<1 CFU/ml). Homogenization resulted in at most a 0.5-log increase in nos. Careful attention to characterizing pasteurizer performance. Decontamination after heating avoided to prevent inactivation of sublethally injured cells,and resuscitation step included.
Raw milk, raw skim milk, cream (30% fat, heated 15–20 s at 105°C). Continuous flow thermal pasteurization. Reynold's no. 2816-7005 depending on holding section used *(58)*.	Similar to Hammer et al. *(57)*, but used a five-MAP strain cocktail inoculum.	Same as Hammer et al. *(57)*.	Skim milk. 67–90°C (152.6–194°F) for 15–60 s: 53/93 (57.0%) positive, 3–6-log reduction by direct plating, 5–7-log reduction by enrichment. Whole milk. 95–100°C (203–212°F) for 15–60 s: 8/30 (26.7%) positive, 5–6-log reduction by direct plating and enrichment. Cream. 85–100°C (185–212°F) for 15–60 s: 9/57 (15.8%) positive, 6-log reduction by direct plating, 5–7-log reduction by enrichment.	Yes	Care taken to prevent cross-contamination between raw and processed milk. In comparative studies with whole, skim, and cream processed at 105–135°C (221–275°F) for 2–5 s, 5–7-log reductions observed. Relative to skim, 4-fold increase in survival in cream and 10-fold increase in survival in whole milk. For skim milk, the no. of tests with survivors tended to decrease with increasing temp and increasing holding time, but not significantly. Lower chance of detecting survivors at higher initial counts could not be explained. Double pasteurization of whole milk provided a 5–7-log reduction, with more positive samples after second treatment and failure to eliminate MAP.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
					Whole milk homogenization trials (72°C [161.6°F] for 15 and 30 s, and 75°C [167°F] for 15 and 30 s with various homogenization pressures [0–700 bar]), logistic regression demonstrated no significant effects of homogenization, either upstream or downstream and irrespective of pressure, on inactivation. However, heating temp had a significant influence, with a 167-fold higher chance of detecting survivors after heating at 75°C (167°F) versus 72°C (161.6°F).
Raw milk. Pilot-scale turbulent flow pasteurizer. 60–90°C (140–194°F) for 6–15 s (Reynold's no. 3405). With and without homogenization *(106)*.	10^2 to 3.5×10^5 CFU/ml	Clear milk with sodium citrate. Pellet cells. Decontaminate using 0.75% HPC, 5h, 37°C (98.6°F); MPN (detection limit 0.023 cells/ml) using dilution series in MGIT with M7H9 broth with OADC enrichment, PANTA antibiotics, and mycobactin J (37°C [98.6°F], 16–50 wk). Confirm with two-target IS*900*-based real-time PCR.	No survivors at 72°C (161.6°F) for 6 s or 70°C (158°F) for 10 s or more stringent conditions (>4.2- to >7.1-log reduction, varies with inoculum concentrations).	No	Feces of Johne's-infected cows used as inoculum.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Fresh, raw milk. Commercial-scale pasteurizer, turbulent flow (Reynold's no. 61,112). 72, 75, or 78°C (161.6, 167, or 172.4°F) for 15, 20, or 25 s, respectively, with homogenization *(81)*.	4.7×10^3 to 1.4×10^5 CFU/ml	Three decontaminations: 4 h HPC (H); 4 h HPC + VAN (HV); overnight HPC + VAN (OHV). Centrifuge milk, resuspend pellet in 0.75% HPC for 4 h at RT or 18–24 h 37°C (98.6°F). Centrifuge and resuspend. For HV and OHV, 18–24 h at 37°C (98.6°F). BACTEC 12B medium without PANTA PLUS (H only); BACTEC 12B with PANTA PLUS and HEYJ (all treatments).	>6-log reduction in 17/20 pasteurization trials at 72, 75, and 78°C (161.6, 167, or 172.4°F) for 15, 20, and 25 s (viable MAP not detected in 1.5 liters of pasteurized milk inoculated at 4×10^2 to 8×10^3 CFU/ml. MAP recovered from three trials: 72°C (161.6°F) for 15 s, 75°C (167°F) for 25 s, and 78°C (172.4°F) for 15 s at concentrations of 0.004 and 0.002 CFU/ml (4–6-log reduction). Recover by H and BACTEC 12B with and without PANTA PLUS.	Yes	MAP field isolates of RFLP strain C1 ($n = 4$) or RFLP strain C3 ($n = 5$).
Raw milk and pasteurized milk. Pilot-scale pasteurizer with turbulent flow (Reynold's no. not reported). Temp: 72.5–84.5°C. Hold times: 15, 25, 60 s. Homogenizer: none, upstream, in-hold *(53)*.	Estimated 10^1–10^5 CFU/ml; MAP not enumerated in untreated raw milk due to overgrowth of other organisms.	BACTEC 12B medium with mycobactin J and PLANTA PLUS. HEYM agar with mycobactin J and VAN. Incubated for up to 18 wk at 37°C (98.6°F). Colonies confirmed by PCR (IS*900*).	>4- to 5-log reduction. In-hold homogenization more effective in reducing counts. No differences between holding times at same temperature.	Yes	

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Ultrahigh temp pasteurized whole homogenized milk. Slug-flow pasteurizer unit and laboratory scale pasteurizer unit. (Reynold's no. not reported). Five time, temp combinations: 62.7°C (144.9°F), 30 min; 65.5°C (149.9°F), 16 s; 71.7°C (161.1°F), 15 s; 71.7°C (161.1°F), 20 s; 74.4°C (165.9°F), 15 s *(124)*.	10^8 and 10^5 CFU/ml with one of three different MAP field strains.	Low-speed centrifugation to pellet bacteria; plate to HEYM (37°C [98.6°F], up to 52 wk) and to supplemented BACTEC medium (37°C [98.6°F], 8 wk). HEYM colonies confirmed by IS*900* PCR, subculture to M7H9, plating to blood agar, and acid-fast staining; BACTEC growth confirmed by acid-fast staining. Enrichment in Dubos broth and subsequent inoculation on HEYM slants and BACTEC medium with confirmation.	Survivors found in 17/45 (38%) of slug-flow pasteurization samples and 12/45 (27%) of HTST trials with inoculum of 10^8 MAP/ml. At 10^5 CFU/ml, survivors present in 9/45 (20%) slug-flow and 8/45 (17.7%) HTST pasteurization samples.	Yes	Log inactivation ranged from <1 to >8, with an average of 5.0- and 7.7-log kill for low and high inoculum levels. Most experiments demonstrated efficient kill except for 65.5°C for 16 s (milk treatment for cheese production), for which inactivation was variable and less effective.
Raw milk and pasteurized milk. APV HXP commercial scale pasteurizer (capacity 2,000 liters/h; Reynold's no. 39,226). 12 trials. In each trial, milks subjected to four pasteurization treatments (73°C [163.4°F] for 15 or 25 s, with and without prior homogenization) *(50)*.	Naturally contaminated	Pellet bacteria, culture, and IMS-PCR. For culture, resuspended pellet decontaminated with CPC the day after treatment, inoculated into HEYM with mycobactin J and supplemented BACTEC Middlebrook 12B radiometric medium (37°C [98.6°F], 18 wk). Presumptive colonies confirmed by acid-fast staining, IS*900* PCR, requirement of mycobactin J for growth. IMS of original pellet, nucleic acid extraction, and PCR.	Viable MAP cultured from 4/60 (6.7%) of raw milk samples and 10/144 (6.9%) of pasteurized samples. MAP DNA was detected in 30/144 (20.8%) pasteurized samples. Fewer pasteurized samples contained viable MAP if milk homogenized prior to pasteurization, although not statistically significant.	Yes	MAP in naturally infected milk survives commercial scale pasteurization (73°C for 15 and 25 s) with and without prior homogenization if bacteria present in sufficient numbers before heat treatment.

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
Raw milk. Continuous flow thermal pasteurization at various time, temp combinations: 68.1–90°C (154.6–194°F) for 17.6–60 s *(57)*.	Free MAP and MAP internalized in macrophages; 10^3–10^5 CFU/ml	Pellet bacteria; (1) enrich in Dubos broth (3–6 mo, 37°C [98.6°F]), detection limit 0.02 CFU/ml; (2) plate to HEYM (6–8 mo, 37°C [98.6°F]), detection limit 0.1 CFU/ml; confirm MAP by viability staining and IS*900* PCR.	68.1–79.1°C (154.6–174.4°F), 18 s: 77/282 (27.3%) experiments positive, survival was always below 1 CFU/ml, 2–6-log reduction; holding time, temp, and inoculum nos. significant, strain insignificant with respect to survival. 72–75°C (161.6–167°F), 18–30 s: 45/45 (100%) experiments positive, 3–6-log reduction; increased holding time did not enhance inactivation. 80–90°C (176–194°F), 18–19 s: 53/53 (100%) experiments positive, 4–6-log reduction. 72–90°C (161.6–194°F), 40–60 s: 48/48 (100%) experiments positive, 4–6-log reduction; holding time and heating temp were negatively correlated (significantly) with survival.	Yes	Five MAP field strains used. Internalization did not significantly influence inactivation; evidence that MAP was heat inactivated at >74°C for >33 s. Survival was independent of initial MAP count and time, temp of heating.
Raw milk. Pilot-scale pasteurizer (Reynold's no. 11,050). 63, 66, 69, and 72°C (145.5, 150.8, 156.2, and 161.6°F) for 15 s, duplicate trials *(102)*.	0.7×10^3 to 16×10^3 CFU/ml	Centrifugation. Pellet treated with 1% CPC (RT, 50 min), centrifuge, inoculate HEYM (20 wk, 37°C [98.6°F]) and BACTEC medium supplemented with PANTA PLUS, egg yolk, and mycobactin J. Confirmation: acid-fast staining, presence of IS*900*, mycobactin dependence.	No survivors at 72°C (161.6°F), 15 s; one strain survived 69°C (156.2°F) for 15 s (1.5 CFU/ml) in one of two trials. $D_{63°C} = 15 \pm 2.8$ s $D_{66°C} = 5.9 \pm 0.7$ s $D_{72°C} = <2.03$ s (>7- log kill at 72°C, 15 s) $z = 8.6$°C >4-log reduction in milk inoculated (3.25×10^3) with fecal material	No	ATCC 19698 (type strain), ATCC 43015 (Linda, human isolate), and three bovine isolates. Properly maintained and operated equipment should ensure absence of viable MAP in pasteurized dairy products. Additional safeguard is commercial practice of operating 1.5–2°C (34.7–35.6°F) above 72°C (161.6°F).

TABLE 7. *Continued*

Product and processing procedures	Inoculum (MAP/ml)	Recovery methods	Results, reduction	MAP survival postpasteurization[b]	Comments
			from high MAP shedder with Johne's (1.3 × 10^7 CFU/g) and heated at 72°C (161.6°F) for 15 s. In milk spiked (20–32 CFU/ml) with feces from moderate shedder, MAP isolated (0.4 CFU/ml) after 69°C (156.2°F) for 15 s in one of two trials.		

[a] MAP, *Mycobacterium avium* subsp. *paratuberculosis*; PBS, phosphate-buffered saline; HEYM, Herrold's egg yolk medium; HPC, hexadecylpyridinium chloride; MPN, most probable number; MGIT, mycobacteria growth indicator tubes; M7H9, Middlebrook 7H9 medium; OADC, oleic acid, dextrose, catalase; PANTA, polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; VAN, vancomycin, amphotericin B, and nalidixic acid; RT, room temperature; PANTA PLUS, polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and 0.5% (vol/vol) glycerol; HEYJ, Herrold's egg yolk medium with mycobactin J; RFLP, restriction fragment length polymorphism; HTST, high temperature short time; IMS-PCR, immunomagnetic separation PCR; CPC, cetylpyridinium chloride.

[b] Treatments meeting legal requirements for pasteurization, e.g., 72°C (161.6°F) for 15 s.

Explanations proposed for survival have included clumping of cells, physiological adaptation resulting in acquisition of thermotolerance, or a change in the physicochemical environment of the organism *(43)*. MAP has a natural tendency to form clumps of cells due to the hydrophobic nature of the cell wall. The organism is likely to be present in clumps in raw milk contaminated with feces from dairy cattle *(1)*. Clumps may be created or dispersed during processing; and this can have an effect on the determination of the initial number of cells as well as the number of cells recovered, which impacts the accuracy of thermal inactivation calculations. Detection of survivors after thermal inactivation is influenced by the enumeration method(s) used. For example, Hammer et al. *(57)* reported that sonication and decontamination resulted in lower recovery of MAP. Grant et al. *(50, 53)* indicated that homogenization increased the lethality of subsequent pasteurization, although Hammer et al. *(58)* found no effect of either upstream or downstream homogenization.

It has been postulated that clumping is responsible for the nonlinear thermal inactivation kinetics in laboratory studies *(45, 48)*. Using viability staining, Hammer et al. *(57)* found surviving MAP only in clumps. The significance of the size and shape of clumps has not been assessed *(58, 80)*. However, heat penetration calculations suggest that the time to reach equilibrium temperature in bacterial clumps is only a few hundredths of a second *(24)*. Klijn et al. *(73)* have suggested that survival may be related to the fraction of clumps with large cell numbers. The number of cells in clumps in fecal material (a potential source of MAP in milk) is low, but clumps from liquid cultures used in thermal resistance studies may have many layers of cells *(58)*. Fecal contamination should be negligible in commercial milk produced under the Pasteurized Milk Ordinance in the United States.

However, clumping alone is unlikely to account for the survival observed in many thermal inactivation studies *(43)*. Adaptation to heat stress has been postulated to account for survival at the high temperatures reported in some studies. However, survival at 90°C (194°F) would require a 1,000-fold increase in heat resistance, which is unlikely *(43)*. Another mechanism for survival could be related to the fact that vegetative bacteria can survive high heat under reduced moisture conditions, which occurs when cells are present in a lipid matrix, e.g., fat globules in milk. One or more of these hypotheses may explain the observed thermal resistance of MAP, but the true mechanism remains elusive.

It has been postulated that microorganisms may be more resistant to heat when sequestered in macrophages. Stabel *(121)* harvested mammary gland macrophages from noninfected control animals and infected them in vitro with MAP. Infected macrophages were subsequently added to raw milk that was treated for 15 s at 72°C (161.6°F). Culturing of this milk after treatment failed to yield viable MAP. Further experiments by Hammer et al. *(57)* utilized an experimentally infected mouse phagocytic cell line; macrophages containing MAP were added directly to milk samples, utilizing an identical aliquot as a control. The

results of this study demonstrated no significant difference between the survival of MAP in macrophages and the control (free bacterial cells) after treatment for 17.6 to 18.8 s at 70.7 to 76.6°C (159.26 to 169.88°F). As with other proposed survival mechanisms, survival of MAP at high temperatures cannot be entirely explained by protection of bacterial cells present in macrophages.

In summary, thermal inactivation studies on MAP have shown conflicting results, while MAP has been detected in retail samples. As noted by Hammer et al. *(58),* ''despite all uncertainties regarding complete inactivation of [MAP] during heat treatment and possible reasons for survival, a reduction of at least 5 to 7 $\log_{10}$ cycles could be demonstrated in whole milk, skim milk and cream. In the framework of the Codex Alimentarius for samples of pasteurized milk, this should be fully sufficient.'' Pearce et al. *(102),* using data from raw milk obtained in New Zealand, the United Kingdom and the Netherlands along with inactivation data from an earlier study *(64),* applied a mathematical modeling approach to assess the probability of MAP occurring in pasteurized milk. These investigators reported in an abstract *(64)* that good milking practices combined with properly operated and validated pasteurizers treating milk at 72°C (161.6°F) for 15 s will result in an extremely low probability of viable MAP being present in the pasteurized product. A publication by Cerf et al. *(13)* described a quantitative model that estimates the probability of finding MAP in pasteurized milk (50-ml samples) in industrialized countries as less than 0.54%, even if interherd and intraherd prevalence are high, provided that MAP numbers in raw milk are low (mean concentration 10 MAP/ml). Thus, it appears that regardless of whether MAP is present in pasteurized milk due to survival of the process or due to recontamination, both the prevalence and the numbers of MAP in pasteurized product will be low. The public health significance of low numbers of survivors or any level of MAP contamination is not known. At this time, there are insufficient data to conclude that increasing the pasteurization times and temperatures would reduce the presence of MAP in milk.

MAP inactivation during cheese manufacture. Spahr and Schafroth *(120)* found that MAP survived for at least 120 days in semihard cheeses manufactured with raw milk inoculated with MAP. The number of organisms present decreased in both hard (Swiss Emmentaler) and semihard (Swiss Tisliter) cheeses over the ripening period. The *D*-value in the hard cheese was 27.8 days, with an expected 10^4 reduction at 120 days. The *D*-value was 45.5 days in semihard cheese, with approximately a 10^3 reduction over the same 120-day period.

Cheddar cheese was made from pasteurized milk that was artificially contaminated with either high (10^4 to 10^5 CFU/ml) or low (10^1 to 10^2 CFU/ml) numbers of MAP and cultured over 27 weeks *(32)*. If levels >3.6 log CFU were present in 1-day cheeses, then in all cases MAP was culturable at 27 weeks. The calculated *D*-values ranged from 90 to 107 days. At low levels of contamination, cheese inoculated with one of three strains of MAP was positive for MAP at 27 weeks.

Sung and Collins *(130)* evaluated rates of MAP inactivation in Hispanic-style, soft white cheese (queso fresco). Pasteurized milk was inoculated with 10^6 MAP/ml (non–heat treated or heat treated at 62°C [143.6°F] for 240 s), and the curd was sampled weekly for up to 4 weeks. MAP was cultured using BACTEC and confirmed by PCR. The investigators reported that the *D*-value in queso fresco was 36.5 days for heat-treated MAP and 59.9 days for non–heat-treated MAP; however, the final counts (4.12 $\pm$ 0.55 log CFU/ml) differed from the initial count (4.64 $\pm$ 0.06 log CFU/ml) by less than 1 log for non–heat-treated samples and for heat-treated samples (4.79 $\pm$ 0.14 log CFU/ml initial and 3.96 $\pm$ 0.16 log CFU/ml final).

Many cheeses in certain countries are made with raw milk, while in the United States cheeses are primarily made with either pasteurized or with thermized milk. Stabel and Lambertz *(124)* studied the effect of thermization (65.5°C [149.9°F] for 16 s) of milk inoculated with 10^5 or 10^8 CFU/ml using a slug-flow pasteurization unit and with an HTST pasteurizer. Survival was observed for the three strains of MAP studied at both inoculum levels (<1- to 4-log reduction with the slug-flow pasteurizer and 2- to 6-log reduction with the HTST unit). Survival was also observed following pasteurization, but to a much lesser extent (depending on inoculum level, 4- to >8-log reduction with the slug-flow pasteurizer and >5- to >8-log reduction with the HTST unit). However, there are a number of factors other than heat treatment that affect microbial survival during cheese manufacture, including salt content, pH, ripening period, and ripening temperature *(120, 124, 130)*.

Inactivation by other processes. Other processes have been explored to determine if they can effectively inactivate MAP. Using high hydrostatic pressure, MAP was reduced by an average 4 log CFU/ml after treatment with 500 MPa for 10 min at 5°C (41°F) and at 20°C (68°F) *(77)*. Donaghy et al. *(29)* obtained a reduction of 6.52 log CFU/ml with 500 MPa for 10 min at 20°C (68°F) and a reduction of 5.03 log CFU/ml for a 5-min treatment.

Stabel et al. *(126)* have shown that viable MAP could not be recovered from milk inoculated with 10^4 and 10^8 CFU/ml and exposed to either 5 or 10 kGy of radiation. Altic et al. *(3)* studied the effects of UV irradiation on the elimination of MAP from whole and semiskim cow's milk. These authors determined only 0.5- to 1.0-log reduction occurred at a dose of 1,000 mJ/ml, which is thought to be the maximum dose before unacceptable organoleptic changes occur in milk. Even doses as high as 2,860 mJ/ml resulted in only a maximum 2.8-log reduction. Thus, the use of UV light treatment of milk is not an acceptable alternative to current pasteurization processes for liquid milk.

Rowan et al. *(113)* studied the effect of pulsed electric fields (PEF) on inactivation of MAP inoculated into sterile cow's milk. PEF treatment at 50°C (122°F) (2,500 pulses at 30 kV/cm) resulted in a 5.9-log reduction, compared to a 0.01-log reduction observed for heating at 50°C (122°F) for

25 min; heating at 72°C (161.6°F) for 25 s (extended HTST pasteurization) resulted in a 2.4-log reduction.

Chlorine inactivation. Given that MAP may contaminate water sources that could be used in foods and food processing, chlorination of water may be an important intervention for reducing exposure. Whan et al. *(146)* determined that when high numbers (10^6 CFU/ml) of MAP were exposed to free chlorine concentrations of up to 2 μg/ml for 15 or 30 min, survivors remained. In this case, log reductions ranged from 1.32 to 2.85 depending on strain, chlorine level, contact time, and the presence or absence of organic matter.

5. What are the research needs?

Based on a review of the literature, including the research needs identified by other groups *(43, 136),* the Committee identified the following research needs. An underlying requirement to facilitate meeting future research needs is the development and standardization of rapid, sensitive, and specific methods to detect and enumerate MAP in a variety of matrices. This means that molecular-based assays will probably be the detection approach of choice. Specifically, the following issues should be addressed: (i) detection of viable versus nonviable cells; (ii) sample preparation prior to detection (concentration and decontamination); (iii) the most efficient manner by which to lyse the cells and extract the nucleic acid(s); (iv) interpretation of positive and negative test results; and (v) standardization and determination of a ''gold standard.''

The Committee decided to combine the two questions below and identified several supporting research needs:

a. What are the additional sources of MAP?
b. What are the frequencies and levels of MAP contamination in specific sources of concern?

The estimated MAP prevalence in U.S. beef herds is 7.9% *(23)*. Beef cattle are the source of the majority of ground beef. MAP prevalence in U.S. dairy herds is estimated at approximately 22% *(137)*. Approximately 10% of ground beef comes from culled dairy cows. Studies are needed to determine the prevalence, numbers of MAP, and thermal inactivation of MAP in meat products. Statistically rigorous studies proportionately representing the herds in the major beef and dairy states are needed to determine herd prevalence.

Of additional interest are cheeses made from raw milk. Studies are also needed on the frequencies and numbers of MAP in fresh produce (fruits and vegetables) and fish from areas of high and low domestic ruminant prevalence.

Water contaminated with MAP can serve as a transport mechanism on-farm to spread infection among domestic ruminants and exposed wildlife and between farms to spread infection to downstream areas and watersheds. Along with direct manure application, water can contaminate animal forage and produce intended for human food at considerable distance from infected herds. Research is needed to clarify the importance of each of these potential water-associated mechanisms in human exposures to MAP. The suitability of agricultural water for its intended use (e.g., animal drinking water, irrigation water, water for application of pesticides) needs to be studied in areas of high and low MAP prevalence and with different soil filtering characteristics.

In addition, the role of water as a direct source of MAP to humans is unknown. For example, there is a need for a large-scale prevalence study of U.S. municipal water supplies to determine the prevalence and number of MAP both pre- and posttreatment. There are no studies that have determined if well water and other ground water sources may serve as potential sources of MAP; however, unprotected wells have been known to become contaminated with pathogens from surface waters. In addition, there are no data on the prevalence or number of MAP in recreation waters, including lakes, ponds, swimming pools, and water parks.

Insects and rodents have been found contaminated with or infected with MAP, but their role in transmitting and/or maintaining the presence of MAP on a premise is unknown and should be further investigated.

Information is needed on the presence and survival of MAP in food processing facilities. If MAP becomes incorporated in biofilms, this could provide a source of pre- or postprocess contamination, but this has not yet been studied.

Additional information is needed about the physiology and metabolic states of MAP in various environments and in response to physical conditions contributing to survival and persistence. The significance of amoebae as a reservoir for MAP should be further investigated.

c. What are the potential processing interventions to eliminate or reduce the levels of MAP contamination?

There is a need for well-controlled studies that will determine the effect of pasteurization on naturally occurring MAP using equipment and conditions representative of commercial processing using standardized laboratory procedures. In this regard, note the Committee's statements on factors that have complicated previous studies.

Standardization of methods used to document efficacy of processes for interlaboratory studies is needed.

If it is determined that municipal water is a source of exposure for humans to MAP, studies are needed to determine effective decontamination procedures.

d. What are the potential pre- and postharvest interventions to prevent MAP from entering the food supply?

Preharvest controls include ways by which to eliminate the transmission of MAP within cattle populations. Identification and quarantine of infected animals is ideal; however, commercial detection methods are much more adept at detecting animals that are clinically infected and/or shedding at high levels. More sensitive clinical detection

methods are necessary. Research on the sensitivity and specificity of various cell-mediated immune diagnostic tests is indicated. Related to this are difficult issues of sampling strategies at both the herd and individual animal level *(19)*.

Strategies to protect newborn and newly acquired calves from contaminated feces, colostrum, milk, and environments have been developed, and following these strategies rigorously has been documented to reduce frequency of infection *(18)*. However, additional work on appropriate implementation strategies is necessary, and additional progress awaits better diagnostic techniques. Identification of methods to control environmental spread (composting, decontamination, water treatment) are needed. A promising control technology would be vaccination, but this is currently in the early stages of implementation. Finally, a standard animal model will be necessary in order to facilitate evaluation of many of the emerging preharvest control strategies.

Filtration is an emerging technology for the production of fluid milk. Currently filtration, especially ultrafiltration, is more commonly used for the treatment of raw milk for ice cream and cheese making. While data are not available specifically for the influence of these technologies on MAP contamination, they have been documented to reduce contamination by other microflora *(116)*.

6. Additional research needs?

The USDA Agricultural Research Service currently is engaged in using information from the MAP genome sequencing project to conduct research on genetic variability, identification of antigens, and transcriptional profiles. This could lead to progress in identification of epitopes from which antibodies or other ligands could be developed for bioseparation strategies that would improve detection and enumeration as well as validate processing interventions to remove MAP from foods and water *(135)*.

The process of risk assessment provides information on the extent and characteristics of the risk attributed to a hazard. An important step preceding a quantitative risk assessment is completion of a risk profile, a document intended to provide background information about the food, the hazard, and the potential risk posed. At the very least, there is a need to complete a risk profile for MAP in milk due to the current availability of data. Similar risk profiles should be conducted for meat, water, produce, fish, and shellfish when data become available. If warranted this could lead to more formal risk assessment efforts in the future. A risk assessment approach to the MAP problem will aid in the identification of information gaps, prioritization of research needs, and, if quantitative, can be used to evaluate, on a preliminary basis, the potential value of candidate intervention strategies.

CONCLUSIONS

The Committee determined that current methods of detection of MAP suffer from significant limitations. A standard method for detection of viable MAP needs to be developed and adopted by researchers in order to accurately determine the presence and numbers of MAP in foods and other potential sources of exposure.

The body of evidence indicates that absent infected domestic ruminant animals, MAP is rarely found. Thus, as with brucellosis, if MAP in cattle is controlled, the source of MAP in other animals, food, and water may largely be eliminated. Based on a comprehensive review of the current literature, the Committee concludes that milk, particularly raw milk, is a potential food source for human exposure to MAP. Thermal processes have been demonstrated to achieve a 4- to 7-log reduction in the number of MAP cells when using milk inoculated with high numbers of MAP. This level of thermal inactivation should be adequate to inactivate the numbers of MAP estimated to be present in raw milk. Nevertheless, several studies, including one using naturally contaminated raw milk, have detected survivors after time and temperature combinations used in commercial pasteurization in the United States and Europe. Several credible published reports described the isolation of MAP from commercially pasteurized milk. At this time, it is not known if such findings are due to MAP surviving the pasteurization process or to postpasteurization contamination. No studies examined retail raw milk for MAP; however, there are several reports that document the presence of MAP in bulk raw milk tanks. Thus, based on current methodology and available studies, it can be concluded that a small percentage ($<3\%$) of commercially pasteurized milk may contain small numbers of culturable MAP cells.

Based on the current detection methodology, cheese made from pasteurized milk is unlikely to be a significant source of exposure to MAP. The Committee believes that pasteurization and ripening combine to greatly reduce the presence of viable MAP in pasteurized milk cheeses. The Committee was unable to assess exposure from raw milk cheeses. The Committee could not find any peer-reviewed data on the presence of MAP in other milk products such as butter, ice cream, cottage cheese, and yogurt. However, if these products are made from pasteurized milk, they are unlikely to be a significant source of MAP.

Ground beef may be another potential food source of MAP; the limited studies available have not detected viable MAP in ground beef. However, culled dairy cattle likely have a significantly higher prevalence of MAP infection compared to beef cattle. The Committee was unable to obtain precise figures for the contribution of meat from culled dairy cattle to the U.S. ground beef supply; however, one source estimated that culled dairy cows account for up to 10% of the ground beef supply. Neither of the studies detected viable MAP in ground beef, and there are no studies on the potential for MAP survival after cooking. The potential for exposure to MAP by consumption of ground beef, particularly containing lymph nodes, needs further study.

Numerous studies report that MAP is prevalent in the environment. It survives in water and soil and is found in many wild animals. Since cattle appear to be a significant source of MAP in the environment, farm runoff may

potentially contaminate irrigation water. For this reason, some food items such as fruits and vegetables that come in contact with an MAP-contaminated environment could result in exposure in humans. The significance of fruits and vegetables as a source of human exposure needs to be further investigated.

There is no information to indicate that treated municipal drinking water is likely to be a significant source of human exposure to MAP; however, further study is needed. Limited studies of water supplies in other countries have not detected MAP in treated water, although MAP has been detected in untreated source water. There are no studies on MAP in the U.S. water supply.

Although it is clear that humans may be exposed to MAP through various routes, including foods and the environment, the frequency and amount of exposure are not known, and research to address these issues is needed. Such research would be critical if it is determined that MAP presents a risk to public health at the likely levels of exposure.

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Modulation of the bovine innate immune response by production of 1α,25-dihydroxyvitamin D_3 in bovine monocytes

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ABSTRACT

In cattle, the kidney has been the only known site for production of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] from 25-hydroxyvitamin D_3 [$25(OH)D_3$] by 1α-hydroxylase (1α-OHase). Based on human studies, it was hypothesized that bovine monocytes could produce $1,25(OH)_2D_3$ upon activation and $1,25(OH)_2D_3$ would regulate expression of vitamin D–responsive genes in monocytes. First, the effects of $1,25(OH)_2D_3$ on bovine monocytes isolated from peripheral blood were tested. Treatment of nonstimulated monocytes with $1,25(OH)_2D_3$ increased expression of the gene for the vitamin D 24-hydroxylase (24-OHase) enzyme by 51 ± 13 fold, but $1,25(OH)_2D_3$ induction of 24-OHase expression was blocked by lipopolysaccharide (LPS) stimulation. In addition, $1,25(OH)_2D_3$ increased the gene expression of inducible nitric oxide synthase and the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) in LPS-stimulated monocytes 69 ± 13 and 40 ± 12 fold, respectively. Next, the ability of bovine monocytes to express 1α-OHase and produce $1,25(OH)_2D_3$ was tested. Activation of monocytes with LPS, tripalmitoylated lipopeptide (Pam3CSK4), or peptidoglycan caused 43 ± 9, 17 ± 3, and 19 ± 3 fold increases in 1α-OHase gene expression, respectively. Addition of $25(OH)D_3$ to LPS-stimulated monocytes enhanced expression of inducible nitric oxide synthase and RANTES and nitric oxide production in a dose-dependent manner, giving evidence that activated monocytes convert $25(OH)D_3$ to $1,25(OH)_2D_3$. In conclusion, bovine monocytes produce $1,25(OH)_2D_3$ in response to toll-like receptor signaling, and $1,25(OH)_2D_3$ production in monocytes increased the expression of genes involved in the innate immune system. Vitamin D status of cattle might be important for optimal innate immune function because $1,25(OH)_2D_3$ production in activated monocytes and subsequent upregulation of inducible nitric oxide synthase and RANTES expression was dependent on $25(OH)D_3$ availability.

Key words: vitamin D, bovine innate immunity, nitric oxide, RANTES

INTRODUCTION

For several decades now, it has been known that there is an endocrine mechanism to regulate renal production of 1,25-dihydroxyvitamin D_3 [$\mathbf{1,25(OH)_2D_3}$] as a way to regulate the concentration of $1,25(OH)_2D_3$ systemically (Horst and Reinhardt, 1983). The primary function of renal $1,25(OH)_2D_3$ production was considered to be maintenance of calcium homeostasis (Horst, 1986). It has become evident that $1,25(OH)_2D_3$ modulates the immune response of several species, including cattle (Waters et al., 2001). Furthermore, activated human macrophages produce $1,25(OH)_2D_3$ as part of the immune response to regulate $1,25(OH)_2D_3$ concentration at the site of inflammation (Liu et al., 2006). Local control of $1,25(OH)_2D_3$ concentration regulates genes involved in immune responses locally rather than systemically (Schauber et al., 2007). Existence of a mechanism to control $1,25(OH)_2D_3$ production and gene expression locally in humans and mice suggests that there might be a similar mechanism in cattle.

Vitamin D, acquired in the diet or by radiation of 7-dehydrocholesterol with UVB light in the skin, is converted to 25-hydroxyvitamin D_3 [$\mathbf{25(OH)D_3}$] in the liver (Horst and Reinhardt, 1983). The major circulating metabolite of vitamin D is $25(OH)D_3$, and the concentration of $25(OH)D_3$ in blood is relatively stable in cattle (Sommerfeldt et al., 1983). Conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ is accomplished by the enzymatic activity of 1α-hydroxylase (**1α-OHase**; Sakaki et al., 2005). The ligand for the vitamin D receptor is $1,25(OH)_2D_3$; the vitamin D receptor is activated upon binding $1,25(OH)_2D_3$ (Reinhardt et al., 1989). The activated vitamin D receptor regulates expression of genes that contain functional vitamin D response elements in their promoters (Lin and White, 2004). It is estimated that greater than 1,000 genes are regulated

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by $1,25(OH)_2D_3$ (Wang et al., 2005), and the vitamin D receptor is present in most tissues and cell types (Lin and White, 2004). Therefore, $1,25(OH)_2D_3$ concentration is regulated tightly to control its effects on gene expression.

The kidney was the only known source for $1,25(OH)_2D_3$ in cattle, and regulation of 1α-OHase expression in the kidney is mainly in response to calcium homeostasis (Horst, 1986). In contrast, 1α-OHase was expressed in human monocytes and macrophages in response to activation by toll-like receptor (**TLR**) recognition of pathogen-associated molecules (Liu et al., 2006). In human macrophages, $1,25(OH)_2D_3$ increases the expression of cathelicidin directly via a vitamin D response element in the cathelicidin promoter (Gombart et al., 2005). It was found that $1,25(OH)_2D_3$ induction of cathelicidin expression in human macrophages was necessary for the killing of intracellular *Mycobacterium tuberculosis* (Liu et al., 2007). In cattle, $1,25(OH)_2D_3$ modulated the immune response in vitro by increasing nitric oxide production and decreasing IFN-γ production in peripheral blood mononuclear cells (Waters et al., 2001). Production of $1,25(OH)_2D_3$ in the kidney does not increase as part of the immune response in cattle (Waldron et al., 2003); so, if $1,25(OH)_2D_3$ modulates the immune response in vivo, there would seem to be a source other than the kidney. It was hypothesized that bovine monocytes express 1α-OHase and produce $1,25(OH)_2D_3$ at the site of infection in response to TLR signaling to direct local regulation of vitamin D–responsive genes. The objectives were to assess the effects of $1,25(OH)_2D_3$ on bovine monocytes isolated from peripheral blood and test the ability of bovine monocytes to express 1α-OHase and convert $25(OH)D_3$ to $1,25(OH)_2D_3$.

MATERIALS AND METHODS

Animals

A group of 12 healthy, midlactation Holstein cows at the National Animal Disease Center were used. The number of cows used for each experiment ranged from 4 to 6 and is specified in the figure legends. The care and treatment of the cows used were approved by the National Animal Disease Center animal care and use committee.

Monocyte Isolation and Culture Conditions

Monocytes were isolated by adherence to tissue culture flasks as described previously (Stabel et al., 1997). Briefly, peripheral blood was collected into 2× acid citric dextrose, and the mononuclear cell fraction was separated by density gradient centrifugation. Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) plus 10% fetal bovine serum (**FBS**; Hyclone, Waltham, MA) and incubated at 37°C for 1 h in tissue culture flasks. Lymphocytes were then removed by washing 3 times with warm PBS. Monocytes were dislodged from the tissue culture flasks using cold PBS plus 20 m*M* EDTA. Monocytes were pelleted and resuspended to a concentration of 10^7 cells/mL in RPMI 1640 containing 50 μg/mL gentamicin (Invitrogen, Carlsbad, CA) and placed in 24-well or 96-well non-tissue-culture-treated polystyrene plates (Becton Dickinson, Franklin Lakes, NJ)

All treatments were added to heat-inactivated FBS at 10× the desired final concentration, and FBS was added to wells containing monocytes to a final concentration of 10%. Monocytes were incubated with the treatments for 24 h at 37°C in 5% CO_2. Lipopolysaccharide from *Serratia marcescens* (Sigma-Aldrich); Pam3CSK4 (InvivoGen, San Diego, CA), a synthetic tripalmitoyl lipopeptide; and peptidoglycan from *Staphylococcus aureus* (InvivoGen) were in endotoxin-free water. Both $25(OH)D_3$ and $1,25(OH)_2D_3$ (Sigma-Aldrich) were diluted to a concentration of 100 ng/μL in 100% ethanol. Concentrations were confirmed by UV spectroscopy using an extinction coefficient of 18,200 M^{-1}/cm. The final concentration of ethanol did not exceed 0.04% for any of the treatments, and a treatment of ethanol alone at 0.04% was used as a control for the effects of ethanol. The lot of FBS used contained no more than 20 ng/mL of $25(OH)D_3$. Ketoconazole (Sigma-Aldrich) was solubilized in PBS at pH 2.5 and diluted to 50 μg/mL in PBS at pH 7.

Measurement of Relative Gene Expression

Ribonucleic acid was isolated from monocytes using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and eluted with 50 μL of RNase-free water. The RNA was reverse transcribed to cDNA in a 20-μL reaction using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 10 μL of RNA sample and 20 units of RNase Inhibitor (Applied Biosystems). Reactions were incubated at 37°C for 2 h and heated to 85°C for 5 s. The cDNA samples were diluted 1:10 in sterile water and stored at −20°C. Quantitative PCR was performed with the 7300 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Reactions consisted of 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), 2.5 μL each of 10 μ*M* forward and reverse primers, and 7.5 μL of diluted cDNA. Primers pairs were designed with Primer3 (http://frodo.wi.mit.edu/primer3) (Rozen

Table 1. Primer sequences for real-time PCR

Gene[1]	Accession no.[2]	Strand[3]	Sequence 5′–3′	Product size (bp)	Primer efficiency
1α-Hydroxylase (1α-OHase)	XM_588481	F	TGGGACCAGATGTTTGCATTCGC	80	1.09
		R	TTCTCAGACTGGTTCCTCATGGCT		
24-Hydroxylase (24-OHase)	XM_591370	F	GAAGACTGGCAGAGGGTCAG	97	1.01
		R	CAGCCAAGACCTCGTTGATT		
Cathelicidin 4 (CATH4)	NM_174827	F	AGAGTGTCATCCTACCCTGGAA	136	0.97
		R	TTTCACTGTCCAGAAGCCCGAA		
Cathelicidin 5 (CATH5)	NM_174510	F	GTGCTTGGAAGAAGTATGGCCCAA	63	1.04
		R	GGCCCACAATTCACCCAATTCTGA		
Cathelicidin 6 (CATH6)	NM_174832	F	TCCAGTCATTCCGCTACTCC	91	1.11
		R	CAGAATCCAAAAGCCTGAGC		
Interleukin-1β (IL-1β)	NM_174093	F	GCCTTGGGTATCAAGGACAA	90	1.04
		R	TTTGGGGTCTACTTCCTCCA		
iNOS[4]	NM_001076799	F	GATCCAGTGGTCGAACCTGC	128	0.97
		R	CAGTGATGGCCGACCTGATG		
RANTES	NM_175827	F	CACCCACGTCCAGGAGTATT	116	1.01
		R	CTCGCACCCACTTCTTCTCT		
Ribosomal protein S9 (RPS9)	NM_001101152	F	GTGAGGTCTGGAGGGTCAAA	108	1.02
		R	GGGCATTACCTTCGAACAGA		
S100A12	NM_174651	F	GCTGAAGCAGCTGATCACAA	102	0.93
		R	TCTTTATCGGCATCCAGGTC		

[1]iNOS = inducible nitric oxide synthase; RANTES = regulated upon activation, normal T-cell expressed and secreted; S100A12 = S100 calcium binding protein A12.

[2]http://www.ncbi.nlm.nih.gov.

[3]F = forward; R = reverse.

[4]Primer sequence from (Aalberts et al., 2007).

and Skaletsky, 2000) to span intron–exon boundaries. Primer sequences along with the efficiency of replication for each primer pair are in Table 1. The efficiency of each primer pair was calculated using the equation Efficiency $= -1 + 10^{(-1/\text{slope})}$, where slope equals the slope of a standard curve generated with known dilutions of cDNA in the PCR reactions. Primer specificity was determined by gel electrophoresis and melting curve analysis. Relative quantification of mRNA transcripts was accomplished using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The gene for ribosomal protein S9 (**RPS9**) was used as the reference gene (Janovick-Guretzky et al., 2007), and stability of RPS9 expression was checked by comparison with β-actin expression. For each experiment, the control sample was used as the calibrator, and expression of each gene is reported as fold increase relative to the control.

Measurement of Nitric Oxide Production

The concentration of nitrite in the culture supernatant at the end of the incubation period was used as an indicator of nitric oxide produced by monocytes. Nitrite concentration was measured by adding 100 μL of culture supernatant or culture media with 0 to 100 μ*M* sodium nitrite to 100 μL of Griess reagent [0.5% sulfanilamide, 2.5% phosphoric acid, and 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride; Sigma-Aldrich] in a 96-well clear bottom plate. The reactions were incubated for 10 min at room temperature. Absorbance at 550 nm in each well was measured using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were converted to micromoles per liter using a standard curve. To ensure that nitrite accumulation in the culture supernatant was a result of nitric oxide synthase activity, 1 m*M* of N^G-monomethyl-L-arginine (Sigma-Aldrich), a nitric oxide synthase inhibitor, was added as a control treatment. There was no accumulation of nitrite in the culture supernatant when N^G-monomethyl-L-arginine was added as a treatment.

Statistical Analysis

Response variables were analyzed as a completely randomized block design with PROC GLM (SAS Institute Inc., Cary, NC). The model accounted for effects of treatment and cow. Experimental units were blocked according to cow to account for variation of monocyte responses between cows. For gene expression, ΔΔCt values were used as the response variables in the analyses. Mean ΔΔCt values ± SE were transformed ($2^{-\Delta\Delta Ct}$) and presented as the mean fold increase relative to control.

Multiple-comparison tests of the means were made with the Tukey adjustment. Differences were considered significant at $P < 0.05$.

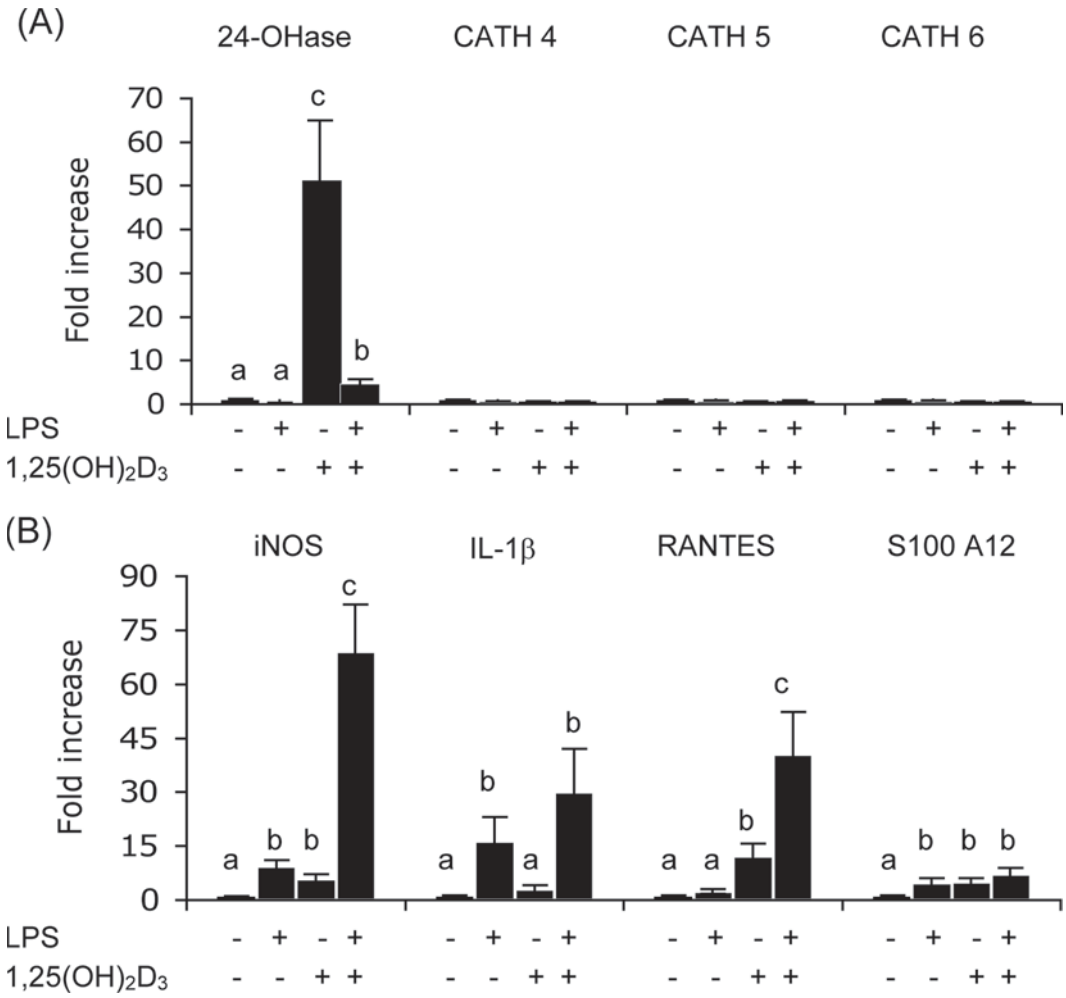

Figure 1. Effects of LPS and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] treatment on gene expression in bovine monocytes. Monocytes were isolated from peripheral blood of 4 cows and treated with 100 ng/mL of LPS and 4 ng/mL of $1,25(OH)_2D_3$ as indicated for 24 h. Relative expression of (A) 24-hydroxylase (24-OHase) and cathelicidin (CATH) 4, 5, and 6 and (B) inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), RANTES (regulated upon activation, normal T-cell expressed and secreted), and S100 calcium binding protein A12 (S100A12) was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown for each gene is relative to the nontreated control. Error bars represent SE, n = 4. Means with different letters are different, $P < 0.05$.

RESULTS

Effects of $1,25(OH)_2D_3$ on Monocytes

Initially the effect of $1,25(OH)_2D_3$ on 24-hydroxylase (**24-OHase**) expression in monocytes was tested because 24-OHase is known to be a vitamin D–responsive gene. The 24-OHase expression in monocytes increased with $1,25(OH)_2D_3$ treatment ($P < 0.05$; Figure 1A). Surprisingly though, the effects of $1,25(OH)_2D_3$ on 24-OHase expression were greatly reduced when monocytes were activated with LPS ($P < 0.05$; Figure 1A). The effects of $1,25(OH)_2D_3$ on cathelicidin gene expression were tested. The expression of CATH4, CATH5, or CATH6 was not increased by $1,25(OH)_2D_3$ treatment in nonstimulated or LPS-stimulated monocytes (Figure 1A).

Of the other genes tested, inducible nitric oxide synthase (**iNOS**), regulated upon activation, normal T-cell expressed and secreted (**RANTES**), and S100 calcium binding protein A12 gene expression were upregulated by treatment with 4 ng/mL of $1,25(OH)_2D_3$ alone ($P < 0.05$; Figure 1B). The combination of LPS and $1,25(OH)_2D_3$ treatments resulted in increases of both iNOS and RANTES gene expression relative to either treatment alone ($P < 0.05$; Figure 1B). There was no synergistic effect of LPS and $1,25(OH)_2D_3$ on S100 calcium binding protein A12 gene expression (Figure 1B). Interleukin-1β gene expression increased slightly ($P > 0.05$) in nonactivated and LPS-activated monocytes treated with 4 ng/mL of $1,25(OH)_2D_3$ (Figure 1B).

In LPS-activated monocytes, RANTES expression was increased by addition of 0.04 ng/mL ($P < 0.05$) of

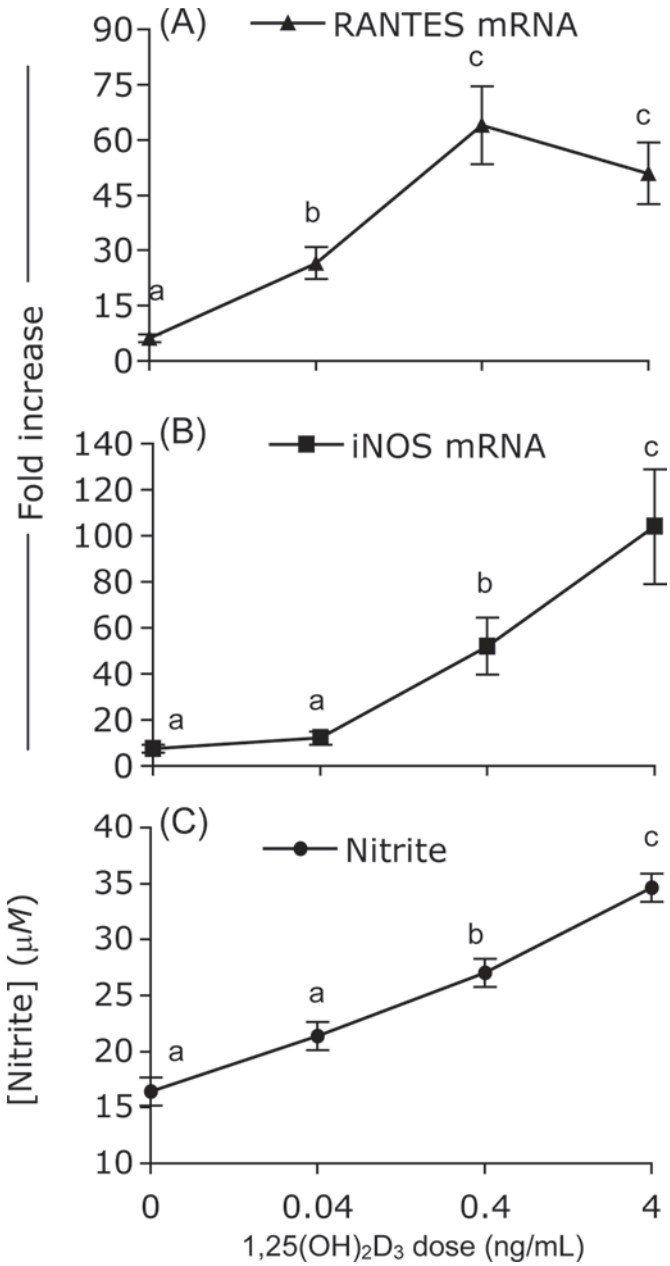

Figure 2. Effects of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] dose on LPS-stimulated monocytes. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL of LPS and 0 to 4 ng/mL of $1,25(OH)_2D_3$ as indicated for 24 h. Relative expression of RANTES (regulated upon activation, normal T-cell expressed and secreted; A) and inducible nitric oxide synthase (iNOS; B) was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown for RANTES and iNOS expression is relative to the nontreated control. (C) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, $P < 0.05$.

$1,25(OH)_2D_3$ and peaked with addition of 0.4 ng/mL of $1,25(OH)_2D_3$ (Figure 2A). Both iNOS expression and nitric oxide production increased with $1,25(OH)_2D_3$ dose in LPS-activated monocytes ($P < 0.05$; Figures 2B and 2C).

Expression 1α-OHase in Monocytes

The ability of bovine monocytes to express 1α-OHase upon activation with TLR ligands was tested. Activation of bovine monocytes with LPS, Pam3CSK4, or peptidoglycan triggered a large increase in 1α-OHase gene expression relative to nonactivated monocytes ($P < 0.001$; Figure 3).

Activity of 1α-OHase in Monocytes

Increasing the concentration of $25(OH)D_3$ in the culture media to physiological concentrations increased RANTES and iNOS gene expression and nitric oxide production in LPS-stimulated monocytes in a dose-dependent manner (linear effect, $P < 0.001$; Figure 4).

To determine if 1α-OHase activity is necessary for the effects of $25(OH)D_3$ on activated monocytes, ketoconazole, a competitive inhibitor of 1α-OHase, was used to block conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. Treatment with ketoconazole decreased the effects of

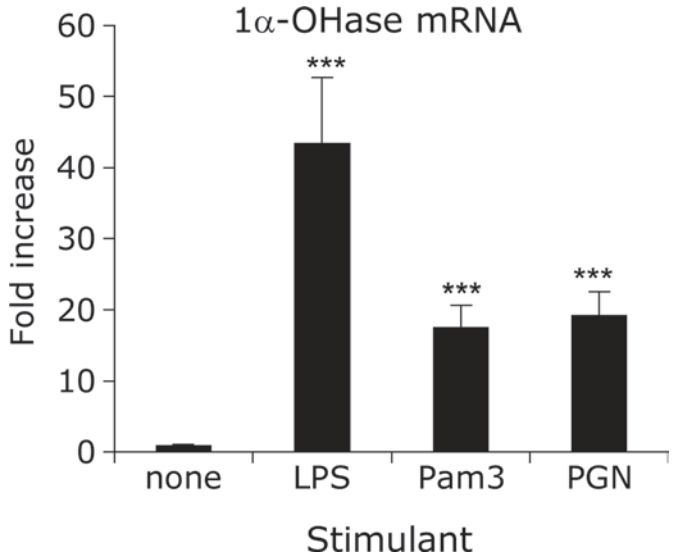

Figure 3. Toll-like receptor signaling induces 1α-hydroxylase (1α-OHase) expression in bovine monocytes. Monocytes were isolated from peripheral blood of 6 cows and treated with 100 ng/mL of LPS, 5 μg/mL of Pam3CSK4 (Pam3), or 5 μg/mL of peptidoglycan (PGN) for 24 h. Relative expression of 1α-OHase was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown for 1α-OHase expression is relative to the nonstimulated control. Error bars represent SE, n = 6. ***Mean is different from control, $P < 0.001$.

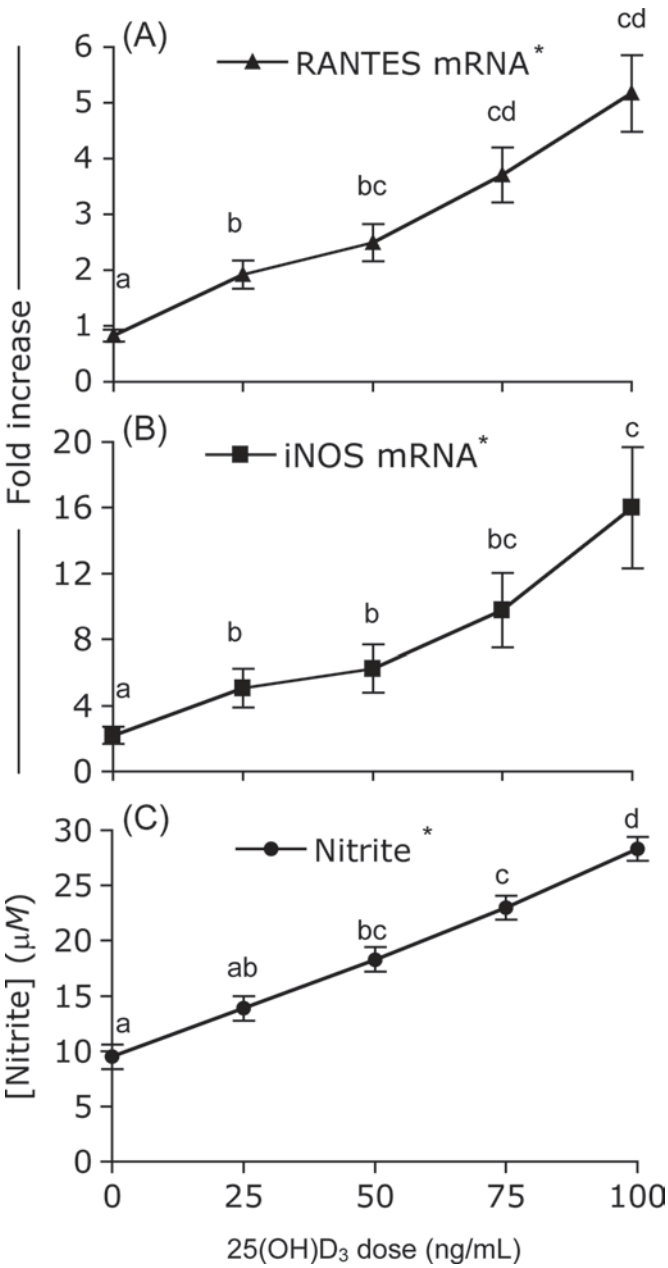

Figure 4. Gene expression of RANTES (regulated upon activation, normal T-cell expressed and secreted) and inducible nitric oxide synthase (iNOS) and nitric oxide production increase with 25-hydroxyvitamin D_3 [$25(OH)D_3$] dose. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL of LPS and 0 to 100 ng/mL of $25(OH)D_3$ as indicated for 24 h. Relative expression of RANTES (A) and iNOS (B) was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown for RANTES and iNOS expression is relative to the nontreated control. (C) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, $P < 0.05$. *Linear effect, $P < 0.001$.

$25(OH)D_3$ on RANTES and iNOS gene expression and nitric oxide production but not 1α-OHase gene expression in LPS-stimulated monocytes ($P < 0.05$; Figure 5). Furthermore, the effects of ketoconazole were reversed when exogenous $1,25(OH)_2D_3$ was added to the culture media ($P < 0.05$). Addition of $25(OH)D_3$ to monocytes that were not activated with LPS increased RANTES gene expression ($P < 0.05$) even though 1α-OHase gene expression was not elevated.

DISCUSSION

It has been known that $1,25(OH)_2D_3$ modulates bovine immune responses by increasing nitric oxide production by peripheral blood mononuclear cells in vitro (Waters et al., 2001). This study more specifically revealed that $1,25(OH)_2D_3$ enhanced iNOS gene expression in activated monocytes. It showed for the first time that RANTES expression was increased by $1,25(OH)_2D_3$. However, the concentration of $1,25(OH)_2D_3$ needed to increase iNOS and RANTES was much greater than the normal concentration of $1,25(OH)_2D_3$ in serum, which is less than 50 pg/mL (Horst and Reinhardt, 1983). Also, the concentration of $1,25(OH)_2D_3$ in serum did not increase during infection in cattle (Waldron et al., 2003). It was shown in this study that bovine monocytes converted $25(OH)D_3$ to $1,25(OH)_2D_3$ in response to TLR signaling, providing $1,25(OH)_2D_3$ at the site of infection. Furthermore, physiological concentrations of $25(OH)D_3$, which typically range from 20 to 50 ng/mL, were sufficient to increase iNOS and RANTES gene expression through the actions of 1α-OHase in monocytes.

An interesting observation in terms of regulating $1,25(OH)_2D_3$ concentration at the site of infection was the regulation of 24-OHase expression. Inactivation of $1,25(OH)_2D_3$ occurred by hydroxylation at the 24 position by 24-OHase (Reinhardt and Horst, 1989). Expression of 24-OHase normally is upregulated by $1,25(OH)_2D_3$ as a means to limit the concentration of $1,25(OH)_2D_3$ (Goff et al., 1992). The same regulation of 24-OHase expression occurs in nonactivated bovine monocytes. Activation of monocytes with LPS blocks induction of 24-OHase expression by $1,25(OH)_2D_3$. Without 24-OHase, $1,25(OH)_2D_3$ produced in monocytes will not be degraded and will continue to regulate gene expression. Inhibition of 24-OHase expression in LPS-activated monocytes seems to amplify the effects of $1,25(OH)_2D_3$ on iNOS and RANTES gene expression. Physiologically, this might be a another means to increase the concentration of $1,25(OH)_2D_3$ at the site of infection and increase the expression of iNOS and RANTES and possibly other genes as well. The specif-

ics of this observation will have to be studied further to better understand the physiological effect.

There is a major difference between humans and cattle in regard to the effects of $1,25(OH)_2D_3$ on the innate immune response. In human monocytes, production of $1,25(OH)_2D_3$ increased the expression of cathelicidin, which enhanced killing of intracellular *M. tuberculosis* (Liu et al., 2007). In contrast, the bovine cathelicidin genes with potential vitamin D response elements in their promoters were not affected by $1,25(OH)_2D_3$.

In cattle, production of $1,25(OH)_2D_3$ by monocytes enhanced production of nitric oxide by increasing the expression of iNOS. Nitric oxide is known to have several effects physiologically and was considered a fundamental component of the antimicrobial response (Bogdan, 2001). Studies with iNOS-deficient mice revealed that production of nitric oxide aided in the resolution of *M. tuberculosis* and *Mycobacterium bovis* infections (MacMicking et al., 1997, Waters et al., 2004). No studies have definitively shown that nitric oxide is necessary for bovine macrophages to kill bacteria. Regardless, nitric oxide production occurred during the course of several major diseases of cattle such as Johne's disease (Waters et al., 2003), mastitis (Blum et al., 2000, Bouchard et al., 1999), and tuberculosis (Palmer et al., 2007). The concentration of $25(OH)D_3$ in cattle might have considerable implications in such diseases because nitric oxide production in monocytes is dependent on $25(OH)D_3$ concentration in vitro.

It was shown that $1,25(OH)_2D_3$ production in bovine monocytes increased RANTES gene expression. Also known as chemokine (C-C motif) ligand 5, RANTES is a chemo-attractant for T-helper cells and monocytes to the site of inflammation (Schall, 1991). In humans, RANTES was implicated in the clearance of viral infections, likely by the recruitment of other immune cells to the site of infection (Levy, 2009). There is little information on the importance of RANTES in the bovine immune response, and most of what is known about the chemokine is drawn from studies in other species. The expression of RANTES was induced by TLR signaling in cattle (Pareek et al., 2005, Widdison et al., 2008). Based on evidence from this study, induction of RANTES expression in bovine monocytes was mediated by production of $1,25(OH)_2D_3$ in monocytes in response to TLR signaling.

Finally, this study provides evidence that vitamin D status of cattle is important for proper immune function. It is clear that RANTES and iNOS expression and nitric oxide production in activated monocytes increased with $25(OH)D_3$ concentration up to 100 ng/mL in vitro. The concentration of serum $25(OH)D_3$ in cattle supplemented with the recommended amount of dietary vitamin D typically ranges from 20 to 50

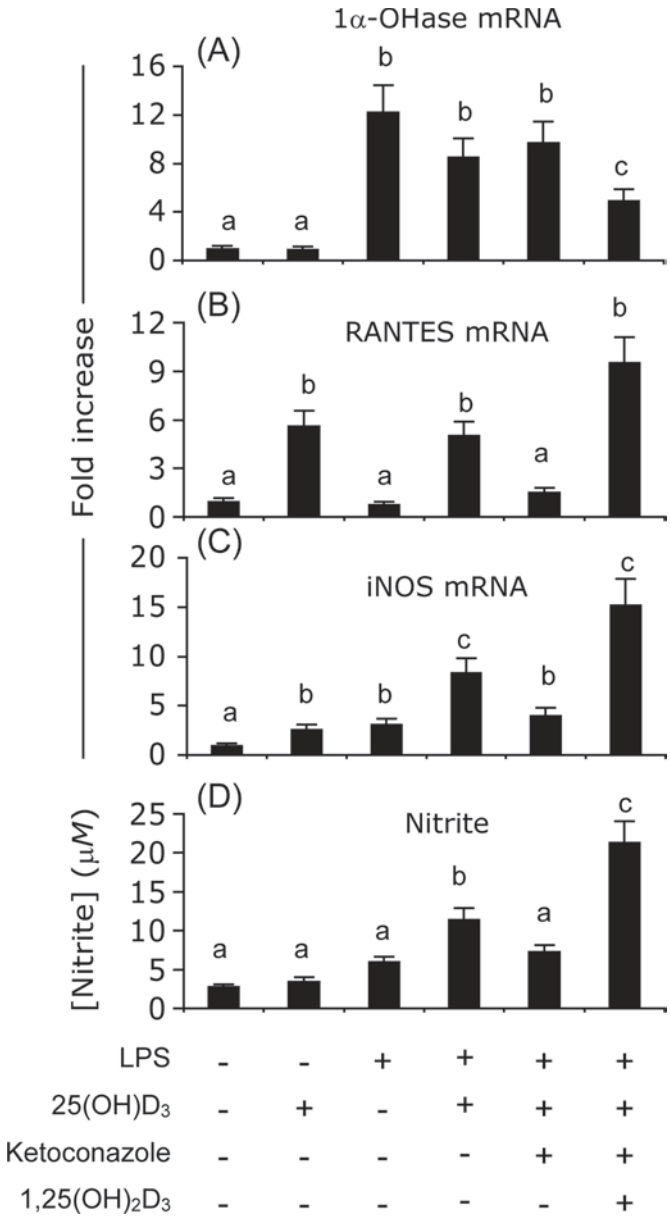

Figure 5. RANTES (regulated upon activation, normal T-cell expressed and secreted), inducible nitric oxide synthase (iNOS), and nitric oxide are dependent on 1α-hydroxylase (1α-OHase) activity. Peripheral blood monocytes were isolated from 6 cows and treated with 100 ng/mL of LPS, 75 ng/mL of 25-hydroxyvitamin D_3 [$25(OH)D_3$], 5 μg/mL of ketoconazole, and 0.4 ng/mL of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] as indicated for 24 h. Relative expression of 1α-OHase (A), RANTES (B), and iNOS (C) was determined using real-time PCR and the $2^{-\Delta\Delta Ct}$ method. The mean fold increase shown for each gene is relative to the nontreated control. (D) Nitric oxide production was determined by measuring the amount of nitrite in the culture supernatant with the Griess assay. Error bars represent SE, n = 6. Means with different letters are different, $P < 0.05$.

ng/mL (McDermott et al., 1985). Serum 25(OH)D_3 concentrations above 50 ng/mL can be reached by additional supplementation; so, it might be possible to boost RANTES expression and nitric oxide production during an immune response in cattle. When the 25(OH)D_3 concentration exceeded 200 ng/mL in serum, calcification of soft tissue occurred (Horst et al., 1994). Current dietary recommendations for vitamin D supplementation of cattle are largely based on the amount of vitamin D needed to maintain proper mineral homeostasis in dairy cattle, not on the amount for proper immune function. Therefore, future studies are needed to determine what concentration of 25(OH)D_3 in cattle is needed for proper immune function.

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In Vivo Activation of the Intracrine Vitamin D Pathway in Innate Immune Cells and Mammary Tissue during a Bacterial Infection

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Abstract

Numerous *in vitro* studies have shown that toll-like receptor signaling induces 25-hydroxyvitamin D_3 1α-hydroxylase (1α-OHase; CYP27B1) expression in macrophages from various species. 1α-OHase is the primary enzyme that converts 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Subsequently, synthesis of $1,25(OH)_2D_3$ by 1α-OHase in macrophages has been shown to modulate innate immune responses of macrophages. Despite the numerous *in vitro* studies that have shown 1α-OHase expression is induced in macrophages, however, evidence that 1α-OHase expression is induced by pathogens *in vivo* is limited. The objective of this study was to evaluate 1α-OHase gene expression in macrophages and mammary tissue during an *in vivo* bacterial infection with *Streptococcus uberis*. In tissue and secreted cells from the infected mammary glands, 1α-OHase gene expression was significantly increased compared to expression in tissue and cells from the healthy mammary tissue. Separation of the cells by FACS9 revealed that 1α-OHase was predominantly expressed in the $CD14^+$ cells isolated from the infected mammary tissue. The 24-hydroxylase gene, a gene that is highly upregulated by $1,25(OH)_2D_3$, was significantly more expressed in tissue and cells from the infected mammary tissue than from the healthy uninfected mammary tissue thus indicating significant local $1,25(OH)_2D_3$ production at the infection site. In conclusion, this study provides the first *in vivo* evidence that 1α-OHase expression is upregulated in macrophages in response to bacterial infection and that 1α-OHase at the site of infection provides $1,25(OH)_2D_3$ for local regulation of vitamin D responsive genes.

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Introduction

Vitamin D has been shown to have a role in regulating immune function in addition to the well-known role it has in regulating calcium homeostasis. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active vitamin D_3 metabolite, regulates the expression of several genes involved in host defense and immune function [1]. Therefore, synthesis of $1,25(OH)_2D_3$ to control vitamin D responsive genes in immune cells is a critical factor in regulating of immune function.

The enzyme that synthesizes $1,25(OH)_2D_3$ from 25-hydroxyvitamin D_3 ($25(OH)D_3$) is 1α-hydroxylase (1α-OHase; CYP27B1) [2]. In the kidney, 1α-OHase expression is induced by parathyroid hormone in response to calcium homeostasis [3,4]. Synthesis of $1,25(OH)_2D_3$ in the kidney regulates the circulating concentration of $1,25(OH)_2D_3$ and the endocrine actions of vitamin D. In monocytes and macrophages, 1α-OHase is expressed in response to activation by IFN-γ or TLR signaling [5,6,7,8]. Conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by 1α-OHase in monocytes regulates the expression of vitamin D responsive genes in an intracrine manner [9]. In human monocytes, production of $1,25(OH)_2D_3$ by 1α-OHase drives cathelicidin gene expression [6]. In the same way, 1α-OHase activity in bovine monocytes enhances iNOS and RANTES gene expression [8]. From in vitro studies, expression of 1α-OHase by macrophages at the site of an infection seems to be an important part of innate immunity. Montoya et al have shown that upregulation of the vitamin D pathway occurs in leprosy lesions of patients with self limiting forms of the disease [10], however, beyond that study there is no evidence that 1α-OHase is expressed by macrophages in vivo as a result of experimental infection [11].

Intra-mammary infections during lactation offers a model of bacterial infection to determine if 1α-OHase is expressed in response to bacterial infection in vivo. Common pathogens that cause mammary infections include *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis* [12,13,14,15]. During mammary infection the number of somatic cells secreted in milk will often exceed 10^6 cells/mL. Approximately 80 to 90 percent of somatic cells in milk from an infected mammary gland are neutrophils and the remainder of the cells are macrophages and lymphocytes [16].

The advantage of using a mammary infection model is that the infiltrating cells during mammary infection can easily isolated from milk using non-invasive procedures; allowing us to study the in vivo immune responses of immune cells to bacterial infection.

TLRs are present in the bovine mammary gland [17] and invasion of the mammary gland by bacteria triggers an innate immune response by TLR signaling [18]. Based on in vitro evidence that 1α-OHase expression in macrophages is induced by TLR recognition of bacteria [6,8,19], we hypothesized that 1α-OHase expression would be upregulated in both macrophages and mammary tissue during a mammary infection. Using an intra-mammary infection as a model in vivo bacterial infection, we present the first in vivo evidence that 1α-OHase expression is upregulated in $CD14^+$ cells that are at the site of a bacterial infection. The subsequent large increased expression of 24-hydroxylase (24-OHase) at the infection site supports local in vivo production of $1,25(OH)_2D_3$ at the site of an infection.

Materials and Methods

Animals

Eight, mid-lactation Holstein cows at the USDA National Animal Disease Center were used for this study. The National Animal Disease Center animal care and use committee approved all procedures used in this study (Protocol ARS-4001). Prior to the study, all cows were healthy and bacteria were not detected in their milk. Mammary infection was induced by infusion of 500 cfu of *Streptococcus uberis* strain 0140 (*S. uberis*; a gift from Dr. Max Paape, USDA, Beltsville, MD) suspended in 3 mL of PBS into one mammary gland. The contra lateral gland was infused with an equal volume of PBS and served as the control. The amount of *S. uberis* in the milk from the control and infected glands was determined by culturing log dilutions of milk samples on blood agar plates for 24 hours at 37°C.

Collection of tissue and cells

Mammary tissue was collected from various locations in the control and infected glands of three cows that were euthanized at the onset of clinical mammary infection. Clinical infection was defined by rectal temperature, noticeable inflammation, and presence of bacteria in the milk. Tissue was placed in RNAlater (Qiagen, Valencia, CA), snap frozen in liquid nitrogen and stored at −80°C.

Cells were isolated from milk from the control and infected glands and peripheral blood of 5 cows before infection with *S. uberis* and at the onset of clinical mastitis. Cells were isolated from milk by centrifuging the milk at 1000Xg for 20 min. Peripheral blood leukocytes were isolated by lysing the erythrocytes with a hypotonic buffer and centrifuging at 650×g for 10 min. The cell pellets from milk and blood were washed 3X by resuspending in cold PBS and centrifuging at 650×g for 10 min. Cells were lysed with RLT buffer (Qiagen) and stored at −80°C or separated by FACS.

For separation of cells from blood and milk by FACS, cells were labeled with monoclonal anti-bovine CD14 IgG_1 (CAM36A; VMRD, Inc., Pullman, WA) and a PE-conjugated anti-mouse IgG antibody (Southern Biotech, Birmingham, AL). Labeled cells were separated based on fluorescence intensity using the BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA). Approximately 10^6 $CD14^+$ and $CD14^-$ cells with greater than 95% purity were isolated from milk from the infected gland and peripheral blood of each animal. The sorted cells were lysed with RLT buffer (Qiagen) and stored at −80°C.

Real-time PCR

RNA was isolated from mammary tissue and cells using an RNeasy Mini Kit (Qiagen). RNA samples were eluted in 50 μL of RNAse-free water. Immediately after elution, RNA was reverse transcribed to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 10 μL RNA sample and 20 units of RNase inhibitor (RNaseOUT, Invitrogen, Carlsbad, CA) in a 20 μL reaction. Reactions were incubated at 37°C for 2 h and heated to 85°C for 5 s. The cDNA samples were diluted 1:10 in water and stored at −20°C. Real-time PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems). The reactions were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction contained 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 2.5 μL each of 10 μ*M* forward and reverse primers, and 7.5 μL of diluted cDNA. Sequences for primer pairs are given in Table 1. Primers were purchased from Integrated DNA Technologies (Corralville, IA). The specificity of each primer pair was determined by gel electrophoresis of cDNA products and efficiency was determined using known dilutions of cDNA. All primer pairs, except for the VDR, have been used previously [8]. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [20]. RPS9 was used as the

Table 1.

Gene (alternate name)	Accesion no.[1]	Strand	Sequence (5′ - 3′)
1α-OHase (CYP27B1)[2]	NM_001192284	Forward Reverse	TGGGACCAGATGTTTGCATTCGC TTCTCAGACTGGTTCCTCATGGCT
24-OHase (CYP24A1)[2]	NM_001191417	Forward Reverse	GAAGACTGGCAGAGGGTCAG CAGCCAAGACCTCGTTGATT
iNOS[2]	NM_001076799	Forward Reverse	GATCCAGTGGTCGAACCTGC CAGTGATGGCCGACCTGATG
RANTES (CCL5)[2]	NM_175827	Forward Reverse	CACCCACGTCCAGGAGTATT CTCGCACCCACTTCTTCTCT
RPS9[2]	NM_001101152	Forward Reverse	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA
VDR	NM_001167932	Forward Reverse	AGCCACCGGCTTCCATTTCA AACAGCGCCTTCCGCTTCAT

[1]Accesion numbers from NCBI database http://www.ncbi.nlm.nih.gov.
[2]Primer sequences have been published previously [8].
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reference gene. RPS9 gene expression also was compared to β-actin gene expression and the relative expression of RPS9 did not differ significantly among treatments.

Statistical analysis

Statistical analysis was performed using PROC GLM of SAS (SAS Institute Inc., Cary, NC). The model used in the analysis accounted for effects of treatment and cow. ΔΔCt values were used to analyze relative gene expression. Mean ΔΔCt values ± SE were transformed ($2^{-\Delta\Delta Ct}$) and shown as the expression relative to the control. The control treatment is designated in the figure legends. Multiple comparison tests of the means were made with the Tukey-Kramer adjustment.

Results

Streptococcus uberis mammary infection

Eight cows were infused with 500 CFUs of *S. uberis* strain 0140 in one mammary gland and sterile PBS in the contra-lateral mammary gland. Bacteria were not detected in milk from any of the mammary glands prior to infection and were not detected in the milk from the control mammary glands during infection (Fig. 1A). The average amount of *S. uberis* in the milk from the infected mammary glands at the peak of the disease was 10^8 CFU/mL (Fig. 1A). Prior to infection cows had normal body temperatures, but during mammary infection body temperatures were elevated (Fig. 1B). The number of somatic cells in the milk from the infected mammary glands rose from an average of 10^5 cells/mL prior to infection to over 10^7 cells/mL during the infection (Fig. 1C). The number of somatic cells in the control mammary glands remained near 10^5 cells/mL on average during infection (Fig. 1C). Examination of mammary tissue by microscopy revealed inflammation and the presence of infiltrating cells in the alveoli of the infected mammary gland (Fig. 1D). The onset of clinical infection was typically 3 days after infection with *S. uberis*; which was consistent with previous studies with *S. uberis* [21]. The mean concentration of 25(OH)D in blood of the cows used in this study was 74.5 ng/ml (SEM +/−4.5) and the concentration of 25(OH)D in blood was not affected by onset of mastitis.

1α-Hydroxylase gene expression during mastitis

In our initial experiment, secretory mammary tissue was collected from the control and infected glands of 3 cows at the onset of clinical mammary infection. In the tissue from the infected mammary glands, 1α-OHase expression was nearly 50 fold greater than 1α-OHase expression in tissue from the contra-lateral uninfected mammary glands ($P<0.001$; Fig. 2A). The alveoli in the infected mammary tissue were packed with infiltrating cells (Fig. 1D) and the

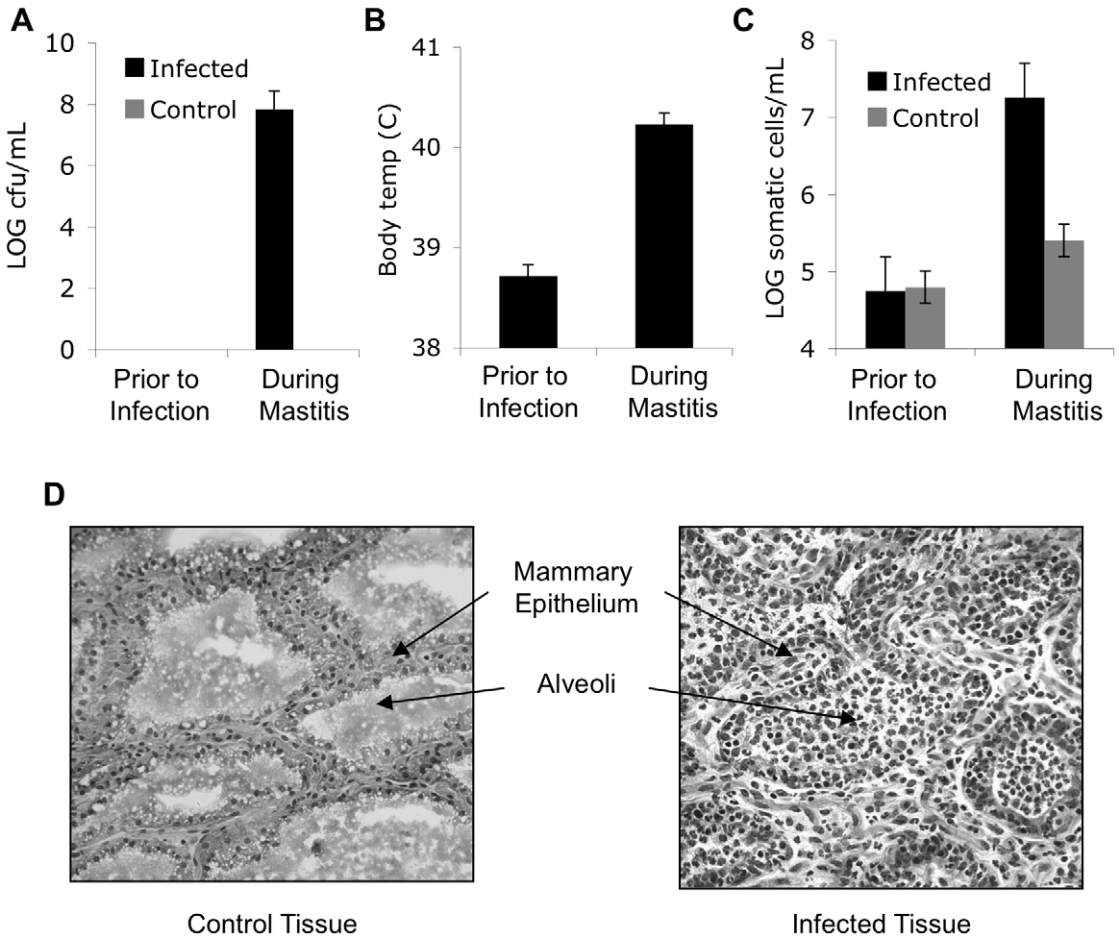

Figure 1. Intra-mammary infection with *Streptococcus uberis*. Five hundred CFU of *S. uberis* strain 0140 were infused into the mammary glands of 8 healthy cows. The contra-lateral glands were infused with 3 mL of PBS and served as the controls. Prior to infection, all glands were free of bacteria. The average CFU of *S. uberis* in milk (**A**), rectal temperatures (**B**), and number of somatic cells in milk (**C**) prior to and during infection are shown. Error bars represent the SE. (**D**) Representative tissue sections at 20X magnification from the control and infected mammary gland.
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infiltrating cells, which are ~10% macrophages during acute mastitis [16], were hypothesized to be the cells expressing 1α-OHase. Therefore, to determine the contribution of infiltrating cells on 1α-OHase gene expression during mastitis we isolated cells from the milk of control and infected mammary glands from 5 cows with *S. uberis*. In cells isolated from milk from the infected mammary glands, 1α-OHase gene expression was 40 fold greater than expression in cells from the contra-lateral uninfected mammary gland ($P<0.001$) and over 300 fold greater than peripheral blood leukocytes ($P<0.001$; Fig. 2B). Finally, separating the cells according to CD14 expression revealed that 1α-OHase was predominantly expressed in the $CD14^+$ cells from the infected mammary glands ($P<0.001$) compared to $CD14^-$ cells from the infected gland, but not in $CD14^+$ cells from peripheral blood (Fig. 2C). CD14 is a marker for monocytes and macrophages [22] and typically 10% of the cells isolated from the milk of the infected mammary gland were $CD14^+$ cells (Fig. 2D–F).

Expression of the VDR and vitamin D responsive genes during mammary infection

The effects of $1,25(OH)_2D_3$ on gene expression depend on the presence of the VDR. We measured VDR gene expression to find if it was more abundant in the infected mammary gland. VDR expression was slightly higher in mammary tissue from the infected mammary gland than in tissue from the contra-lateral uninfected mammary gland (Fig. 3A). However, VDR expression in the cells isolated from milk of the infected mammary gland was 8 fold higher than VDR expression in cells from the contra-lateral uninfected mammary gland ($P<0.05$) and 75 fold higher than VDR expression in peripheral blood leukocytes ($P<0.001$; Fig. 3B). In the cells from the infected mammary gland, there was no difference of VDR expression between the $CD14^+$ and $CD14^-$ populations (Fig. 3C). In both populations though, VDR expression was at 50 fold greater in the cells from the infected mammary gland than in peripheral blood leukocytes ($P<0.05$) (Fig. 3C).

The 24-OHase gene expression was 50 fold higher in the infected mammary glands relative to 24-OHase expression in the contra-lateral uninfected mammary glands ($P<0.001$; Fig. 4A). The 24-OHase expression in cells isolated from milk was 4 fold higher in cells from the infected mammary gland than in cells from the contra-lateral uninfected mammary gland or peripheral blood ($P<0.05$; Fig. 4B). During bacterial infection, 24-OHase expression was not significantly higher in $CD14^+$ cells from the infected glands compared to the $CD14^+$ cells from blood, but was higher in $CD14^-$ cells from the infected glands compared to $CD14^-$ cells from blood ($P<0.05$; Fig. 4C).

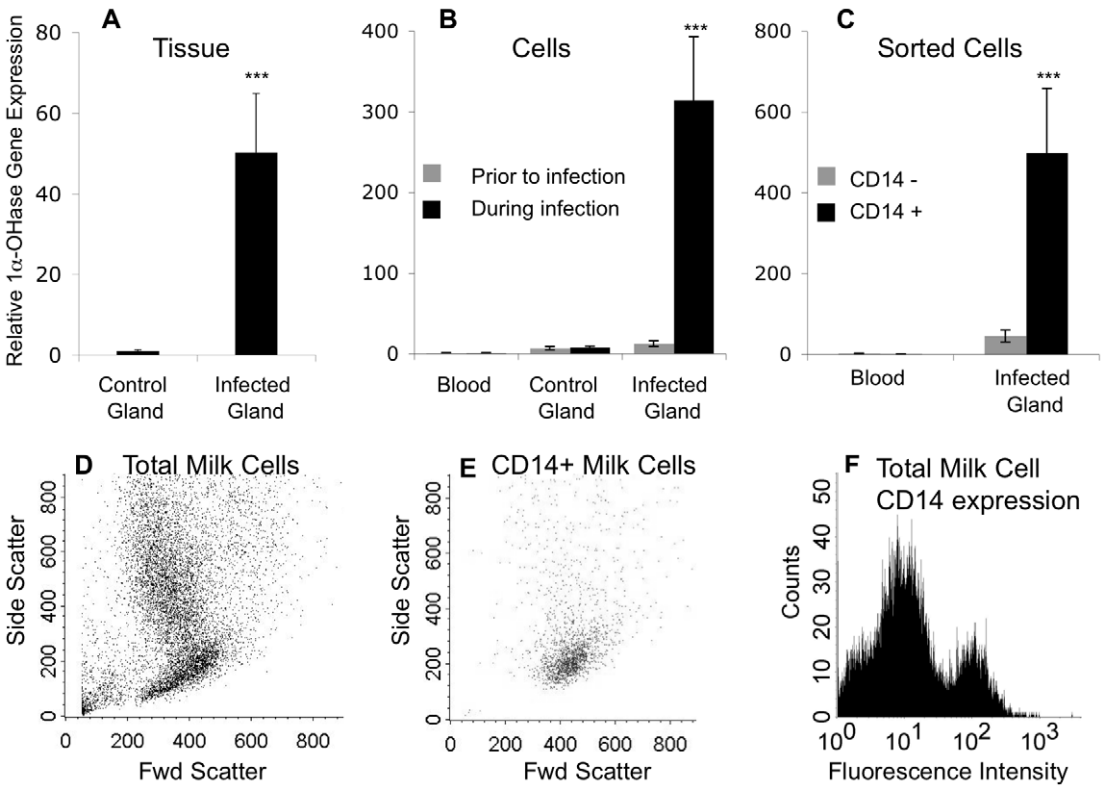

Figure 2. 1α-OHase gene expression during *S. uberis* mammary infection. (**A–C**) Relative 1α-OHase gene expression in mammary tissue during infection (A), total cells from blood and milk prior to and during mammary infection (B), and $CD14^+$ cells and $CD14^-$ cells from blood and milk during mammary infection. (**A**) Three cows were infected with *S. uberis* and mammary tissue was collected from control and infected glands. (**B and C**) Five other cows were infected with *S. uberis* and cells (mononuclear and polymorphonuclear) were collected from blood and milk from the control and infected glands prior to and during infection. (**C**) Blood and milk cells from the infected gland were separated according to CD14 expression on the cell surface using FACS. The amount of 1α-OHase mRNA in each sample was determined by quantitative real-time RT-PCR and normalized to RPS9 mRNA. The relative amount of 1α-OHase mRNA was determined using the $2^{-\Delta\Delta Ct}$ method. Data represent the mean ± SE expression of 1α-OHase relative to 1α-OHase expression in control tissue (**A**), or peripheral blood leukocytes (**B and C**). ANOVA was performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment; ***mean is different from other means, $P<0.001$. (**D and E**) Scatter plots of all cells and $CD14^+$ cells isolated from milk from an infected gland. (**F**) Representative histogram of CD14 expression on cells isolated from the milk from the infected gland.
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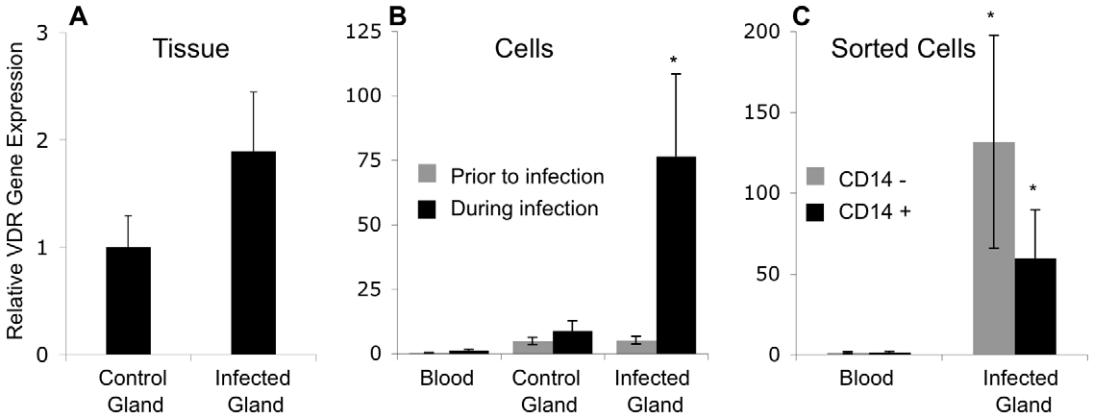

Figure 3. VDR gene expression during mammary *S. uberis* infection. Relative VDR gene expression in mammary tissue during an infection (**A**), total cells from blood and milk prior to and during mammary infection (**B**), and $CD14^+$ cells and $CD14^-$ cells from blood and milk during mammary infection (**C**). Tissue (n = 3 cows) and cells (n = 5 cows) were collected as in Figure 2. The amount of VDR mRNA in each sample was determined by quantitative real-time RT-PCR and normalized to RPS9 mRNA. The relative amount of VDR mRNA was determined using the $2^{-\Delta\Delta Ct}$ method. Data represent the mean ± SE expression of VDR relative to VDR expression in control tissue (**A**), or peripheral blood leukocytes (**B and C**). ANOVA was performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment; *mean is different from other means, $P<0.05$.
doi:10.1371/journal.pone.0015469.g003

In vitro activated bovine monocytes, $1,25(OH)_2D_3$ increases expression of iNOS and RANTES [8]; so, we measured the expression of both genes in mammary tissue and cells from the control and in vivo infected mammary glands (Fig. 4D–I). iNOS and RANTES expression was greater ($P<0.001$ for iNOS and $P<0.05$ for RANTES) in tissue and cells from the infected mammary glands than in tissue or cells from the contra-lateral uninfected mammary glands. Like 1α-OHase, iNOS was predominantly expressed in the $CD14^+$ cells from the infected gland ($P<0.001$ compared to $CD14^-$ cells from the infected glands). RANTES, however, was expressed more in the $CD14^-$ population from the infected glands compared to $CD14^+$ cells from the infected glands ($P<0.05$).

Discussion

Numerous in vitro studies have shown that 1α-OHase expression and subsequent $1,25(OH)_2D_3$ induction of 24-OHase in monocytes and macrophages is induced by TLR signaling in vitro [6,8,19]. However, there was a lack of in vivo evidence that the vitamin D pathway was induced in macrophages in response to infection. Genes of the vitamin D pathway were elevated in macrophages in lesions of leprosy patients [10] but that evidence could not confirm whether or not the pathway was upregulated in response to infection. In this study, we give in vivo confirmation that genes of the vitamin D signaling pathway were upregulated in response to bacterial infection. Furthermore, macrophages at the site of the bacterial infection were the predominant cells that expressed 1α-OHase, which confirms the many in vitro studies that have shown that 1α-OHase is expressed in macrophages upon pathogen recognition.

Induction of 1α-OHase gene expression in the infected mammary gland has major implications because it allows for local control of $1,25(OH)_2D_3$ synthesis. In addition to upregulation of 1α-OHase, VDR gene expression was elevated in cells from the infected mammary gland. Increased VDR expression would have enhanced the sensitivity of those cells to $1,25(OH)_2D_3$. Induction of 1α-OHase and VDR expression, consequently allowed for control of vitamin D responsive genes in the infected mammary glands. During bacterial infection, 24-OHase, iNOS and RANTES were expressed in the infected mammary glands. Cathelicidin genes have previously been shown to not be regulated by $1,25(OH)_2D_3$ in cattle, in contrast to other species, and therefore were not tested [8]. However, induction of 1α-OHase gene expression in bovine monocytes in the presence of $25(OH)D_3$ resulted in upregulation of iNOS and RANTES [8]. Accordingly, upregulation of iNOS and RANTES expression in monocytes is in part depended on local 1α-OHase activity and $1,25(OH)_2D_3$ production. Likewise, the expression of 24-OHase is known to be highly upregulated by $1,25(OH)_2D_3$ [23,24]. The upregulation of 24-OHase in the infected mammary glands indicates that $1,25(OH)_2D_3$ was synthesized locally in the infected mammary glands. Altogether, our data provides in vivo evidence that a vitamin D signaling mechanism is activated as part of the innate immune response to pathogens in order to provide local control of vitamin D-dependent immune responses.

The substrate for 1α-OHase is $25(OH)D_3$, so production of $1,25(OH)_2D_3$ and subsequently regulation of gene expression by $1,25(OH)_2D_3$, depends on the availability of $25(OH)D_3$. The circulating concentration of $25(OH)D_3$ depends on dietary intake of vitamin D and exposure to sunlight [25,26]. In cattle supplemented with the recommended amount of vitamin D, the circulating concentration of $25(OH)D_3$ typically ranges from 20 to 50 ng/mL [27]. Circulating concentrations of 25(OH)D above 20 ng/mL have been considered adequate for calcium homeostasis [28], however, circulating 25(OH)D concentrations below 30 ng/mL are now considered insufficient for proper immune function in humans [1,29,30]. The target range for 25(OH)D in humans still remains elusive, however, because of the inability to perform tightly controlled experiments with human subjects. Similarities between cattle and humans in regards to vitamin D metabolism and immune function [31,32,33] indicate that cattle are a useful model to study vitamin D requirements for proper immune function in humans. Experiments to find an optimal range of circulating 25(OH)D for proper immune function in cattle, however, have not been performed. Therefore, efforts to find the optimal range of 25(OH)D concentration for proper immune function in cattle has implications for bovine and human health.

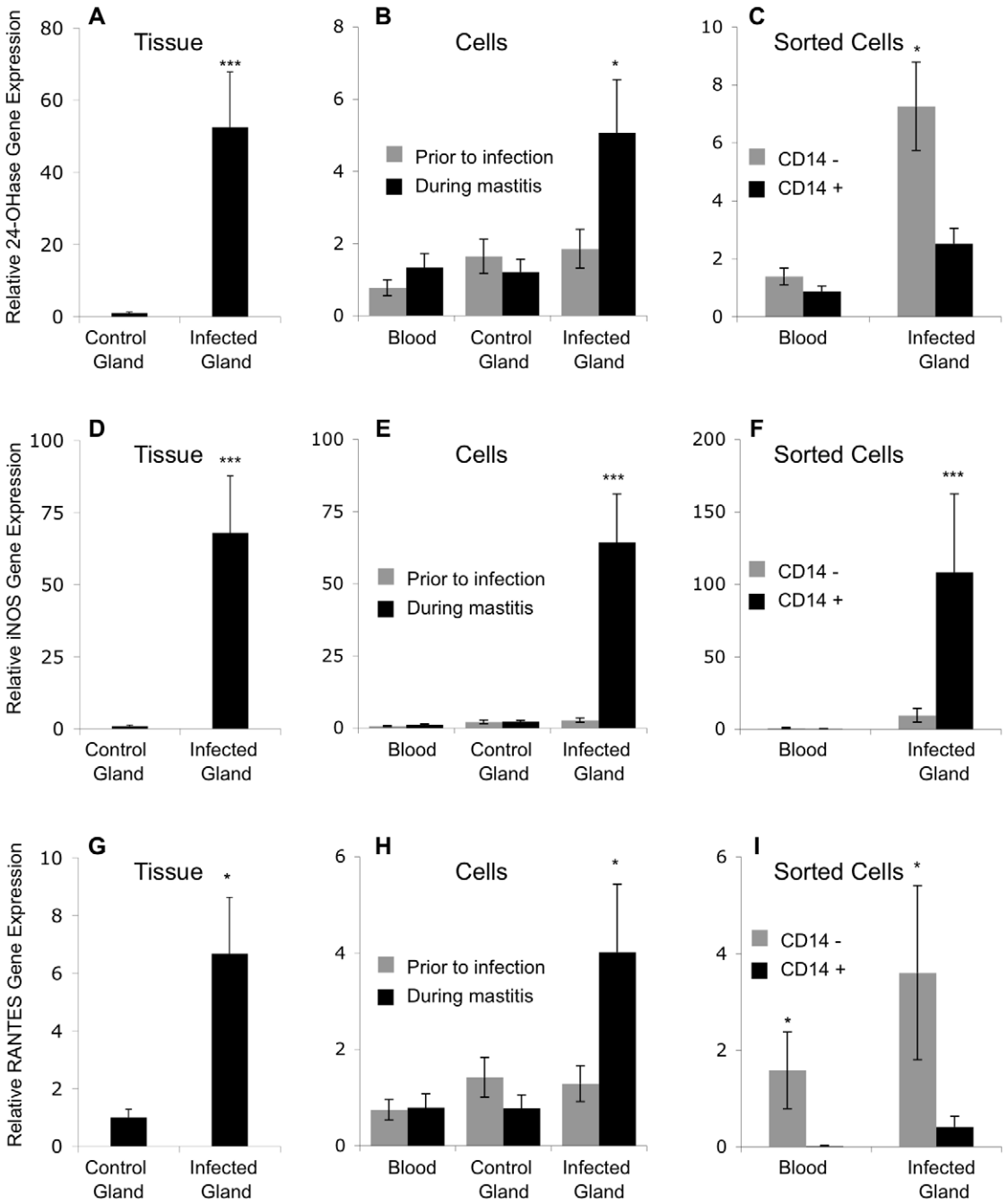

Figure 4. Expresssion of $1,25(OH)_2D_3$ inducible genes during mammary infection. Relative 24-OHase (**A–C**), iNOS (**D–F**), and RANTES (**G–I**) gene expression in mammary tissue, cells from peripheral blood and cell from milk during mammary infection. Tissue (n = 3 cows) and cells (n = 5 cows) were collected and sorted as in Figure 2. The amount of mRNA for each gene in each sample was determined by quantitative real-time RT-PCR and normalized to RPS9 mRNA. The relative amount of mRNA for each gene was determined using the $2^{-\Delta\Delta Ct}$ method. Data represent the mean ± SE expression of each gene relative to expression in control tissue (**A**), or peripheral blood leukocytes (**B and C**). ANOVA was performed by SAS using the general linear model and multiple comparison tests were made using the Tukey adjustment; mean is different from other means* $P<0.05$, *** $P<0.001$.

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We did measure the concentration of $1,25(OH)_2D$ in milk during mammary infection but the there was not a detectable increase of $1,25(OH)_2D$ in milk from the infected mammary gland compared to milk from an uninfected mammary gland. In fact milk $1,25(OH)_2D_3$ was at or below the detection limits of the assay (data not shown) This was expected as the concentration of vitamin D metabolites is are low in milk compared to plasma [27,34], and the $1,25(OH)_2D_3$ produced by macrophages would have been diluted as milk accumulated in the cistern of the mammary gland.

The mean blood concentration of 25(OH)D in the cows used in this study was 74.5 ng/ml (SEM +/−4.5), which is above the $25OHD_3$ threshold needed for innate immune cells to produce $1,25(OH)_2D_3$ [8,35]. The increased expression of the 1α-OHase

we observed in innate immune cells from infected mammary tissue along with the subsequent induction of 24-OHase in these cells supports the conclusion of local in vivo production of $1,25(OH)_2D_3$ during an infection. We show for the first time that an in vivo infection results in activation of vitamin D with the concomitant downstream vitamin D dependant genes of the innate immune system activated similar to that previously observed in vivo.

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Author Contributions

Conceived and designed the experiments: CN TR DB JL. Performed the experiments: CN JL. Analyzed the data: CN TR DB JL. Contributed reagents/materials/analysis tools: CN TR DB JL. Wrote the paper: CN TR JL.

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Short communication: Fat-soluble vitamin and mineral status of milk replacer-fed dairy calves: Effect of growth rate during the preruminant period[1]

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ABSTRACT

Effects of growth rate on fat-soluble vitamin and macro- and micromineral concentrations in the circulation of preruminant dairy calves were evaluated. Dietary treatments were designed to achieve 3 targeted rates of gain [no growth (NG) = 0.0 kg/d; low growth (LG) = 0.55 kg/d; or high growth (HG) = 1.2 kg/d] over a 7-wk period. Milk replacer (MR) intakes necessary to achieve these growth rates were estimated using the National Research Council's *Nutrient Requirements of Dairy Cattle* calf model computer program. All of the calves were fed a 30% crude protein, 20% fat MR reconstituted to 14% dry matter. The diets were formulated to ensure that protein was not a limiting nutrient. No-growth and LG calves were supplemented additionally with vitamins A, D, and E to compensate for treatment differences in dry matter intake relative to the HG calves; however, no attempt was made to adjust mineral intake based on MR consumption. Growth rates for NG (0.11 kg/d), LG (0.58 kg/d), and HG (1.16 kg/d) calves differed during the study. Health was minimally affected by growth rate and this was reflected by comparable and relatively low serum haptoglobin concentrations in all calves during the 7-wk period. Concentrations of serum retinol, 25-(OH)-vitamin D_3, and zinc were unaffected by growth rate. The HG calves had lower *RRR*-α-tocopherol concentrations than NG and LG calves at wk 7, suggesting that the increased growth rate of HG calves was associated with increased utilization of vitamin E. Serum concentrations of all vitamins increased with age. Copper, calcium, and phosphorous concentrations in HG calves exceeded those in LG and NG calves during the latter weeks of the study, likely because of increased MR intake by HG calves. Fat-soluble vitamin and mineral concentrations for all treatment groups remained within ranges considered normal for preruminant calves.
Key words: preruminant calf, vitamins A, D, and E, mineral status

The importance of nutrient supply on body composition and growth performance of preruminant calves has been evaluated extensively. Consequences of increased growth rate, associated with enhanced early nutrition, on fat-soluble vitamin status and mineral status of dairy calves fed milk replacer (**MR**) have not been investigated.

Vitamin A plays a vital role in vision, growth, and bone formation; vitamin E is a potent lipid-phases antioxidant; and vitamin D is a key regulator of calcium and phosphorus homeostasis. All 3 vitamins are recognized as being essential in ensuring optimal immune function and infectious disease resistance. At birth, dairy calves have very low plasma concentrations of vitamin A (retinol, <40 ng/mL), vitamin E (*RRR*-α-tocopherol, <200 ng/mL; Nonnecke et al., 1999), and vitamin D [25-hydroxyvitamin D_3 (25(OH)D_3), 20–25 ng/mL; Rajaraman et al., 1997; Nonnecke et al., 2009] compared with adult dairy cows (retinol, 400–700 ng/mL, *RRR*-α-tocopherol >500 ng/mL, and 25(OH)D_3, 45–80 ng/mL; Horst et al., 1994; Goff et al., 2001). Feeding colostrum within hours after birth induces a substantial increase in circulating concentrations of retinol and *RRR*-α-tocopherol but has no effect on 25(OH)D_3 status (Rajaraman et al., 1997). Producers feed MR containing high concentrations of these vitamins to ensure adequate vitamin status and may administer, parenterally, fat-soluble vitamins within hours after birth. The most recent recommendations (NRC, 2001) regarding the nutrient requirements of dairy calves increased the vitamin A (9,000 IU/kg of DM) and vitamin E (50 IU/kg of DM) requirements for MR-fed calves. The vitamin D requirement (600 IU/kg of DM) was not increased from the previous recom-

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mendation, although commercial MR typically provide 10- to 20-fold more vitamin D than the current NRC (2001) recommendation.

The essential roles of Ca, Mg, and P in nerve and muscle function, bone formation, and metabolic disorders of dairy cattle were reviewed by Goff (2004). The normal range for plasma Ca is 9 to 10 mg/dL in the adult cow and slightly higher in calves (NRC, 2001). Deficiency in growing calves is manifested by suboptimal growth and, to a lesser extent, rickets. Normal plasma Mg concentrations in the calf range from 2.2 to 2.7 mg/dL (Roy, 1990) compared with 1.8 to 2.4 mg/dL for adult dairy cows (NRC, 2001). Deficiency usually occurs in rapidly growing calves fed large amounts of milk or milk substitute and with no access to other supplementary feeds (Roy, 1990). Clinical symptoms are similar to those of Mg-deficient cows (i.e., excitability, uncoordinated gate, tetany) and may not be evident until plasma concentrations are <0.7 mg/dL. Plasma P concentrations in replete cattle range from 4 to 8 mg/dL, with growing cattle typically having concentrations at the higher end of this range (6 to 8 mg/dL; NRC, 2001). Nonspecific signs of chronic deficiency (2 to 3.5 mg/dL) in the calf may be poor growth and possibly rickets in cases of severe deficiency.

Copper functions as a cofactor for enzymes involved with aerobic respiration, cross-linking of collagen, detoxification of oxygen metabolites, and transport of ferric iron in peripheral blood. Plasma Cu concentrations in normal preruminant calves range from 0.7 to 1.3 μg/mL, whereas marginal deficiency occurs when concentrations are <0.6 μg/mL. Concentrations in the newborn calf are low (<0.30 μg/mL) but increase to near-adult levels (approximately 1 μg/mL) within the first week of life. Symptoms of deficiency may not be evident until concentrations are <0.2 μg/mL (Suttle, 1986). Zinc is intimately associated with numerous metalloenzymes affecting carbohydrate, protein, and lipid metabolism and is a component of thymosin, a key regulator of T-cell-mediated immunity. Although the normal range for plasma Zn concentrations in the preruminant calf is not established, the range in adult dairy cows is 0.7 to 1.3 μg/mL, with deficiency occurring at <0.4 μg/mL (NRC, 2001).

The objective of this study was to evaluate the effect of growth-rate during the neonatal period on serum concentrations of retinol (**VitA**), *RRR*-α-tocopherol (**VitE**), and 25(OH)D_3 (**VitD**) and minerals (Ca, P, Mg, Zn, Cu) in calves fed MR at different rates to achieve targeted daily rates of gain representing no growth (**NG**), low growth (**LG**), and high growth (**HG**).

Calf-related procedures were approved by the Animal Care and Use Committee of the National Animal Disease Center (Ames, IA). Holstein bull calves (n = 24) received at least 3.9 L of colostrum within the first 6 h postpartum. Navels were dipped in iodine and an *Escherichia coli* vaccine (Genecol-99, Schering Plough Animal Health, Union, NJ) was administered orally at birth. Calves were housed individually in elevated pens (1.52 m long × 0.914 m wide × 0.914 m high) in a temperature-controlled (18°C) barn. Calf health was monitored and recorded daily. The nutritional management of the calves has been described previously (Foote et al., 2007). On d 0, calves (mean age ± SEM: 9.1 d ± 2.4 d) were weighed and assigned randomly to 3 treatment groups (8 calves per treatment) to achieve 3 targeted daily rates of gain (NG = 0.0 kg/d, LG = 0.55 kg/d, or HG = 1.2 kg/d) in live weight over a 7-wk period. Milk replacer intakes required to achieve the targeted growth rates were estimated using the NRC computer program (NRC, 2001). All calves were fed a 30% CP, 20% fat, all milk protein MR (Land O'Lakes Inc., Shoreview, MN) reconstituted to 14% DM as described previously (Foote et al., 2007). Composition of the MR is provided in Table 1.

Table 1. Partial listing of milk replacer components[1]

Component	Analysis
CP, % of DM	30.10
Crude fat, % of DM	20.30
Lactose, % of DM	32.65
Calcium, % of DM	0.90
Phosphorous, % of DM	0.84
Magnesium, % of DM	0.12
Copper, mg/kg	10.08
Zinc, mg/kg	41.79
Vitamin A, IU/kg (IU/lb.)	45,415 (20,600)
Vitamin D_3, IU/kg (IU/lb.)	11,356 (5,151)
Vitamin E, IU/kg (IU/lb.)	220.46 (100)

[1]Manufactured by Land O'Lakes Animal Milk Products Co. (Shoreview, MN).

Calves were bucket-fed twice daily (0700 and 1800 h) and offered water ad libitum. Starter grain was not offered. Amounts of MR offered and refused were recorded at each feeding. Calves were weighed weekly and the amount of MR fed was adjusted at these times to compensate for changes in live weight. Because vitamin concentrations in the MR were based on DMI of HG calves, the NG and LG calves were supplemented with additional vitamins A, D, and E once weekly to compensate for their reduced consumption of MR. In contrast, mineral intakes by NG and LG were not adjusted for their reduced consumption of MR.

Sera for haptoglobin, vitamin, and mineral analyses were obtained weekly by jugular venipuncture. These were collected in the morning after a fasting period of approximately 12 h. Haptoglobin concentrations (μg/mL) were determined using a immunoperoxidase-based

ELISA kit (Immunology Consultants Laboratory Inc., Newberg, OR) according to the manufacturer's instructions. Assay standards were 1,000, 500, 250, 125, and 62.5 ng/mL. Test samples were undiluted to ensure absorbance of samples was within the standard curve.

Serum VitA and VitE concentrations were determined by reverse-phase HPLC (Ametaj et al., 2000) using a modification of a method originally described by Kaplan et al. (1987). Serum VitD was quantified by radioimmunoassay using a method described by Hollis et al. (1993).

Serum Ca and Mg concentrations were determined by atomic absorption spectrometry as described previously (Waldron et al., 2003). Serum P was determined using a procedure modified from Parekh and Jung (1970), validated in our laboratory, and described in detail by Waldron et al. (2003). Serum Zn and Cu concentrations were analyzed by atomic absorption spectrometry (AAnalyst 100 atomic absorption spectrometer, Perkin Elmer Inc., Wellesley, MA). Zinc standards were prepared by dissolving zinc metal (125 mg) in HCl (6 *M*, 6 mL) and then increasing the volume to 250 mL using 1% HCl. A 1:100 dilution of this was diluted further in 22.5% glycerol to yield 0.5, 1.0, and 1.5 μg/mL standards. The 0 μg/mL standard consisted of 22.5% glycerol without Zn. In the assay, 0.5 mL of standards and test sera were diluted in 1 mL of water. Standards, test samples, and an internal laboratory standard (0.82 μg/mL) were run in duplicate. The spectrometer was operated with a 2-nm slit-width, 213.9 nm wavelength, and lamp current of 15 mA. Zinc concentrations in test samples were expressed as micrograms of Zn per milliliter. Copper standards were prepared by dissolving Cu metal (250 mg) in HNO_3 (8.5 *M*, 6 mL) and then increasing the volume to 250 mL using water. A 1:100 dilution of this was diluted further in 20% glycerol to yield 0.5, 1.0, and 1.5 μg/mL standards. The 0 μg/mL standard consisted of 20% glycerol without Cu. In the assay, 0.5 mL of standards and test sera were diluted in 0.5 mL of water. Standards, test samples, and an internal laboratory standard (0.85 μg/mL) were run in duplicate. The spectrophotometer was operated with a 0.7-nm slit-width, 324.8 nm wavelength, and lamp current of 15 mA. Copper concentrations in test samples were expressed as micrograms of Cu per milliliter.

Data were analyzed as a completely randomized design (Statview software, version 5.0, SAS Institute Inc., Cary, NC). Calf served as the experimental unit in the analysis of all data. Fat-soluble vitamin, mineral, and haptoglobin concentrations were analyzed as a split-plot with repeated-measures ANOVA. The model included fixed effects of growth rate (NG, LG, or HG), time (week of experiment), and treatment × time interaction, and calf was included in the model as the random effect. Fisher's protected LSD test was applied when effects ($P < 0.10$) were detected.

Growth performance, health, and immune function data for these calves have been reported previously (Foote et al., 2007). To summarize, calf weights were not different at the beginning of the study (wk 0) and averaged 45.7 kg. Mean growth rates for NG (0.11 kg/d), LG (0.58 kg/d), and HG (1.16 kg/d) calves during the 7-wk study differed ($P < 0.0001$); at the conclusion of the treatment period, BW of NG, LG, and HG calves were 51.4 (SEM ± 0.7), 78.6 (±2.6), and 111.6 (±1.9) kg, respectively. Several calves were treated for scours or respiratory illness during the experimental period. Five HG calves and 2 NG calves required treatment for scours, whereas LG calves did not manifest symptoms of diarrhea. Three HG calves were also treated for respiratory illness. The LG or NG calves did not manifest symptoms of respiratory illness during the study. Symptoms typically considered indicative of fat-soluble and mineral deficiencies were not evident in any of the calves during the experimental period.

Haptoglobin is one of several acute-phase proteins produced in response to inflammatory changes associated with infection, making it a potentially useful indicator of calf health (Grell et al., 2005). Serum haptoglobin concentrations were unaffected by growth rate ($P > 0.05$; Figure 1) and the haptoglobin × time interaction was not significant ($P = 0.54$), suggesting that the symptoms of respiratory illness in HG calves and scours in LG and HG calves were not of sufficient severity to induce measurable treatment differences in haptoglobin concentrations.

Both NG and LG calves were supplemented orally with additional amounts of VitA, VitD, and VitE weekly to compensate for their reduced consumption of MR relative to that of HG calves. Using this approach, all calves received approximately the same amounts of these vitamins. Serum VitA, VitE, and VitD concentrations were determined weekly. These data are presented in Figure 2, with Table 2 providing a summary of treatment and time effects and their interaction for all variables. Both VitA and VitD concentrations were unaffected by growth rate. At wk 0 (i.e., pretreatment), concentrations were comparable across the treatment groups and averaged 127.2 ng/mL for VitA and 42.9 ng/mL for VitD. These concentrations are typical of those of newborn calves fed adequate colostrum (Rajaraman et al., 1997; Nonnecke et al., 1999). Retinol concentrations increased (182 ng/mL; $P < 0.001$) during the first week and averaged 178 ng/mL by the conclusion of the study. Plasma VitD increased progressively during the experimental period and averaged 79.8 ng/mL by wk 7. Both VitA and VitD concentrations were within the normal ranges for 2-mo-old Holstein calves.

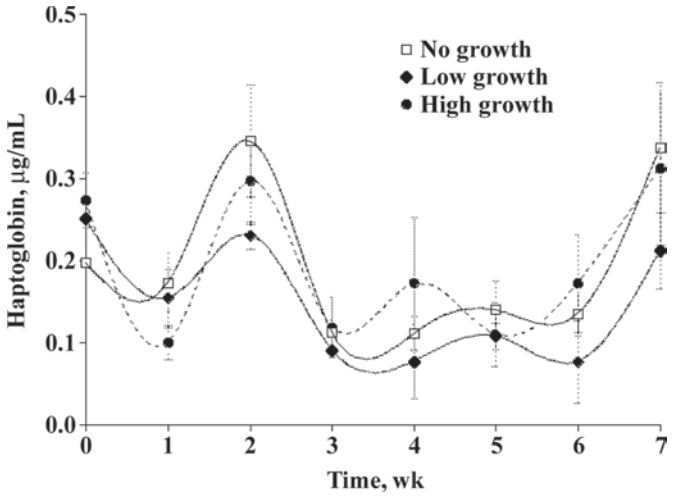

Figure 1. Serum concentrations (μg/mL, means ± SEM) of haptoglobin in preruminant dairy calves fed to achieve no, low, and high growth rates (n = 8/treatment). Calf age (mean ± SE) at the beginning of the treatment period (wk 0) was 9.1 ± 2.4 d. Treatment effect and treatment × time interaction were not significant, $P > 0.05$.

Serum VitE concentrations (Figure 2b), however, were affected by growth rate. Concentrations, by treatment group, were not different at the beginning of the study and averaged 1,934 ng/mL for all calves; however, by wk 6 the mean concentration in LG calves exceeded the mean concentration in HG calves (4,348 ng/mL vs. 2,049 ng/mL) and by wk 7 concentrations in LG (3,442 ng/mL) and NG (3,090 ng/mL) calves exceeded ($P < 0.05$) that in HG calves (1,774 ng/mL). The wk 7 value in HG calves was substantially below the 3,000 ng/mL (Weiss et al., 1997) considered necessary for optimal health in adult cows. Results from a previous study (Foote et al., 2003) considering the effects of growth on fat-soluble vitamin status of preruminant calves suggest that the increased growth rate associated with feeding an intensified diet results in lower serum VitE concentrations. Calves in the latter study (Foote et al., 2003) were fed a standard (0.45 kg/d of a 22% CP, 20% fat MR) or an intensified (1.14 kg/d of a 28% CP, 20% fat MR) diet until 8 wk of age, with calf starter fed ad libitum to intensified calves and limit-fed to calves on the standard diet. Using this approach, gain of the intensified-fed calves (0.58 kg/d) exceeded by more than 2-fold the gain of standard-fed calves (0.26 kg/d). Although the approaches used to control growth rate differed between studies, results from both studies suggest an association between growth rate and VitE status and may indicate a need for increasing the amount of VitE in MR-fed calves in nutrition programs promoting high growth rates. Another consideration is the role of colostrum in promoting optimal VitE status in newborn calves (Rajaraman et al., 1997). In the pres-

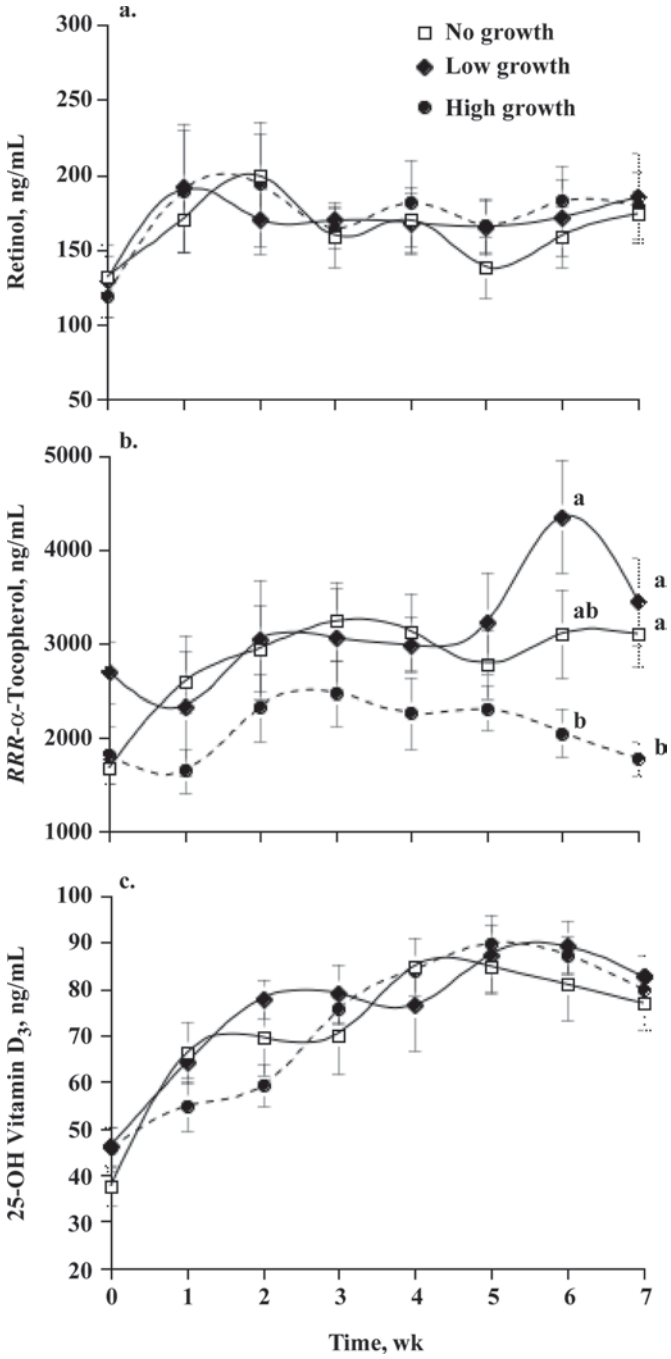

Figure 2. Serum concentrations (ng/mL, means ± SEM) of (a) vitamin A (retinol), (b) vitamin E (*RRR*-*α*-tocopherol), and (c) vitamin D (25-hydroxyvitamin D_3) in preruminant dairy calves fed to achieve no, low, and high growth rates (n = 8/treatment). No-growth and low-growth calves received additional supplementation with vitamins A, D, and E once weekly to compensate for their reduced consumption of milk replacer relative to high-growth calves. [a,b]Treatment means at specific times with different letters differ, $P < 0.05$.

Table 2. Results (P-values) from repeated-measures ANOVA for metabolic response variables[1]

Variable	Treatment effect	Time effect	Interaction
Retinol, ng/mL	NS[2]	<0.0001	NS
RRR-α-Tocopherol, ng/mL	NS	<0.0001	<0.001
25-(OH)-vitamin D_3, ng/mL	NS	<0.0001	NS
Copper, mg/dL	0.05	<0.05	<0.01
Zinc, mg/dL	NS	<0.001	NS
Calcium, mg/dL	<0.05	<0.0001	<0.001
Magnesium, mg/dL	NS	<0.01	<0.05
Phosphorous, mg/dL	<0.001	NS	<0.05
Haptoglobin, mg/mL	NS	<0.0001	NS

[1]Variables characterized responses of preruminant dairy calves to 3 dietary regimens producing no, low, and high growth rates during the 8-wk study.

[2]NS = $P \geq 0.05$.

ent study, all calves received adequate colostrum that contributed substantially to the relatively high VitE concentrations at the beginning of the study. Conceivably, the VitE status of HG calves in the present study would have been compromised further if they had not received colostrum. Mechanisms underlying the association between growth rate and vitamin E status warrant investigation.

Serum mineral concentrations in NG, LG, and HG calves from wk 0 through wk 7 are shown in Figures 3 and 4. Because no attempt was made to adjust mineral supplementation in NG and LG calves to approximate levels consumed by HG calves due to increased MR intake, the expectation was that serum mineral concentrations would be higher in HG calves during the latter stages of the study. This was the case for serum Ca (Figure 3a), P (Figure 3c), and Cu (Figure 4a). Serum Ca concentrations in HG calves were higher than those in NG calves from wk 4 to wk 7, and higher than those in LG calves at wk 6 and wk 7; however, concentrations for all treatment groups remained within the normal range for young calves (approximately 9 to 10.5 mg/dL; NRC, 2001). Mean Ca concentrations in HG, LG, and NG calves at wk 7 were 10.01, 9.62, and 9.21 mg/dL, respectively. Serum P concentrations for all treatment groups (Figure 3c) remained within the normal range (4 to 8 mg/dL; NRC, 2001) during the study period; however, concentrations in HG calves exceeded those in NG and LG calves from wk 2 to wk 7. Mean P concentrations in HG, LG, and NG calves at the conclusion of the study were 6.79, 5.72, and 5.08 mg/dL, respectively. Serum Cu concentrations in all calves remained within the normal range for healthy preruminant calves (0.7 to 1.3 μg/mL; Suttle, 1986), although concentrations in NG and LG calves approached the lower end (<0.71 μg/mL) of this range on several occasions during the study (Figure 4a). Mean Cu concentrations in HG, LG, and NG calves at wk 7 were 0.87, 0.72, and 0.74 μg/mL, respectively, and were several fold higher than concentrations (<0.2 μg/mL; NRC, 2001) associated with symptoms of deficiency.

In contrast to the pattern observed for serum Ca, P, and Cu concentrations, serum Mg (Figure 3a) and Zn (Figure 4b) concentrations in HG calves were not higher than those in LG and NG calves at any time during the 7-wk study, even though amounts of MR consumed by HG calves were substantially greater than those fed to LG and NG calves. The Mg concentrations in HG calves were not different from those in LG calves at any time and were lower than concentrations in NG calves from wk 2 to wk 5. The mean Mg concentration (1.84 μg/mL) in HG calves during this period approached the lower limit of the normal range for adult dairy cattle (1.8 to 2.4 μg/mL) and was substantially lower than the normal range for young dairy calves (2.2 to 2.7 μg/mL). The sustained, low Mg concentrations in HG calves, however, were considerably higher than concentrations associated with clinical deficiency (<0.7 μg/mL; Roy, 1990). Given the importance of Mg in bone mineral formation (i.e., early skeletal growth) and the comparatively low Mg concentrations in HG calves in the present study, reevaluation of Mg levels in milk replacers used in nutrition programs promoting high growth rates in preruminant calves may be warranted.

Serum Zn concentrations in HG, LG, and NG calves were comparable throughout the study and not different at any sampling time (Figure 4b). For all treatment groups, Zn concentrations remained within the middle to higher end of the normal range for growing calves (0.7 to 1.3 μg/mL) throughout the study period.

In conclusion, serum vitamin and mineral concentrations in NG, LG, and HG calves remained within ranges considered normal for young growing calves. Serum *RRR*-α-tocopherol concentrations were lowest in HG calves, suggesting an association between growth rate and vitamin E status. Additional research is necessary to determine if there is a need for increased amounts of vitamin E in milk replacers fed to calves managed

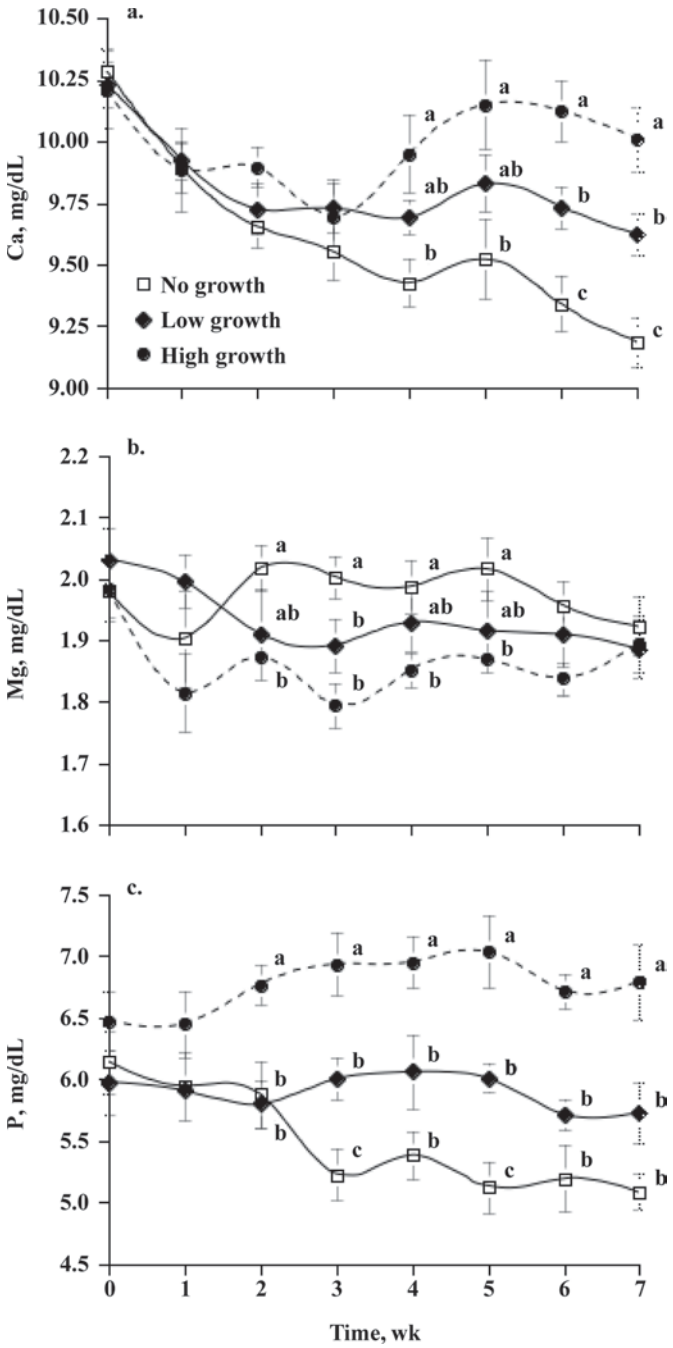

Figure 3. Serum concentrations (mg/dL, means ± SEM) of (a) calcium, (b) magnesium, and (c) phosphorous in preruminant dairy calves fed to achieve no, low, and high growth rates (n = 8/treatment). Calcium, Mg, and P intakes by no-growth and low-growth calves were not adjusted for their reduced consumption of milk replacer relative to high-growth calves. [a–c]Treatment means at specific times with different letters differ, $P < 0.05$.

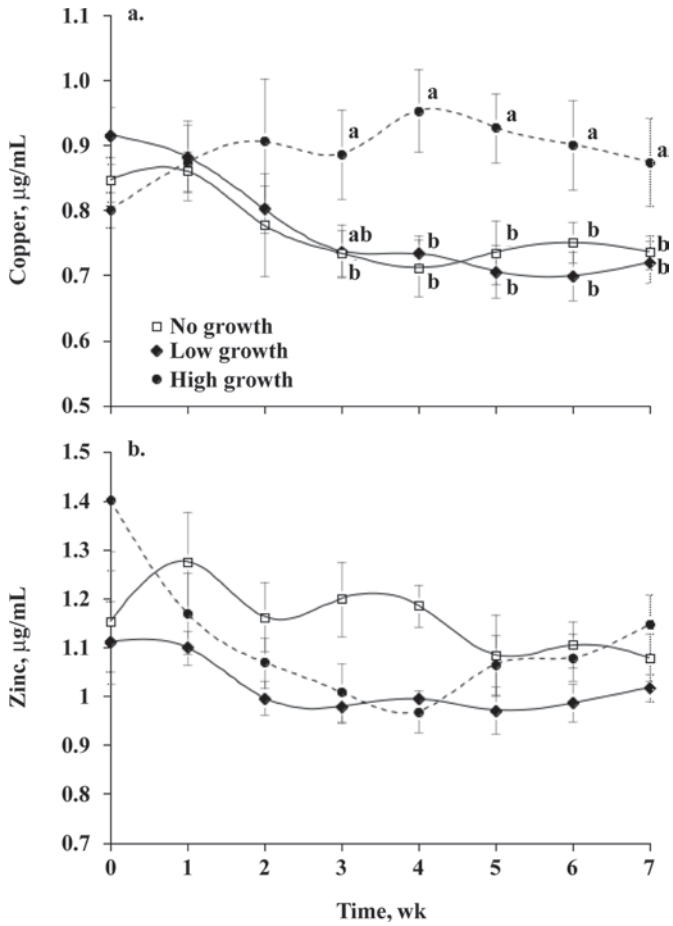

Figure 4. Serum concentrations (μg/mL, means ± SEM) of (a) copper and (b) zinc in preruminant dairy calves fed to achieve no, low, and high growth rates (n = 8/treatment). Copper and Zn intakes by no-growth and low-growth calves were not adjusted for their reduced consumption of milk replacer relative to high-growth calves. [a,b]Treatment means at specific times with different letters differ, $P < 0.05$.

nutritionally to promote high growth rates. Experimental evidence regarding the contribution of colostrum to the vitamin E status of the newborn calf (Rajaraman et al., 1997) suggests that feeding fresh, high quality colostrum to newborns destined for nutritional programs promoting a high growth rate may be essential in ensuring that vitamin E status is adequate during the preruminant phase of development.

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Metabolism

Effect of chlorate, molybdate, and shikimic acid on *Salmonella enterica* serovar Typhimurium in aerobic and anaerobic cultures

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ABSTRACT

Experiments were conducted to determine factors that affect sensitivity of *Salmonella enterica* serovar Typhimurium to sodium chlorate (5 mM). In our first experiment, cultures grown without chlorate grew more rapidly than those with chlorate. An extended lag before logarithmic growth was observed in anaerobic but not aerobic cultures containing chlorate. Chlorate inhibition of growth during aerobic culture began later than that observed in anaerobic cultures but persisted once inhibition was apparent. Conversely, anaerobic cultures appeared to adapt to chlorate after approximately 10 h of incubation, exhibiting rapid compensatory growth. In anaerobic chlorate-containing cultures, 20% of total viable counts were resistant to chlorate by 6 h and had propagated to 100% resistance ($>10^9$ CFU mL^{-1}) by 24 h. In the aerobic chlorate-containing cultures, 12.9% of colonies had detectable resistance to chlorate by 6 h, but only 1% retained detectable resistance at 24 h, likely because these cultures had opportunity to respire on oxygen and were thus not enriched via the selective pressure of chlorate. In another study, treatment with shikimic acid (0.34 mM), molybdate (1 mM) or their combination had little effect on aerobic or anaerobic growth of *Salmonella* in the absence of added chlorate. As observed in our earlier study, chlorate resistance was not detected in any cultures without added chlorate. Chlorate resistant *Salmonella* were recovered at equivalent numbers regardless of treatment after 8 h of aerobic or anaerobic culture with added chlorate; however, by 24 h incubation chlorate sensitivity was completely restored to aerobic but not anaerobic cultures treated with shikimic acid or molybdate but not their combination. Results indicate that anaerobic adaptation of *S.* Typhimurium to sodium chlorate during pure culture is likely due to the selective propagation of low numbers of cells exhibiting spontaneous resistance to chlorate and this resistance is not reversible by molybdenum supplementation.

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1. Introduction

Salmonella is the second most common bacterial cause of foodborne illness in the U.S., causing a third of reported bacterial foodborne illness [1]. An estimated 1.4 million cases of *Salmonella* food-poisoning occur each year in the U.S., with approximately 15,000 hospitalizations and 400 deaths, and an estimated annual cost of 0.5–2.3 billion dollars [1,2]. Antibiotic resistant *Salmonella* strains are a cause of growing concern worldwide. Multidrug resistant (MDR) *Salmonella enterica* serovar Typhimurium have increased from 15% of all tested isolates in 1992 to 42% in 2001 according to a survey of 29 mostly industrialized countries of Europe, the Americas, Africa, Asia, and Oceania [3]. Of concern is the recent development of resistance by *S.* Typhimurium to fluoroquinolones, since these are the drugs of choice for treatment of severe *Salmonella* infection in humans [3]. Because of concerns about resistance to antibiotics, alternative means of controlling *Salmonella* in food animals need to be explored.

A recently developed experimental chlorate-containing product has shown promise in the pre-slaughter reduction of the human pathogens *Salmonella* and *Escherichia coli* O157:H7 from the gastrointestinal tracts of food animals. Chlorate, a valence state analog of nitrate, is thought to be metabolized to cytotoxic chlorite

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by the molybdoenzyme component of respiratory nitrate reductase [4–6]. Consequently, it was hypothesized and subsequently demonstrated that chlorate selectively targets bacteria possessing membrane bound respiratory nitrate reductase activity but not beneficial anaerobes lacking the enzyme thereby conserving the competitive exclusion potential of the host's normal flora [7]. The selective bactericidal activity of chlorate has been demonstrated in several laboratory and field studies with both ruminants and monogastrics, achieving significant reductions in *Salmonella* and *E. coli* thus supporting its conceptual use as a feed additive to reduce gut concentrations of foodborne pathogens [4]. The effect of chlorate treatment on numbers of *Salmonella* and *E. coli* O157:H7 varied during these and other studies [8,9], with reductions ranging from approximately 100 to 100,000 cells suggesting that the possibility that the bactericidal activity of chlorate may be limited depending on cultural and feeding conditions. Additionally, in some batch culture studies the consumption of chlorate or its bactericidal effect appeared to be transient, with considerable regrowth of the pathogens occurring by 24 h of incubation, thereby suggesting the possible acquisition of resistance or other adaptive mechanisms [10,11]. The development of chlorate resistance within the gut of animals could potentially reduce the efficacy of chlorate products administered as feed additives. Consequently, we conducted several studies to quantify the effects of chlorate on growth and acquisition of chlorate resistance by *S.* Typhimurium and to investigate potential factors such as oxygen, molybdate and shikimic acid that may potentially mitigate the acquisition of chlorate resistance.

2. Materials and methods

2.1. Media and culture conditions

Brain heart infusion (BHI) broth and agar (Difco; Becton, Dickinson and Company; Sparks, MD) were reconstituted according to manufacturer's instructions. Glucose limited minimal medium contained (mg L^{-1}): K_2HPO_4, 225.0; KH_2PO_4, 225.0; $(NH_4)_2SO_4$, 450.0; NaCl, 450.0; $MgSO_4 \cdot 7H_2O$, 45.0; $CaCl_2$, 22.5; resazurin, 1.0; cysteine-HCl, 500.0; Na_2CO_3, 4000.0; Bacto peptone, 3700.0. Cysteine-HCl and Na_2CO_3 were added after the medium containing the other ingredients was adjusted to pH 7.0. Anaerobic broth was prepared by boiling and then cooling the medium on ice while flushing with oxygen-free 100% N_2 [12]. The medium was anaerobically distributed (9 mL) to 18 × 150 mm glass walled crimp top culture tubes which were immediately closed with rubber stoppers, crimped with aluminum seals and autoclaved. Aerobic broth was prepared similarly except without N_2 and was distributed to 18 × 150 mm straight walled glass tubes and closed with stainless steel closures. Respective additions of sodium chlorate, sodium nitrate, sodium molybdate, shikimic acid or glucose were made to sterilize media via aseptic addition (0.1–0.2 mL) of concentrated filter sterilized stock solutions. Final volumes of all tubes were equalized via addition of appropriate volumes of sterile reverse osmosis water. All incubations were carried out at 39 °C.

2.2. Effect of chlorate on growth and resistance during anaerobic and aerobic growth

S. enteric serovar Typhimurium was incubated aerobically or anaerobically in triplicate BHI broth tubes supplemented with 5 mM nitrate and with or without 5 mM chlorate (Experiment 1). Cultures were inoculated with 0.1 mL of an overnight-grown culture of *S.* Typhimurium. Growth was determined by measuring absorbance at 600 nm (A600) on a spectrophotometer (Spectronic 20D+, Spectronic Instruments, Inc., Rochester, NY) during a 24 h incubation period. Lag times varied for different treatments; therefore, maximum specific growth rates were calculated during the steepest part of logarithmic growth [13].

To enumerate proportions of chlorate susceptible and chlorate resistant *S.* Typhimurium, samples (1 mL) were collected from each culture tube at 0, 3, 6 and 24 h and serially diluted (10-fold) in phosphate buffer. One-tenth mL of each 10^{-2}–10^{-6} dilution and 0.5 mL of each 10^{-1} dilution were spread onto BHI agar (prepared according to manufacturer's instructions) and colonies were enumerated after 24 h aerobic incubation at 39 °C. Our limit of detection was 600 cells (2.8 $\log_{10}$) mL^{-1} (based on a plate spread with 0.5 mL of 10^{-1} dilution yielding a minimum of 30 CFU). Up to three successively diluted plates were selected from each sample at each sampling time for determination of nitrate reductase activity using the agar overlay method of Glaser and DeMoss [14], in which colonies capable of reducing nitrate to nitrite turn purple, while nitrate reductase deficient (chlorate resistant) colonies remain the original ivory color. Chlorate susceptible and resistant colonies were counted.

2.3. Stability of a chlorate resistant phenotype

To determine the stability of a chlorate resistant phenotype, a colony originating from a 24 h anaerobic culture grown in BHI broth supplemented with 5 mM nitrate and 5 mM chlorate was picked from a colony propagated after 24 h incubation in a Bactron Anaerobic Chamber (Sheldon Labs Manufacturing Inc., Cornelius, OR) on BHI agar supplemented likewise. Chlorate resistance was defined as the ability of the *S.* Typhimurium to grow and survive in broth containing 5 mM chlorate. The chlorate resistant isolate was then conditioned to growth with or without 5 mM chlorate via anaerobic subculture for two successive 24 h passages in anaerobic glucose limited minimal broth supplemented accordingly. Thereafter, the conditioned cultures were each used to inoculate (in duplicate) tubes containing glucose limited minimal broth supplemented with or without 5 mM chlorate (Experiment 2) or with and without 5 mM nitrate (Experiment 3). Growth and mean specific growth rates in the presence or absence of 5 mM added chlorate were determined as described above. Growth and mean specific growth rates of a chlorate susceptible *S.* Typhimurium cultured similarly were measured for comparison.

To determine if reduced expression of nitrate reductase activity by the chlorate resistant phenotype persisted following culture in the absence of chlorate, cells of the chlorate resistant phenotype conditioned to growth with or without 5 mM chlorate after 2 consecutive passages (24 h each) in 30 mL anaerobic BHI broth supplemented with 5 mM nitrate were harvested by centrifugation (20 min at 10,000 g). Chlorate and nonchlorate-conditioned cells were each resuspended separately in 5 mL of anaerobic dilution solution [15] containing 5 mM nitrate and distributed (1.4 mL) to 18 × 150 mm crimp top glassed wall tubes (in triplicate). Tubes were immediately closed with rubber stoppers and a H_2:CO_2 (1:1) gas phase was used with H_2 serving as the reducing substrate for reduction of nitrate. Cells of a chlorate susceptible *S.* Typhimurium grown in nitrate supplemented BHI broth without added chlorate were harvested and distributed likewise. The tubes were incubated at 39 °C and samples (0.2 mL) collected at 0, 3, 6 and 24 h were analyzed colorimetrically for determination of nitrate concentration [16]. Protein content of the cell suspensions was determined using a commercial modified Lowry Protein Assay Kit (Pierce, Rockford, IL, USA). Specific nitrate reductase activity was calculated as the linear rate of nitrate disappearance divided by protein concentration within the reaction fluids.

2.4. Effect of shikimic acid, molybdate or their combination on growth and resistance of S. Typhimurium during aerobic and anaerobic growths with or without added chlorate

To determine the effect of additions of molybdate, shikimic acid or their combination on the bactericidal activity of chlorate against *S.* Typhimurium, cultures were incubated in triplicate aerobically and anaerobically (Experiment 4) in BHI broth supplemented with 5 mM nitrate and with or without 5 mM chlorate, 0.34 mM shikimic acid, 1 mM molybdate or their combinations. Growth and viable cell counts were determined as previously described.

2.5. Determination of rates of chlorate metabolism

3 mL samples of the 10^{-1} dilution from each culture were frozen until subsequent analysis of chlorate by HPLC. Samples were thawed at room temperature, and prepared and chromatographed according to the method of Beier et al. [10]. Rates of chlorate disappearance were calculated using linear regression.

2.6. Statistical analysis

Data from each experiment were analyzed separately to avoid confounding effects between experiments using Statistix 9 Analytical Software (Tallahassee, FL). Maximum specific growth rates, maximum optical densities and specific nitrate reductase activities were analyzed to determine effects of treatments using a general analysis of variance. Treatment means were compared to means from controls (cultures incubated without chlorate and treatment) using a Dunnett's procedure and were considered significant at $P < 0.05$. Log_{10} transformations of viable cell counts (CFU mL^{-1}) were likewise analyzed using general analysis of variance at each respective sampling time during incubation but due to the greater variability associated with viable cell counts a more conservative $P < 0.01$ was considered significant for these mean comparisons. Rates of chlorate metabolism determined in chlorate-supplemented cultures were also analyzed by analysis of variance but because rates were nonexistent from controls lacking chlorate, least squares' means of all chlorate-supplemented cultures were compared using a Tukey's multiple comparison procedure. Regression analysis was used to compare slopes of estimated and observed growth curves.

3. Results and discussion

3.1. Effect of chlorate on growth and resistance during anaerobic growth

In our first experiment, chlorate inhibition was apparent early during anaerobic culture of *S.* Typhimurium with added chlorate, as evidenced by an extended 9–10 h lag in growth as well as by a decrease ($P < 0.05$) in viable numbers of *S.* Typhimurium compared to nonchlorate-supplemented controls (Fig. 1A, B). The maximum specific growth rate during logarithmic growth for the anaerobic cultures incubated with chlorate was > 50% slower ($P < 0.05$) than that of aerobic controls or that of anaerobic cultures incubated without chlorate (Table 1).

The ability of *Salmonella* and *E. coli* to grow in the presence of chlorate has long been recognized and attributed to genetic defects in *chl* alleles which more recently have been renamed to more accurately describe deficiencies in molybdenum uptake and metabolism (*moa, mob, nar, mod, moe, mog*) [17]. These genetic defects often are expressed as a deficiency in membrane bound respiratory nitrate reductase activity, but inactivation of other molybdenum containing enzymes such as dimethyl sulfoxide reductase, trimethylamine N-oxide reductase and thiosulfate

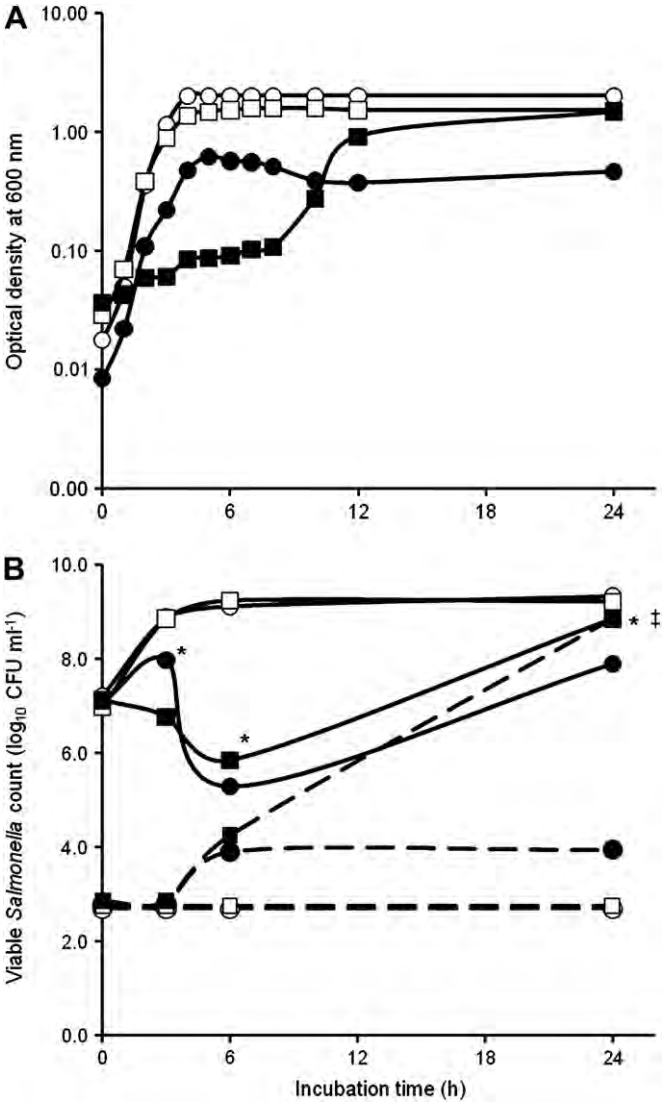

Fig. 1. Growth curves (A) and viable cell counts (B) of *Salmonella* Typhimurium grown aerobically (circles) or anaerobically (squares) in nitrate supplemented (5 mM) tryptic soy broth without (open symbols) or with (closed symbols) 5 mM added chlorate (Experiment 1). Total viable counts (solid lines) with values ≤ * denotation and nitrate reductase deficient counts (dashed lines) with values ≥ ‡ denotation differ from aerobic controls incubated without added chlorate; SEM were ≤ 0.83 and 0.24 for total viable counts and for nitrate reductase deficient counts, respectively.

Table 1
Comparative mean specific growth rates and maximum optical density during aerobic and anaerobic culture of *Salmonella* Typhimurium without or with chlorate[a]

	Comparative response during aerobic and anaerobic growths (Experiment 1)			
	Mean specific growth rate (ΔA_{600} h^{-1})		Maximum optical density (A_{600})	
Supplement	Aerobic	Anaerobic	Aerobic	Anaerobic
None	1.59	1.28	1.99	1.58[b]
Chlorate	1.16[b]	0.60[b]	0.61[b]	1.46[b]

[a] Cultures in triplicate were grown aerobically or anaerobically (100% N_2) in nitrate supplemented (5 mM) tryptic soy broth without or with 5 mM added chlorate. SEM = 0.09 and 0.01 from a general analysis of variance of mean specific growth rates and maximum optical densities, respectively.

[b] Least squares means differ ($P < 0.05$) from aerobic controls incubated without added chlorate.

reductase may also occur [18]. Results from the present study are consistent with the most widely accepted view that chlorate toxicity affects molybdoenzymes such as membrane bound nitrate reductase A (NarA) which catalyze the reduction of chlorate to chlorite, the later which is toxic to the bacteria, probably by causing oxidative damage [5–7,18–20]. The NarA enzyme, which is expressed under anaerobic conditions, is purported to be the primary contributor to this reduction, although the constitutively expressed NarZ which can account for approximately 10% the nitrate reductase activity may also contribute to chlorate toxicity [11].

Tests for nitrate reductase proficiency (which was our measure of chlorate sensitivity) in the current study revealed that 20 and 100% of the total viable non-$\log_{10}$ transformed *S.* Typhimurium counts after 6 and 24 h anaerobic incubation, respectively, with added chlorate were deficient in nitrate reductase activity (Fig. 1B). The appearance of chlorate resistant *S.* Typhimurium observed in our study is likely due to the selective propagation of low numbers of cells expressing spontaneous resistance to chlorate within the population rather than to a mutagenic effect as chlorate has been reported to be nonmutagenic when tested via the Ames test [21]. For instance, in cells grown without selective pressure of nitrate or chlorate the frequency of recovering chlorate resistant *S.* Typhimurium was estimated to occur at 1.56×10^{-6} [22]. Based on this frequency, we calculate that 21 ± 5.9 ($1.32 \log_{10}$) mL^{-1} resistant cells would have been present at the start of our incubations. These low numbers of resistant cells would have been unobserved as our limit of detection was 600 ($2.8 \log_{10}$) mL^{-1} cells. A comparison of regression lines obtained from theoretical logarithmic growth curves estimated from the propagation of these low numbers of chlorate resistant cells to the line plotted from counts observed during anaerobic culture with added chlorate revealed that the lines did not significantly ($P > 0.05$) differ (not shown). In our cultures incubated without added chlorate, nitrate reductase deficient *S.* Typhimurium were not observed at any time (Fig. 1B), but this is not unexpected as these cultures contained nitrate and thus would have selected for nitrate proficient phenotypes.

Results from our first experiment do not allow us to discount the possibility that the long lag phase that occurred during anaerobic incubation with chlorate may alternatively reflect an adaptive response manifested by a chlorate-stimulated down-regulation of Nar gene expression. However, when a genetically uncharacterized chlorate resistant *S.* Typhimurium isolate obtained from our study was harvested from subcultures grown 2 successive passages without or with exposure to chlorate they similarly expressed low nitrate reductase activity (0.024 ± 0.04 and 0.019 ± 0.03 μmol mg prot.$^{-1}$ h^{-1}, respectively). This finding indicates that the resistant phenotype persisted regardless of chlorate exposure. By comparison, nitrate reductase activity expressed by cells of a chlorate sensitive isolate grown without chlorate exposure (0.83 ± 0.06 μmol mg prot.$^{-1}$ h^{-1}) was much higher ($P < 0.05$). Moreover, in Experiment 2 we observed that propagation of the chlorate resistant isolate for two consecutive 24 h transfers in a glucose minimal medium lacking added chlorate did not restore the cells to a sensitive or non-adaptive state. For instance, growth curves (Fig. 2A) and maximum specific growth rates (Table 2) were nearly equivalent whether subsequently cultured with or without chlorate. The chlorate resistant cultures did grow more slowly during the first 3 h of culture than the parental chlorate susceptible *S.* Typhimurium grown without added chlorate which suggests that some adaptation or differential regulation may occur during early growth of the chlorate resistant *S.* Typhimurium but this lag was observed independent of whether or not the resistant cultures were grown with or without chlorate. By 3 h of incubation the chlorate resistant cultures began to grow more rapidly than the non-chlorate-supplemented susceptible cultures and achieved an equivalent maximum optical density after 22 h incubation indicating that the energetic cost associated with chlorate resistance was minimal during fermentative growth in this energy restricted medium (Fig. 2).

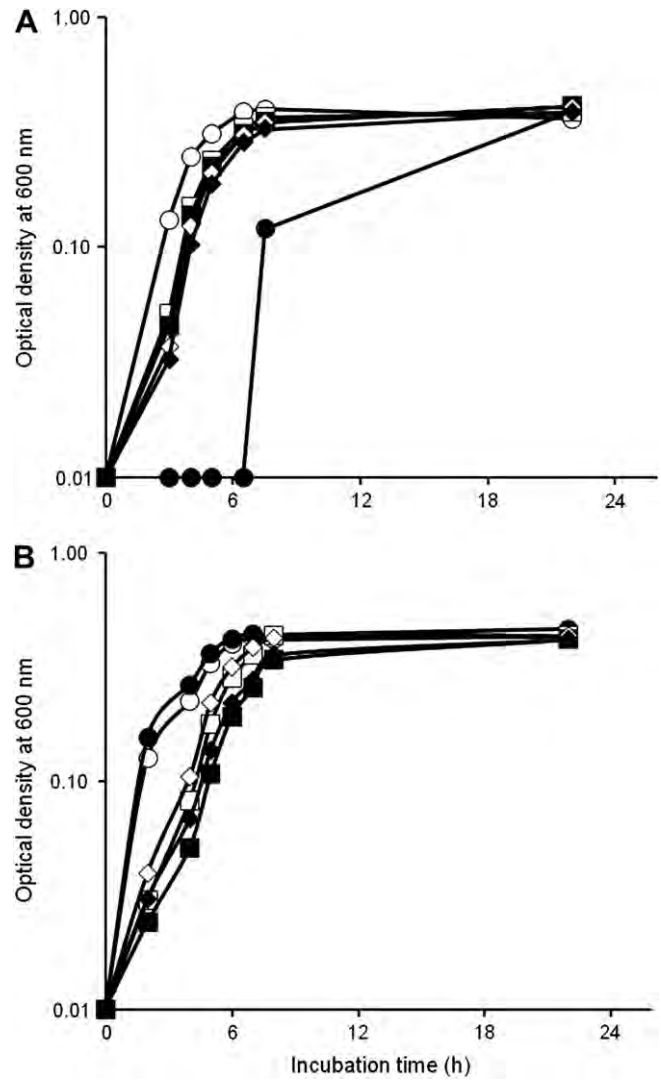

Fig. 2. Growth curves of a chlorate susceptible (circles) and an uncharacterized nitrate reductase deficient (chlorate resistant) *S.* Typhimurium grown anaerobically in glucose minimal broth without (open symbols) or with (closed symbols) 5 mM added chlorate (A, Experiment 2) or nitrate (B, Experiment 3). The chlorate resistant isolate had been conditioned immediately prior via two 24 h cultures in medium lacking (diamonds) and containing added chlorate (squares).

When the chlorate resistant *S.* Typhimurium was grown in a glucose minimal medium with or without added nitrate (Experiment 3) it also grew more slowly during the first 3 h of culture but subsequently grew more rapidly than the parental chlorate susceptible *S.* Typhimurium grown without added nitrate (Fig. 2B). The resistant isolate also achieved higher maximum specific growth rates than the parental chlorate susceptible *S.* Typhimurium grown without added nitrate, although the rate was not significantly higher for cultures previously conditioned in the absence of chlorate and then grown with added nitrate (Table 2). In this case, however, chlorate resistant cultures grown with added nitrate achieved lower ($P < 0.05$) maximum optical density than resistant cultures grown without nitrate and the susceptible cultures grown without added nitrate thus indicating an energetic cost of chlorate

Table 2
Comparative mean specific growth rates and maximum optical densities during anaerobic culture of a chlorate susceptible and a preconditioned chlorate resistant *S.* Typhimurium during subsequent growth with or without chlorate or nitrate [a]

	Mean specific growth rate (ΔA_{600} h^{-1})			Maximum optical density (A_{600})		
Supplement	Susceptible isolate	Resistant isolate		Susceptible isolate	Resistant isolate	
		Grown prior without chlorate	Grown prior with chlorate		Grown prior without chlorate	Grown prior with chlorate
Comparative response to growth with or without chlorate (Experiment 2)[b]						
None	0.47	0.76[d]	0.77[d]	0.39	0.38	0.40
Chlorate	0.26	0.78[d]	0.75[d]	0.39	0.41	0.38
Comparative response to growth with or without nitrate (Experiment 3)[c]						
None	0.44	0.60[d]	0.64[d]	0.42	0.42	0.42
Nitrate	0.43	0.50	0.53[d]	0.44	0.38[d]	0.39[d]

[a] Cultures in duplicate were incubated anaerobically (100% N_2) in glucose minimal medium supplemented without or with 5 mM sodium chlorate (Experiment 2) or sodium nitrate (Experiment 3). Chlorate resistant cells used as inocula had been conditioned via two prior 24 h cultures in similar medium supplemented with or without chlorate; chlorate susceptible cells used as inoculum had been grown similarly without chlorate.
[b] SEM = 0.02 and 0.01 from a general analysis of variance of mean specific growth rates and maximum optical densities, respectively.
[c] SEM = 0.04 and 0.01 from analysis of mean specific growth rates and maximum optical densities, respectively.
[d] Least squares means within experiment differ ($P < 0.05$) from chlorate susceptible control incubated without added chlorate or nitrate.

resistance when nitrate was present, possibly due to induction of incomplete respiratory function. Chlorate susceptible cultures grown with added nitrate achieved the highest maximum optical density measurement which is as expected because nitrate respiration is considered to be more efficient than fermentation [18]. As observed in our first experiment, chlorate susceptible *S.* Typhimurium cultured with added chlorate did not exhibit logarithmic growth until after 7 h of incubation, achieving a slower ($P < 0.05$) maximum specific growth rate (0.26 h^{-1}) but an equivalent maximum optical density than chlorate susceptible controls incubated without chlorate (Fig. 2B). This observation is consistent with our conclusion that cells from both cultures were conserving energy via fermentative metabolism rather than respiration.

3.2. Effect of chlorate on growth and resistance during aerobic growth

In contrast to that observed during anaerobic culture with added chlorate, the maximum specific growth rate, which was achieved within the first 4 or 5 h of incubation, by aerobic cultures incubated with added chlorate did not differ significantly from that of control cultures incubated aerobically without chlorate (Table 1). However, by 6 h of incubation a persistent inhibition of growth was evident during aerobic culture with added chlorate as the maximum optical density (Table 1) and viable cell counts achieved at this time or thereafter were significantly lower than aerobic controls incubated without chlorate (Fig. 1B). As was observed during anaerobic culture without added chlorate, viable nitrate reductase deficient *S.* Typhimurium were not recovered at any time from cultures incubated without the selective pressure of added chlorate (Fig. 1B). Nitrate reductase deficient *S.* Typhimurium were recovered after 6 h aerobic culture with added chlorate at numbers (12.9% of the total viable non-$\log_{10}$ transformed population) nearly equivalent to those recovered during anaerobic culture, but numbers recovered after 24 h culture with chlorate accounted for only 1% of the total viable *S.* Typhimurium population (Fig. 1B). Substantial propagation of nitrate reductase deficient *S.* Typhimurium would not be expected with oxygen-respiring cultures, however, as NarA nitrate reductase activity is suppressed in favor of more energetically preferred respiration on oxygen, which has a higher redox couple potential than nitrate (pϵ of +12.7 and +13.8, respectively) [5,23–25]. In our study as well, aerobic respiration was more efficient than anaerobic respiration on nitrate in cultures lacking added chlorate as evidenced by higher optical density values (Fig. 1A). Under aerobic conditions, an intermediate product produced from nitrate reductase reduction of chlorate has been proposed to exert a toxic effect on *E. coli* by inhibiting an early step in the biosynthesis of aromatic amino acids or in ubiquinone-8 biosynthesis via the shikimic acid pathway [26–29]. Giordano et al. [30] also suggested that the mechanism of chlorate toxicity during aerobic growth may involve the oxidation of quinols as *E. coli* were also able to reduce nitrate under aerobic conditions. Inhibition of

Table 3
Comparative mean specific growth rates and maximum optical density during aerobic and anaerobic culture of *S.* Typhimurium without or with chlorate and treatments with or without molybdate, shikimic acid or their combination[a]

	Comparative response during growth with potential mitigating treatments (Experiment 4)					
	Mean specific growth rate (ΔA_{600} h^{-1})		Maximum optical density (A_{600})		Rate of chlorate disappearance (μmol mL^{-1} h^{-1})	
Supplement	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Without chlorate						
None	0.97	0.79	1.19	1.08	–	–
Molybdate	1.09	0.76	1.19	1.09	–	–
Shikimic acid	0.99	0.74	1.18	1.08	–	–
Combination	1.03	0.75	1.19	1.09	–	–
With chlorate						
None	0.57[b]	0.52[b]	0.52[b]	0.39[b]	4.39	5.51
Molybdate	0.64[b]	0.57[b]	0.48[b]	0.36[b]	6.61	4.21
Shikimic acid	0.65[b]	0.54[b]	0.50[b]	0.34[b]	4.18	4.79
Combination	0.61[b]	0.61	0.47[b]	0.37[b]	7.38	4.50

[a] Cultures in triplicate were grown aerobically in nitrate supplemented (5 mM) tryptic soy broth without or with 5 mM added chlorate and with or without treatments of 1 mM sodium molybdate, 0.34 mM shikimic acid or their combination. SEM = 0.04, 0.01 and 1.84 from a general analysis of variance of mean specific growth rates, maximum optical densities and rates of chlorate disappearance, respectively.
[b] Least squares means within columns differ ($P < 0.05$) from controls incubated without added chlorate.

the electron carrier function of various quinols may explain the decreased growth efficiency observed during respiratory growth in the absence of added chlorate but with nitrate as an electron acceptor (Fig. 2B). It is possible, however, that chlorate may have been reduced in our aerobic cultures by aerobically expressed nitrate reductase NarZ, which is also purported to reduce chlorate [31], or possibly via expression of NarA in oxygen deficient areas of the media as the tubes were not continually agitated.

3.3. Effects of molybdate, shikimate or their combination on growth and resistance during anaerobic and aerobic growth

To further investigate the toxicity of chlorate, *S.* Typhimurium was cultured similar to that in our first experiment except with or without additions of shikimic acid, molybdate or a combination of these compounds. In this study (Experiment 4), all cultures with added chlorate, regardless of whether treated or not with shikimic acid, molybdate or their combination, had achieved equivalent lower maximum specific growth rates (Table 3), lower maximum optical densities and lower total viable *S.* Typhimurium than controls cultured without chlorate (Fig. 3). Consistent with observations from our first experiment, nitrate reductase deficient *S.* Typhimurium were not detected in any of the nonchlorate-supplemented cultures or from any of the chlorate-supplemented cultures at the 0 h sampling time (Fig. 3). In aerobic cultures, nitrate reductase deficient *S.* Typhimurium were not detected after 4 h of incubation and were recovered at nearly equivalent numbers from all the cultures with added chlorate at 8 h of incubation (Fig. 3A). After 24 h of aerobic incubation, nitrate reductase deficient *S.* Typhimurium were recovered only from chlorate-supplemented cultures treated with neither or with both shikimic acid and molybdenum (Fig. 3A). However, only in the cultures treated with both shikimic acid and molybdate were numbers of nitrate reductase deficient *S.* Typhimurium significantly higher than the limit of detection value determined in controls grown without added chlorate (Fig. 3A). In cultures incubated anaerobically, nitrate reductase deficient *S.* Typhimurium were recovered in significantly higher numbers than from controls incubated without added chlorate after 8 h and 24 h incubation but were similar between treatments (Fig. 3B). As done in Experiment 1, a comparison of the regression line obtained from a theoretical logarithmic growth curve, estimated via the propagation of 12 ± 6.8 ($1.1 \log_{10}$) mL^{-1} resistant cells initially present, to that from counts observed during anaerobic culture with added chlorate revealed that the lines did not differ ($P > 0.05$).

Our observation of decreased recovery of nitrate reductase deficient *S.* Typhimurium from chlorate-supplemented aerobic cultures treated with molybdate alone (Fig. 3B) is consistent with earlier findings that when provided in excess, as done here, molybdate restored nitrate reductase activity in *E. coli* mutants deficient in molybdenum uptake *modABCD* (*chlD*) thereby overcoming chlorate resistance [14].

The transient appearance of nitrate reductase deficient *S.* Typhimurium at 8 h but not at 24 h may be reflective of greater proportional amounts of functional enzyme produced during later stages of growth as NarZ expression is increased as cells approach or are in stationary phase [32]. Molybdate did not restore chlorate sensitivity during anaerobic culture with chlorate but the reason for this is not clear. We speculate that the more abundant expression of NarA during anaerobic growth may have rapidly consumed available molybdenum or overwhelmed the capacity of the culture to incorporate molybdenum. Alternatively, it is possible that differences exist in the uptake and incorporation of molybdenum during the different growth conditions or that anaerobiosis may differentially select for chlorate resistance not caused by defects in molybdenum uptake. Our finding that initial rates of chlorate

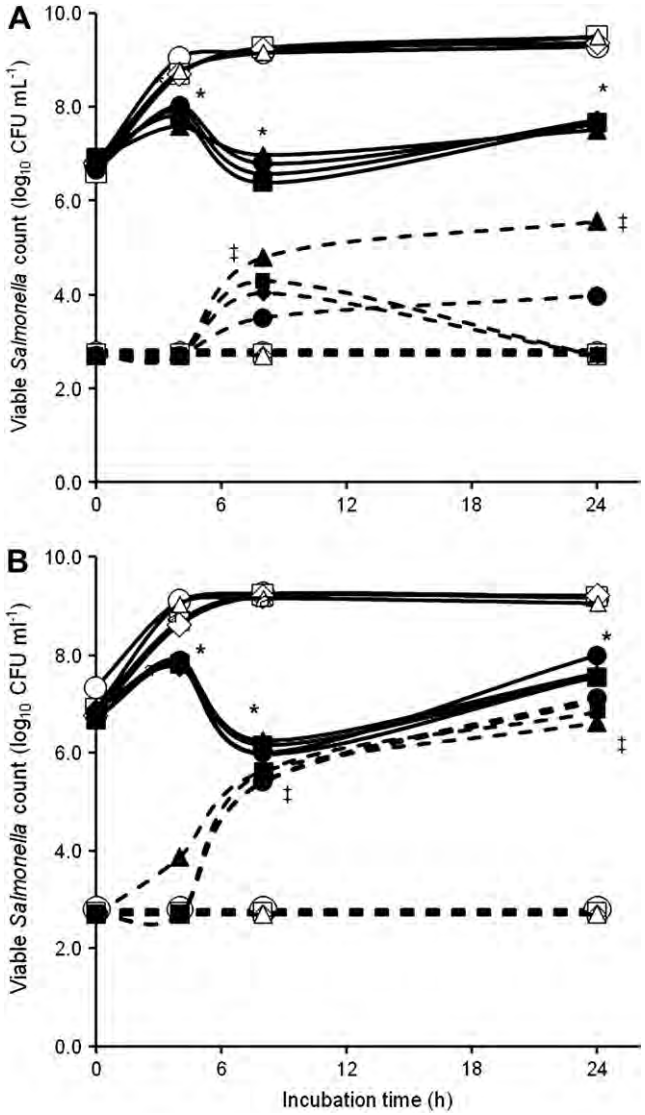

Fig. 3. Viable cells' counts of *S.* Typhimurium grown aerobically (A) or anaerobically (B) in nitrate supplemented (5 mM) BHI broth without (open symbols) or with (closed symbols) 5 mM added chlorate (Experiment 4). Cultures were also treated without (circles) or with 0.34 mM shikimic acid (squares), 1 mM molybdate (diamonds) or their combination (triangles). Total viable counts (solid lines) with values $\leq$ * denotation and nitrate reductase deficient counts (dashed lines) with values $\geq$ ‡ denotation differ from controls incubated without added chlorate; SEM were ≤ 0.14 and 0.24 for total viable counts and ≤ 0.43 and 0.38 for nitrate reductase deficient counts during aerobic and anaerobic growths; respectively.

disappearance (Table 3) and amounts of chlorate consumed (20% of initial 457.5 ± 59.3 ppm) did not differ between aerobic or anaerobically grown cultures argues against that former hypothesis. However, quantitative aspects of chlorate metabolism within *Salmonella* and *E. coli* have not been thoroughly investigated and it is possible that our measurements of chlorate disappearance reflect intracellular uptake of chlorate rather than its reduction to chlorite. Unlike nitrate, uptake of chlorate is not influenced by oxygen [33].

Newman et al. [26] also showed that molybdate additions were able to restore nitrate reductase activity and hence chlorate susceptibility in aerobically grown *E. coli*. They further showed that additions of shikimic acid alleviated chlorate toxicity presumably by restoring aromatic amino acid biosynthesis otherwise inhibited by nitrate reductase-mediated reduction of chlorate [26]. In our study, however, shikimic acid did not lessen chlorate toxicity either aerobically or anaerobically and appeared to counteract molybdate restoration of

nitrate reductase activity during aerobic growth as evidenced by higher recovery of nitrate reductase deficient *S.* Typhimurium from cultures co-treated with both shikimic acid and molybdate (Fig. 3B). A possible explanation for the antagonism observed with co-addition of shikimic acid and molybdate may be that biosynthesis of the functional quinone electron carriers may also be enhanced as shikimic acid is an intermediate for the production of chorismate. Chorismate is a common intermediate for production of phenylalanine, tryptophan, tyrosine and for ubiquinone and menaquinone [29,34]. Alternatively, as a central intermediate in the biosynthetic shikimic acid pathway, which includes branches for the synthesis of the aromatic amino acids, quinols, folate and other compounds [35], it is conceivable that different metabolic demands could dictate how shikimic acid is used. It is also possible that molybdate supplementation could shift the direction of shikimic acid use from one biosynthetic branch to another that contains a key molybdoenzyme. In addition, a major difference between our study and that of Newman et al. [26] is the species of bacteria investigated: we tested *S.* Typhimurium while the Newman group examined *E. coli*.

In summary, chlorate differentially inhibited growth of *S.* Typhimurium during anaerobic and aerobic culture, with inhibition occurring more rapidly in anaerobically grown cultures. Chlorate resistant *S.* Typhimurium, as defined by a deficiency in nitrate reductase activity, were recovered from all chlorate-supplemented cultures but propagated to a much higher level during anaerobic culture, most likely due to the selective propagation of low numbers of *S.* Typhimurium exhibiting spontaneous resistance to chlorate. The propagation of chlorate resistant populations within the gut of animals administered chlorate would naturally be problematic as far as maintaining efficacious control of targeted pathogens. From a practical perspective, however, this occurrence may be unlikely in mixed populations as Callaway et al. [36] reported that a chlorate resistant *E. coli* O157:H7 strain did not persist when experimentally inoculated into continuous flow cultures of mixed ruminal microbes. In the presence of chlorate, an uncharacterized nitrate reductase deficient *S.* Typhimurium appeared to be as fit as nitrate proficient *S.* Typhimurium grown in glucose limited medium lacking chlorate as long as energy was conserved via fermentative metabolism. However, Callaway et al. [36] observed that when pure cultures of *E. coli* O157:H7 were desensitized to chlorate during broth culture in an energy rich medium containing added chlorate, the population was rapidly resensitized upon subsequent transfer to broth lacking chlorate, presumably via enrichment of chlorate sensitive populations within the culture. Thus, it is reasonable to suspect that chlorate resistance may impede an organism's competitive fitness during growth in a less restrictive medium or in the presence of competing organisms. From a theoretical perspective, this seems likely as genetic defects in molybdenum uptake or molybdoenzyme synthesis responsible for chlorate resistance can also affect the function of other molybdenum containing enzymes such as formate dehydrogenase [6,18]. Selective propagation of chlorate resistant *S.* Typhimurium was not observed to the same extent during aerobic culture presumably because these bacteria had opportunity to respire on oxygen. Molybdate or shikimic acid added individually, but not in combination, was able to restore chlorate sensitivity in aerobic cultures, but not in anaerobic cultures. The latter result is consistent with earlier observations reported by Callaway et al. [36].

4. Conflict of interest

Mention of a trade name, proprietary product, or specific equipment do not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

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PATHOGENESIS OF BACTERIAL INFECTIONS IN ANIMALS

Fourth Edition

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22
Brucella

S. C. Olsen, B. H. Bellaire, R. M. Roop II, and C. O. Thoen

INTRODUCTION

Brucellae are intracellular pathogens that localize in lymphoreticular tissues and primarily cause reproductive losses in natural hosts. In many parts of the world, brucellosis continues to be, or is reemerging as, a significant zoonosis. As with other intracellular pathogens, *Brucellae* utilize multiple molecular mechanisms to enhance pathogenesis *in vivo*.

CHARACTERISTICS AND SOURCES OF THE ORGANISMS

The genus *Brucella* encompasses a group of gram-negative bacteria that survive almost exclusively in infected hosts with preference for localization in intracellular compartments of phagocytic, reticulo-endothelial, and specialized epithelial cells. Based on microbial characteristics and host specificity, the *Brucella* genus has traditionally been characterized as having six species: *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella suis*. Genomic analysis indicates that *Brucella* strains comprise a single genetic "species" (Vizcaino et al. 2000), but the epidemiologic and diagnostic benefits of assigning *Brucella* strains to separate "nomenspecies" based on their distinctive phenotypic characteristics are readily apparent. Relatively recent isolations of new and phenotypically diverse *Brucella* strains from sea mammals, voles, and a prosthetic breast implant have been reported, and this will certainly expand the number of nomenspecies included in the genus *Brucella* (De et al. 2008).

Brucella abortus, *B. melitensis*, *B. neotomae*, *B. suis*, and marine isolates are designated as smooth strains because of their surface expression of the O polysaccharide on their lipopolysaccharide (LPS). *B. canis*, *B. ovis*, and some vaccine strains are designated as rough due to their lack of surface expression of the O polysaccharide. Most *Brucella* strains have two circular chromosomes encoding approximately 3.2 kb. The genome sequences of the *Brucella* nomenspecies display an average of >94% identity at the nucleotide levels (Vizcaino et al. 2000; Chain et al. 2005), with *B. abortus* and *B. melitensis* being the most closely related. A close relationship has also been detected between *B. canis* and *B. suis*, whereas *B. neotomae* and *B. ovis* demonstrate greater divergence levels from the other *Brucella* species. With the exception of biovar 5, *B. suis* isolates cluster together (Whatmore et al. 2007). Bacteriophages have been isolated from *Brucella* strains (Corbel 1987), but plasmids have not. The extent to which these bacteria are able to undergo genetic exchange among themselves or with other bacteria is unknown, and it has been proposed that intracellular niches inhabited by *Brucella* during residence in the host limit their opportunities for genetic exchange with other bacteria. The genetic homogeneity of *Brucella* strains and the close genetic relationships they maintain with other members of the α-proteobacteria, including the *Sinorhizobium* and *Agrobacterium* species (which infect plants), support this proposition. Traditionally, after isolation of gram-negative coccobacilli with cultural and biochemical characteristics consistent with identification as a *Brucella* spp., species

differentiation has been based on phage and dye sensitivity, CO_2 dependence for growth, production of H_2S, and serum agglutination tests. However, since microbial isolation of *Brucella* can be slow and tedious, other more rapid DNA-based methods have been developed for identification.

Some of the classical nomenspecies (*B. abortus*, *B. melitensis*, and *B. suis*) are divided into biovars based on biochemical, phenotype, and antigenic properties. Biotyping has been used for epidemiologic purposes, but it is somewhat subjective because it is based on subtle differences. Genetic mutations responsible for the biovar phenotypes remain to be determined (Whatmore et al. 2007).

Although *Brucella* strains have traditionally been given their nomenspecies designations based on their preferred host, all the pathogenic strains have the capability to infect other hosts. For example, under field conditions, *B. suis* and *B. melitensis* have been isolated from cattle and *B. abortus* from feral swine. Clinical symptoms of brucellosis are generally more severe in incidental hosts, including humans. At least four of the *Brucella* nomenspecies are pathogenic in humans, with *B. abortus*, *B. melitensis*, and *B. suis* considered to be highly pathogenic and *B. canis* to be less virulent. Clinical symptoms of recurrent fever, malaise, general debility, and hepatosplenomegaly are most common in human brucellosis, although symptoms may differ dependending upon the site of bacterial localization (i.e., meningitis and myocarditis). Although *B. canis* is considered less pathogenic, it can still induce typical clinical symptoms of brucellosis and more severe clinical syndromes if localized in aberrant locations. *B. ovis* and *B. neotomae* are not considered zoonotic. Zoonotic infections with marine *Brucella* strains have been documented as producing typical brucellosis symptoms. Although one marine *Brucella* strain did not cause disease when delivered conjunctivally to pregnant cattle (Rhyan et al. 2001); the limited data currently available would suggest that the virulence of these strains in aberrant hosts remains to be characterized.

Transmission

Members of the genus *Brucella* exist as intracellular pathogens in mammalian hosts. They do not exist as commensals nor are they found free-living in the environment (Roop et al. 2003). Although they can temporarily be recovered from environmental samples associated with infected animals, environmental persistence is generally accepted to be of no epidemiological importance since direct or close contact is required for transmission. Therefore, maintenance of brucellosis in an animal or a human population requires continual infection of susceptible hosts with *Brucella* spp.

Mechanisms of transmission for *Brucella* can generally be described by dividing the genus into virulent (*B. abortus*, *B. melitensis*) and low pathogenic (*B. ovis*, *B. canis*) strains. For the virulent strains, transmission primarily occurs through fluids or tissues associated with the birth or abortion of infected fetuses or to offspring through milk (Alton 1990; Thoen et al. 1993; Corbel 1997; Godfroid et al. 2004a, 2004b; Olsen and Stoffregen 2005). Since colonization in fetal fluids or placenta after *B. abortus* abortion can be as high as 10^9–10^{10} CFU/g and minimum infectious dosages are estimated to be in the 10^3–10^4CFU range, abortion events can readily infect many animals with brucellosis. Venereal transmission is not considered to be important for transmission of *B. abortus* or *B. melitensis*. By contrast, venereal transmission is an important route of infection for *B. canis*, *B. ovis*, and *B. suis* (Godfroid et al. 2004b). *B. canis* can also be shed in urine, milk or from mucosal surfaces and has also been recovered from feces. Bitches may shed approximately 10^{10}CFU/ml in a serosanguinous vaginal discharge for 4–6 weeks after abortion. In general, animals infected with *B. canis* effectively shed *Brucella* for longer times from mucosal surfaces and urine when compared with animals infected with *B. abortus* or *B. melitensis* (Alton 1990).

B. suis strains are an exception to the categorical division described above because, although these strains are highly pathogenic in terms of the disease they produce in the host, they share transmission routes with both the virulent and low pathogenic strains of *Brucella*. Venereal transmission, for example, is important in the spread of *B. suis* infections in swine, but the bacterium can also be spread by contaminated milk, urine, and across mucosal surfaces. Like *B. canis*, infected swine effectively shed *B. suis* for long periods from mucosal surfaces. Although abortion storms have been reported with *B. suis*, data from experimental challenges suggest that it is more common for swine to maintain pregnancy for normal gestation periods as long as several fetuses remain alive. Experimental data suggest that shedding of *B. suis* can occur from the reproductive tract

even prior to parturition in infected sows (Stoffregen et al. 2006). The possibility of transmission of *B. suis* through consumption of dead fetuses or fetal membranes can also not be eliminated.

Brucellosis has been reported in several wildlife species, including bison, elk, wild boar, caribou, feral swine, and marine mammals (Godfroid 2004). Transmission between land mammals is similar to that reported for domestic animals (Olsen et al. 2004). The means by which *Brucella* are transmitted between marine mammals are unknown. However, the almost exclusive localization of these bacteria within the intestinal lumen and/or uterus of lungworms (*Parafilaroides*, *Phocoena*) in the pulmonary systems of an infected Pacific harbor seal and harbor porpoise suggests the intriguing possibility that parasites may play a role in disease transmission (Garner et al. 1997).

BACTERIAL VIRULENCE FACTORS

The capacity of *Brucella* to maintain long-term residence within macrophages serves as the basis for their ability to establish and maintain chronic infection (Roop et al. 2003). Within macrophages, *Brucella* remain enclosed in the phagosomal compartment. The extent to which *Brucella*-containing phagosomes mature along the endosomal–lysosomal pathway in host macrophages is an area of intense study. During long-term residence in the phagosomal compartment of host macrophages, *Brucella* are exposed to reactive oxygen intermediates (ROIs), acid pH, and nutrient deprivation (Roop et al. 2003). *Brucella* require acidification of the phagosomal compartment to a pH < 4.5 before they display wild-type intracellular replication. This requirement for low pH is transient and only extends through the initial stages of intracellular infection. Data suggest that low pH stimulates expression of genes, such as the VirB operon, which is required for successful intracellular adaptation. Several *Brucella* cell components have been linked to the resistance of these bacteria to exposure to low pH *in vitro*, but only CydB (a component of d-type cytochromes; Endley et al. 2001), Asp24 (a calcium binding protein of unknown function; Kahl-McDonagh and Ficht 2006), and urease (Bandara et al. 2007; Sangari et al. 2007) have been shown to contribute to the virulence of *Brucella* strains in experimental or natural hosts.

Bacteria with unlimited nutrients typically display exponential or balanced growth, whereas when nutrients become limiting, they enter a stationary phase (Roop et al. 2003). In the stationary phase, metabolism is primarily oriented toward maintaining cell viability over prolonged periods with little or no net increase in cell numbers. *Brucella* probably use both stationary and exponential stages for survival *in vivo*. Stationary-phase physiology provides *Brucella* potential benefits for successfully adapting to the harsh environmental conditions encountered in the phagosomal compartment. Evidence supporting the use of stationary physiology by *Brucella* includes the following: (1) During experimental infections in mice, *Brucella* tissue concentrations plateau quickly, with little or no replication demonstrated beyond the first 2 weeks post-infection; and (2) despite long-term antibiotic treatment of human brucellosis, relapses occur frequently, suggesting a stationary, non-replicative bacterial state (Roop et al. 2003). Molecular regulation of *Brucella* gene expression in the phagosomal compartment may differ as *Brucella* enter the stationary phase. VirB has been found to be maximally expressed during exponential growth, but its expression is repressed upon entry into the stationary phase (Roop et al. 2003).

One molecular mechanism that *Brucella* may utilize to survive nutrient limitations in the phagosome is *hfq* (host factor I). In other bacteria, production of *hfq* is required for translation of mRNA encoding the stationary-phase σ factor RpoS, which is required for efficient transition into stationary-phase physiology. *Brucella hfq* mutants demonstrate stationary physiology defects, including greater sensitivity to H_2O_2 and acidic pH, quicker loss of viability in minimal media, and accelerated clearance from the spleens of mice after the first week of infection (Roop et al. 2003).

Since oxidative killing is the primary mechanism employed by host phagocytes to control replication of intracellular pathogens, *Brucella* have multiple molecular mechanisms to detoxify free radicals. Expression of *sodC* is important for detoxification of O_2 radicals generated by the oxidative burst of macrophages. The *Brucella* gene *ahpC* is controlled by *hfq* and may also protect against oxidative killing. In other bacteria, *ahpC* protects against low levels of H_2O_2 and can detoxify peroxynitrite ($ONOO^-$), an oxidizing compound from macrophages believed to play an important bacteriocidal role. A *dps* gene,

a gene that plays a critical role in stationary-phase resistance to oxidative killing and acidic pH in other bacteria, has also been identified in *Brucella*. The LPS O chain also appears to protect against cellular cationic peptides and oxygen metabolites.

Effective acquisition of transition metals (Fe, Mn, Zn, and Mg) in the nutrient-restricted environment of the host is an important determinant of virulence. Transition metals are important for bacterial metabolism in the host. *Brucella* mutants that are unable to use high-affinity transport systems to capture Mn (E. S. Anderson, unpublished data), Zn (Kim et al. 2004; Yang et al. 2006), or Mg (Lavigne et al. 2005) are attenuated in mice. Studies with the mouse model also indicate that heme serves as an important iron source for *Brucella* strains in the host (Paulley et al. 2007). Several *Brucella* have been demonstrated to utilize heme as an iron source *in vitro* and in trophoblasts and macrophages (Roop et al. 2003; Cutler et al. 2005). *Brucella* scavenge iron through siderophores, such as 2,3 dihydoroxybenzoic acid or brucebactin (Bellaire et al. 2003), which are encoded by the *dhb*CEBA operon and regulated by iron response regulators and AraC-like transcriptional activators (Anderson et al. 2008). Mutation of iron regulatory genes has been demonstrated to attenuate virulent *Brucella* strains in mammalian hosts and make *Brucella* more sensitive to oxidative killing.

Successful adaptation to the differing environmental conditions *Brucella* strains encounter as they transit between hosts and extracellular and intracellular locations within the host requires programmatic changes in gene expression in these bacteria (Letesson and de Bolle 2004). *Brucella* have a quorum-sensing pathway involved in regulating the VirB type IV secretion system (Rambow-Larson et al. 2008) and a two-component system for environmental sensing of light (Swartz et al. 2007). BvrR and BvrS comprise a two-component regulator that controls the expression of genes encoding components of the cell envelope of *Brucella* strains (López-Goñi et al. 2002). *Brucella bvrRS* mutants are highly attenuated, indicating that cell envelope modifications carried out by BvrRS regulated genes play critical roles in interactions with the host. VjbR is a homolog of the LuxR-type regulators that recognize homoserine lactone-type quorum-sensing molecules in other gram-negative bacteria (Delrue et al. 2005). VjbR regulates expression of the genes encoding the type IV secretion machinery, and *vjbR* mutants are attenuated in experimental hosts. *Brucella* strains produce a C12 homoserine lactone (Taminiau et al. 2002), and this compound modulates VjbR activity *in vitro* (Uzureau et al. 2007), but exactly how "quorum sensing" by the brucellae contributes to virulence in the host has yet to be determined. Many bacteria employ small noncoding regulatory RNAs (sRNAs) to control the expression of genes at the posttranscriptional level (Gottesman 2004). This allows for rapid modulation of gene expression encoding gene products that are needed for quick adjustments to changing environmental conditions. The activity of the RNA chaperone Hfq is required for the vast majority of the bacterial sRNAs that have been examined. *Brucella hfq* mutants are highly attenuated in mice (Robertson and Roop 1999), goats (Roop et al. 2000), and nonhuman primates (Nikolich, unpublished data), suggesting that sRNAs play an important role in controlling the expression of the *Brucella* genes required for virulence in the host.

PATHOGENESIS

Overview

Brucella are intracellular pathogens that localize in lymphoreticular cells throughout the body and have a predilection for reproductive tissues. Upon entry through the plasma membrane, they modify the endosomal compartment of phagocytic cells to allow replication and long-term survival, use various mechanisms to modify the host environment, resist oxidative killing, and modify their metabolism to survive in their preferred intracellular environment. Cell-mediated immunity and Th1 cytokines, rather than humoral immunity, are important for protective immunity against brucellosis. Unknown molecular and/or physiologic mechanisms may contribute to species preference, recrudescence, and targeting of reproductive tissues in mammalian hosts.

Interaction between the Bacterium and Host Defenses

Brucella are highly adapted to survive within the hostile intracellular environment of monocytes. However, the host employs countermeasures that transform monocytes from permissive into resistant hosts capable of significant anti-*Brucella* activity. The most potent inducer of anti-*Brucella* activity is interferon-γ (IFN-γ). Stimulating monocytes/macrophages with IFN-γ triggers many antimicrobial activities, including increased production of

reactive oxygen (ROI) and nitrogen (RNI) radicals. However, in the case of *B. abortus*, ROI production was found to play a larger role in intracellular resistance to the bacteria than RNI (Jiang et al. 1993), whereas others have found that *B. suis*-infected monocytes reduced bacterial load primarily through the iNOS activity (Gross et al. 2005). Interestingly, pretreatment of monocytes with IFN-γ yielded the strongest anti-*Brucella* activity, which was characterized as a bacteriostatic mechanism that prevented intracellular replication rather than simply increased bacterial killing (Jiang et al. 1993). Recent discoveries in monocyte biology reveal that IFN-γ stimulates phagosome maturation and phagosome/lysosome fusion and increases rates of autophagy, which degrade intracellular vesicles and organelles. Under these conditions, it is difficult for even the most successful intracellular pathogens, such as *Mycobacterium tuberculosis* and *Leishmania*, to avoid phagosome/lysosome fusion.

In vivo or *in vitro* infections of mammalian hosts with *Brucella* is often associated with production of IFN-γ and Th1 cytokines, but human infections may switch predominantly to Th2 cytokines with chronicity (Rafiei et al. 2006). Data suggest the *virB* type IV secretion system is important for inducing IFN-γ and Th1 cytokine responses under *in vivo* conditions (Rolán and Tsolis 2008). Exogenous administration of IFN-γ or IL-12 to macrophage cell lines under *in vitro* conditions or *in vivo* to laboratory animals can reduce colonization with *Brucella*.

Like other intracellular pathogens, *Brucella* have adapted to their intracellular lifestyle and no longer require the accumulation of energy-storing molecules (Chain et al. 2005). The presence of cytochromes (cytochrome bc1 complex or quinol oxidase) with a high oxygen affinity may represent an important adaptation of *Brucella* to their intracellular lifestyle. As discussed, *Brucella* have a complete and functional type IV secretion system that is highly conserved at the nucleotide level. *Brucella* do not possess classic virulence factors, such as toxins, but rather those identified apparently facilitate cell invasion and survival by subverting innate cellular defense mechanisms.

Entry into Host Cells

Entry of non-opsonized smooth *Brucella* into cells relies on the cytoskeleton of the host cell for internalization. Smooth strains of *Brucella* interact with cholesterol-rich lipid rafts within the plasma membrane to facilitate contact with the host cell and mediate internalization by phagocytes and nonprofessional phagocytes. Lipid rafts, which contain glycosphingolipids, cholesterol, and glycosyl-phosphatidylinositol anchored proteins (Cutler et al. 2005), facilitate membrane-associated sorting events, such as the formation of multi-subunit membrane complexes, and signaling across membranes and membrane fusion. Lipid rafts are found in intracellular organelles, vesicles, and the plasma membrane. The *Brucella* LPS O polysaccharide appears to be a key molecule for interaction with lipid rafts on host cells but also prevents complement-mediated bacterial lysis and host cell apoptosis (Bagüés et al. 2004). Opsonization of smooth strains of *Brucella* increases entry tenfold; however, entry is targeted to the phagolysosomal compartment. Opsonization targets *Brucella* to specific receptors on the surface of phagocytes (Fc receptor for bound IgG, complement receptors for C3b and C4b) and diverts smooth bacteria from entry via lipid rafts. Receptor-mediated phagocytosis leads to greater killing of internalized *Brucella* by monocytes.

Entry of rough strains of *Brucella* differs from smooth strains. Because rough strains of *Brucella* are unable to sustain interactions with lipid rafts, they are readily phagocytosed following either Toll-like receptor 4 (TLR4) or mannose receptor recognition of the LPS-deficient bacterial surface. As a result, rough strains demonstrate elevated invasion possibly due to exposure of ligands that are normally hidden by the O chain and may have increased capability to adhere to macrophages (Bagüés et al. 2004). Consequently, non-opsonized rough *Brucella* are internalized as efficiently as opsonized *Brucella*, are rapidly targeted to the phagolysosomal compartment, and are generally unable to replicate (Rittig et al. 2003). Entry of smooth and rough *Brucella* strains into the cells through different pathways may also involve receptors with a distinct ability to regulate the level of phagocytosis. In contrast to rough strains that are defective at intracellular replication, the intracellular smooth *Brucella* surviving after opsonin-mediated phagocytosis are capable of significant intracellular replication (Bellaire et al. 2005). Genes necessary for O side chain synthesis (i.e., *manB*, *wboA*) play a significant role in establishing the intracellular replicative compartment for smooth strains of *Brucella*.

Intracellular Pathways Utilized

Internalized *Brucella* quickly traffic through the early endosomal compartment in host macrophages and depart the phagosome to form the modified phagosome ("brucellosome") by acquiring components of the endoplasmic reticulum (ER) in a manner similar to autophagosome biogenesis. The modifications of the phagosome prevent its fusion with lysosomes.

Brucella initially localize within acidified phagosomes (Rittig et al. 2001), where they are exposed to free oxygen radicals generated by the respiratory burst. Early localization in an acidified environment is important for *Brucella* replication and survival by inducing expression of the *VirB* operon (*virB* 1–10), an operon that controls expression of genes associated with a type IV secretion system. The VirB operon interacts with the ER in a way that neutralizes the pH of the phagosome (Celli 2006) and transports effector molecules, such as VceA and VceC (de Jong et al. 2008), into the cytoplasm of the infected macrophages to interfere with phagosomal maturation. The modified phagosome environment provides conditions of nutrient depletion and limited oxygen availability.

Another intracellular survival mechanism for *Brucella* involves modification of the lipid content of the phagosome-limiting membrane. Virulent *Brucella* strains express a cyclic glucan synthase (*cgs*) that produces and secretes low-molecular-weight cyclic glucans. These molecules disrupt the lipid raft microdomain structures within intracellular membranes surrounding the bacteria, a modification that inhibits phagosome maturation and prevents fusion with lysosomes and is independent from *VirB* and type IV secretion systems (Arellano-Reynoso et al. 2005).

Lesions and Disease Characteristics

Brucella primarily enter through penetration of mucous membranes, although infection through breaks in the epithelium is possible. Licking or sniffing aborted fetuses and placental membranes or ingesting contaminated milk introduces the *brucellae* to the oral and nasal mucosa, tonsils, and gastrointestinal mucosa. *Brucella* can penetrate the epithelium-covering domes of ileal Peyer patches. With *B. canis B. ovis*, and *B. suis*, entry can also occur through the mucous membranes of the reproductive tract.

Following penetration of mucosa, bacteria are transported, either free or within phagocytic cells, to regional lymph nodes, which become enlarged due to lymphatic and reticuloendothelial hyperplasia and inflammation. If bacteria do not become localized, and are not killed in regional lymph nodes draining the site of infection, they may replicate and spread to other lymphoreticular tissues and organs via the lymph and blood. With most *Brucella* strains, the bacteremia is short-lasting, such that live bacteria are not readily isolated from blood samples of infected individuals. Particularly with *B. canis* and to a lesser extent with *B. suis*, the bacteremia is longer in duration or occurs frequently during infection.

Localization in the reproductive or mammary glands is associated with the most severe pathology and capability to transmit infection. Hosts are more susceptible to infection with *Brucella* strains during pregnancy, although host mechanisms responsible for the increased susceptibility are not fully characterized. Infection with the brucellae during mid-gestation is associated with the greatest pathogenicity in cattle, although the probability of isolation of *B. abortus* at parturition increases as the days of gestation at the time of exposure increase (Crawford et al. 1987). *Brucella* gain access to the uterus and fetus via a hematogenous route, and the bacteria initially localize within erythrophagocytic trophoblasts of the placentome. Adjacent chorioallantoic trophoblasts become infected, leading to an inflammatory response. Infected trophoblasts may eventually rupture and ulcerate the chorioallantoic membrane. In addition to the hematogenous route, fetuses may also become infected via ingestion of amniotic fluid containing *Brucella*. Other than a diffuse submucosal inflammatory reaction, the endometrium is not infected with *Brucella* and remains largely intact.

The physical and hormonal characteristics of the placenta that allow immune suppression and prevent maternal immune-based rejection of the developing fetus likely play an important role in this susceptibility since the bulk of the bacterial replication that takes place during *Brucella* infections occurs within placental trophoblasts, host cells that are considered to be "immune privileged." In ruminants, the timing of maximum production of the four-carbon sugar alcohol erythritol by placental trophoblasts coincides with the period when these animals exhibit their greatest susceptibility to *Brucella* infections.

Erythritol is the preferred carbon source for *Brucella* strains, and it has been proposed that the presence of this compound is an important factor driving the extensive intracellular replication exhibited by the brucellae in placental trophoblasts during the latter trimester of pregnancy. Data suggest that mutations in erythritol uptake and metabolism genes contribute to the attenuation of *B. abortus* strains *in vivo* (Crasta et al. 2008). This postulated relationship between erythritol metabolism and the virulence of *Brucella* strains in ruminants has not been directly tested experimentally.

Brucella may also reside in a dormant, nonreplicative state in phagocytic cells. Calves infected with *B. abortus* at a young age often do not demonstrate seroconversion until after puberty. Recrudescence of clinical brucellosis is also a problem in human patients. Molecular mechanisms controlling recrudescence or host physiology influencing *in vivo* replication of *Brucella* have not been identified.

The most common clinical manifestation of brucellosis in natural hosts is reproductive loss through abortions, birth of weak offspring, or infertility. Other clinical signs associated with brucellosis in these hosts are relatively rare, but occasionally *Brucella* spp. can localize in joints, bones, or other aberrant locations producing inflammation and associated pathology. In contrast, *Brucella* infections in humans are frequently associated with clinical illness, including recurrent fever ("undulant fever"), general debility, hepatosplenomegaly, and other symptoms associated with inflammatory responses to infection.

Immunity

Although *Brucella* strains induce a vigorous immune response in infected animals, the chronic nature of the *Brucella* infections indicates that that these immune responses are insufficient to eliminate *Brucella* from their intracellular niche. Indeed, there is considerable evidence suggesting that a critical determinant in the pathogenesis of *Brucella* infections is the capacity of the bacteria to inhibit or avoid components of the host's immune defenses. Lipid A of the LPS of *Brucella* strains stimulates a greatly reduced inflammatory response in mammalian hosts than does the endotoxin of other gram-negative bacteria (Barquero-Calvo et al. 2007). This results in reduced infiltration of neutrophils and reduced cytokine production by infected animals during the early stages of infection, conceivably giving the brucellae a chance to reach their intracellular niches, where they would be protected from polymorphonuclear leukocytes (PMNs), complement, and antibodies. The unusual chemical composition of the O chain of the LPS of smooth *Brucella* strains also plays a direct role in inhibiting host immune responses. Specifically, macrophages have great difficulty in degrading the perosamine O chain of the *Brucella* LPS, and undigested O chain directly inhibits the ability of infected macrophages to present antigens to T cells via the major histocompatibility (MHC) class II pathway (Forestier et al. 2000).

Adaptive immunity, involving interactions between CD4+ and CD8+ T cells, macrophages, and dendritic cells are key to providing immunity against *Brucella*. The antigen-presenting cells (APCs) present antigens with MHC class I or II molecules to T cells, which undergo clonal expansion to develop responsive populations that can react to current and future infections with that pathogen (Wyckoff 2002). It is the $\alpha\beta$ T-lymphocyte subsets that undergo antigen-specific clonal expansion in response to vaccination or infection and generally manifest specific immunity. In general, antigens derived from the outside and that enter the APC through phagocytosis for subsequent degradation in the phagolysosome are presented through the class II MHC, exogenous pathway. By contrast, antigens synthesized within the APC cytoplasm and transported to the ER are presented with class I MHC and processed through endogenous pathways. Recent work has suggested that pathogens such as *Listeria monocytogenes* and *Salmonella* spp. can lead to trafficking of proteins out of the phagolysosome and into the endogenous antigen pathway (Wyckoff 2002). The intracellular location or entry of antigens into the cell is important since processing via the exogenous pathway is typically associated with a Th2 response, which is predominantly humoral. However, antigens presented on the surface of the APCs via the endogenous pathway tend to evoke a Th1 response associated with cell-mediated immune responses.

Optimal immunity to intracellular bacteria, such as *Brucella*, is generally believed to be mediated by the Th1 subset of CD4+ lymphocytes and is associated with the production of IFN-γ and tumor necrosis factor α (TNF-α). Secretion of IL-12 and/or IL-18 by APCs works synergistically through different signaling pathways (STAT 4 and p65/

p50 NF-κB, respectively) to enhance the production of IFN-γ, TNF-α, and IL-2 from T cells (Tizard 2009). IFN-γ and TNF-α activate macrophages, leading to enhanced production of ROI and RNI by these phagocytes, and experimental studies have documented that activated macrophages have an increased capacity to kill intracellular brucellae compared with their unactived counterpartments.

Development of cytotoxic T lymphocytes may also play a role in protection. Following killing of infected cells by activated CD8+ T cells, intracellular bacteria within the cells may be released and be available for uptake and destruction by activated macrophages (Wyckoff 2002). Infected host cells killed by cell-mediated processes may also be killed by apoptosis and display signals that trigger phagocytosis and degradation without release of intracellular bacteria. Microarray data from infections of macrophages with smooth *Brucella* strains indicate that infection is associated with a down-regulation of genes associated with apoptosis (Rajashekara et al. 2006). Prevention of host cell apoptosis may be one mechanism used by *Brucella* to facilitate intracellular survival and persistence.

In general, antibodies are considered to play a minor role in long-term protection against brucellosis, although they may opsonize bacteria and facilitate phagocytosis by macrophages, leading to antigen processing/presentation and clonal expansion/activation of T cells. Entry of opsonized bacteria into phagocytes mediated by the Fc receptor also exposes these bacteria to a stronger oxidative burst. Stimuli that induce Th2 cytokines, such as interleukins 4 (IL-4) and 10 (IL-10), are aligned with humoral responses and do not lead to macrophage activation and protective immunity against *Brucella*. Inoculation with killed *Brucella* bacteria tends to induce primarily a non-protective Th2 response. This is most likely due to the processing of dead bacteria via an exogenous antigen presentation pathway.

The role of Toll-like receptors (TLRs) in an innate and adaptive immunity to microbes is a subject of considerable recent interest. Dendritic cells are of particular interest since they express TLRs that function *in vivo* as immunological sensors for the detection of pathogens. When pathogen-recognition receptors are triggered, the dendritic cells integrate the signals and migrate to the T cell areas of secondary lymphoid organs to stimulate naive T cells.

In mice, heat-killed *B. abortus* can stimulate dendritic cells to secrete both TNF-α and IL-12 (Huang et al. 2005). Dendritic cells secrete TNF-α after stimulation of the TLR4 pathway, whereas secretion of IL-12p40 is dependent on stimulation of the TLR9 and the MyD88 signal transduction molecule. The TLR4 pathway (fig. 22.1) may be of importance for bacterial pathogens since this TLR recognizes LPS as a ligand, can induce signaling through TLR-signaling adaptor proteins (MyD88, TRIF, TRAM, and MAL), and is capable of inducing potent immune responses (Takeda and Akira 2004). There is considerable evidence indicating that the lipid A component of the *Brucella* LPS has a reduced capacity to induce inflammatory responses compared with lipid A molecules from other gram-negative bacteria, such as the *Salmonella* spp., and this lower biological activity may stem from a reduced interaction of the *Brucella* lipid A with TLR4.

The TLR9 pathway may also be of importance in immunological responses to *Brucella*. TLR9 interacts with CpG DNA and has been shown to translocate from the ER to the endosome after cell activation. In mouse dendritic cells, IFN-γ secretion is TLR9-dependent and requires endosomal acidification, suggesting that interactions between TLR9 and heat-killed *B. abortus* occur at the endosome. In murine macrophages, unlike dendritic cells, secretion of IFN-γ is dependent on the MyD88 adaptor molecule but only partially dependent on TLR9 (Huang et al. 2005).

The TLR2 pathway may also contribute as *Brucella* lipoproteins (Omp16, Omp19) can interact with TLR2 and stimulate cytokine (TNF-α, IL-6, IL-10, IL-12) release from dendritic cells.

Activation of TLR pathways is not only regulated by the specificity of ligand binding to appropriate receptors but also by diverse regulatory mechanisms for adaptors (i.e., MyD88, TRIP, TRAM, MAL) within signaling pathways. The *B. melitensis*, *B. abortus*, and *B. suis* genomes encode a gene (TcpB) and corresponding protein (Btp1) that is homologous to the Toll/Interleukin-1 receptor (TIR) domains found in host cell adaptor proteins. The TcpB protein interferes with MyD88-dependent TLR signaling through TLR4 and TLR2 pathways and impairs activation and maturation of dendritic cells (Cirl et al. 2008; Salcedo et al. 2008).

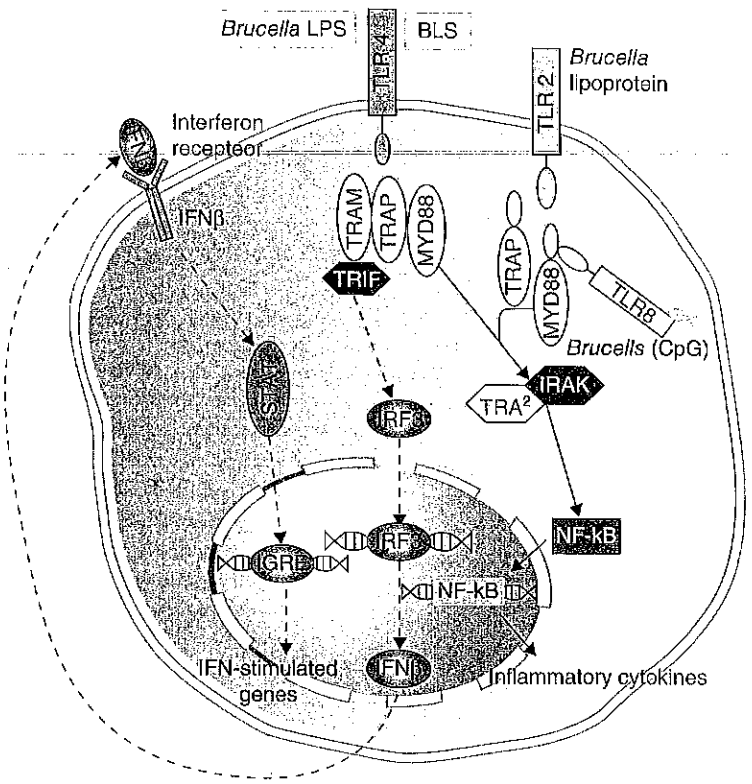

Figure 22.1. TLRs and signaling pathways through which *Brucella* can interact. (See color plate)

DISEASE CONTROL AND EPIDEMIOLOGY

Many countries have programs to control or eradicate highly pathogenic *brucellae* (*B. abortus*, *B. melitensis*, and *B. suis*). Although brucellosis can cause severe economic losses to livestock producers, most regulatory programs for brucellosis were developed to reduce brucellosis in animal reservoirs as a mechanism to prevent human infections. Multiple studies have demonstrated this approach to be the most cost-efficient mechanism for public health (Roth et al. 2003).

Regulatory programs can include sanitation programs to prevent disease transmission, vaccination programs to reduce herd susceptibility, and test-and-removal programs to eliminate animals infected with *Brucella*. Efficacious vaccines are available for *B. abortus* and *B. melitensis* but not for other *Brucella* spp. Although vaccination is highly effective in reducing production losses (i.e., abortions or weak offspring), it is less effective at preventing infection after exposure to field strains of *Brucella* spp. With efficacious vaccines, most vaccinated animals are probably only transiently infected but do develop antibody responses that react to brucellosis serologic tests used for surveillance. Although vaccination has been a very effective tool in controlling brucellosis, vaccination alone has never been effective in eradicating brucellosis. The most effective brucellosis programs incorporate combinations of sanitation, vaccination, and test-and-removal procedures.

Since brucellae are intracellular pathogens, microbial isolation is not always reliable, and most test-and-removal programs are based on removal of animals that demonstrate antibody responses to *Brucella*. Most serologic tests are based on the detection of antibodies against the O side chain of the *Brucella* LPS as it is a very immunodominant

antigen for humoral responses. Although the antibodies indicate that the individual's immune response has responded to infection with *Brucella*, it should be emphasized that seropositive responses do not necessarily indicate that the animal is currently infected or capable of transmitting brucellosis.

Factors accounting for differences between countries in brucellosis regulatory programs include prevalence of the disease within livestock or human populations, economic considerations, and the strength and prominence of livestock regulatory structures within the country. If there is a high prevalence of brucellosis within domestic livestock, regulatory programs may be designed to reduce prevalence rather than eradicate disease. Use of extensive test-and-removal programs in areas of high brucellosis prevalence may have unacceptable economic costs and may be devastating to livestock production in that area. In a similar manner, successful test-and-removal programs require that a country has strong regulatory and diagnostic structures for livestock disease control and sufficient resolve such that adequate numbers of livestock are being tested for brucellosis, livestock movement or change in ownership within the country is not occurring without brucellosis testing, and seroreactors are rapidly removed to prevent disease transmission. Since non-pasteurized dairy products are the primary mechanism for food-borne transmission, emphasis on testing of milk-producing livestock would be the most effective mechanism to prevent human infections that are not occupationally associated.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Although progress has been made in characterizing the mechanisms that *Brucella* utilize for intracellular entry and persistence, there are many gaps regarding genes responsible for survival, regulatory control, and immunogenicity in the intracellular environment. It is anticipated that microarray techniques will provide valuable data on gene expression in *Brucella* that will address these knowledge gaps. Comparative studies of gene expression *in vivo* may also explain the observed phenotypic differences in host species preferences by different *Brucella* species.

Knowledge of specific genes or gene products that mediate protective immunity in various species is also lacking. A better definition of protective antigens may allow development of more efficacious vaccines for domestic livestock, which may include nonliving vaccines. In a similar manner, greater characterization of mechanisms by which brucellae subvert immunologic responses may also be beneficial for vaccine development. Increased knowledge in this area might allow development of a human vaccine, which could be beneficial for public health in areas with a high prevalence of brucellosis.

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Brucellosis in the United States: Role and significance of wildlife reservoirs

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ABSTRACT

Regulatory programs for brucellosis in domestic livestock have been active in the U.S. for almost 80 years. Wildlife reservoirs of brucellosis include bison (*Bison bison*) and elk (*Cervus elaphus nelsonii*) for *Brucella abortus* whereas *Brucella suis* is the predominant species infecting feral swine. The persistence of brucellosis in wildlife reservoirs poses a risk for reintroduction of *Brucella* into domestic livestock. Reducing the prevalence of brucellosis in wildlife reservoirs is anticipated to be complicated and costly, and the problem is unlikely to be quickly resolved. Although some tools are currently available for use in the wildlife reservoirs, development of new vaccines, diagnostics, and management procedures will most likely be needed for effective control of brucellosis.

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Regulatory programs to control brucellosis in the U.S. were first initiated in 1934 as part of a program to reduce the cattle population during severe drought conditions [1]. Congress appropriated additional funds in 1954 to establish a national eradication program for brucellosis which is administered by the Animal and Plant Health Inspection Service (APHIS) in the U.S. Department of Agriculture [1]. In 1998 it was estimated that $3.5 billion in federal, state, and private funds had been spent on brucellosis eradication programs in domestic livestock [2]. Beginning with approximately 11.5% seroprevalence in 1934, brucellosis regulatory programs have essentially eliminated brucellosis from domestic cattle in the U.S. [1]. A significant event occurred in 2008 in which all 50 states were simultaneously classified as free of brucellosis in cattle. However, re-infection of cattle with *Brucella abortus* from wildlife reservoirs has subsequently caused the loss of brucellosis-free status of one state. The ability of the U.S. to totally eliminate brucellosis from domestic livestock and protect its financial investment will be dependent upon resolving the persistence of disease in free-ranging wildlife reservoirs.

Brucellosis in domestic livestock is of regulatory importance due to the capacity of *B. abortus*, *Brucella suis*, and *Brucella melitensis* to infect people and cause clinical disease. From a public health perspective, studies have demonstrated that addressing the disease in domestic livestock is more cost-effective than treatment of human brucellosis [3,4]. Symptoms of brucellosis in humans are generally non-specific and influenza-like, including intermittent fever, chills, malaise, arthralgia, diaphoresis, myaligia, headache, anorexia, and fatigue [5]. Humans are most commonly infected by consumption of dairy productions made from nonpasturized milk, or by direct contact with infected animals. In general, the U.S. has averaged approximately 100–200 cases of human brucellosis since the 1980s as compared to 6321 human cases of brucellosis reported in 1947 [6]. Due to the reduction in brucellosis in domestic livestock in the U.S., most human cases are related to travel or consumption of food products from outside the country. However, human cases resulting from exposure to wildlife reservoirs of brucellosis have been reported [5].

This manuscript will discuss the epidemiology of *B. abortus* and *B. suis* in free-ranging wildlife reservoirs in the U.S. and review the current status of relevant knowledge on vaccines, diagnostics, and management procedures that are needed to address the prevalence of disease in these reservoirs. For the purposes of this manuscript, only wildlife in which the disease is manifested and transmission occurs (maintenance hosts) will be discussed, as compared to species in which disease is not manifested and transmission does not occur (dead-end or spill-over hosts).

1. Wildlife reservoirs of *Brucella abortus*

The U.S. has two primary reservoir hosts of *B. abortus*, free-ranging bison (*Bison bison*) and elk (*Cervus elaphus*). Both species are in the Greater Yellowstone Area (Yellowstone National Park and surrounding areas) and can have close association with cattle during winter months when available forage is limited. Current epidemiologic data suggest that recent infections of cattle in the Greater Yellowstone Area occurred via transmission of brucellosis from elk (A. Gertonson, personal communication).

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2. Bison (*Bison bison*)

As Yellowstone National Park (YNP) is arguably the flagship of national parks in the U.S., the *Brucella* infection in the historically important, free-ranging bison herd remains controversial. Bison populations have increased from 397 to peak populations of 4500–5000 since the elimination of hands-on management in 1967. Until 1984 when the population was estimated at 2114, few bison attempted to leave YNP [7]. Despite the high seroprevalence in YNP bison, approximately 50%, the population does continue to increase but brucellosis does appear to impact population growth [8].

The National Park Service is responsible for protecting and maintaining the unique ecology of Yellowstone National Park. Current brucellosis management plans for YNP bison do not include capture of bison within the park, or test and removal procedures [7]. For this reason research has focused on epidemiologic characterization of brucellosis in free-ranging bison, development of efficacious vaccines that can be remotely delivered, and development of minimally invasive management procedures that may reduce disease prevalence.

Epidemiologic data suggest the concept that brucellosis in the YNP bison is similar to the characteristics of a chronically infected cattle herd [9–11]. Abortion, reproductive failure, retained placenta, and inflammatory lesions in the male reproductive tract have been reported in *B. abortus*-infected bison [9,10,12–14]. One study reported that 46% of seropositive bison were culture positive; which is similar to data from cattle from a chronically infected herd [11].

Under experimental conditions, clearance of the *B. abortus* strain RB51 (RB51) vaccine takes approximately twice as long to be eliminated from lymphoreticular tissues of bison as compared to cattle [15,16]. Parenteral vaccination of RB51 induces humoral and cellular immune responses [17,18] although cellular immunity is considered to be more important for long-term protection against intracellular pathogens like *Brucella*. When compared to nonvaccinates, bison vaccinated as calves with 10^{10} colony-forming units (CFU) of RB51 have reductions ($P < 0.05$) in abortions, reproductive colonization, or mammary gland colonization after experimental challenge with a virulent *B. abortus* strain during pregnancy [18,19]. This is in contrast to studies which indicated that the *B. abortus* strain 19 (S19) vaccine does not protect bison against experimental challenge and is abortigenic in pregnant bison [20,21]. Data suggest that protection induced by RB51 vaccination of bison is slightly reduced ($P > 0.05$) in bison as compared to protection induced in RB51-vaccinated cattle [22,23]. Reduced protection from experimental challenge may in part reflect greater susceptibility of bison to brucellosis as nonvaccinated bison have higher ($P < 0.05$) rates of abortion and infection after experimental challenge when compared to data from nonvaccinated cattle (S. Olsen, unpublished).

At the current time, dart delivery of RB51 liquid vaccine or ballistic delivery of biodegradable biobullets® containing RB51 payloads are the only remote vaccination techniques that have been evaluated in bison [17]. Ballistic delivery of RB51 biobullets® is associated with slight reductions ($P < 0.05$) in immunologic responses [17,24] and protection against experimental challenge exposure during pregnancy (Olsen, unpublished). Use of darts to deliver RB51 induces similar immunologic responses as compared to parenteral vaccination (Olsen, unpublished), although efficacy of RB51 delivered by this method has not yet been determined. Dart delivery also offers the ability to deliver vaccine at greater distances as compared to ballistically delivered biobullets®.

Due to the population size and management constraints, it is anticipated that a vaccination program for YNP bison will be expensive, labor intensive, and require long-term commitment. Therefore, a vaccine strain and delivery method that induces the greatest efficacy in bison is desired. For these reasons, studies continue to evaluate new vaccine strains or delivery methods as they become available in an effort to find the most highly efficacious method to protect bison against brucellosis.

Currently, temporal and spatial separation is being used to prevent transmission of brucellosis from bison to cattle [7]. Brucellosis vaccination will be utilized when responsible agencies determine that a suitable vaccine and delivery method is available. Immunocontraception has been proposed as a management tool that could be used to prevent seropositive bison from transmitting brucellosis [25]. With the exception of vaccination and immunocontraception, no other management techniques have been proposed for reducing brucellosis prevalence in bison. In an effort to prevent transmission to cattle, proposals have been made in regard to population control and expanding areas into which bison are allowed to migrate. Evaluations of quarantine procedures for detecting *Brucella*-infected bison are ongoing in an effort to allow movement of noninfected bison from the Greater Yellowstone Area.

3. Elk (*Cervus elaphus nelsonii*)

Although North American elk populations were estimated at 10 million prior to arrival of Europeans on the North American continent, they were reduced to 40,000–100,000 animals at the start of the 1900s with an estimated 40,000 located in the Yellowstone area [26]. Early in the 1900s, human development had compromised traditional elk migration routes causing starving elk to feed on livestock hay and causing conflict with humans [27]. In response to starving elk during a severe winter in 1909, the Wyoming legislature appropriate $5000 to purchase hay to feed elk in the Jackson, WY area [27]. In 1912, the U.S. government established the first feedground at Jackson Hole, WY. Currently, Wyoming Game and Fish Department manages 22 feedgrounds in western Wyoming and the U.S. Fish and Wildlife Serve manages the National Elk Refuge in Jackson Hole, WY. In 2006, it was estimated that 22,000 elk overwintered on the 23 feedgrounds in Wyoming [28]. In response to brucellosis associated with elk feedgrounds, the states of Idaho and Montana banned artificial feeding of elk.

Brucellosis was first reported in elk in 1930 after aborted fetuses were found on the National Elk Refuge [29]. Wyoming Game and Fish has monitored seroprevalence for brucellosis in elk since the 1970s. Overall data suggest that seroprevalence for brucellosis in elk on feedgrounds is significantly higher than in elk that do not winter on feedgrounds [30]. Artificial winter feeding appears to be primarily responsible for the maintenance and transmission of brucellosis in elk in the Greater Yellowstone Area [30]. In the neighboring state of Idaho, seroprevalence in areas of artificial feeding ranged from 12 to 80% whereas hunter surveys indicated that population seroprevalence was only 2–3% [31]. Data suggest that disease prevalence in elk may be associated with population density on feedgrounds [28], length of artificial feeding [32], or congregation on feedgrounds during February to June when the majority of brucellosis-induced abortions occur [33]. The literature is currently lacking in descriptions of pathologic lesions associated with *Brucella* infection in elk. However, as in bison, *B. abortus* has been documented to cause abortions, stillbirths, and weak calves in elk [31].

As with bison, efforts have been made to develop a safe and efficacious vaccine for elk. Although the RB51 vaccine is safe in elk, repeated studies have demonstrated that RB51 vaccination does not protect elk against experimental challenge with virulent *B. abortus* [34,35]. Based on preliminary data, Wyoming Game and Fish initiated ballistic vaccination of elk with *B. abortus* strain 19 (S19) on feedgrounds in 1985 [30]. Currently available data suggest that vaccination of elk with S19 provides partial pro-

tection against experimental challenge [36,37]. Characterization of immunologic responses of elk after inoculation with tuberculosis and brucellosis vaccines has indicated that elk primarily develop humoral responses to vaccination and have poor anamestic responses to booster vaccination with *Brucella* vaccines [38,39]. As cellular immunity is required for long-term protection against brucellosis, the failure of brucellosis vaccines to provide strong protection in elk may be related to intrinsic regulation or limitations of immunologic responses to intracellular pathogens in elk. Development of new vaccines that stimulate strong cellular immune responses is needed to facilitate resolution of brucellosis in elk.

Although controversial, changes in elk management to reduce elk populations over a long period of time should indirectly reduce numbers on feedgrounds and be beneficial in reducing transmission and disease prevalence. Feedground management procedures to decrease length of feeding, and more widely disperse feedstuffs during artificial feeding may also reduce brucellosis in elk. Habitat improvement programs to increase feed sources off feedgrounds and thereby reduce population densities on feedgrounds are ongoing. Wyoming has recently initiated an experimental test and removal pilot program on a feedground to determine if this would be effective in reducing serologic prevalence of brucellosis. However, current data suggest that only 33–50% of seropositive elk are culture positive, and financial costs for per removed animal are extremely high (Kreeger, personal communication).

4. Feral swine (*Sus scrofa*) brucellosis

In the U.S., *B. suis* remains endemic in feral swine although it has been nearly eradicated from domestic swine [40,41]. Primarily by human intervention, feral swine continue to expand their ranges across the U.S. with at least 38 of 50 states reported to have resident populations [42]. The rapid expansion into new areas has primarily been caused by unregulated human transportation of feral swine to establish new populations for hunting. The feral swine population in the U.S. is currently estimated at 4–5 million animals with highest populations in the southeast [5]. Because serology methods are based on tests developed to detect antibodies to *B. abortus* in cattle, current tests are not as sensitive or specific for detection of swine antibodies to *B. suis*. However, available serologic methods have documented brucellosis in feral swine populations in at least 14 states [43,44]. Although *B. suis* is the greatest concern with feral swine, data from a feral swine herd that had been segregated from domestic livestock for decades documented the maintenance and transmission of *B. abortus* [40].

Data characterizing the epidemiologic characteristics of brucellosis in feral swine are extremely limited. In a naturally infected feral swine herd, a high percentage of animals were culture positive and males were more likely to be seropositive or infected than females [40]. Inflammatory lesions in reproductive or lymphatic tissues were noted in male and female swine. Under experimental conditions, infected swine can shed *B. suis* from mucosal surfaces for at least 4 weeks after infection with a virulent field strain (Stoffregen, unpublished). In contrast to *B. abortus* challenges in uniparous species, swine experimentally challenged with virulent *B. suis* are not as likely to abort. Rather, swine appear to maintain pregnancy while viable offspring are present *in utero*.

Tools to manage feral swine brucellosis are currently very limited. When a domestic swine herd is identified as being infected, the herd is depopulated. However, removal of feral swine populations from an area is currently not feasible. There is a definitive need to develop a vaccine that can be delivered in oral baits or through other methods of delivery that do not require capture of swine or targeted vaccination of individuals. Data have demonstrated that parenteral vaccination of swine with the *B. abortus* strain RB51 vaccine does not protect against experimental challenge, primarily because the strain did not appear to colonize lymphoreticular tissues of pigs [45]. Recent work with a natural rough mutant of *B. suis*, strain 353-1, has shown that oral or parental vaccination induces significant immune responses and protection in pigs (Stoffregen, unpublished). However, additional studies with this strain are needed.

5. Summary

The maintenance of brucellosis in wildlife reservoirs continues to be a threat for reintroduction of disease into domestic livestock. Both feral swine and elk have been strongly implicated for causing infection and seroconversion in cattle. Feral swine have also been shown to infect domestic swine. Resolution of brucellosis in wildlife reservoirs will be difficult, costly, and require long-term commitment. However, the alternative is to risk significant economic losses to domestic livestock producers and imperil the financial investment made in brucellosis eradication by state, federal, and private sources. It is unlikely that the prevalence of brucellosis in wildlife reservoirs will significantly change without introduction of management activities that specifically address the disease.

A case can be made that control or eradication of brucellosis in wildlife reservoirs is beneficial for the wildlife species. For bison and elk it is hypothesized that brucellosis was originally introduced by domestic livestock. Therefore, the pathogen is not indigenous to these North American species and from an ecological standpoint, should be removed even though it is not impairing population growth. However, new diagnostics, vaccines, and other tools need to be developed to facilitate control of brucellosis in wildlife. Even then, it is anticipated that brucellosis control programs for wildlife will be far more complicated and challenging than current programs designed for domestic livestock.

Conflict of Interest Statement

All authors declare they have no conflict of interest

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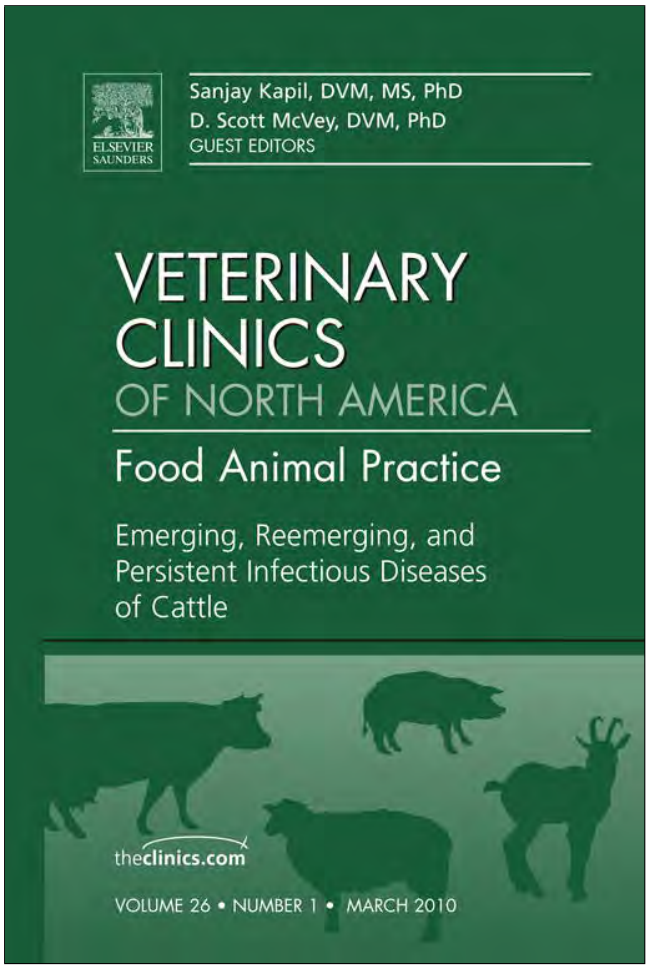

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Bovine Brucellosis

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KEYWORDS

• *Brucella abortus* • Brucellosis • Vaccination • Strain

Infection of cattle caused by *Brucella abortus* (ie, bovine brucellosis) has been of political importance in the United States for many decades. Regulatory programs to control or eradicate brucellosis in cattle were first initiated in the United States in 1934 as part of a recovery program to reduce cattle populations during severe drought conditions.[1] In 1954, the Congress appropriated funds to change the brucellosis control program to an eradication program as a joint effort between federal and state governments and cattle producers.

In the United States, the Animal and Plant Health Inspection Service of the US Department of Agriculture is responsible for managing national eradication or control programs that target livestock diseases of economic or zoonotic importance. Because the brucellosis eradication program has been ongoing for decades, a significant financial investment has been made in an effort to eliminate brucellosis. Although several new infected cattle herds have recently been identified, the United States achieved a milestone in 2008 in that, for the first time, all states were simultaneously declared free of cattle brucellosis.

DISEASE CHARACTERISTICS

The most common clinical manifestation of brucellosis in natural hosts is reproductive loss resulting from abortion, birth of weak offspring, or infertility.[2] In particular, third trimester abortion of dead or weak, nonviable calves is the hallmark of brucellosis. Aborted fetuses are generally fresh with minimal autolysis. Although mammary gland infection may not result in visible clinical symptoms or gross lesions, *Brucella* spp frequently localize in the mammary gland and cause mastitis.[2] Brucellosis can negatively affect lactation as one study demonstrated that a reduction in disease prevalence was associated with increased milk production.[3] Other clinical signs associated with brucellosis in cattle are rare. Occasionally, *Brucella* spp can localize in joints, bones, male reproductive tissues, or other aberrant locations where inflammation and associated pathology induce lesions and clinical signs.

Gross lesions in cattle are not pathognomonic but can include variable necrosis of cotyledons across the placenta and thickening of intercotyledonary areas.[4] Common

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histologic lesions of maternal tissues include necrotic placentitis and lymphosuppurative or lymphohistiocytic interstitial mastitis. The most frequent fetal lesion is a multifocal, histiocytic bronchopneumonia with or without suppurative infiltrates. Fetal lesions may also include variable foci of necrotizing arteritis, necrosis, and granulomas in the lung and other tissues.

THE PATHOGEN

B abortus, like other members of the genus *Brucella*, is a facultative intracellular pathogen that infects mammalian hosts. Brucellae are small (0.4–3 μm), gram-negative, coccobacillary organisms within Alphaproteobacteria. *B abortus* has 2 circular chromosomes encoding approximately 3.2 Mb and has been divided into 7 biovars based on biochemical, phenotypic, and antigenic properties.[5] Only biovars 1, 2, and 4 of *B abortus* have been reported in the United States. Although biotyping is used for epidemiologic purposes, it is somewhat subjective because it is based on subtle differences, including requirements for higher CO_2 tensions for growth, production of hydrogen sulfide, growth on media containing dyes (thionine or basic fuchsin), and agglutination with monospecific A and M antisera.[5,6]

Because brucellae use multiple mechanisms to protect against DNA mutations, there is a 94% identity at the nucleotide level across the genus. The high homology in the *Brucella* genus has led some scientists to propose that brucellae are actually one species.[7] Because of the stability of *Brucella* genomes, molecular assays designed to assess genetic relationships between isolates (ie, restriction fragment length polymorphism) are ineffective for epidemiologic investigations or strain comparisons. A recent molecular technique, variable number tandem repeat, which evaluates strings of nucleotide repeats in noncoding areas of the chromosome, has been used to compare genetic relationships among strains.[8]

Transmission of *B abortus* is primarily through direct or aerosolized mucosal contact with fluids or tissues associated with the birth or abortion of infected fetuses.[9] Because bacterial concentrations in fetal fluids or placenta after abortion can be as high as 10^9 to 10^{10} colony-forming units (CFUs)/g and minimum infectious doses are estimated in the 10^3 to 10^4 CFU range, abortion events can laterally transmit brucellosis to many cattle that have contact with birthing materials. Vertical transmission to offspring can occur through shedding of *B abortus* in milk. Although *Brucella* can colonize the male reproductive tract and cause seroconversion, bulls are considered dead-end hosts, as venereal transmission is not considered to be of significance for *B abortus*.[10] *Brucella* can temporarily be recovered from environmental samples (ie, soil) associated with infected animals. However, *B abortus* is not considered to be a commensal bacterium, and environmental persistence is generally accepted to be of no epidemiologic importance because direct or close contact with aborted material or infected animals is required for transmission.[11] Therefore, maintenance of *B abortus* in cattle requires continual infection of susceptible hosts.

Once *B abortus* has entered a susceptible host, bacteria initially localize in lymphatic tissues draining the site of entry. After a brief period of bacteremia, brucellae localize in phagocytic and reticuloepithelial cells throughout the body.[9] Bacteremia occurs periodically and transiently in chronically infected cattle, but the number of bacteria in the blood is at low numbers as *B abortus* is infrequently isolated from blood samples obtained from seropositive cattle. Although controversial, the preferential localization of *B abortus* in the reproductive tract has been hypothesized to be related to the high concentration of erythritol in fetal placenta because this sugar promotes the growth of *B abortus* and is metabolically preferred over glucose.[12]

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The entry of nonopsonized smooth *Brucella* into phagocytic cells relies on the cytoskeleton of the host cell for internalization and occurs through interactions of the bacterial lipopolysaccharide O side chain with cholesterol-rich microdomains, termed lipid rafts, within the phagocyte plasma membrane.[13–16] Internalized *Brucella* initially localize within phagocytes in an acidified membrane-bound compartment (phagosome) where they are exposed to free oxygen radicals generated by the respiratory burst.[17] Early localization in an acidified environment is important for the replication and survival of *Brucella*, and it induces the expression of the *virB* operon, a type IV secretion system. Activation of the *virB* operon neutralizes the pH of the phagosome, and other *Brucella* genes modify the phagosomal compartment to prevent phagosome maturation and lysosome fusion.[18] This allows *Brucella* to establish an intracellular niche with limited exposure to oxygen- or nitrogen-free radicals but limited in available nutrients. Most brucellae (approximately 70%–85%) are eliminated by phagolysosome fusion, but creation of the modified phagosomal compartment allows intracellular survival of some.[19] *Brucella* uses stationary-phase physiology and siderophores to scavenge iron as a mechanism for long-term survival within the nutrient-poor intracellular environment of the modified phagosome.[11] This unique internal environment is also responsible for limiting antibiotic action and explains some of the discrepancies between in vitro and in vivo studies on the survival of brucellae.[19]

In contrast, opsonization of *Brucella* strains increases the entry 10-fold. However, such an entry is targeted to phagosomes that mature and fuse with lysosomes to form phagolysosomes. Localization within phagolysosome compartments leads to intracellular killing of brucellae by monocytes.

DISEASE PREVENTION/REGULATORY PROGRAMS

Brucellosis regulatory programs were primarily developed as the most efficient way to prevent human infections.[20–23] These programs also assist producers in preventing economic losses that are associated with fetal losses, decreased milk production, and reduced fertility. The brucellosis eradication program in the United States is based on (1) vaccination of prepubescent heifers, (2) serologic testing at the first point of assembly (livestock markets, stockyards), (3) traceback of reactor animals to the herd of origin, (4) quarantine of infected herds, and (5) test and removal of seropositive animals or herd depopulation.[1] Because eradication programs are expensive and require good record keeping and infrastructure, brucellosis programs in some countries may be designed to control brucellosis rather than to eradicate it.

Although antibody responses do not correlate with protection, identifying humoral responses is a valid method to determine if an animal has been exposed to *Brucella* spp. Conventional brucellosis surveillance tests are primarily based on the detection of antibodies against an immunodominant epitope on the *Brucella* lipopolysaccharide, the O side chain (perosamine residue). In the United States, serologic methods that combine screening tests (high sensitivity, lower specificity) with confirmatory tests (high specificity, lower sensitivity) are designed to detect reactor animals economically and accurately. The presence of antibodies against *Brucella* does not necessarily mean that the animal is infected at the time of testing but does indicate that the animal has been previously infected with *Brucella*. Regulations consider any seropositive animal to be infected because brucellae are intracellular pathogens and isolation by noninvasive microbiologic methods is not always reliable. Accurate assessment of *Brucella* infection requires sampling of internal lymphatic tissues, which can only be obtained postmortem at necropsy or during processing at an abattoir.

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CATTLE VACCINATION

Vaccination is a critical tool to control or eradicate brucellosis; it primarily prevents clinical effects of the disease (ie, abortions or infected calves) that lead to transmission.[24] In the United States, routine brucellosis vaccination is administered via intramuscular or subcutaneous injection and limited to prepubescent heifers (4–12 months). Vaccination recommendations were originally designed to minimize the retained titers from strain 19 vaccination. Vaccination programs have not included males because they are not considered to have a significant role in disease transmission. In infected herds, vaccination of adult cows with a reduced vaccine dosage has been conducted with regulatory approval.

Long-term protection of cattle against brucellosis is associated with the stimulation of cellular immunity, whereas antibodies are considered to play a minor role in protecting cattle against *B abortus*.[25] Although specific correlates of protective immunity are currently not known in cattle, it is believed that protection is mediated by the T_H1 subset of $CD4^+$ lymphocytes and is associated with the production of interferon-γ.[26] Despite efforts to develop a nonliving brucellosis vaccine, killed vaccines are generally deficient in the stimulation of cellular immune responses and are less efficacious than live vaccines in cattle and other animals.[25] Delivery of brucellosis vaccines by alternative routes (ie, oral and intraconjunctival) has been reported to be efficacious against *B abortus*, but protection is less consistent or repeatable as compared with parenteral vaccination.

Most effective regulatory programs combine vaccination and serologic testing. Vaccination programs are very effective in reducing clinical effects and disease transmission, but vaccination alone has never been effective in eradicating brucellosis from any animal population. Even when the prevalence of brucellosis is low, elimination of vaccination with reliance on test and removal programs can be associated with resurgence of human or livestock brucellosis. Efforts to reduce brucellosis vaccination in the United States in the early 1960s and early 1970s resulted in an increased number of *Brucella*-infected cattle herds (Dr Mike Gilsdorf, personal communication, 2009). Currently available brucellosis vaccines for cattle (strains RB51 and 19) are highly effective in reducing production losses caused by brucellosis and in reducing transmission but are less effective in preventing livestock from being infected with field strains of *Brucella* spp or seroconverting after exposure. Vaccination is not effective in preventing seroconversion if cattle are exposed to infectious dosages of *B abortus* field strains. Vaccinated cattle can test seropositive after exposure irregardless of infection status.

Experimental efficacy studies ensure that all animals receive a known infectious dose of a virulent *Brucella* strain at the most susceptible time (midgestation). However, vaccine-induced protection under field conditions is most likely greater than that in experimental studies because exposure times and dosages under field conditions may not be optimal for infection.[24] Field efficacy may be influenced by factors (nutrition, environment, stress, or concurrent infections) that deleteriously affect immunity. It has been demonstrated that abortion and infection rates are directly related to the exposure dose of a virulent *B abortus* strain,[27,28] and high challenge dosages can overwhelm vaccine-induced protection.[29] Because of the large number of variables that may influence vaccine-induced protection against brucellosis, assignment of a specific numeric value for *Brucella* vaccine efficacy under field conditions would be unreliable. Rather, numeric estimates of protection should be viewed as being approximations and as reflecting differences in effects between paired vaccinated and nonvaccinated animals under controlled conditions. It should also be noted that

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abortion or infection rates in field or experimental studies may be influenced by known or unknown factors.

Strain 19

The *B abortus* strain 19 vaccine (S19) was the principal vaccine used for cattle vaccination in the United States until 1996. It was first isolated in 1923, experimentally evaluated in the 1930s, and introduced for field use in the United States in 1941. Derived from a virulent isolate of *B abortus*, S19 was naturally attenuated when the virulent culture was inadvertently left at room temperature for 1 year. As a smooth strain, S19 expresses the O side chain on its lipopolysaccharide. The O side chain of S19 induces serologic responses on brucellosis surveillance tests, which cannot be differentiated from antibody responses caused by infection with field strains of *B abortus*. The persistence of S19 until adulthood in an estimated 2 animals per 100,000 calfhood vaccinates can contribute to retained titers that interfere with serologic surveillance. Retained titers from S19 vaccination can be problematic in countries, such as the United States, where serologic surveillance is emphasized. Some countries in South America continue to use the original full dosage of S19 (2.5–12 $\times$ 10^{10} CFUs) for calfhood vaccination, whereas the United States and other countries switched to a reduced dosage (3–10 $\times$ 10^9 CFUs) in the 1980s in an effort to reduce the number of calfhood vaccinates having retained antibody titers.

Numerous studies over many years have demonstrated that calfhood vaccination with full and reduced dose of S19 protects cattle against brucellosis. In a large field trial in the United States in 1936, full-dose S19 vaccination of more than 18,000 calves in 260 *Brucella*-infected herds led to normal parturition in more than 96% of the calves when they reached adulthood.[30] Similar efficacy results were demonstrated in cattle vaccinated with reduced dosages of S19.[31] Retrospective data have also demonstrated that S19 calfhood vaccination in *Brucella*-infected herds reduces the number of seroreactors and facilitates the elimination of brucellosis from the herd.[32] Based on experimental data that used the full calfhood dose and evaluated protection in cattle up to 9 years of age, it was estimated that approximately 65% to 75% of all vaccinated animals were completely protected for their productive lifespan.[28,33] Similar data for the duration of immunity has not been reported for the reduced calfhood dosage of S19.

S19 has also been effectively used as an adult vaccine in *Brucella*-infected herds and has facilitated reductions in brucellosis prevalence.[34–36] Low dosages (0.3–3 $\times$ 10^9 CFUs) of S19 in adult cows reduce brucellosis seroprevalence and fetal losses under field conditions. A large adult vaccination study in cattle demonstrated a reduction in human infections.[34] Adult vaccination seems beneficial particularly for larger herds in which test and slaughter methods alone might not be effective, and a large and perhaps prohibitive portion of seroreactors might have to be removed.[36] Although beneficial for controlling or eradicating *B abortus* from cattle, adult vaccination with S19 can be associated with adverse effects. In 2 studies in which calfhood vaccination status was not clarified, 22% of pregnant cattle aborted after vaccination with 5.8 $\times$ 10^9 CFUs of S19[35] and 14.3% of cattle between 1 and 9 months of gestation aborted after vaccination with 1.2 $\times$ 10^7 CFUs of S19.[37] In a large field study involving more than 10,000 dairy cattle in herds that routinely used calfhood vaccination, less than 1% of cattle aborted after vaccination with a standard dosage of S19.[36] In the later study, significant reductions in milk production for 8 to 12 days after vaccination were noted.

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Strain RB51

In 1996, the United States essentially eliminated the use of S19 and switched to the *B abortus* strain RB51 vaccine. The RB51 strain is rough because it has minimal expression of the lipopolysaccharide O side chain. Because of the lack of O-side-chain expression, RB51 does not induce antibody responses that are detected by conventional brucellosis serologic tests. Cattle that were calfhood vaccinated with S19 did not seroconvert as adults when boostered with calfhood dosages of RB51. Under experimental conditions, cattle vaccinated with 1 to 3.4×10^{10} CFUs of RB51 have reduced incidents of abortion or *Brucella* infection at necropsy when compared with nonvaccinated cattle.[38,39] Ongoing experiments suggest that high levels of immunity persist for 4 to 5 years of age in animals that are calfhood vaccinated with RB51 (Olsen, unpublished, 2009).

As with S19, adult vaccination with RB51 has been used to protect cattle against brucellosis. Under experimental conditions, parenteral vaccination of pregnant cattle with 1 to 3×10^9 CFUs of RB51 is safe and efficacious for the subsequent pregnancy.[40] Like S19, RB51 can induce abortions under field conditions.[41] Current data suggest that calfhood vaccinated cattle can be safely booster vaccinated with RB51 (1–3×10^9 CFUs) as pregnant adults.[42,43]

Comparison of S19 with RB51

At present, the greatest controversy in regard to *B abortus* vaccination is whether calfhood vaccination with RB51 induces equivalent protection in cattle as compared with S19 vaccination. Although several experimental and field studies have demonstrated that RB51 is efficacious in protecting cattle against experimental infection, there are only limited data in which the experimental design included simultaneous comparisons of both vaccines. In a series of calfhood vaccination studies at the National Animal Disease Center involving relatively small numbers of cattle, a nonsignificant reduction in the abortion rates was observed in S19 vaccinates (0 of 19) when compared with RB51 vaccinates (1 of 29). Cattle vaccinated with S19 had slight reductions in the infection rates at necropsy (1 of 22, 5%) when compared with those vaccinated with RB51 (3 of 29, 10%).

The prevalence of brucellosis within the livestock population, the intensity of serologic surveillance, the regulatory pressure, and economics are factors that may influence vaccine selection. In the absence of intense serologic surveillance, retained titers from S19 vaccination would be unlikely to present significant regulatory problems. In contrast, in the late 1980s and early 1990s in the United States, most seropositive or suspect responses detected at market surveillance were found by epidemiologic investigation to have resulted from S19 vaccination. In the United States, switching to RB51 vaccination has been beneficial in reducing economic costs associated with reactor traceback efforts, and it has facilitated the identification of remaining *Brucella*-infected herds. In countries with a high prevalence of brucellosis and/or limited regulatory programs, S19 may be the vaccine of choice because it may be slightly more protective and many countries can produce commercial S19 vaccines. However, in the United States, where prevalence of brucellosis is low and serologic surveillance is high, the RB51 vaccine is preferred because of its lack of interference with serologic surveillance and comparable efficacy in protecting against brucellosis.

Other Vaccines

A heat-killed *B abortus* strain 45/20 vaccine was developed in the 1920s and introduced for field use. As a rough strain, it offers serologic advantages like RB51 but cannot be used

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as a live vaccine because of its reversion to a smooth form in vivo.[44] Because it requires 2 injections and is not as protective as S19, its use worldwide has been very limited.[45]

Several potential new vaccine strains, predominantly genetically engineered strains,[46–48] have been evaluated in laboratory animal models. In addition, new encapsulation methods that may increase immunogenicity have been evaluated. The lack of development of new vaccine candidates may be partly because of the lack of progression from laboratory animals to the host species of interest, the high cost of efficacy studies in large ruminants, and the limited economic return on new brucellosis vaccines. In general, candidates that have progressed into ruminant studies at this time have not demonstrated protection that exceeds currently available vaccines.

The use of laboratory animal models, such as mice, for *B abortus* research is inexpensive when compared with studies done in the natural host. Inherent deficiencies in laboratory animals as predictive models for natural hosts may also explain conflicting results between mice and large ruminant studies[46,49] and may also have negatively impaired development of new vaccines. As laboratory animal models are generally used as positive predictors of efficacy in the host species of interest, the possibility that protective cattle vaccines may have been discarded because of negative results in murine models cannot be eliminated.

OTHER RESERVOIR HOSTS

Regulatory programs have been very effective in reducing or eliminating the prevalence of targeted diseases in domestic livestock, but spillover of the disease from domestic livestock to wildlife has allowed the establishment of new reservoir hosts. The establishment of wildlife reservoirs of disease has complicated regulatory efforts by allowing transmission back to domestic livestock and has allowed the persistence of *B abortus*.[24] It is ironic that most experts agree that brucellosis in wildlife reservoirs originated from *Brucella* infections in domestic livestock. Numerous studies have documented spillover into hosts in whom the disease is not manifested and transmission does not occur (dead-end or spillover hosts). However, current problems in the UnitedStates are associated with wildlife species in which the disease is manifested and transmission does occur (maintenance hosts).

The United States currently has at least 2 wildlife species that function as maintenance hosts for *B abortus*: elk (*Cervus elaphus nelsoni*) and bison (*Bison bison*). Brucellosis in bison remains controversial because of its infection of a historically important free-ranging herd in arguably the most eminent park in the United States. Since the elimination of hands-on management of bison in Yellowstone National Park in 1967,[50] the herd has increased from 397 animals to peak populations of approximately 3000 to 5000 bisons. Wolves have been reintroduced into the environment, but predation does not have a significant effect on the bison population.[50] Seroprevalence of brucellosis in the Yellowstone National Park bison is approximately 50%,[51] and data suggest that *B abortus* can be isolated from approximately 46% of seropositive bisons.[52] Bulls have a high seroprevalence for brucellosis, and recent data have suggested that behavior may play a role in the transmission to males. Experimental studies have demonstrated that bisons can transmit *B abortus* to co-housed cattle,[53] although documentation of transmission under field conditions is lacking. Lack of documented transmission is most likely because of regulatory efforts to maintain spatial and temporal separation of free-ranging bison from cattle. The risk of interspecies transmission may also be prevented by the rapid removal of aborted fetuses from the landscape by numerous predators in the environment. At present, no cases of human brucellosis have been linked to brucellosis in bison.

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In the Greater Yellowstone Area (GYA), the total population of elk (*C elaphus nelsoni*) is estimated at 120,000 animals.[54] Because of the loss of traditional winter-feeding ranges due to development and agriculture, beginning in 1909, the state of Wyoming and the US Fish and Wildlife Service established 23 feedgrounds, which provide hay during winter months. Seroprevalence of brucellosis averages approximately 35% in adult female elk on Wyoming feedgrounds compared with 2% to 3% in elk that overwinter off the feedgrounds.[55] In the neighboring state of Idaho, seroprevalence in areas of artificial feeding ranged from 12% to 80%, whereas hunter surveys suggested population seroprevalence of only 2% to 3%.[56] Epidemiologic data have suggested that the 10 *Brucella*-infected cattle herds identified in the GYA since 2002 have resulted from wildlife transmission, with elk implicated as the most likely source. Brucellosis in elk has been epidemiologically linked to 2 zoonotic infections in which the only significant risk factor was contact with elk carcasses.[57]

Although feral swine are most commonly infected with *Brucella suis*, recent work has suggested that they may also serve as maintenance hosts for *B abortus*.[58] Field transmission of *B abortus* from feral swine to cattle has not been documented at present. However, Texas and other southeastern states report numerous cases of seropositive cattle, which are shown by bacterial culture to be infected with *B suis*.

OTHER BRUCELLA SPP IN CATTLE

Feral swine are rapidly expanding their ranges across the United States, with at least 44 of 50 states reporting populations.[59] Feral swine populations were estimated at 4 million animals in 1999 by one report,[60] whereas another report estimated in 2000 that Texas alone had 3 million.[61] In at least 14 states, brucellosis has been documented to be present in feral swine populations,[62] with some populations demonstrating high seroprevalence.[63] Feral swine shed *B suis* for extended periods of time in urine and mucosal secretions. Numerous field cases have been documented in which cattle were infected with *B suis* from feral swine. Cattle infected with *B suis* have positive responses on brucellosis serologic tests, which cannot be differentiated from responses after *B abortus* infection. Differentiating *B suis* from *B abortus* requires bacteriologic isolation, which is not always successful.

In some areas of the world, *Brucella melitensis* has become endemic in cattle populations.[64] Although extremely rare in the United States, *B melitensis* was isolated from a cow in southern Texas in 1999.[65] Previously, *B melitensis* was last detected in sheep and goats in southern Texas in 1969. Limited data are currently available on vaccines to protect cattle against *B melitensis*. Currently, the World Organization for Animal Health, formerly known as the Office International des Epizooties, does not recommend the use of the *B melitensis* Rev.1 vaccine in cattle.[66] Data to support the use of other vaccines in protecting cattle against *B melitensis* are currently lacking.

New and phenotypically diverse strains of *Brucella* have been isolated from sea mammals (eg, dolphins, porpoises, seals, beluga whales).[67] Some isolates have caused zoonotic infections in people in England, Peru, and New Zealand. Under experimental conditions, cattle infected conjunctivally or intravenously with a seal *Brucella* isolate from Puget Sound, Washington, did seroconvert on brucellosis serologic tests. Abortion occurred in 2 of 3 animals after intravenous injection but not in cattle conjunctivally exposed.[68] The marine *Brucella* are intriguing, as current data suggest that lungworms (*Parafilaroides* and *Phocoena*) may play a role in natural transmission in seals and porpoises.[69] However, current knowledge suggests that marine strains of *Brucella* are of low risk for causing brucellosis in cattle.

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HUMAN INFECTION

Although reproductive losses caused by *B abortus* can be expensive to cattle producers, the primary impetus for regulatory programs to control brucellosis in cattle is to prevent zoonotic infections in humans. Multiple studies have demonstrated that addressing brucellosis in animal reservoirs is the most cost-efficient mechanism for controlling human brucellosis.[20–23] Human infection with *B abortus* can occur from direct contact with infected animals or tissues or fluids associated with abortion. However, consumption of nonpasteurized dairy products from *Brucella*-infected animals is the most frequent route of human infection. Entry can occur across mucosal surfaces by aerosolization into respiratory tissues, by oral consumption, or by penetration through breaks in the epidermis. Inadvertent exposure to live vaccine strains, most commonly via needle sticks, has also been a frequent source of human infections, especially in the veterinarian profession.[70] *Brucella* spp are probably also the most common zoonotic agent for causing laboratory-associated infections. Human-to-human transmission through breast milk or coitus has been occasionally described for *B melitensis*[71] but does not appear to be of significance for *B abortus*.

Human infection with *B abortus* can cause chronic, debilitating clinical illness with nonpathognomonic symptoms.[72,73] Typically, infected humans have recurrent episodes of fever (undulant fever), splenomegaly, hepatomegaly, and asthenia and frequently have malodorous perspiration. Although generally targeted to lymphoreticular tissues, *Brucella* can localize in almost any tissue, including joints, brain, and cardiac muscle, with inflammatory responses leading to complications, such as osteoarthritis, meningitis, and myocarditis. Bone and joint involvement is the most common complication of human brucellosis,[73] whereas endocarditis is most frequently associated with fatalities.

Between the years of 1996 and 2005, the United States had 1057 cases of brucellosis reported to the Centers for Disease Control and Prevention (105.7 cases/year; www.cdc.gov/mmwr). This number is less when compared with the reported average of 218 human cases per year in 1967 to 1972 when brucellosis was widespread in domestic livestock. Before the initiation of the State/Federal Cooperative Brucellosis Control Program, the Centers for Disease Control and Prevention reported 6321 human cases of brucellosis in 1947.

SUMMARY

Brucellosis is a zoonotic disease that causes reproductive losses in cattle and has international trade implications. During the last 80 years, the United States has invested considerable financial resources in eliminating this disease. With the exception of the introduction of the *B abortus* RB51 strain in 1996, tools to control or eradicate brucellosis have not significantly changed for decades. Vaccination and serologic detection remain important for regulatory programs. Although brucellosis is associated with economic losses for producers, justification for the cost of regulatory programs is based on data that indicate that addressing the disease in animals is the most cost-effective mechanism to prevent human disease. As the United States nears completion of programs to eradicate brucellosis from domestic livestock, the persistence of *Brucella* spp within wildlife reservoirs will increase in importance. Although eradicating brucellosis in wildlife reservoirs will be difficult and costly, it is unlikely that the United States can be considered totally free of brucellosis in cattle, without resolution of the disease in free-ranging wildlife reservoirs.

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Investigations on Deer to Deer and Deer to Cattle Transmission of the Vaccine *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)

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Abstract

Mycobacterium bovis is the causative agent of tuberculosis in animals and can cause tuberculosis in humans clinically indistinguishable from that caused by *Mycobacterium tuberculosis*. Efforts to eradicate bovine tuberculosis have significantly decreased prevalence in developed countries. However, some countries have found it impossible to eradicate bovine tuberculosis due to the presence of a wildlife reservoir of *M. bovis*. In Michigan, USA there exists a reservoir of *M. bovis* in free-ranging white-tailed deer. Vaccination with *M. bovis* BCG is one approach to controlling tuberculosis in wildlife. Nevertheless, use of a live vaccine generates concerns about exposure risk to non-target species, including domestic livestock. Unintentional exposure of cattle to BCG may result in increased numbers of false positive tuberculin skin test reactions. Twenty-nine white-tailed deer received 1 SC dose of 10^7 colony-forming units of *M. bovis* BCG Danish 1331 (n=19) or no vaccination (n=10). Vaccinated and non-vaccinated deer were co-mingled with opportunity for direct and indirect contact. Twelve unvaccinated Holstein calves were housed in a separate paddock with no means of direct contact with deer; however, indirect contact through the sharing of feed and water was permitted. After 180 days, 11 out of 15 vaccinated deer and 4 out of 8 non-vaccinated deer were classified as reactors using the tuberculin skin test. All 12 calves were categorized as non-reactors, by both the tuberculin skin test and BOVIGAM™ assay. Vaccination of free-ranging white-tailed deer with BCG Danish is unlikely to have a deleterious effect on tuberculosis surveillance measures in cattle.

Keywords: Deer; Mycobacteria; Tuberculosis; Vaccination; Wildlife

Introduction

Mycobacterium bovis is the causative agent of tuberculosis in animals and has one of the broadest host ranges of any pathogen. *Mycobacterium bovis* can cause tuberculosis in humans clinically indistinguishable from that caused by *Mycobacterium tuberculosis*. In the early 20th century, public health concerns posed by transmission of *M. bovis* from cattle to humans, through consumption of contaminated milk or meat, prompted the United States, and other countries, to implement national programs to eradicate tuberculosis from cattle. Efforts have centered on slaughter of cattle reacting positively to a tuberculin skin test, and most eradication campaigns have been successful in decreasing the prevalence of bovine tuberculosis. However, some countries have found it impossible to eradicate bovine tuberculosis due to the presence of a wildlife reservoir of *M. bovis*. In most cases, wildlife originally acquired tuberculosis from cattle; however, the disease is now spilling back from wildlife to cattle, impeding the progress of eradication [1,2]. In an effort to reduce wildlife to cattle transmission of *M. bovis* some countries are investigating the possible role of wildlife vaccination.

In 1994, a free-ranging white-tailed deer (*Odocoileus virginianus*) in Michigan, USA was diagnosed with tuberculosis due to *M. bovis* [3]. Subsequent surveys identified the first known reservoir of *M. bovis* in free-ranging wildlife in the United States and a significant impediment to ongoing efforts to eradicate bovine tuberculosis from domestic livestock [4,5]. Current disease control measures include, among other things, decreasing deer density through increased hunting. Control and surveillance measures have now been in place in Michigan for over 10 years and a significant reduction in apparent prevalence of tuberculosis in deer has been achieved; however, public support for further population reduction is waning [6]. Vaccination of deer could be used in areas of sustained high disease prevalence, in order to prevent infection, disease, or transmission. Recently, protection to experimental infection with *M. bovis*, was demonstrated by subcutaneous (SC) and oral vaccination of white-tailed deer with *M. bovis* Bacillus Calmette Guerin (BCG) Danish [7,8]. However, transmission of BCG from vaccinated deer to co-mingled unvaccinated deer was also documented [7].

Potential vaccination of wildlife with a live vaccine generates various concerns, not least of which is the exposure risk to non-target species, including domestic livestock. The purpose of this study was to evaluate the potential for transmission of BCG from vaccinated deer to unvaccinated deer or cattle through direct or indirect contact, respectively. Unintentional exposure of cattle to BCG may result in increased numbers of false-positive tuberculin skin test reactions. An increase in false-positive reactions would confound current bovine tuberculosis surveillance efforts and lead to the unnecessary slaughter of otherwise normal animals.

Materials and methods

Animals and vaccination

Twenty-nine white-tailed deer (6-18 months old) were obtained

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from a captive breeding herd (tuberculosis free) at the National Animal Disease Center (Ames, Iowa, USA). All deer were housed and cared for according to institutional guidelines. Deer were randomly assigned to one of two groups, 1 SC dose of 10^7 colony-forming units (CFU) *M. bovis* BCG Danish 1331 (n=19) or no vaccination (n=10). Deer were vaccinated subcutaneously on the right side of the neck, midway between the head and shoulder. Vaccinated and non-vaccinated deer were housed together in an outdoor paddock with shared feeding and watering sites. Twelve Holstein calves (6 months old, castrated males) were not vaccinated and housed in a similar, but separate, paddock with no means of direct contact with deer. On a daily basis, deer were moved to the cattle paddock and cattle were moved to the deer paddock in such a manner as to prevent direct deer-cattle contact. Deer feeding was done such that excess feed remained in feeders when cattle entered the deer paddock. Switching of pens to afford an opportunity for indirect deer-cattle contact proceeded for 180 days.

Vaccine

The *M. bovis* BCG Danish 1331 strain was grown in Middlebrook's 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) as described for virulent *M. bovis* [9]. Mid log-phase growth bacilli were pelleted by centrifugation at 750 x *g*, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, MD). A single vaccine dose consisted of 10^7 CFU *M. bovis* BCG in 2.0 ml PBS.

Evaluation of vaccine shedding

Immediately prior to the study, and again at the conclusion of the study, all deer and calves were tested for exposure to *M. bovis* by use of the comparative cervical tuberculin skin test (CCT) as described previously [10,11]. Deer and calves were categorized as reactors or non-reactors, according to USDA guidelines [12]. At the same time, cattle were also evaluated using a commercially available interferon-gamma (IFN-γ) assay (BOVIGAM™, Prionics, Zurich, Switzerland) as described previously [10]. Both the CCT and the BOVIGAM™ assay are approved tests for cattle in the United States Department of Agriculture (USDA) bovine tuberculosis eradication effort. The USDA has also approved the CCT for use in deer. The CCT requires injection of purified protein derivative (PPD) from *M. bovis* (National Veterinary Services Laboratories, USDA, Ames, IA) and *Mycobacterium avium* (National Veterinary Services Laboratories) at 2 different sites on the lateral neck [12]. Exposure to *M. bovis* is indicated when the change in skin thickness at the *M. bovis* PPD injection site is greater than that measured at the *M. avium* PPD injection site. Conversely, exposure to non-tuberculous mycobacteria is indicated by a change in skin thickness at the *M. avium* injection site greater than that measured at the *M. bovis* PPD injection site [12].

All deer were monitored for vaccine shedding by collection of oronasal swabs and feces. Samples were collected prior to vaccination and on days 14, 28, 42, 56, 89 and 179 after vaccination. One swab from each nostril and one oral swab were processed together representing a single oronasal sample from each deer at each time point. Approximately 2 gm feces was obtained from each deer at each time point and processed for isolation of *M. bovis* BCG.

Necropsy and tissue processing

All deer and all calves were euthanized by intravenous sodium pentobarbital and examined at the termination of the study (180 days after vaccination). At necropsy, the following tissues were examined grossly; liver, lung; mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and superficial cervical lymph nodes; and palatine tonsil. Samples of mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic and mesenteric lymph nodes were collected for bacteriological isolation of BCG as described [13]. Tissues from an individual deer were submitted for bacteriological culture in 3 pools; head pool (medial retropharyngeal, mandibular and parotid lymph nodes), thoracic pool (tracheobronchial and mediastinal lymph nodes) and abdominal pool (mesenteric and hepatic lymph nodes). Tissues with gross lesions compatible with tuberculosis were further processed for microscopic analysis as described [14,15].

Isolation and identification of mycobacterial isolates

Tissue and swab samples were processed for isolation of *M. bovis* BCG as previously described [13] using both the BACTEC 460 radiometric system and BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson and Company, Sparks, MD). Isolates of *M. bovis* BCG were identified by a combination of Ziehl-Neelsen acid-fast staining, biochemical tests and nucleic acid probes (ACCUPROBE, Gen-Probe, San Diego, CA). Further identification was done using 16S ribosomal DNA sequencing as described previously [16]. Sequences were then identified through use of a mycobacterial species sequence database [17].

From some oronasal swabs a mycobacterial species was isolated; however, identification by the above methods was not possible due to contamination and overgrowth by non-mycobacteria. These mycobacterial isolates were further analyzed by PCR using the following protocol. Two milliliters of MGIT culture were centrifuged for 3 min at 10,000 x *g* to pellet mycobacteria. Pellets were washed with 1 ml Tris-EDTA (TE) buffer. The pellet was suspended in 200 µl Buffer AL (Qiagen, Valencia, CA) and lysed by boiling for 8 min. After cooling to less than 56°C, 20 µl Proteinase K (Qiagen) was added and incubated overnight at 56°C or until completely lysed. Liberated DNA was isolated using QIAMP DNA Mini Kit (Qiagen) according to the manufacturer's directions. Detection of IS6110 was performed in 50 µl reactions using FASTSTART Taq (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions using primers for IS6110 as described previously [18]. Duplicate reactions were run using primers Sp1 and Sp2 [19] to serve as a PCR positive control and to confirm the presence of mycobacteria. Samples were analyzed by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide.

Results

Tuberculin skin testing and BOVIGAM™

Prior to the study all deer and all calves were classified as non-reactors (negative for exposure to *M. bovis*) according to USDA guidelines for interpretation of CCT results [12]. Likewise all calves were categorized as negative using the BOVIGAM™ assay and manufacturer's recommendations for interpretation of results. During the course of the study 6 deer died of causes unrelated to the experimental procedure (trauma, necrobacillosis, or *Pasteurella multocida* induced pneumonia). Two of six were non-vaccinated deer and 4 of 6 were vaccinated deer. From these 6 deer, tissues were collected and processed as described above.

At the conclusion of the study (n=23 deer) 11 out of 15 vaccinated

deer and 4 out of 8 non-vaccinated deer were classified as reactors using the CCT. All other deer were categorized as non-reactors (Table 1). All 12 calves were categorized as non-reactors or negative, at the conclusion of the study, by both the CCT and BOVIGAM™ assay, respectively.

Vaccine shedding

From vaccinated and non-vaccinated deer, a total of 160 oronasal swabs and 150 fecal specimens were processed for bacteriological culture. At no time during the course of the study was BCG isolated from fecal samples. Bacteriological culture of oronasal samples yielded a number of isolates of non-tuberculous mycobacteria. *Mycobacterium fortuitum* was isolated from 5 of 19 vaccinated deer (Table 2) and 9 of 10 non-vaccinated deer (Table 1). Isolations were from a single time point from 9 out of the 14 deer and multiple time points from 5 out of the 14 deer (Table 1 and Table 2). *Mycobacterium smegmatis* was isolated from oronasal swabs from 2 vaccinated deer at one time point each and *Mycobacterium alvei* was isolated from 2 non-vaccinated deer at a single time point. From 84 of the 160 oronasal samples processed, an acid fast bacillus belonging to the genus *Mycobacteria* was isolated, but further speciation was prohibited due to contamination and overgrowth by non-mycobacteria. PCR analysis of these isolates identified 2 out of 84 as BCG; isolated from 1 vaccinated deer 89 days after vaccination and 1 non-vaccinated deer 56 days after vaccination (Table 1 and Table 2). Final CCT results identified the vaccinated deer as a reactor and the non-vaccinated deer as a non-reactor.

Necropsy and tissue processing

Focal caseonecrotic granulomas (1-1.5 cm) were seen in the medial retropharyngeal lymph nodes of 1 vaccinated deer (skin test reactor) and 1 non-vaccinated deer (skin test non-reactor). Microscopically, lesions were characterized by coalescent caseonecrotic granulomas composed of macrophages, lymphocytes and multinucleated giant cells, all surrounded by thin bands of collagen fibers. Ziehl-Neelsen staining did not reveal acid-fast bacteria in any lesions. BCG was isolated from tissue samples of 6 out of 19 vaccinated deer and 1 out of 10 non-vaccinated deer. Specifically BCG was isolated from tissues of the thoracic pool from each of the 6 vaccinated deer and the single non-vaccinated deer. Additionally, BCG was isolated from tissues of the head pool from the non-vaccinated deer (Table 1). BCG was not isolated from either deer with caseonecrotic granulomatous lesions in the medial retropharyngeal lymph nodes. In addition to BCG, *Mycobacterium avium*, *Mycobacterium kansasii* and *Mycobacterium simiae* were also isolated from a single deer each.

Discussion

The present study suggests that indirect contact would not result in transmission of BCG Danish from vaccinated deer to cattle, even under circumstances of repeated and sustained indirect contact. If indirect transmission were to occur, the magnitude of shedding would likely be critical. Shedding of BCG was seen inconsistently and in a limited fashion from oronasal samples, and not from feces. In humans with suspected tuberculosis, sputum and gastric fluids may be examined for acid-fast bacilli. Oronasal samples, as collected in the present study, would be most analogous to sputum samples from humans. Samples of rumen or abomasal fluids (analogous to gastric fluid) were not examined in the present study.

Previous studies have shown that oral exposure of calves to BCG does not reliably induce positive tuberculin reactivity as measured by the intradermal skin test or IFN-γ assays [20]. Reports of oral vaccination of cattle with 10^8 or 10^{10} CFU BCG demonstrated a positive skin test response in 3 of 9 and 6 of 10 calves, respectively. Four of nineteen orally vaccinated calves developed positive responses to the standard IFN-γ assay. In contrast all calves vaccinated SC with 10^6 CFU of BCG developed positive skin test responses. Clearly, in cattle, route of vaccination and vaccine dose affect tuberculin skin test status. Although the CCT and the BOVIGAM™ assays may not identify all cattle exposed to BCG, especially those exposed by the oral route; in the current study, bacteriological culture of tissues from non-vaccinated calves also failed to yield BCG, suggesting that BCG was not transmitted and did not colonize cattle.

Natural behavior of deer and cattle will impact the degree of contact, either direct or indirect. In the present study a form of indirect contact through sharing of feed and exposure to soiled paddocks

			Bacteriological Culture			
Deer ID	Pre CCT[1]	Post CCT[2]	Feces[3]	Oronasal[3]	Tissue[4]	Tissue Pool[5]
13	Neg	Neg	Neg	BCG[6] (56) *M. fortuitum* (14) UIM (14, 28, 42, 56, 89)	Neg	NA
35	Neg	Neg	Neg	*M. fortuitum* (28, 56, 89) *M. alvei* (14) UIM (42)	Neg	NA
1072	Neg	Neg	Neg	*M. fortuitum* (179) UIM (28, 42, 56, 179)	Neg	NA
32M	Neg	Neg	Neg	*M. fortuitum* (14) UIM (14, 28, 56, 89)	Neg	NA
4M[7]	Neg	ND	Neg	*M. fortuitum* (14) UIM (14, 28, 42, 56)	*M. simiae*	Head
6M[7]	Neg	ND	Neg	*M. fortuitum* (14) UIM (42)	*M. kansasii*	Thoracic
1M	Neg	Reactor	Neg	UIM (14, 42, 56, 89)	BCG	Head, Thoracic
14	Neg	Reactor	Neg	*M. fortuitum* (14) *M. alvei* (28) UIM (14, 42)	Neg	NA
29	Neg	Reactor	Neg	*M. fortuitum* (89) UIM (28, 42)	Neg	NA
20M	Neg	Reactor	Neg	*M. fortuitum* (14) UIM (14, 28, 42, 56, 89, 179)	Neg	NA

[1]Comparative cervical tuberculin skin test (CCT) prior to comingling. Neg = no evidence of prior exposure to *M. bovis*.
[2]CCT after 180 days of comingling.
[3]Feces and oronasal swab samples collected prior to comingling and on days 14, 28, 42, 56, 89 and 179 days of comingling. Isolate (sampling day after comingling).
[4]Isolation of mycobacteria from tissues collected at necropsy after 180 days of comingling. Neg = no mycobacteria isolated.
[5]For bacteriological isolation of mycobacteria, tissues were allocated into one of three pools; head (mandibular, parotid and medial retropharyngeal lymph nodes), thoracic (lung, mediastinal and tracheobronchial lymph nodes), or abdominal (mesenteric and hepatic lymph nodes).
[6]Unspeciated mycobacteria later identified by PCR as *M. bovis* BCG Danish.
[7]Deer died due to trauma unrelated to the experimental protocol prior to end of study.
UIM = Unspeciated mycobacteria not found not to be *M. bovis* BCG Danish by PCR.
NA = not applicable.
ND = not done.

Table 1: Summary of intradermal tuberculin test and bacteriological culture results from unvaccinated deer co-mingled with *Mycobacterium bovis* BCG Danish vaccinated deer for 180 days.

			Bacteriological Culture			
Deer ID	Pre CCT[1]	Post CCT[2]	Feces[3]	Oronasal[3]	Tissue[4]	Tissue Pool[5]
38	N	N	N	*M. fortuitum* (14, 28, 42, 56)	Neg	NA
21M	N	N	N	*M. fortuitum* (179) UIM (28, 42, 89)	Neg	NA
23M	N	N	N	UIM (14, 42, 89)	Neg	NA
24M	N	N	N	*M. smegmatis* (14) UIM (28, 42)	BCG	Thoracic, Abdominal
15	N	R	N	*M. fortuitum* (14) UIM (28, 42)	Neg	NA
36	N	R	N	*M. fortuitum* (179) UIM (28, 42, 56)	BCG	Thoracic
28M	N	R	N	UIM (14, 28, 42, 56, 89)	Neg	NA
2M	N	R	N	UIM (28, 42, 56, 89)	Neg	NA
30M	N	R	N	UIM (14, 28, 42)	Neg	NA
34M	N	R	N	BCG[6] (56) UIM (28, 42, 56)	*M. avium*	Thoracic
38M	N	R	N	*M. fortuitum* (14, 179) UIM (28, 42, 56)	BCG	Thoracic
33M	N	R	N	*M. fortuitum* (14) UIM (28, 42, 89)	Neg	NA
3M	N	R	N	*M. fortuitum* (179) UIM (14, 89, 179)	Neg	NA
5M	N	R	N	UIM (14, 28, 42)	Neg	NA
7M	N	R	N	UIM (14, 28, 42, 56)	Neg	NA
22[6]	N	ND	N	*M. fortuitum* (14) UIM (42, 89)	BCG	Thoracic
28[7]	N	ND	N	UIM (14, 28, 42)	Neg	NA
19M[7]	N	ND	N	*M. fortuitum* (56) UIM (28, 42, 89)	BCG	Thoracic
18M[7]	N	ND	N	*M. fortuitum* (56) UIM (14, 28, 42, 89)	BCG	Thoracic, Abdomen

[1]Comparative cervical tuberculin skin test prior to comingling. Neg = no evidence of prior exposure to *M. bovis*.
[2]Comparative cervical tuberculin skin test after 180 days of comingling.
[3]Feces and oronasal swab samples collected prior to comingling and on days 14, 28, 42, 56, 89 and 179 days of comingling. Isolate (sampling day after comingling).
[4]Isolation of mycobacteria from tissues collected at necropsy after 180 days of comingling. Neg = no mycobacteria isolated.
[5]For bacteriological isolation of mycobacteria, tissues were allocated into one of three pools; head (mandibular, parotid and medial retropharyngeal lymph nodes), thoracic (lung, mediastinal and tracheobronchial lymph nodes), or abdominal (mesenteric and hepatic lymph nodes).
[6]Unspeciated mycobacteria later identified by PCR as *M. bovis* BCG Danish.
[7]Deer died due to trauma unrelated to the experimental protocol prior to end of study.
UIM = Unspeciated mycobacteria found not to be *M. bovis* BCG Danish by PCR.
NA = not applicable.
ND = not done.

Table 2: Summary of intradermal tuberculin test and bacteriological culture results from *Mycobacterium bovis* BCG Danish vaccinated deer co-mingled with unvaccinated deer for 180 days.

was used to simulate a commonly observed form of deer to cattle contact. Admittedly, it is not possible to rule out the potential for more intimate contact to occur between deer and cattle in a natural setting rather than the restrictive indirect contact allowed in the present study. However, one study done in the natural surroundings of northeastern Michigan showed direct deer-cattle interactions to be exceedingly rare; conversely, indirect contact was common [21].

As previously observed white - tailed deer are exposed to and harbor a wide variety of non-tuberculous mycobacteria [22,23]. Sensitivity to non-tuberculous mycobacteria may result in false positive results upon intradermal tuberculin testing. In the present study, to differentiate intradermal tuberculin reactions due to non-tuberculous mycobacterial sensitivity from true *M. bovis* infection, the CCT was used in cattle and deer. The addition of *M. avium* PPD increases specificity of the CCT as compared to a single cervical test (SCT) using only *M. bovis* PPD. Deer or cattle sensitized to non-tuberculous mycobacteria may be positive using a SCT; however, by using the CCT these sensitized animals can often be identified by reason of a greater change in skin thickness at the *M. avium* PPD injection site compared to the change in skin thickness at the *M. bovis* PPD injection site. In cattle, an IFN-γ release assay is also available and may be used in place of the CCT. Unfortunately, efforts to create a similar IFN-γ assay for deer have shown such an assay to be unreliable [24], leaving intradermal tuberculin testing the only means of antemortem diagnosis of exposure of *M. bovis* (including *M. bovis* BCG) in deer.

Shedding of BCG from vaccinated deer to non-vaccinated deer has been documented previously [7,23]. To date, studies have not evaluated the mechanism of spread. In the current study, BCG was not isolated from feces of vaccinated deer and rarely from oronasal swabs of vaccinated deer. Proof of shedding is evident in the conversion of 4 out of 10 non-vaccinated deer from non-reactor to reactor status as identified by the CCT. Moreover, shedding is further evidenced by isolation of BCG from a non-vaccinated deer. Although transmission of BCG did occur, it is not possible, from the present study, to determine if deer to deer transmission occurred through direct or indirect contact.

Few studies have examined post-vaccinal spread of BCG in animals or humans. Post-vaccinal spread of BCG in humans is generally limited to shedding of BCG from ulcerated lesions at the site of vaccination. In one study 47 of 48 healthy, immunocompetent, volunteers vaccinated SC with BCG Connaught or Tice strains developed ulcerated lesions at the vaccination site. Viable BCG could be isolated from lesion exudate for up to 4 weeks [25]. Interestingly, similar to results in the present study, biopsies of ulcerated vaccination sites revealed granulomas, but no acid-fast bacteria [25].

Shedding has been evaluated in other species of wildlife. Shedding of virulent *M. bovis* has been documented in naturally infected badgers, [26] however; shedding of BCG is not seen in BCG vaccinated European badgers (*Meles meles*) after SC or intramuscular vaccination [27]. Moreover, unvaccinated badgers housed in the same enclosure, with ample opportunity for direct contact, did not become infected with BCG, or show immunologic responses suggestive of exposure to BCG. Oral vaccination of brushtail possums (*Trichosurus vulpecula*) with BCG showed fecal shedding of vaccine for 7 days after vaccination [28]. The magnitude of shedding was found to be

low, with mean peak shedding of $1x10^4$ CFU/gm feces 2 days after vaccination. In spite of inconsistent and transient shedding, BCG was isolated from mesenteric lymphoid tissue up to 8 weeks after vaccination [28]. In general, studies of BCG vaccinated wildlife have concluded that the risk for transmission of BCG from vaccinated wildlife to cattle is low; especially shedding sufficient to induce false positive tuberculin skin test reactions in cattle [27,28].

The current study demonstrates that while BCG Danish may be shed and transmitted in a limited fashion from vaccinated to unvaccinated deer, the risk of transmission to cattle through indirect contact is low. The significance of BCG shedding from vaccinated deer is not trivial. Guidelines for the assessment of live biological agents suggest that persistence and shedding must be considered if the vaccine is shed as a live agent [29]. Moreover, if the agent is shed, the magnitude and duration of shedding should be investigated. Before implementation of a wildlife vaccination policy, further studies and both vaccine safety and efficacy will be required.

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ORIGINAL ARTICLE

Persistence of *Mycobacterium bovis* Bacillus Calmette-Guérin in White-Tailed Deer (*Odocoileus Virginianus*) after Oral or Parenteral Vaccination

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Impacts

- Bovine tuberculosis is an important zoonotic disease, for which most industrialized nations have eradication programmes.
- A serious obstacle to many eradication programmes worldwide is the presence of wildlife reservoirs of *Mycobacterium bovis*. Vaccination is one tool that may mitigate transmission from wildlife to domestic animals.
- Bacillus Calmette-Guérin (BCG) was not isolated from the muscle; however, after oral or SC vaccination of deer with BCG Danish, the live attenuated vaccine could be isolated from various lymphoid tissues 3 and 9 months after vaccination, respectively.

Keywords:
BCG; Danish; deer; *Mycobacterium bovis*; persistence; tuberculosis; vaccination; zoonosis

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Summary

Mycobacterium bovis is the cause of tuberculosis in cattle and a serious zoonotic pathogen, most commonly contracted through consumption of unpasteurized dairy products. To control this zoonosis, many countries have developed bovine tuberculosis eradication programmes. Although relatively successful, efforts are hindered in many regions by spillover from wildlife reservoirs of *M. bovis* to cattle. Such is the case in the United States where spillover of *M. bovis* from free-ranging white-tailed deer to cattle occurs. One approach to control such inter-species transmission is vaccination of wildlife. The live, attenuated human vaccine *M. bovis* Bacillus Calmette-Guérin (BCG) has been shown to reduce disease severity in white-tailed deer; however, vaccine persistence within tissues has also been noted. Consumption of venison containing BCG by hunters may present a public health concern as BCG exposure, although unlikely to cause disease, could cause false positive tuberculin skin test results. To examine BCG persistence further, 42 white-tailed deer were vaccinated orally or subcutaneously (SC) with BCG Danish. Three deer from each group were killed and examined at periods ranging from 2 weeks to 11 months after vaccination. BCG was recovered from orally vaccinated deer as late as 3 months after vaccination, while BCG persisted in SC vaccinated deer for as long as 9 months. At no time was BCG isolated from meat; however, prolonged persistence was seen in lymphoid organs. Although vaccine persistence was noted, especially in SC vaccinated deer, the distribution of culture-positive tissues makes human exposure through consumption unlikely.

Introduction

Mycobacterium bovis is the causative agent of tuberculosis in animals and has one of the broadest host ranges of any pathogen. *Mycobacterium bovis* can cause tuberculosis in humans clinically indistinguishable from the disease caused by *Mycobacterium tuberculosis*. Public health concerns posed by potential transmission of *M. bovis* from cattle to humans prompted many countries to implement national programmes to eradicate tuberculosis from

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cattle. Most eradication campaigns have been successful in decreasing the prevalence of bovine tuberculosis. However, some countries have found it impossible to eradicate bovine tuberculosis. One factor responsible for many faltering eradication campaigns has been the presence of a wildlife reservoir of *M. bovis*. In most cases, wildlife originally acquired tuberculosis from cattle; however, the disease is now spilling back from wildlife to cattle, impeding the progress of eradication (Daszak et al., 2000; Miller and Kaneene, 2006). In an effort to reduce wildlife to cattle transmission of *M. bovis*, some countries are investigating the possible role of vaccination.

In 1994, a free-ranging white-tailed deer (*Odocoileus virginianus*) in Michigan was diagnosed with tuberculosis caused by *M. bovis* (Schmitt et al., 1997). Subsequent surveys identified a focus of *M. bovis* in free-ranging white-tailed deer in northeast Michigan (O'Brien et al., 2001, 2002). This represented the first known reservoir of *M. bovis* in free-ranging wildlife in the United States and a significant impediment to the ongoing effort to eradicate bovine tuberculosis from domestic livestock. Current disease control measures include decreasing deer density through increased hunting. Control and surveillance measures have now been in place in Michigan for over 10 years and a significant reduction in apparent prevalence of tuberculosis in deer has been achieved (O'Brien et al., 2006). However, public support for further population reduction is waning (O'Brien et al., 2006). Vaccination of deer could be used in specific areas of sustained high disease prevalence, in order to prevent infection, disease, or transmission. Recently, protection has been demonstrated by subcutaneous and oral vaccination of white-tailed deer with *M. bovis* Bacillus Calmette-Guerin (BCG) (Palmer et al., 2007, 2009; Nol et al., 2008). These studies also demonstrated the ability of BCG to persist within host tissue for up to 250 days after vaccination and to be shed to non-vaccinated deer.

As venison is frequently consumed by deer hunters (Wilkins et al., 2003), the persistence of BCG in vaccinated deer presents a potential public health issue. Although BCG is generally considered avirulent for immunocompetent humans, exposure to BCG could cause a false positive response to the tuberculin skin test, interfering with public tuberculosis surveillance efforts. The purpose of the present study was to evaluate vaccine distribution and persistence within white-tailed deer vaccinated orally or SC with BCG.

Materials and Methods

Animals, vaccination and challenge

Forty-two white-tailed deer (~1 year old, 31 females and 11 castrated males) were obtained from a captive breeding herd (tuberculosis- and paratuberculosis-free) at the National Animal Disease Center (Ames, Iowa, USA). All deer were housed and cared for according to institutional guidelines. Deer were randomly assigned to one of two groups; one subcutaneous dose of 10^7 colony-forming units (CFU) *M. bovis* BCG Danish ($n = 21$) or one oral dose of 10^9 CFU *M. bovis* BCG Danish ($n = 21$). Deer were vaccinated SC on the right side of the neck, midway between the head and shoulder. Oral vaccination, including dosage, was performed as described (Nol et al., 2008) using a liquid suspension of BCG Danish. Three deer from each group were killed by IV sodium pentobarbital and examined 2 weeks, 1, 3, 5, 7, 9 and 11 months after vaccination. At necropsy, the following tissues were collected from SC and orally vaccinated deer lung; liver; and mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and superficial cervical lymph nodes. Skin and subcutis from the injection site were collected from SC vaccinated deer. Additional tissues collected from orally vaccinated deer included palatine tonsil; nasopharyngeal tonsil; lingual tonsil; tonsil of the soft palate; duodenum; proximal, middle and distal jejunum; proximal middle and distal ileum; ileocaecal valve; caecum; spiral colon; transverse colon; descending colon; duodenal lymph node; proximal, middle and distal jejunal lymph nodes; and ileal lymph node, colic lymph node and iliac lymph node. From all deer, samples of muscle from the following areas were collected for bacteriological isolation of BCG: epaxial, sublumbar, shoulder (supraspinatus and triceps muscles), and thigh (semimembranosis, semitendinosis and biceps femoris muscles). All samples from all deer were processed for isolation of BCG and microscopic analysis as described (Palmer et al., 2002a,b; Hines et al., 2006).

Vaccine

The *M. bovis* BCG Danish strain was grown in Middlebrook's 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI, USA) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA) as described for virulent *M. bovis* (Bolin et al., 1997). Mid log-phase growth bacilli were pelleted by centrifugation at 750 ***g***, washed twice with phosphate-buffered saline (PBS) (0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, MD, USA). A single vaccine dose consisted of 10^7 CFU *M. bovis* BCG in 1.5 ml PBS for SC vaccinated deer and 10^9 CFU in 2 ml PBS for orally vaccinated deer.

Isolation and identification of mycobacterial isolates

Tissues were processed for the isolation of *M. bovis* BCG as described previously (Hines et al., 2006) using both the BACTEC 460 radiometric system and BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson and Company, Sparks, MD, USA). Isolates of *M. bovis* BCG were identified by a combination of Ziehl-Neelsen acid-fast staining, biochemical tests and nucleic acid probes (AccuProbe, Gen-Proe, San Diego, CA, USA). Further identification was carried out using 16S ribosomal DNA sequencing as described previously (Kierschner and Bottger, 1998). Sequences were then identified through the use of a mycobacterial species sequence database (Harmsen et al., 2003).

Results

SC vaccinated deer

At no time during the study was BCG isolated from any sample of muscle; mandibular, parotid, medial retropharyngeal or mediastinal lymph nodes; lung or liver. *Mycobacterium bovis* BCG was isolated from tissues as late as 9 months after subcutaneous vaccination (Table 1) with one of three deer sampled contributing all positive tissues sampled at 9 months. In SC vaccinated deer, the most common site from which BCG was isolated was the right superficial cervical lymph node (7/21) followed by the hepatic lymph node (4/21). Although the latest date from which BCG could be isolated from the right superficial cervical lymph node (draining the site of vaccination) was 3 months after vaccination, BCG was isolated from sites such as the hepatic lymph node and mesenteric lymph node as late as 9 months after vaccination. Various species of non-tuberculous mycobacteria (NTM) were isolated from various lymph nodes and liver (Table 1).

Gross lesions compatible with a granulomatous process, such as *M. bovis* infection, were not seen in any deer during any period. Microscopic lesions were seen at 2 weeks, 1 and 7 months in subcutis at the injection site (one deer at each period). Microscopic lesions were also seen in the tracheobronchial lymph node of one deer each at 2 weeks and 1 month after vaccination and the mediastinal lymph node of one deer at 1 month after vaccination. Regardless of the location, lesions were characterized by infiltrates of epithelioid macrophages and lymphocytes, and with the exception of subcutis lesions, were absent a caseonecrotic core. Acid-fast staining revealed low to moderate numbers of intralesional acid-fast bacilli.

Orally vaccinated deer

At no time during the study was BCG isolated from any sample of muscle; nasopharyngeal tonsil; tonsil of the soft palate; mandibular, parotid, tracheobronchial, superficial cervical, duodenal, proximal jejunal, distal jejunal, colic or iliac lymph nodes; lung; liver; duodenum; mid-jejunum; distal jejunum; proximal ileum; distal ileum; ileocaecal valve; spiral colon; transverse colon or distal colon. *Mycobacterium bovis* BCG was isolated from orally vaccinated deer as late as 3 months after vaccination (Table 2), with the medial retropharyngeal lymph node being the

Table 1. Results of mycobacterial isolation from tissues collected from deer vaccinated SC with 1×10^6 CFU *Mycobacterium bovis* BCG Danish and examined at various times after vaccination

	Months after vaccination (isolation of *M. bovis* BCG Danish)						
Tissue	0.5	1	3	5	7	9	11
Mandibular LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Parotid LN	0/3	0/3[a]	0/3	0/3	0/3	0/3	0/3
Med retropharyngeal LN	0/3	0/3[b]	0/3	0/3	0/3	0/3	0/3
Tracheobronchial LN	0/3	1/3	0/3	0/3	0/3	1/3	0/3
Mediastinal LN	0/3[c]	0/3[b]	0/3	1/3	0/3	0/3[b]	0/3[b]
Lung	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Liver	0/3	0/3	0/3	0/3	0/3[d]	0/3	0/3
Hepatic LN	0/3	0/3	1/3	1/3	1/3	1/3	0/3
Mesenteric LN	0/3	0/3	1/3	0/3	0/3	1/3	0/3
Right Sup Cervical LN	3/3	2/3	2/3	0/3	0/3	0/3	0/3
Injection site	0/3	1/3	0/3	0/3	0/3	0/3	0/3

LN, lymph node.
[a]Isolation of *Mycobacterium fortuitum* complex.
[b]Isolation of *Mycobacterium avium* complex.
[c]Isolation of *Mycobacterium gastri/kansasii* complex.
[d]Isolation of *Mycobacterium aurum.*

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Table 2. Results of mycobacterial isolation from tissues collected from deer vaccinated orally with 1×10^9 CFU *Mycobacterium bovis* BCG Danish and examined at various times after vaccination

	Months after vaccination (isolation of *M. bovis* BCG Danish)						
Tissue	0.5	1	3	5	7	9	11
Palatine tonsil	0/3	0/3	1/3	0/3	0/3	0/3[a]	0/3
Nasopharyngeal tonsil	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Soft palate tonsil	0/3[b]	0/3	0/3	0/3	0/3	0/3	0/3
Lingual tonsil	0/3	0/3	1/3	0/3	0/3	0/3	0/3
Mandibular LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Parotid LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Med retropharyngeal LN	0/3[c,d]	0/3	3/3	0/3	0/3	0/3	0/3[d]
Tracheobronchial LN	0/3	0/3	0/3	0/3	0/3	0/3[d]	0/3[d]
Mediastinal LN	0/3	0/3	1/3	0/3	0/3	0/3	0/3[e]
Lung	0/3[f,g]	0/3	0/3	0/3	0/3	0/3	0/3[h]
Liver	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Hepatic LN	0/3	0/3	1/3	0/3	0/3	0/3	0/3
Mesenteric LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Sup Cervical LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Duodenum	0/3[g]	0/3	0/3	0/3	0/3	0/3	0/3
Duodenal LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Proximal jejunum	0/3[g]	0/3	1/3	0/3	0/3	0/3	0/3
Proximal jejunal LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Mid-jejunum	0/3[g]	0/3	0/3	0/3	0/3	0/3	0/3
Mid-jejunal LN	0/3	0/3	1/3	0/3	0/3	0/3	0/3
Distal jejunum	0/3[i]	0/3	0/3	0/3	0/3	0/3	0/3[j]
Distal jejunal LN	0/3	0/3	1/3	0/3	0/3	0/3	0/3
Proximal ileum	0/3[g]	0/3	0/3	0/3	0/3	0/3	0/3
Distal ileum	0/3[g]	0/3	0/3	0/3	0/3	0/3	0/3
Ileocaecal LN	0/3	0/3	1/3	0/3	0/3	0/3	0/3
Ileocaecal valve	0/3[g,k,l]	0/3	0/3[b]	0/3	0/3	0/3	0/3
Caecum	0/3[k]	0/3	1/3	0/3	0/3	0/3	0/3
Spiral colon	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Colic LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Transverse colon	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Distal colon	0/3[f,k]	0/3	0/3	0/3	0/3	0/3	0/3
Iliac LN	0/3	0/3	0/3	0/3	0/3	0/3	0/3

LN, lymph node.
[a]Isolation of *Mycobacterium moriokaense* (*Mycobacterium fortuitum* complex).
[b]Isolation of *Mycobacterium acapulcensis.*
[c]Isolation of *Mycobacterium fortuitum* (*Mycobacterium fortuitum* complex).
[d]Isolation of *Mycobacterium avium* complex.
[e]Isolation of *Mycobacterium gastri/kansasii complex.*
[f]Isolation of *Mycobacterium neoaurum.*
[g]Isolation of *Mycobacterium mucogenicum.*
[h]Isolation of *Mycobacterium septicum* (*Mycobacterium fortuitum* complex).
[i]Isolation of *Mycobacterium pulveris.*
[j]Isolation of *Mycobacterium terrae.*
[k]Isolation of unidentified mycobacterial sp.
[l]Isolation of *Mycobacterium vaccae.*

site most commonly involved (3/21), followed by the palatine tonsil (1), lingual tonsil (1), mediastinal lymph node (1), hepatic lymph node (1), proximal jejunum (1), mid-jejunal lymph node (1), distal jejunal lymph node (1), ileocaecal lymph node (1) and caecum (1). BCG was isolated from all deer samples sampled at 3 months; however, one deer contributed seven of the 12 positive samples with other deer contributing one or two positive samples each. Similarly, this was the only period at which BCG was isolated from orally vaccinated deer. Numerous species of NTM were isolated from the tonsil, lung, various lymph nodes and segments of intestine (Table 2).

Gross lesions were not seen in orally vaccinated deer at any period. Microscopic lesions were rare and seen in one deer 3 months after vaccination in both the medial retropharyngeal and mediastinal lymph nodes. Lesions were characterized by the absence of necrosis and by focal to multifocal infiltrates of epithelioid macrophages and low numbers of intralesional acid-fast bacilli.

Discussion

Mycobacterium bovis BCG persistence within host tissues is an important safety concern in animals that may be consumed for food. Few studies have examined BCG persistence in domestic or wild ruminant species (Slobbe et al., 1999; Palmer et al., 2007). Previous studies in white-tailed deer demonstrated BCG within host tissues up to 250 days after subcutaneous vaccination (Palmer et al., 2007). After vaccination, those deer had been challenged with virulent *M. bovis* and it remains unclear what influence, if any, active tuberculosis had on vaccine persistence. In red deer, BCG was still present in tissues from the site of injection, as well as draining lymph nodes, in 2/6 and 3/6 deer, respectively, 3 months after subcutaneous vaccination with 2×10^6 CFU of *M. bovis* BCG Pasteur (Slobbe et al., 1999).

It is clear from the present study that not only does BCG persist for prolonged periods in SC vaccinated deer, but also that vaccine disseminates from the site of vaccination, colonizing sites such as hepatic, mesenteric and tracheobronchial lymph nodes. Such dissemination suggests systemic spread via vascular or lymphatic channels. Similar dissemination has not been a feature in previous studies involving red deer or cattle (Griffin et al., 1993). Furthermore, gross or microscopic lesions in regional lymph nodes have not been features of studies involving red deer or cattle (Griffin et al., 1993). Prolonged vaccine persistence, dissemination and lesion development may be features unique to white-tailed deer. However, studies in mice have demonstrated persistence within the spleen for up to 30 weeks after SC vaccination and persistence within the mesenteric lymph node for up to 30 weeks after oral vaccination with a lipid encapsulated form of BCG Pasteur. Non-lipid encapsulated forms persisted only up to 12 weeks after oral vaccination (Aldwell et al., 2006).

In the present study, prolonged persistence of BCG was seen in lymphoid organs. Humans generally avoid consumption of lymphoid organs and usually cook meat before consumption, reducing the risk of ingesting large numbers of organisms (Wilkins et al., 2003). Thorough cooking at 60°C (140°F) for 6 min has been shown to kill virulent *M. bovis* (Merkal and Whipple, 1980). Therefore, potential for human exposure to BCG through consumption is low.

Although a possible safety concern in food animals, persistence within host tissue may be necessary for development of an effective immune response. Studies in mice show that persistence of BCG is vital in sustaining long-lasting immunological memory. BCG vaccinated mice receiving chemotherapy to eliminate residual post-vaccinal BCG demonstrated inferior cell-mediated immune responses and inferior protection against challenge with virulent *M. tuberculosis,* as measured by colonization of the spleen by *M. tuberculosis,* when compared with vaccinated but untreated mice still harbouring low numbers of BCG (Olsen et al., 2004; Cross et al., 2007). Thus, persistence of BCG within tissues of white-tailed deer may prove beneficial to sustained protective vaccine efficacy in this species.

The importance of meticulous identification of mycobacterial isolates is emphasized by the isolation of numerous species of NTM from several deer in the present study. This finding illustrates the ubiquitous nature of NTM and their ability to colonize susceptible hosts. Absence of lesions supports their non-pathogenic nature in deer. Various species of NTM have been isolated previously from deer (De Lisle and Havill, 1985, Palmer et al., 1999, 2001, 2007; Hall et al., 2005). With the exception of *Mycobacterium avium* subsp. *paratuberculosis* (Mackintosh et al., 2004) and rare cases of disease caused by *M. avium* subsp. *avium* (De Lisle and Havill, 1985) and *Mycobacterium kansasii* (Hall et al., 2005), most NTM are considered non-pathogenic in deer.

Similar NTM have been isolated from cattle, and with few exceptions, have not been associated with disease (Peterson, 1965; Jarnagin et al., 1983; Hughes et al., 2005). A caveat comparing past and present cases involving isolation of NTM is that current molecular analysis demonstrates greater sensitivity in the identification of mycobacterial species. Moreover, speciation by molecular analysis and that performed by traditional methods (standard phenotypic and biochemical) do not always agree (Hughes et al., 2005).

Isolation of NTM underscores the challenges posed by exposure to NTM present in the environment in tuberculosis vaccine development. Studies in cattle have shown that sensitization of calves to NTM in the environment adversely affects the protective efficacy of BCG vaccination (Buddle et al., 2002). Similarly, one explanation for the highly variable efficacy observed in human BCG vaccine trials has been exposure to environmental NTM (Brandt et al., 2002). BCG vaccination of neonatal calves has been used as a strategy to avoid prior sensitization by environmental NTM. Indeed, vaccination of neonatal calves induces a higher level of immunity than that seen in calves vaccinated at 5-6 months of age (Buddle et al., 2003a,b; Hope et al., 2005). The optimal age at which to

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vaccinate deer will be critical, not only to optimize protective immune responses, but also to time field vaccination and minimize potential exposure of humans to BCG in hunter-harvested venison.

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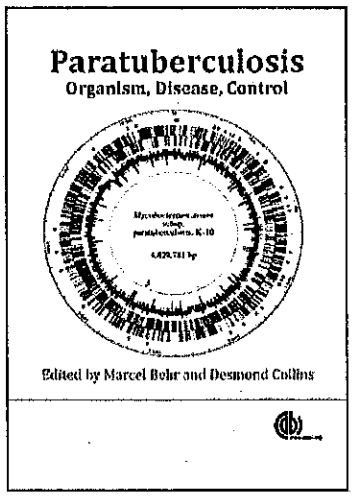

Paratuberculosis
Organism, Disease, Control

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Paratuberculosis, also referred to as Johne's disease, affects cattle, goats, sheep, buffalo, deer and other ruminants. It is common worldwide and responsible for significant economic losses in the dairy industry. Recent advances in detection, vaccination and microbial genetics make this a timely book that examines the epidemiology of paratuberculosis, the organism that causes the disease, and practical aspects of its diagnosis and control. It also addresses the link between paratuberculosis in the food chain and human health implications, including Crohn's disease.

Audience:
Researchers, practitioners and students in veterinary and animal science, veterinary microbiology, environmental biology, food microbiology and mycobacteriology and livestock industry personnel.

Contents:

1. History of paratuberculosis
2. Global Prevalence and Economics of Infection with Mycobacterium avium subsp. paratuberculosis in ruminants
3. Epidemiology of paratuberculosis
4. Mycobacterium avium subsp. paratuberculosis in animal-derived foods and the environment
5. Paratuberculosis and Crohn's disease
6. Genetics of host susceptibility to paratuberculosis
7. Mycobacterium avium complex
8. Mycobacterium avium subsp. paratuberculosis genome
9. Molecular genetics of Mycobacterium avium subsp. Paratuberculosis
10. Proteome and antigens of Mycobacterium avium subsp. Paratuberculosis
11. Host-pathogen interactions and intracellular survival of Mycobacterium avium subsp. Paratuberculosis
12. Comparative differences between strains of Mycobacterium avium subspecies paratuberculosis
13. Mycobacterium avium subsp. paratuberculosis and antimicrobial agents
14. Paratuberculosis in Cattle
15. Paratuberculosis in sheep
16. Paratuberculosis in goats
17. Paratuberculosis in deer, camelids and other ruminants
18. Infection of non-ruminant wildlife by Mycobacterium avium subsp. paratuberculosis
19. Experimental ruminant models of paratuberculosis
20. Experimental small animal models of paratuberculosis
21. Immunology of paratuberculosis infection and disease
22. Cultivation of Mycobacterium avium subsp. Paratuberculosis
23. Diagnosis of paratuberculosis by PCR
24. Immune-based diagnosis of paratuberculosis
25. Strain characterisation of Mycobacterium avium subsp. Paratuberculosis
26. Paratuberculosis control measures in Europe
27. Paratuberculosis Control in the USA
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8 *Mycobacterium avium* subsp. *paratuberculosis* Genome

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8.1 Introduction

The post-genomic era of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) research began in 2005 with the publication of the complete, annotated genome sequence of isolate K-10, which was isolated from a Wisconsin dairy herd in 1990 (J. Hansen, Iowa, 2009, personal communication). The 4,829,781 base pair sequence was determined by preparing and sequencing a random small insert library and PCR products totalling 66,129 unique sequences (L. Li *et al.*, 2005). The *MAP* K-10 genome was originally annotated as having 4350 predicted open reading frames. Subsequent automated analyses of the genome sequence have annotated up to 4587 total genes (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=atma03). The *MAP* genome is characterized by a relatively high proportion of guanine and cytosine nucleotides within the genome (69%), as well as an abundance of insertion sequences and PE/PPE proteins, similar to other sequenced mycobacteria. Genomic regions with altered nucleotide compositions are in many cases associated with mobile genetic elements and have been shown to be sites of large sequence polymorphisms (Fig. 8.1). Recent work using optical mapping technology to compare *MAP* genomes has identified a 648 kb inversion near the origin of replication in the *MAP* K-10 genome that may be due to an error in sequence assembly (Wu *et al.*, 2009).

The completion of the *MAP* K-10 genome sequence has opened the door to many new avenues of research. In the few years since the publication of the genome sequence, the manuscript describing the completed sequence has been cited in the scientific literature more than 85 times. The public availability of a completed genome sequence has allowed researchers to include *MAP* in comparative genomic studies with other mycobacteria, which in turn has facilitated the transfer of information from other fields of research. This has led to new insights into mechanisms of virulence,

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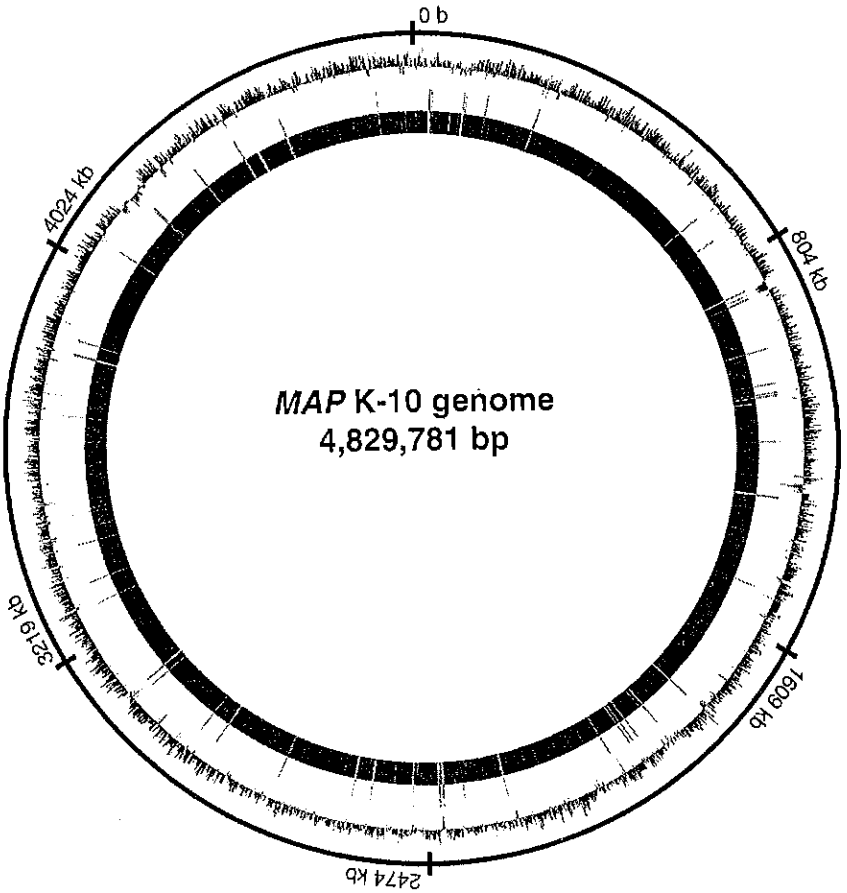

Fig. 8.1. *Mycobacterium avium* subsp. *paratuberculosis* K-10 genome. Beginning at the outer ring, the first circle displays the nucleotide position, starting at the origin of replication. The next circle is a graph of the percentage of guanine and cytosine nucleotides within the genome, while the innermost ring consists of bars representing individual coding sequences, in which those sequences predicted to encode mobile genetic elements are highlighted by moving those bars towards the outside of the ring. The image was generated using the Microbial Genome Viewer (Kerkhoven *et al.*, 2004).

diagnostic targets and the molecular evolution of *MAP* as a pathogen. The genome sequence has facilitated the development of DNA microarrays for use in genome-wide comparative genomic hybridizations and transcriptional analyses. The systematic cloning and expression of a large subset of *MAP* proteins from the annotated genome sequence has been initiated. In addition, the use of proteomic techniques such as tandem mass spectrometry has been enabled by the availability of a reference genome from which to identify the genes that encode observed peptide sequences. The anticipated completion of additional mycobacterial genome projects in the near future (Table 8.1) should further add to the utility of the *MAP* genome sequence.

Other *MAP* genomes have also been subjected to whole-genome sequencing. Draft sequence coverage has been obtained from a human *MAP* isolate (V. Kapur, 2009, unpublished results), while genome closure and annotation are currently under way for a sheep isolate (M. Paustian, 2009, unpublished

Table 8.1. Sequenced mycobacterial genomes.

Completed genomes	Size (Mb)	% G+C	Accession
Mycobacterium abscessus	5.12	64.1	NC_010397.1
Mycobacterium avium subsp. *hominissuis* 104	5.5	69.0	NC_008595.1
Mycobacterium avium subsp. *paratuberculosis* K-10	4.8	69.3	NC_002944.2
Mycobacterium bovis AF2122/97	4.35	65.6	NC_002945.3
Mycobacterium bovis BCG str. Pasteur 1173P2	4.4	65.6	NC_008769.1
Mycobacterium gilvum PYR-GCK	5.96	67.7	NC_009338.1
Mycobacterium leprae TN	3.27	57.8	NC_002677.1
Mycobacterium marinum M	6.62	65.7	NC_010612.1
Mycobacterium smegmatis str. MC2 155	7	67.4	NC_008596.1
Mycobacterium sp. JLS	6	68.4	NC_009077.1
Mycobacterium sp. KMS	6.22	68.2	NC_008705.1
Mycobacterium sp. MCS	5.92	68.4	NC_008146.1
Mycobacterium tuberculosis CDC1551	4.4	65.6	NC_002755.2
Mycobacterium tuberculosis F11	4.4	65.6	NC_009565.1
Mycobacterium tuberculosis H37Ra	4.4	65.6	NC_009525.1
Mycobacterium tuberculosis H37Rv	4.4	65.6	NC_000962.2
Mycobacterium ulcerans Agy99	5.6	65.5	NC_008611.1
Mycobacterium vanbaalenii PYR-1	6.5	67.8	NC_008726.1
Genomes in progress			
Mycobacterium africanum GM041182			
Mycobacterium avium subsp. *avium* ATCC 25291			
Mycobacterium avium subsp. *paratuberculosis* (sheep)			
Mycobacterium avium subsp. *paratuberculosis* (human)			
Mycobacterium bovis BCG (2 isolates)			
Mycobacterium canetti (2 isolates)			
Mycobacterium chelonae CIP 104535			
Mycobacterium chlorophenolicum			
Mycobacterium intracellulare ATCC 13950			
Mycobacterium kansasii ATCC 12478			
Mycobacterium leprae Br4923			
Mycobacterium liflandii 128FXT			
Mycobacterium marinum DL240490			
Mycobacterium microti OV254			
Mycobacterium parascrofulaceum ATCC BAA-614			
Mycobacterium sp. Spyr1			
Mycobacterium tuberculosis (42 isolates)			
Mycobacterium ulcerans (2 isolates)			

results). Preliminary results indicate that the human *MAP* isolate is essentially identical to the bovine K-10 isolate, while the sheep isolate contains several large sequence polymorphisms, some of which have been described previously (Marsh and Whittington, 2005; Marsh *et al.*, 2006; Semret *et al.*, 2006; Wu *et al.*, 2006; Paustian *et al.*, 2008). Phenotypic differences between *MAP* strains are discussed elsewhere (see Stevenson, Chapter 12, this volume). The draft genome sequence of the *MAP* sheep isolate has confirmed the presence of these polymorphisms and provided their precise location. In addition, the genome sequencing project has identified novel features within the genome, such as a 5000 bp segment of which 3500 bp are homologous to sequences from the *Frankia* sp.

EAM1pec genome (M. Paustian, 2009, unpublished results). This region is flanked by a coding sequence homologous to MAP2180c, which itself is annotated as a hypothetical protein with homology only to sequences from the *Frankia* genome. Thus this region may represent a genome insertion event that was later partially excised from the genome of *MAP* cattle isolates. The *MAP* sheep genome sequence has also identified a glycopeptidolipid biosynthesis operon with homology to a similar gene cluster in *M. avium* subsp. *avium* (Paustian *et al.*, 2008), although production of glycopeptidolipids by *MAP* sheep isolates has not yet been experimentally verified.

8.2 Metabolic Pathway Comparison

The complete genome sequence of *MAP* has facilitated functional comparisons of mycobacterial metabolic pathways. Relative to the *M. tuberculosis* genome, *MAP* is lacking the fumarate reductase complex members *frdBCD* as well as the nitrate reductase gene *narX* (Marri *et al.*, 2006). These genes are thought to be important for survival under anaerobic conditions. Three members of the aspartate family (*asnB*, *dapA* and *lysA*) are duplicated in the *MAP* genome (Marri *et al.*, 2006). The genes *dapA* and *lysA* encode proteins involved in lysine biosynthesis and their duplication may therefore be indicative of an increased requirement for lysine, while *asnB* has been shown to facilitate natural resistance to antibiotics in *Mycobacterium smegmatis* (Ren and Liu, 2006). The *MAP* genome is lacking the urease operon (*ureABC* and *ureDFG*), which enables the utilization of ammonia as a nitrogen source (L. Li *et al.*, 2005). Compared with other mycobacteria, the *MAP* genome is lacking the genes *atsBDFH*. These genes are predicted to encode arylsulfatases that are used to generate sulfated glycolipids. Relative to mycobacteria other than *M. avium* subspecies, the *MAP* genome contains an abundance of functionally redundant lipid metabolism genes. *MAP* is missing 19 genes that are present in *M. tuberculosis* but has an additional 35 due to gene duplications (Marri *et al.*, 2006). Overall, there are significant differences in the types of membrane-protein encoding genes present in the *M. tuberculosis* and *MAP* genomes, which probably reflects their distinct differences in pathogenesis. The complete genome sequence of *MAP* has provided a possible explanation for the *in vitro* growth requirement of supplemental mycobactin. The gene cluster encoding the biosynthetic enzymes used to build the mycobactin siderophore (*mbtA-J*) is similar to other mycobacteria, with the notable exception that *mbtA* is truncated by approximately 150 amino acids (L. Li *et al.*, 2005). This observation remains to be experimentally verified. The comparison of the *MAP* genome with other sequenced mycobacteria has enabled researchers to more accurately and efficiently translate research findings from other fields into their studies.

8.3 Virulence Factors

The PE/PPE family of proteins are putative virulence factors originally identified in *M. tuberculosis* (Cole *et al.*, 1998). These proteins are distinguished by their specific domains (Pro-Glu and Pro-Pro-Glu), which often contain polymorphic sequences and are thought to be expressed on the cell surface (Fleischmann *et al.*, 2002). Thus far, PE/PPE proteins have only been found in mycobacteria, where they comprise anywhere from 1% of the genome (*MAP*) to nearly 10% (*M. tuberculosis*). Hypothesized functions for these proteins include host cell binding, antigenic variation, inhibition of antigen processing, macrophage survival and iron uptake. The relative homology between members of this gene family suggests that some perform redundant functions, which would complicate efforts to study individual genes. The genome sequence of *MAP* K-10 has identified 10 PE and 37 PPE homologues, 18 of which are specific to *MAP* (L. Li *et al.*, 2005; Gey van Pittius *et al.*, 2006). A comparative analysis of PE and PPE gene families from sequenced mycobacterial genomes indicates that all but one of the genes present in the *MAP* genome are part of an evolutionary distant group from which other subfamilies later evolved

in other mycobacteria (Gey van Pittius *et al.*, 2006). Experimental data have shown that a *M. avium* subsp. *hominissuis* mutant defective in the production of a PPE protein homologous to MAP1505 was found to be restricted for growth within macrophages (Y. Li *et al.*, 2005). Subsequent analyses of *MAP* PPE proteins have indicated that MAP3420c and MAP1506 are expressed at the cell surface and are immunogenic in cattle (Newton *et al.*, 2009); additionally, MAP1506 sequence polymorphisms were shown to distinguish between different subtypes of *MAP* (Griffiths *et al.*, 2008).

The *MAP* genome contains many homologues of mammalian cell entry (*mce*) genes, which were originally identified in *M. tuberculosis* and have been associated with survival within macrophages and increased virulence (Arruda *et al.*, 1993; Flesselles *et al.*, 1999; Chitale *et al.*, 2001; Haile *et al.*, 2002; Harboe *et al.*, 2002; Kumar *et al.*, 2003; Sassetti and Rubin, 2003; Gioffre *et al.*, 2005). The *MAP* genome encodes 62 proteins containing conserved domains that have been associated with Mce proteins, while the *M. tuberculosis* genome contains 32 (Casali and Riley, 2007). These coding sequences are organized into eight clusters scattered around the *MAP* K-10 genome and share some of the characteristics of ATP-binding cassette (ABC) transport systems. Nonpathogenic environmental mycobacteria also contain *mce* genes, which further suggests that this gene family originally arose as part of a transport system that was later adapted for facilitating cellular invasion. The *mce* genes in pathogenic mycobacteria have been hypothesized to play a role in modulating the cell envelope (Casali and Riley, 2007). Notably, one of the *mce* gene clusters present in *MAP* (MAP2189 to MAP2194) is part of a large sequence polymorphism that is absent from the genomes of other closely related *M. avium* subspecies (Paustian *et al.*, 2005; Semret *et al.*, 2005; Wu *et al.*, 2006). The remainder of this polymorphic region comprises genes homologous to sequences in the *Frankia* strain EAN1-pec genome. The contribution of *mce* genes to *MAP* pathogenesis is not fully understood, but studies on homologous sequences in other mycobacteria suggest that they are likely to be important contributors.

8.4 Repetitive Sequences

The *MAP* genome encodes 12 proteins that are homologous to the REP13E12 family of repetitive elements originally described in *M. tuberculosis* (Gordon *et al.*, 1999). These elements have been shown to be targets of phage integration, and one of the copies (MAP1432) is absent from sheep isolates of *MAP* (Paustian *et al.*, 2008).

Insertion sequences (IS) are short segments of DNA that act as transposable elements. A total of 58 insertion sequences have been identified within the *MAP* genome, including 17 copies of IS*900*, 7 copies of IS*1311* and 3 copies of ISMav2 (L. Li *et al.*, 2005). In addition, two novel insertion sequences were originally identified within the *MAP* genome, although subsequent genome sequence projects have identified homologous sequences in other organisms. IS_MAP02 is present at six locations within the *MAP* genome and is approximately 80% identical to insertion sequences in *Mycobacterium vanbaalenii* and *Mycobacterium gilvum*, although the sequences in these organisms contain a deletion of approximately 100 bp relative to IS_MAP02. ISMAP_04 is present in four copies and is 80% identical to IS elements reported in the genomes of *Mycobacterium* sp. KMS, *Mycobacterium* sp. MCS, *M. avium* subsp. *hominissuis* 104, *Rhodococcus jostii*, *M. smegmatis* and *M. gilvum*.

The *MAP* genome was found to contain 185 mono-, di- and trinucleotide repeats (Amonsin *et al.*, 2004). While no biological implications due to these repetitive sequences have been observed, a subset have proven to be a useful tool for molecular epidemiology studies of *MAP* isolates (Motiwala *et al.*, 2004; Harris *et al.*, 2006) (see Collins, Chapter 25, this volume).

8.5 Unique Genes

The *MAP* K-10 genome contains several genes that are not found in any other organisms for which sequence information is currently available within public databases. Many of these unique coding sequences are grouped together in clusters and some include phage-related

sequences, which points to the route by which they were probably acquired. Initial efforts have been made both to identify these sequences and to determine if any encode proteins that are capable of eliciting an immune response (Bannantine *et al.*, 2002, 2004; Klitgaard Nielsen and Ahrens, 2002; Paustian *et al.*, 2004; Stabel and Bannantine, 2005). The unique nature of these sequences makes them fertile ground for the discovery of novel diagnostic targets and virulence factors. The sequences currently identified as being uniquely present within the *MAP* genome are listed in Table 8.2, and several of these coding sequences are discussed below.

MAP0094 has very low homology to a transcriptional regulator and is immediately adjacent to two genes encoding putative ABC transport proteins (MAP0092 and MAP0093). This suggests that these genes comprise an ABC transport system for a yet-to-be-determined substrate. Near this location is another unique ORF, MAP0099, which has only low homology to a hypothetical protein within the *M. smegmatis* genome.

A large collection of unique genes is located from MAP0851 through MAP0865. This gene cluster contains two genes with homology to phage sequences (MAP0854 and MAP0859c), as well as genes with low homology to an *Acidothermus cellulolyticus* hypothetical protein (MAP0862) and an FtsK-like protein (MAP0865). The remaining genes in this cluster have no homology to any publicly available sequences. Among these unique ORFs, protein domain searches identify functions related to sugar transport (MAP0852) and DNA repair (MAP 0855). The remaining genes encode proteins with no obvious functional domains and thus fail to shed further light on the role of this large cluster of unique sequences. MAP0860c and MAP0862 were cloned and expressed as fusion proteins, but only the latter was found to be immunogenic with sera from animals naturally and experimentally infected with *MAP* (Paustian *et al.*, 2005). This cluster of sequences is flanked on the 5′ end by two insertion sequences (IS*1311* and IS_MAP03) and on the 3′ end by a phage integrase gene (MAP0866) and a tRNA. In addition, the average G+C nucleotide composition of this cluster is 61%, which is significantly lower than the genome average of 69%. These features strongly suggest that this gene cluster was horizontally acquired by a phage-mediated recombination event.

MAP1220c has no homology to currently available sequences. It is part of a cluster of genes that extends from MAP1216c through MAP1219c, which are homologous to genes in the *M. avium* subsp. *hominissuis* 104 genome (MAV_3285 to MAV_3288). These genes encode hypothetical proteins and one putative lipoprotein (MAP1216c).

Another cluster of unique genetic features is present from MAP2149 to MAP2158. This group of sequences includes one with homology to a hypothetical protein from the *Pseudomonas viridiflava* genome (MAP2153). This region also contains four insertion sequences: IS*1311* (MAP2150), ISMAP13 (MAP2155), ISMav2 (MAP2156) and IS*900* (MAP2157), and is flanked on the 5′ end by a phage integrase gene. Similar to other unique gene clusters, the combination of mobile genetic elements and lower average G+C nucleotide composition (61% in this case) suggest a horizontal route of acquisition.

The region between MAP2751 and MAP2767 contains additional coding sequences uniquely present in the *MAP* K-10 genome. Like the other novel gene clusters, this group of sequences contains several phage-related genes (MAP2752, MAP2756c and MAP2766c) and is flanked at the 5′ and 3′ ends by four tRNAs and a phage integrase gene, respectively. The average G+C nucleotide composition is 65%. Several ORFs are homologous to genes encoding hypothetical proteins in other organisms (MAP2755, MAP2758, MAP2759 and MAP2765c), while the remainder have no homology to any publicly available sequences. Purified MAP2751 fusion protein was detected by sera from animals naturally and experimentally infected with *MAP*, while the protein encoded by MAP2753 was not (Bannantine *et al.*, 2004).

Another group of unique sequences is present between MAP3815 and MAP3817c. MAP3817c has sequence features similar to membrane proteins, while the other two ORFs are not homologous to any described sequences. Although a tRNA sequence is nearby, this cluster of sequences does not

Table 8.2. Unique coding sequences present in the *MAP* K-10 genome.

ORF	Size (bp)	Conserved domains
MAP0094	285	None
MAP0852	546	Sugar transport
MAP0853	660	Aldehyde dehydrogenase
MAP0855	945	DNA repair
MAP0856c	1728	None
MAP0857c	318	None
MAP0858	549	None
MAP0860c	891	None
MAP0861	342	None
MAP0863	675	FAD/NAD binding domain
MAP0864	426	None
MAP1220c	336	FAD/NAD binding domain
MAP1636c	474	None
MAP1731c	270	Decarboxylase
MAP2149c	645	None
MAP2151	438	None
MAP2152c	375	None
MAP2154c	576	None
MAP2158	582	None
MAP2751	582	None
MAP2753	759	None
MAP2754	258	Repressor
MAP2757	282	None
MAP2761c	717	None
MAP2762c	441	None
MAP2763c	309	None
MAP2764c	450	None
MAP2767c	552	Ferredoxin
MAP3437c	843	DNA binding
MAP3815	855	None
MAP3816	243	Phage integrase
MAP4267	363	None

display the hallmarks of phage insertion events that were observed for some of the other unique gene groups.

IS_MAP02 is a novel insertion sequence present at six locations within the *MAP* genome (MAP0338c, MAP2416c, MAP2502, MAP2566, MAP3357 and MAP3467c). While the transposase encoded within IS_MAP02 is similar to those found in other insertion sequences, the remainder of the sequence is unique to the *MAP* genome.

Other than the initial identification and immunological characterization, these unique genome features have not been subjected to a

rigorous examination by the scientific community. It remains to be determined whether or not these sequences represent 'genetic baggage' from previous genetic transfer events or keys to understanding the pathogenesis of paratuberculosis. The latter may be true, considering that these sequences distinguish *MAP* from other closely related and less pathogenic *M. avium* subspecies.

8.6 Summary

The *MAP* genome sequence has quickly been utilized in many areas of paratuberculosis research. While great strides have been made with the available sequence information, there is still a large segment of the genome (greater than 30%) that encodes sequences with no identified function. Thus much work remains to be done in order to elucidate further insights into *MAP* virulence and metabolism.

One of the trends observed in the *MAP* genome (and other non-tuberculosis mycobacteria) is the presence of genes with homology to sequences found in other high-G+C, Gram-positive *Actinomycetales*, including species of *Frankia*, *Acidothermus*, *Clavibacter*, *Streptomyces* and *Corynebacterium*. This reflects the shared soil environment inhabited by these organisms and is a reminder that many pathogenic mycobacteria such as *MAP* are not far removed from closely related, non-pathogenic organisms.

Advances in sequencing technology and functional genomics techniques should continue to provide opportunities for new insights into the biology of *MAP*. Additional *MAP* and mycobacterial sequencing projects are under way and should yield valuable information for comparative genomic studies. The challenge for researchers is to wade through the ongoing flood of data in order to identify the critical links that will lead to the development of novel and improved diagnostics, vaccines and management strategies.

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Experimental validation of a nested polymerase chain reaction targeting the genetic element ISMAP02 for detection of *Mycobacterium avium* subspecies *paratuberculosis* in bovine colostrum

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Abstract. Colostrum samples experimentally inoculated with *Mycobacterium avium* subsp. *paratuberculosis* (MAP; strain K-10) at increasing concentrations between 1×10^1 and 1×10^9 cells/ml were tested for recovery of MAP DNA using a nested ISMAP02 target polymerase chain reaction initially developed for detecting MAP DNA in fecal samples. The following detection rates were achieved for sample replicates inoculated with unsonicated MAP pure stock: 100% between 1×10^7 and 1×10^9 cells/ml, 75% between 1×10^3 and 1×10^6 cells/ml, and 50% between $1 \times 10^1$and 1×10^2 cells/ml replicates. Detection rates achieved for the colostrum sample replicates inoculated with sonicated MAP cell suspension were 75% for 1×10^9 cells/ml, 100% between 1×10^7 and 1×10^8 cells/ml, 75% for 1×10^6 cells/ml, 0 for 1×10^4 cells/ml, and 25% between 1×10^1 and 1×10^3 cells/ml. When negative control colostrum samples were tested, 16 of 18 (89%) samples were correctly detected as negative for MAP DNA using the current assay. In conclusion, the MAP DNA detection rates of the present assay improved with increasing concentrations of MAP in the colostrum sample replicates, although MAP DNA was also detected in 2 of 18 (11%) negative control samples, suggesting an undefined technical problem with the assay or, perhaps, sample contamination during preparation. Overall, the present findings suggest a potential role of the proposed polymerase chain reaction assay to detect MAP in colostrum. However, adoption of this test for use in routine screening of field colostrum for MAP awaits findings from an ongoing field validation study.

Key words: Bovine colostrum; ISMAP02; *Mycobacterium avium* subsp. *paratuberculosis*; nested polymerase chain reaction.

Mycobacterium avium subsp. *paratuberculosis* (MAP) infection in cattle is characterized by chronic enteritis, progressive diarrhea, weight loss, and eventual death. Although the most common source of MAP is thought to be feces from infected adult cattle, with most transmissions occurring via the fecal–oral route,[13] MAP has also been detected in milk and colostrum collected from cows subclinically infected with Johne's disease,[12,14] suggesting that raw colostrum and waste milk might be some of the earliest sources of MAP by which calves become infected.

Although a number of factors are known to hinder efforts to control and ultimately eliminate Johne's disease from MAP-infected herds, including the long incubation period of the disease in cattle, the situation is compounded by limited availability of diagnostic assays with sufficient sensitivity to detect MAP-infected cattle early in the course of the disease.[3,9] The current commercially available diagnostic tests have been developed and occasionally field tested for direct and indirect detection of MAP in serum, milk, and fecal clinical specimens.[4,7,16,17] However, no validated diagnostic test for the direct or indirect detection of MAP in colostrum samples exists to date. The recent mapping of the complete MAP gene sequence[8] has provided an alternative involving the possible use of unique elements within the MAP genome for development of diagnostic assays. The genetic elements IS900[6] and ISMAP02[8] are considered specific to MAP and are detectable in clinical specimens by polymerase chain reaction (PCR).[3,11,15] In a 2005 study,[11] researchers developed and validated a nested PCR method targeting the multiple copy element ISMAP02[8] for detection of MAP in fecal samples.

In the present study, a previously developed nested ISMAP02 PCR[11] technique was validated for the detection of MAP in colostrum samples. The objectives of the current study were to estimate the limits of detection and to determine the ability of the nested ISMAP02 PCR to discriminate between known MAP-positive and -negative colostrum samples (negative controls). This technique could then be used in a future field-based study to determine the sensitivity and specificity of the assay.

Approximately 250 ml of fresh raw colostrum sample was collected from a single cow in a Minnesota dairy herd

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(45 adult cows) enrolled in the Minnesota Johne's Disease Control Program. The herds in the program are classified based on cattle types (i.e., beef or dairy) and program participation category (i.e., test-negative program or management program). Herds enrolled in the management program include those implementing a Johne's disease control effort, with or without herd testing for Johne's disease, and without classified herd status. Herds enrolled in the test-negative program include herds at levels 1, 2, 3, and 4 of the U.S. Voluntary Bovine Johne's Disease Control Program with each increase indicating a lower probability of Johne's disease presence in the herd. The herd selected in the present study had a level 4 herd status classification.

Prior to collection of the colostrum sample, blood and fecal samples were collected from the donor cow (adult cow ≥36 months old) and tested to determine her true MAP infection status as follows: 10 ml of blood was drawn from the coccygeal vein using a 20-gauge, 1-inch needle into a 16 mm × 100 mm blood collection tube[a] and tested for MAP antibodies using a previously described serologic method.[4] Additionally, 10 g of feces were collected from the rectum using disposable plastic rectal examination gloves and tested by a previously described conventional culture method for MAP.[17]

The colostrum sample was collected as follows: 6 hr after calving, the teat skin of the donor cow was disinfected using 0.05% povidone iodine detergent and swabbed with 70% ethyl alcohol–impregnated pledgets prior to actual collection of colostrum samples. The fore stripping colostrum was discarded, and 250 ml of colostrum was collected into a sterile glass tube from all 4 quarters and stored at −20°C. The colostrum was transported to the National Animal Disease Center (U.S. Department of Agriculture, Agricultural Research Service, Ames, IA), for further processing and analysis. The ability of the nested ISMAP02 PCR to correctly discriminate between known MAP-positive and -negative colostrum samples was evaluated using a predetermined set of experimentally infected colostrum samples while uninfected colostrum samples were used as controls.

The method for preparation of strain K-10 MAP pure cell suspensions used to experimentally inoculate the colostrum samples in the current study has been previously described.[11] Briefly, pure isolates of MAP were collected during the log growth phase (Abs_{540nm} = 0.2–0.4). Isolates were pelleted through centrifugation at 7,500 × *g*, washed twice with phosphate buffered saline (pH 7.4), and resuspended in phosphate buffered saline to a concentration of 1×10^9 cells/ml. Half the preparation was sonicated[b] through short bursts at 35 W for between 10 and 15 sec to disperse potentially clustered bacterial particles. This was then followed by a 10-fold (1×10^1–1×10^9 cells/ml) serial dilution of both sonicated and unsonicated stocks in phosphate buffered saline. One milliliter of colostrum samples in quadruplicate were then inoculated with the inocula preparations containing MAP in concentrations that ranged between 1×10^1 and 1×10^9 cells/ml of sample.

Seventy-two colostrum samples were inoculated with strain K-10 cell suspension (i.e., 4 samples for each MAP [strain K-10] concentration level between 1×10^1 and 1×10^9 cells/ml). The MAP (strain K-10) pure isolate used to inoculate 36 colostrum samples was sonicated to reduce potential clumping of the bacteria while the remaining 36 colostrum samples were spiked using the unsonicated MAP (strain K-10) pure isolate. Eighteen colostrum samples were not inoculated with MAP (strain K-10) and were used as negative controls.

For extraction of MAP DNA, 1 ml of colostrum was added to a sterile Eppendorf tube. Tubes were centrifuged at 15,000 × *g* for 5 min, and the whey was discarded. To each cell pellet, 1 mg of proteinase K solution[c] (10 mg/ml) was added, and samples were vigorously vortexed to resuspend the pellets. Samples were incubated overnight (15 hr) at 50°C in a shaking water bath. To each sample, 44 µl of TEN (10 mM Tris–HCl, pH 8.0; 1 mM ethylenediamine tetra-acetic acid, pH 8.0; 0.1 M NaCl) buffers[d] and 20 µl of 0.4 M NaOH were added, and samples were mixed by vortexing. Tubes were then placed in a boiling water bath for 30 min. After cooling, DNA was extracted by adding an equal volume of phenol, chloroform, and isoamyl alcohol[e] (25:24:1). The samples were vortexed followed by immediate centrifugation at 15,000 × *g* for 5 min. The aqueous layer was transferred to a new tube, and the DNA was precipitated by adding 2.2 volumes of cold 100% ethanol[f] and 0.1 volume of 3 M sodium acetate (pH 5.2). Samples were mixed gently by inversion and then placed in a −20°C freezer for 1 hr. The tubes were centrifuged at 15,000 × *g* for 15 min followed by decantation of the supernatants. The DNA pellets were air dried for 2–5 min, and then the pellets were resuspended in 20 µl of sterile water. The DNA was either used immediately or frozen at −20°C to be analyzed later.

Specific primers for the ISMAP02 element were selected for use in a nested PCR format for amplification of DNA isolated from the colostrum samples. The primer sequences for the initial amplification included 5′-GCACGG-TTTTTCGGATAACGAG-3′ (forward primer) and 5′-TCAACTGCGTCACGGTGTCCTG-3′ (reverse primer). In the first round, PCR was run using a conventional thermal cycler.[g] The reaction mixture consisted of ultrapure distilled water (free of DNase and RNase), GeneAmp[h] 10× PCR buffer II, 2.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates, 0.3 µM primers, and 2 U DNA polymerase.[i] Negative controls consisted of the reaction mixture alone and the nonspiked colostrum sample collected from the cow previously confirmed to be MAP negative as mentioned earlier. The positive controls consisted of genomic DNA from MAP (strain K-10 isolates) and the colostrum samples spiked with the same MAP strain. Five microliters of DNA was added for each sample, and samples were run in triplicate in 96-well plates according to the following protocol: initial denaturing cycle at 94°C for 5 min, followed by 20 cycles at 94°C for 45 sec, 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 7 min.[11] The second amplification was performed using real-time PCR[j] and included a fluorophore 6-carboxyfluorescein–labeled probe specific for the ISMAP02 target sequence for the semiquantitative evaluation of the test samples. Primers nested within the first set included 5′-GGATAACGAGACCGTGGATGC-3′ (forward primer)

and 5′-AACCGACGCCGCCAATACG-3′ (reverse primer) for this second amplification, yielding a 117-bp product.[11] One microliter of DNA from the first amplification was added to a reaction mixture consisting of PCR mastermix,[k] ultrapure distilled water (free of DNase and RNase), 0.05 μM primers, and 0.05 μM of the fluorophore 6-carboxyfluorescein–labeled probe (5′-/56-fluorophore 6-carboxyfluorescein/CAACCCGCACGCTG/3BHQ-1/-3′). A standard was constructed by amplifying the ISMAP02 target from MAP strain K-10 genomic DNA and cloned into the topoisomerase I expression vector[l] followed by transformation in *Escherichia coli*. The insert was analyzed for accuracy after plasmid digestion with EcoRI and verification of size on a 4% agarose gel. Further verification of the cloned insert was conducted by sequencing the product using a commercial DNA analyzer[m] after labeling the product with BigDye Terminator v3.1 cycle sequencing kit.[n] Primers used for sequencing were M13F: 5′-CGTTGTAAAACGACGGCCAGT-3′ (forward) and M13R: 5′-CAGGAAACAGCTATGAC-3′ (reverse). Optimal concentrations of the plasmid for use as a real-time PCR standard ranged from 1 ng to 100 attg. Real-time PCR conditions for the amplification of test samples and standards were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 25 sec, and 60°C for 1 min. Positive samples were visualized in the form of sigmoid curves plotted on 2-dimensional grids, with the x-axis representing the PCR cycle number and the y-axis representing the relative fluorescence of the signal. Sample runs with threshold cycles in the range of 15–30 cycles were declared positive.

The detection rates for MAP were 100% between 1×10^7 and 1×10^9 cells/ml (4/4), 75% between 1×10^3 and 1×10^6 cells/ml (3/4), and 50% between 1×10^1 and 1×10^2 cells/ml (2/4) replicates for the colostrum samples inoculated with unsonicated strain K-10 cell suspensions. For the colostrum samples inoculated with sonicated strain K-10 cell suspensions, the detection rates were 75% for 1×10^9 cells/ml (3/4), 100% between 1×10^7 and 1×10^8 cells/ml (4/4), 75% for 1×10^6 cells/ml (3/4), 0% for 1×10^4 cells/ml (0/4), and 25% between 1×10^1 and 1×10^3 cells/ml (1/4) replicates. Lack of detection of DNA in colostrum samples inoculated with 1×10^4 cells/ml of sonicated MAP cell suspension might have been due to an apparent absence of MAP organisms during serial dilution of the original inoculum due to the known clumping tendency of MAP and the fact that the sonication conditions may have been less than ideal to disperse clustered MAP cells.

The overall proportion of known MAP-positive colostrum samples (replicates) based on the unsonicated strain K-10 cell suspensions as the inocula correctly detected as positive for MAP DNA by the current assay was 28 of 36 (78%). However, when the colostrum samples inoculated with sonicated strain K-10 were evaluated, the proportion of colostrum samples (replicates) correctly detected as positive for MAP DNA was 19 of 36 (53%). The proportion of negative control samples correctly detected as negative for MAP DNA using the current protocol was 16 of 18 (89%).

Under laboratory conditions specified in the current study, the nested PCR protocol had detection limits for MAP that ranged between 1×10^1 and 1×10^9 cells/ml in spiked colostrum samples, although the sensitivity was compromised at lower concentrations of the inoculum (i.e., only 25% and 50% of the colostrum samples containing between 1×10^1 and 1×10^2 cells/ml of sonicated and unsonicated strain K-10 cell suspensions were correctly identified as positive for MAP DNA using the present protocol).

Although 16 of 18 (89%) negative control samples were correctly identified as negative for MAP DNA using the present protocol, false-positive test outcomes were observed in 2 of 18 (11%) negative control samples. This finding, although not unexpected, was definitely disappointing. The nested aspect of the PCR assay was designed to improve specificity and sensitivity by amplifying target DNA in a 2-step procedure using 2 distinct primer pairs. However, it should be noted that the attempt at improving sensitivity comes at a great price in that the nested design detracts from specificity, increasing the likelihood of false-positive test outcomes as a result of the increased risk of crossover contamination.[1,2] In practice, running positive and negative controls through the entire process, including the DNA extraction process, is highly recommended to exclude potential contamination.[1] In the present study, negative controls consisting of the reaction mixture alone and positive controls consisting of genomic DNA from strain K-10 MAP isolates were run as controls to monitor the possibility of contamination and soundness of the current assay. Given that running a parallel test involving negative and positive controls in addition to the known MAP-positive colostrum samples did not reduce the likelihood of cross-contamination (i.e., false-positive outcomes), the results could have been different had the conventional PCR been employed instead. Therefore, further investigation is required regarding potential contamination of the assay.

Sonication through short bursts at 35 W for 10–15 sec to disperse potentially clustered mycobacterial cells appeared to compromise sensitivity of the current assay. This observation was contrary to initial expectation, because sonication has been previously shown to improve the efficiency of PCR detection of Gram-positive bacteria DNA under laboratory conditions by causing disruption of the cellular walls and subsequent release of DNA.[5] A previous study[10] demonstrated that sonication of MAP for 2 min at 100 W did not affect the viability of MAP and allowed for maximum colony-forming unit counts when cultured on Herrold's egg yolk medium. It is therefore likely that the sonication conditions used in the current study were insufficient to disperse clustered mycobacterial cells or improve efficiency of PCR detection, thus explaining the inconsistent and unexpected findings of the present study. Perhaps the results would have been different had the samples been sonicated at 100 W for 2 min.[10]

Lastly, although the advantage of the present study lay in its laboratory-controlled nature, a major limitation was that the MAP (strain K-10) cell suspension concentrations

(1×10^1–1×10^9 cells/ml) used to spike the colostrum samples prior to analysis and the frequency of detection at the different concentration levels might not necessarily mimic the levels of MAP shed naturally in colostrum. Although data on the efficacy of this test based on field colostrum samples are currently lacking, work is being undertaken to determine the diagnostic sensitivity of this test on colostrum samples obtained from Minnesota dairy herds naturally infected with Johne's disease.

The present findings suggest a potential role of the proposed PCR assay to detect MAP in colostrum. However, adoption of this test for use in routine screening of field colostrum for MAP awaits findings from an ongoing field validation study.

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Sources and manufacturers

a. Vacutainer® Blood Collection Tubes, BD, Franklin Lakes, NJ.
b. Teledyne Tekmar, Mason, OH.
c. Qiagen Inc., Valencia, CA.
d. Sigma-Aldrich, St. Louis, MO.
e. Amresco Inc., Solon, OH.
f. Pharmco-AAPER, Brookfield, CT.
g. Peltier Thermal Cycler, MJ Research Inc., Waltham, MA.
h. GeneAmp® 10X PCR Buffer, Applied Biosystems, Foster City, CA.
i. AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Foster City, CA.
j. 7500 Real-Time PCR System, Applied Biosystems, Foster City, CA.
k. TaqMan® Universal PCR Master Mix, Applied Biosystems, Foster City, CA.
l. Invitrogen Corp., Carlsbad, CA.
m. 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA.
n. BigDye® Terminator v3.1, Applied Biosystems, Foster City, CA.

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Evaluation of Specificity of BP3385 for *Bordetella pertussis* Detection[▽]

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BP3385 has been proposed as a diagnostic PCR target for discriminating between *Bordetella pertussis* and other *Bordetella* species that also infect humans. Our results demonstrate that this gene is also present in some strains of *Bordetella hinzii* and *Bordetella bronchiseptica*.

Bordetella pertussis is an obligate human pathogen and the causative agent of whooping cough. Identification based on traditional culture and biochemical testing is the current diagnostic standard but is hampered by poor sensitivity and a slow turnaround time. Primers targeting a variety of genes have been proposed and evaluated for PCR-based identification strategies (11). These methods provide rapid and highly sensitive detection, but specificity is suboptimal since other species of *Bordetella* sometimes associated with respiratory disease in humans, including *Bordetella bronchiseptica* and *Bordetella hinzii*, may also possess the targeted sequences (4, 7, 9). Based on a comparison of the genomes of 22 isolates of *B. bronchiseptica*, 1 of human origin, and 10 isolates each of *B. pertussis* and *B. parapertussis*, it was suggested that open reading frame BP3385 of *B. pertussis* may be a highly specific diagnostic target (1). The authors of a subsequent study that included an unspecified number of *B. bronchiseptica* and *B. hinzii* isolates similarly concluded that BP3385 is specific for *B. pertussis* (3).

The goal of the present study was to screen a large and genetically heterogeneous group of *Bordetella* strains, including many of human origin, to further evaluate the specificity of BP3385 as a diagnostic target for *B. pertussis*.

A total of 142 *Bordetella* isolates originating from the United States, Europe, or Australia were evaluated using the previously described BP3385-specific real-time (RT)-PCR assay (3). These included 112 *B. bronchiseptica* isolates representing 31 unique PvuII ribotypes, 23 isolates of *B. hinzii* representing 4 PvuII ribotypes, and 7 *B. avium* isolates representing 6 PvuII ribotypes (5, 6, 8; K. B. Register, unpublished data). Twenty-five *B. bronchiseptica* isolates and 6 *B. hinzii* isolates were of human origin. The Tohama strain of *B. pertussis*, in which BP3385 was identified previously (1), was used as a positive control. The RB50 strain of *B. bronchiseptica*, which is missing BP3385 (1), was used as a negative control. Bacteria were cultured on Bordet-Gengou agar supplemented with 10% sterile, defibrinated sheep's blood at 37°C for 18 to 36 h. Chromosomal DNA was purified using a commercially available kit (Promega, Madison, WI) and quantified with PicoGreen reagent (Invitrogen, Carlsbad, CA).

Excepting the *B. pertussis* positive control, only *B. bronchiseptica* strain PV6 gave unequivocally positive results in the BP3385 RT-PCR assay. Results for an additional 8 isolates of *B. bronchiseptica* and 18 isolates of *B. hinzii* were interpreted as inconclusive on the basis of measurable, but elevated, cycle threshold (C_T) values (Table 1). All remaining isolates tested gave negative results.

Isolates with either positive or inconclusive RT-PCR results were further evaluated by Southern blotting. Three micrograms of purified DNA from each isolate was digested with EcoRV, for which no recognition sites are present in BP3385, and the resulting fragments were separated on 0.6% agarose gels. Blots were prepared and hybridized under highly stringent conditions, as described previously (5), with a probe encompassing the entire 450-bp BP3385 open reading frame. The probe was amplified from purified *B. pertussis* Tohama DNA and labeled with digoxigenin by using a commercially available kit (Roche Applied Science, Indianapolis, IN) and PCR primers BP3385-1 (5′-ATGGCACGACCATCCAAATACCA-3′) and BP3385-2 (5′-TTAAGCGTCGGCATCAGGGA-3′). Following brief exposure times (5 to 20 min), films displayed a single fragment hybridizing to the BP3385 probe for *B. pertussis*, 4 isolates of *B. bronchiseptica* (including PV6), and 11 isolates of *B. hinzii* (Table 1). A total of 4 uniquely sized fragments, none of which displayed the same mobility as the *B. pertussis* BP3385-containing fragment, was detected among all positive isolates. Representative results are included in Fig. 1. When films were overexposed, an additional, faintly detectable fragment of ~4.2 kb was evident in *B. hinzii* strains 1277 and F-1.

To further verify the presence of a BP3385 ortholog in *B. bronchiseptica* and *B. hinzii* isolates that were positive by Southern blotting, conventional PCR was carried out in a model 9700 thermal cycler (Applied Biosystems, Foster City, CA) using 100-ng samples of purified DNA from the appropriate strains and primers BP3385-1 and BP3385-2. Reaction mixtures included 0.4 μM primers, 1 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μl of 10× buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM $MgCl_2$, 10% dimethyl sulfoxide (DMSO), and 200 μM deoxynucleoside triphosphates (dNTPs) in a final volume of 25 μl. Cycling conditions were 2 min at 95°C, 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 7 min. Five microliters of each PCR mixture was analyzed by agarose gel electrophoresis in a 3:1 NuSieve gel (Cambrex

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TABLE 1. *Bordetella* isolates with positive or inconclusive RT-PCR results for BP3385[a]

Species	Isolate or strain	C_T	Hybridization to BP3385 probe/approx fragment size (kb)	Amplicon obtained with BP3385-1/BP3385-2	BP3385 allelic variant
B. pertussis	Tohama	26.2	+/4.7	+	1
B. bronchiseptica	RB50	ND	−	−	
B. bronchiseptica	PV6	26.1	+/8.0	+	4
B. bronchiseptica	OSU-083	42.9	+/3.8	+	5
B. bronchiseptica	OSU-189	35.0	−	ND	
B. bronchiseptica	OSU-553	34.6	−	ND	
B. bronchiseptica	OSU-585	32.9	−	ND	
B. bronchiseptica	ISU-CA90 BB1187	37.8	−	ND	
B. bronchiseptica	ISU-CA90 BB135	30.1	−	ND	
B. bronchiseptica	ISU-WI91 BB062/T5	40.8	+/3.8	+	5
B. bronchiseptica	ISU-WI91 BB064/T3	40.9	+/3.8	+	5
B. hinzii	ATCC 51783	36.8	+/7.9	+	3
B. hinzii	4134	35.5	+/7.9	+	3
B. hinzii	4135	36.0	+/11.2	+	2
B. hinzii	4140	32.3	−	ND	
B. hinzii	4159	35.5	+/7.9	+	3
B. hinzii	4161	35.3	+/7.9	+	3
B. hinzii	4449	33.3	−	ND	
B. hinzii	4595	35.2	+/7.9	+	3
B. hinzii	OH87 BAL006II	34.8	+/11.2	+	2
B. hinzii	OH87 BAL007II	44.2	−	ND	
B. hinzii	OH87 BAL028II	34.7	+/7.9	+	3
B. hinzii	OH87 BAL030II	35.2	+/7.9	+	3
B. hinzii	CA90 BAL33	35.6	+/7.9	+	3
B. hinzii	CA90 BAL1384	38.9	−	ND	
B. hinzii	BAL168II	35.6	+/7.9	+	3
B. hinzii	F-1	33.6	−	−	
B. hinzii	1277	34.4	−	−	
B. hinzii	DBL191	34.2	−	ND	

[a] ND, not determined; +, present; −, absent.

BioScience, Rockland, ME) containing a 10^{-4} dilution of GelRed stain (Phenix Research Products, Candler, NC). An amplicon of the size predicted for *B. pertussis* BP3385 was obtained from each of the 4 isolates of *B. bronchiseptica* and 11 isolates of *B. hinzii* previously noted to strongly hybridize with the BP3385 probe, as well as *B. pertussis* Tohama. No amplicons were obtained when DNA from *B. hinzii* strain 1277 or F-1 was used, although parallel testing with a 16S rRNA-specific PCR (10) confirmed the integrity of the DNA used as a template. Amplicons were purified using spin columns (Qiagen, Valencia, CA) and sequenced directly (with two reactions from each strand) by fluorescence-based cycle sequencing with AmpliTaq and BigDye Terminator kits on an ABI 377 sequencer at the National Animal Disease Center Genomics Unit. Sequences were analyzed using the Vector NTI suite (Invitrogen).

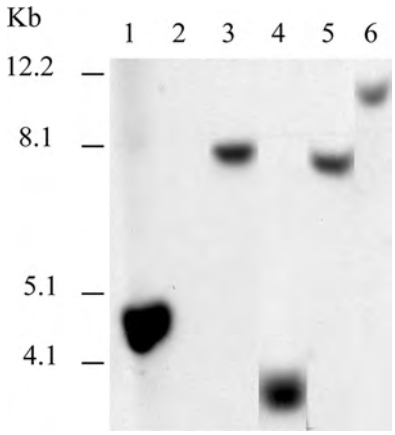

FIG. 1. Southern blot with EcoRV-digested *Bordetella* genomic DNA hybridized to a BP3385 probe. Lanes: 1, *B. pertussis* Tohama; 2, *B. bronchiseptica* RB50; 3, *B. bronchiseptica* PV6; 4, *B. bronchiseptica* OSU-083; 5, *B. hinzii* ATCC 51783; and 6, *B. hinzii* 4135. Relative positions of DNA size markers are indicated to the left.

The sequence we obtained from the BP3385-1/BP3385-2 amplicon from *B. pertussis* Tohama is identical to the corresponding sequence already reported for both Tohama (GenBank accession no. NC_002929) and *B. pertussis* isolate ATCC 9797 (3). Four additional allelic variants were identified among the *B. hinzii* and *B. bronchiseptica* strains evaluated, with two each found exclusively in one species (Fig. 2). Every variant is associated with an EcoRV fragment of unique size (Table 1), suggesting that strains constituting variant-specific groups may have additional genetic similarities. The predicted amino acid sequences of all variants are distinct from one another. The amino acid sequence predicted for variant 4, found only in *B. bronchiseptica* strain PV6, most closely approximates the sequence for *B. pertussis* BP3385 (with 98.0% amino acid identity). The amino acid sequences of the remaining variants are 89.1 to 91.1% identical to the corresponding sequence from *B. pertussis*.

The prevalence of BP3385 reported here for *B. hinzii*, 47.8%, suggests that the usefulness of this locus as a detection

```
           62
variant 1  TCGGTGCCAC CGACAAGGAC CTGGCCGATT TCTTCCATGT GACCGAGCGC ACGCTGAACA
variant 2  ----C----- ------A--- -----T---- -----GC--- ---G------ -----C----
variant 3  ----C----- ------A--- ---------- -----GC--- ---G------ -----C----
variant 4  ----C----- ---------- ---------- -------C-- ---------- ----------
variant 5  ----C----- ------A--- ---------- -----GC--- ---G------ -----C----

           122
variant 1  CTTGGAAGAA GCAGATCCCC GGGTTTCTTC AGGCCCTAAA TGGGGGCAAA GTCATGGCGG
variant 2  ---------- ----TA-G-- -A-------- ---------- --C------G AC-T----C-
variant 3  -C-------- ----CA-G-- -A-------- ---------- --C------G AC-C----C-
variant 4  ---------- ---------- -A-------- ---------- ---------- ----------
variant 5  -C-------- ----CA-G-- -A-------- ---------- --C------G AC-C----C-

           182
variant 1  ACGCCGAGGT GGCCGACAGG CTCTACCAAC GCGCTCTGGG CTACACACAC GTCGAGGACG
variant 2  -T-------- ------T--- --------G- ----C----- ------G--T -C--------
variant 3  -T-------- ---------- --------G- ----C----- ------G--T -C--------
variant 4  ---------- ---------- ---------- ---------- ---------- ----------
variant 5  -T-------- ---------- --T-----G- ----C----- ------G--- -C--------

           242
variant 1  ACATCCGGGT ATGCGACGGG GTGATCATCA CGACGCCCAC CACTAGGCAC TACCCGCCGG
variant 2  ---------- G--------T ------G-T- ----C--G-- G--C-A---- ----------
variant 3  ---------- T--------C ------G--- ---------- ---C------ ----------
variant 4  ---------- G--------T ------G--- ---------- ---C------ ----------
variant 5  -T-------- G--------C ------G-T- -C-------- G--C-A---T ----------

           302
variant 1  ACACGACGGC CTGCATCTTC TGGCTGAAGA ACCGGCGTCC GGATCTCTGG CGCGACAAGC
variant 2  -T--TGT--- ---------- ---------- -------C-- ---------- ----------
variant 3  ----C--A-- ---T------ ---------- ----A--C-- ---------- ----------
variant 4  ---------- ---------- ---------- -------C-- ---------- A-G-------
variant 5  -----GT--- G--------- ---T------ -------C-- ---C------ ----------

           362
variant 1  CGGACCC
variant 2  -------
variant 3  -------
variant 4  ----T--
variant 5  -------
```

FIG. 2. Comparison of *B. pertussis*, *B. bronchiseptica*, and *B. hinzii* BP3385-1/BP3385-2 amplicon sequence variants. The top line shows the sequence obtained from *B. pertussis* Tohama (BP3385); an identical sequence has also been reported for *B. pertussis* ATCC 9797 (3). Variants 2 and 3 were found only in *B. hinzii*, while variants 4 and 5 were found only in *B. bronchiseptica*. Dashes represent conserved bases; substitutions are indicated by the appropriate letter. Bases in bold indicate nonconservative substitutions. RT-PCR primer sequences are shaded, and the RT-PCR probe sequence is underlined. Nucleotide positions are numbered relative to the BP3385 start codon.

target for *B. pertussis* is limited. We found its prevalence in *B. bronchiseptica* to be considerably lower, 3.6%, but additional study may reveal a higher prevalence within specific subpopulations. With the exception of *B. bronchiseptica* PV6, all *B. hinzii* and *B. bronchiseptica* isolates in which we identified a BP3385 homolog are of avian origin. Approximately 70% of the *B. hinzii* strains we evaluated here were obtained from an avian host, compared to only 15% of the *B. bronchiseptica* strains tested. It is currently unknown whether *B. hinzii* isolates arising from particular hosts share unique genetic characteristics. However, sequence data derived from housekeeping genes indicate that most avian *B. bronchiseptica* strains, including many evaluated here, fall within the phylogenetic lineage from which *B. pertussis* is proposed to have evolved and which comprises largely human isolates (2; Register, unpublished). Although originally cultured from a pig, *B. bronchiseptica* PV6 is also in this lineage. A search for BP3385 in additional strains of the lineage may more accurately predict the frequency with which human *B. bronchiseptica* isolates would be mistakenly identified as *B. pertussis* when BP3385 is used as a diagnostic target.

Allelic variants 2, 3, and 5 have multiple base mismatches with the BP3385 RT-PCR primer and probe sequences, including the 3′-terminal position of the forward primer (Fig. 2). Although both Southern blotting and sequencing of amplicons obtained by conventional PCR prove the existence of a BP3385 homolog in isolates with these alleles, mispriming at some other locus in their genomes is a more likely explanation for the high C_T values obtained with the BP3385 RT-PCR. In contrast, except for a mismatch near the 5′ terminus of the RT-PCR forward primer, the variant 4 sequence corresponding to the RT-PCR probe and primers is identical to that from *B. pertussis*, thus explaining the lower C_T value and positive RT-PCR result obtained with *B. bronchiseptica* PV6.

Our results demonstrate that BP3385 is not restricted solely to *B. pertussis* and suggest that diagnostic assays targeting this

open reading frame are unlikely to provide a degree of specificity any higher than that achieved with currently used PCR assays. Clinical *Bordetella* isolates with positive BP3385 RT-PCR results require additional confirmatory testing for definitive identification as *B. pertussis*. Ongoing *Bordetella* genome sequencing projects and global genome comparisons will perhaps identify promising alternative targets.

Nucleotide sequence accession numbers. GenBank accession numbers for sequences obtained in this study are HM189275 to HM189289.

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Bordetella

K. Register and E. Harvill

INTRODUCTION

The genus *Bordetella* includes eight formally recognized species, of which *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Bordetella avium*, and *Bordetella hinzii* are of veterinary interest. *Bordetella pertussis*, the type species, is an obligate human pathogen and the causative agent of whooping cough. *B. parapertussis*, *B. bronchiseptica*, and *B. avium* are also associated with respiratory disease. *B. parapertussis* comprises two genetically and phenotypically distinct subgroups, one causing a pertussis-like illness in humans and the other a chronic, nonprogressive pneumonia in sheep. *B. bronchiseptica* causes tracheobronchitis (kennel cough) in dogs, bronchopneumonia in numerous laboratory, companion, and wild animals, and neonatal pneumonia in piglets and is an important contributor to swine atrophic rhinitis. Infection is often chronic and/or asymptomatic. The organism also rarely causes disease in humans. *B. avium* is the etiologic agent of turkey coryza and, with the exception of two recent isolations from humans (Spilker et al. 2008; Harrington et al. 2009), has been found exclusively in avian hosts. *B. hinzii*, recognized as a species since 1994, has been identified primarily from humans and poultry. It is an opportunistic pathogen in immunocompromised humans, and some strains induce mild to moderate respiratory disease in turkeys (Register and Kunkle 2009).

The remaining species of the genus, recognized since 1995 or later, include *Bordetella holmesii*, *Bordetella petrii*, and *Bordetella trematum*. All are associated with opportunistic human infections of the respiratory tract, blood, or other body sites. An additional species isolated from humans, *Bordetella ansorpii*, has been described but not yet accorded standing in nomenclature (www.bacterio.cict.fr/nonvalid.html).

CHARACTERISTICS OF THE GENUS

Bordetella are gram-negative coccobacilli of the family *Alcaligenaceae* and closely related to *Achromobacter* and *Alcaligenes* (Gerlach et al. 2001). Comparative studies suggest that *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*, the so-called "*B. bronchiseptica* cluster," or "classical" *Bordetella*, are highly clonal and represent subspecies of a single species. *B. bronchiseptica* has been proposed to be the ancestral progenitor from which two independent, host-restricted clones, *B. pertussis* and *B. parapertussis*, evolved. Genome sequences recently obtained from these species support this hypothesis (Parkhill et al. 2003).

All *Bordetella* grow under aerobic conditions; only *B. petrii* also grows anaerobically. The *Bordetella* are unable to utilize carbohydrates and depend on amino and carboxylic acids as primary carbon and energy sources. Growth of *B. pertussis* is inhibited by fatty acids and other components found in many media, and specialized formulations are required for isolation. Most other *Bordetella* species grow readily on a variety of agar media, including blood or MacConkey agar, and form mature colonies in 1–3 days. *Bordetella* of veterinary importance are resistant to a number of antibiotics, and selective media are preferred for isolation since overgrowth of other, faster growing bacteria

present in higher numbers otherwise interfere with detection. The optimal temperature for cultivation of *Bordetella* is 37°C, although all but *B. pertussis* grow reasonably well at temperatures as low as 25°C. Speciation of *Bordetella* isolates may generally be accomplished by consideration of the host of origin and the results of traditional biochemical tests (Songer and Post 2005). Polymerase chain reaction (PCR) assays for identification of *B. bronchiseptica* and *B. avium* have been favorably evaluated for use by veterinary diagnostic laboratories (Register and Yersin 2005; Register and DeJong 2006), but clinical validation has so far not been reported.

SOURCES OF THE BACTERIA

Bordetella species of veterinary interest are associated with mammals and/or birds and have a worldwide distribution. In their natural hosts, they inhabit the respiratory tract and are spread by direct contact or aerosol. *B. bronchiseptica* is most commonly found in pigs, dogs, rabbits, guinea pigs, and cats and has also been isolated from poultry and many other farm, companion, or wild animals. There is evidence for occasional transmission across host species, particularly between dogs and cats (Gaskell et al. 2006), but the full extent to which this occurs is unclear. Potential sources of *B. avium* include many species of domesticated and wild birds (Hopkins et al. 1990; Raffel et al. 2002). Transmission from free-living avian populations to poultry production farms has yet to be demonstrated. Poultry are the primary natural hosts of *B. hinzii*; isolation from a rabbit and a mouse has also been reported (Register et al. 2003; Hayashimoto et al. 2008). *B. parapertussis* is found exclusively in sheep and humans, and the two host-specific subgroups are not known to cross species.

Many animal hosts may be asymptomatically infected with *Bordetella*, and these carriers are a significant source of exposure and spread, especially under conditions associated with intensive food animal production or companion animal shelters. Excepting the human-adapted lineage of *B. parapertussis*, *Bordetella* species of veterinary significance only rarely cause disease in healthy adult humans. It is unclear whether humans may act as asymptomatic carriers of *B. bronchiseptica* following exposure to infected or immunized animals. There are only a few reports of adverse consequences secondary to such exposure, but this is an area in need of further investigation (Berkelman 2003).

VIRULENCE FACTORS

The classical *Bordetella* share an array of known or putative virulence genes, some of which are species specific. Most can be broadly categorized as colonization factors or toxins; additional products that otherwise alter host functions or assist in survival have also been described. Historically, most investigations have focused on *B. pertussis* due to its importance as a human pathogen. The extensive sequence identity generally shared among virulence gene orthologs of the classical *Bordetella* suggests that the corresponding proteins may perform equivalent functions, and related investigations generally confirm this. Nonetheless, the importance and function of some virulence factors differ among species, likely reflecting host-specific adaptation, and cross-species inferences should be viewed as presumptive. Initial efforts to identify common virulence factors in *B. avium* were largely unsuccessful. More recent studies reveal that these failures are attributable to both limited DNA sequence similarity of orthologous genes and diversity in the complement of *B. avium* virulence factors (Spears et al. 2003; Sebaihia et al. 2006). Information is limited regarding virulence factor identity and function in *B. hinzii*, but a few classical *Bordetella* virulence factors have been detected in some, but not all, isolates (Gerlach et al. 2001; Donato et al. 2005). Table 21.1 summarizes existing knowledge with respect to major virulence factors expressed by *Bordetella* species of veterinary interest; *B. pertussis* is included for comparison.

Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA), encoded by the *fhaB* gene, is a large, rod-shaped, ~220 kDa protein that functions as a major adhesin and is both associated with the bacterial cell surface and released into the culture medium during growth *in vitro*. Several distinct domains of FHA have been implicated in attachment to a variety of cell and tissue types (Mattoo and Cherry 2005). These include a carbohydrate recognition domain involved in binding to ciliated cells and macrophages, a heparin-binding domain of potential importance in the initial interaction with components of the extracellular matrix of the respiratory tract (also involved in hemagglutination), and an Arg-Gly-Asp (RGD) amino acid triplet that promotes binding to leukocytes, stimulates phagocytosis, and alters leukocyte signaling such that bacterial binding via complement receptor type

Table 21.1. Virulence Factor Expression in *B. pertussis* as Compared with Species of Veterinary Importance

Virulence factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	*B. hinzii*
BvgAS	+	+	+	+	+
Filamentous hemagglutinin	+	+	+	+	–
Fimbriae	+	+	+	+	ND
Pertactin	+	+	+	–	–
BrkA	+	+	+	–	ND
BatB	–	+	+	–	ND
Adenylate cyclase toxin	+	+	+	–	+/–[a]
Dermonecrotic toxin	+	+	+	+	–
Tracheal cytotoxin	+	+	+	+	ND
Osteotoxin	+	ND	+	+	ND
Alcaligen	+	+	+	–	ND
Enterobactin receptor	+	+	+	+	ND
Heme uptake system	+	+	+	+	ND
Type III secretion system	+[b]	+	+	–	–
Pertussis toxin	+	–	–	–	–

[a]Results differ for the only two strains so far evaluated.
[b]Demonstrated only in low-passage isolates.
ND = not determined.

3 (CR3) is increased. FHA is essential for tracheal colonization by *B. bronchiseptica* in rats, although one or more other adhesins are also required (Cotter et al. 1998), and it plays a primary, but not exclusive, role in biofilm formation (discussed later). The functional domains of the predicted proteins are highly conserved across the classical *Bordetella* species, but alterations are also present that may underlie, in part, differences in host specificity, cell- and tissue-specific colonization, and host receptor binding.

The *fhaB* gene directs synthesis of a 367-kDa precursor that is modified at both the N- and C-termini to produce a 220-kDa mature protein. An accessory gene in the same operon, *fhaC*, is required for translocation of FHA across the outer membrane by the two-partner secretion (TPS) pathway. Recent studies provide a detailed model for TPS transport and demonstrate that the C-terminus of cell-associated FHA is critically important for function *in vivo*, likely due to its orientation away from the cell surface and consequent availability for binding host cell receptors (Hodak et al. 2006; Mazar and Cotter 2006).

The *B. avium fhaB* and *fhaC* orthologs are required for virulence in turkeys, but the precise role of FHA in infection is unclear since mutation of either gene does not significantly impair attachment to tracheal rings (Spears et al. 2003; Sebaihia et al. 2006). Hemagglutination by *B. avium*, also associated with virulence, is likewise not a function of FHA but has been attributed to the proteins encoded by the *hagA/hagB* genes, which are novel among *Bordetella* but share some similarity with *fhaB/fhaC* (Sebaihia et al. 2006).

Additional genes encoding proteins highly similar to FHA have been identified among *Bordetella*, including those of veterinary importance (Julio and Cotter 2005; Sebaihia et al. 2006). None have so far been implicated in attachment or virulence.

Fimbriae

Fimbriae, produced by a variety of bacteria including *Bordetella*, are hair-like filaments extending from the cell surface that mediate attachment. The fimbrial shaft is composed of a repeating major subunit protein. Six major fimbrial subunit genes, scattered throughout the chromosome, have been identified among the classical *Bordetella*, but expression of only two, *fim2* and *fim3*, is shared among all species in the group (Mattoo and Cherry 2005). Fimbriae are highly immunogenic, and the Fim2 and Fim3 proteins are the basis for the serological typing system for *B. pertussis*.

The remaining major subunit genes are either absent from one or more species or present as pseudogenes that are not expressed due to a frameshift in the coding region or a deletion in the promoter. For example, the *fimA* subunit gene is found in all three species, but expression appears to be limited to *B. bronchiseptica* and ovine strains of *B. parapertussis* (Parkhill et al. 2003; Mattoo and Cherry 2005). The *fimA* gene is found within the *fimABCD* cluster, itself located between the *fhaB* and *fhaC* genes. *FimB* and *fimC* are involved in transport and assembly of the fimbrial structural proteins; *fimD* encodes the minor structural protein that forms the fimbrial tip. Synthesis of FHA and fimbriae is tightly coupled since the *fimBCDfhaC* genes are co-transcribed from a single promoter upstream of *fimB*.

In addition to control by the *bvgAS* locus (see discussion later), expression of most fimbriae is regulated by phase variation, a random, reversible event resulting from slipped-strand mispairing during DNA replication. Misalignment of the parent and daughter strands along a continuous stretch of cytosine residues (C stretch) upstream of the promoter leads to alterations in the length of the C stretch, thereby abolishing or activating transcription. Since the fimbrial structural genes are unlinked on the chromosome, phase variation of those with a C stretch occurs independently, and any combination of phase-variable fimbriae may be expressed simultaneously. On-off switching of fimbrial expression may be important for the maintenance of fimbrial function in the face of an immune response or other host or environmental selective pressures.

Bordetella avium also produces fimbriae, and 11 putative fimbrial subunit genes have been identified (Spears et al. 2003; Sebaihia et al. 2006). All but one, an ortholog of *fimA*, are novel to this species. The *B. avium* fimbrial structural subunit genes have no C stretch, but several contain a repeated sequence at the 5' end, which is potentially amenable to phase variation.

Both the major and minor fimbrial subunit proteins bind to sulfated sugars common on cell surfaces and in the mucus and extracellular matrix of the respiratory tract. Additionally, FimD binds to the monocyte receptor VLA-5, leading to increased FHA-mediated attachment through interaction with CR3. Although not requisite for colonization of the nasal cavity, fimbriae play an important role in colonization of the trachea and are required for persistence at that site.

Pertactin and Other Autotransporter Proteins

Pertactin is an outer membrane protein adhesin initially described as a protective immunogen in mice challenged with *B. bronchiseptica* and later shown to have a similar effect in pigs (Kobisch and Novotny 1990). The pertactin gene *prn* is found in all species of the *B. bronchiseptica* cluster but not in *B. avium* (Sebaihia et al. 2006). Pertactin is a member of the autotransporter family of proteins, known to play important roles in the pathogenesis of many gram-negative bacteria (Wells et al. 2007). Autotransporter proteins are characterized by a conserved C-terminal translocator domain that forms a pore through which the N-terminal passenger domain is directed for transit across the outer membrane. Following cleavage, the passenger domain remains cell-associated through a non-covalent interaction with the translocator, which is itself retained within the membrane. Mature pertactin, the fully processed N-terminal domain, migrates at ~68–70 kDa depending on the species of *Bordetella* that produces it.

The protein contains two regions of repetitive amino acids that form separate loops extending from a helical backbone (Emsley et al. 1996). Alterations in the size of pertactin are due to variance in the number of repeat units at these sites (Boursaux-Eude and Guiso 2000; Register 2001). Immunoprotective epitopes reside within both regions, and it has been postulated that repeat sequence polymorphism serves as a mechanism for immune evasion, with the antigenically variable immunodominant epitopes acting as decoys to prevent recognition of functional domains (Hijnen et al. 2007). Located adjacent to the N-proximal repeat is an RGD motif involved in binding to Chinese hamster ovary (CHO) cells. Numerous other *in vitro* studies suggest a role for pertactin in attachment, but direct evidence for its specific role *in vivo* is lacking, and it may be that pertactin functions as a subordinate or accessory adhesin during infection (Hibrand-Saint Oyant et al. 2005).

Twenty-one additional predicted or known autotransporter genes have been identified among the classical *Bordetella* (Parkhill et al. 2003; Wells et al. 2007), with some absent from or not expressed by all species. The BrkA autotransporter confers resistance to complement-mediated killing in *B. pertussis* but appears not to function similarly in *B. bronchiseptica*, although it is produced by most strains (Mattoo and Cherry 2005). The recently

identified BatB autotransporter plays a role in subversion of innate immune clearance mechanisms by *B. bronchiseptica* (Williams et al. 2008). Specific roles in virulence have been identified for only a few other autotransporters, primarily in *B. pertussis* (Mattoo and Cherry 2005; Wells et al. 2007). *B. avium* appears to possess seven intact autotransporter genes, none of which are orthologs to those of the classical *Bordetella* (Sebaihia et al. 2006). The function or importance of autotransporters other than pertactin and BatB in *Bordetella* of veterinary interest is largely unknown.

Adenylate Cyclase Toxin

The adenylate cyclase toxin (ACT) belongs to the "repeat in toxin" (RTX) family of bacterial pore-forming toxins, so named on the basis of conserved, calcium-binding glycine- and aspartate-rich repeats (Masin et al. 2006; Vojtova et al. 2006). It is a multifunctional, single polypeptide chain of 1706 amino acids with an N-terminal adenylate cyclase (AC) domain and a C-terminal hemolysin and pore-forming domain. The hemolysin may function independently of the AC domain, but both domains are required for AC activity. Internalization of ACT occurs through a receptor-independent mechanism (Martin et al. 2004) as well as a specific interaction with a β-2 integrin on the surface of many cells of the immune system. Unlike most RTXs, the majority of ACT remains associated with the bacterial cell surface, likely through interaction with FHA. However, it is the portion actively secreted into the external milieu that appears to be important for target cell intoxication. Yet, ACT does not function as a typical secreted toxin since intoxication additionally requires a close physical association between the bacterium and target cell. Once delivered to the eukaryotic cell cytosol, the AC domain is activated by the regulatory protein calmodulin. This results in unregulated conversion of cellular ATP to cyclic adenosine monophosphate (AMP), thereby disrupting cell signaling. Recently ACT was also demonstrated to elevate the level of calcium in target cells, potentially enhancing the activity of the toxin (Fiser et al. 2007).

ACT is essential for virulence and plays a critical role in infection by disabling innate immunoprotective functions, including phagocytosis, chemotaxis, and superoxide production, and by modulating immune responses through alteration of cytokine secretion. It also triggers apoptosis in macrophages, but cytotoxicity is likely effected through multiple pathways and depends on both pore-forming and AC activities (Hewlett et al. 2006). ACT has been suggested to contribute to colonization, but this is perhaps a manifestation of the functional stabilization of FHA rather than direct participation as an adhesin (Perez Vidakovics et al. 2006).

The structural gene for ACT, *cyaA*, is part of the *cyaABCDE* operon; CyaC catalyzes a posttranslational modification required for full activity, and cyaB, D, and E are required for secretion. ACT is produced by all classical *Bordetella* species, but each is antigenically distinct (Gueirard and Guiso 1993). Notwithstanding its demonstrated importance in virulence, the toxin appears nonessential for the success of *B. bronchiseptica* in certain niches based on the recent discovery of a unique phylogenetic lineage, hypovirulent in mice, missing the *cya* operon (Buboltz et al. 2008). Investigators have variously reported the absence (Gerlach et al. 2001) or presence (Donato et al. 2005) of *cyaA* and production of ACT in *B. hinzii*, a discrepancy likely due to interspecies differences (K.B. Register, unpublished data). No ACT-like activity has been detected in *B. avium*, and the *cyaA* operon is not present in the genome (Sebaihia et al. 2006).

Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is an intracellular, heat-labile, ~160-kDa protein that causes necrotic lesions, splenic atrophy, or death, depending on the dose and route of administration, in a variety of laboratory animals (Hoffmann and Schmidt 2004; Masin et al. 2006). The level of DNT expressed by *B. bronchiseptica* has been correlated with the severity of nasal and lung lesions in experimentally infected pigs, and purified toxin impairs bone formation, likely through inhibition of osteoblast differentiation. Studies using *B. bronchiseptica* DNT mutants established unequivocally that the toxin is required for the induction of pneumonia and turbinate atrophy in both mice and pigs. In contrast, *B. pertussis* DNT appears to have a minimal role in whooping cough. DNT is produced by all members of the *B. bronchiseptica* cluster, and the amino acid sequences are highly conserved. *B. avium* also produces DNT and likewise requires it for virulence (Temple et al. 1998), although there is considerable divergence in the deduced amino acid sequence as compared with the DNT of other *Bordetella* species (Sebaihia et al. 2006). DNT mutants of *B. bronchiseptica* and *B. avium* display reduced ability to colonize the upper

respiratory tract (Temple et al. 1998; Brockmeier et al. 2002), but this is most likely an indirect effect related to a lesser degree of tissue damage rather than any adhesive property of the toxin. Sequence-dependent approaches suggest that DNT is absent in *B. hinzii* (Gerlach et al. 2001).

Delivery of DNT to target cells is initiated through binding of the N-terminus to an unknown receptor. The toxin is subsequently cleaved by a host cell protease, and the enzymatically active C-terminus is released into the cytoplasm, where it catalyzes modifications of regulatory guanosine triphosphate (GTP)-binding proteins, leading to aberrations in cytoskeletal organization, protein and DNA synthesis, and other processes important for homeostasis and normal cellular function.

Tracheal Cytotoxin

Initially characterized as a component of *B. pertussis* culture supernatants toxic for ciliated epithelial cells, tracheal cytotoxin (TCT) was subsequently determined to be a muramyl dipeptide fragment of peptidoglycan resulting from normal cell wall remodeling during growth in many gram-negative bacteria (Luker et al. 1995). Such fragments are typically transported from the periplasm back to the cytoplasm by the AmpG protein, where they are reused in the biosynthesis of newly forming peptidoglycan. AmpG is also expressed in *Bordetella*, but for reasons currently unclear, the vast majority of TCT is released extracellularly. TCT causes ciliostasis and extrusion of ciliated cells from the respiratory epithelium via induction of IL-1 in associated mucus-secreting cells and consequent accumulation of the free radical nitric oxide. TCT alone may elicit cytokine production from other cell types, but its effect on epithelial cells requires, and is synergistic with, endotoxins (Flak et al. 2000). *B. bronchiseptica*, *B. parapertussis*, and *B. avium* (Gentry-Weeks et al. 1988) also produce TCT.

Osteotoxin

A cytotoxic protein expressed by *B. avium*, distinct from DNT, was demonstrated to be lethal for cell lines derived from the trachea and bones (Gentry-Weeks et al. 1993). Originally referred to as an osteotoxin, cloning and sequencing of the gene and characterization of the purified protein revealed the gene to be an ortholog of *metC*, which encodes a β-cystathionase involved in the biosynthesis of methionine. Toxic effects appear to be due to reactive derivatives arising from β-cystathionase-catalyzed cleavage of extracellular L-cystine. The gene and corresponding toxin are also present in *B. pertussis* and *B. bronchiseptica*, but *metC* appears to be a pseudogene in *B. parapertussis* (Gentry-Weeks et al. 1993; Parkhill et al. 2003). A role in virulence has been postulated, but no studies to date have directly addressed this question for any *Bordetella* species.

Lipopolysaccharide

Bordetella lipopolysaccharide (LPS) is a highly immunogenic, major constituent of the outer cell membrane and an important bacterial defense against host immune responses including antibodies, complement, antimicrobial peptides, and surfactants (Preston and Maskell 2001; Pilione et al. 2004; Schaeffer et al. 2004; Goebel et al. 2008). In addition, it shapes both innate and adaptive immune responses through modulation of host TLR4-mediated signaling. *Bordetella* express species- and even strain-specific lipid A structures through variable patterns of fatty acid substitution (Caroff et al. 2002). Structural variation within the core oligosaccharide has also been described. The O-antigen polysaccharides of *B. bronchiseptica* and the human cluster of *B. parapertussis* are homopolymers of a diaminohexuronic acid in which the terminal residue is highly modified. Interestingly, these modifications vary between subsets of strains and may represent a form of antigenic diversity (Preston et al. 2006). The O-antigens of *B. hinzii* and *B. avium* are composed of one or two additional diaminouronic acids (Preston and Maskell 2001; Caroff and Karibian 2003). Numerous LPS biosynthetic loci have been identified in species of the *B. bronchiseptica* cluster, and the function of at least some genes has been directly demonstrated or may be reasonably inferred from sequence similarity (Preston and Maskell 2001; Parkhill et al. 2003). Variation occurring in these loci corresponds to altered LPS structures in some strains (e.g., *B. parapertussis* strains of ovine origin, Cummings et al. 2004; Brinig et al. 2006), suggesting that the full repertoire of *Bordetella* LPS structures has yet to be defined. Many orthologs for LPS biosynthesis genes have been found in *B. avium*, but variable gene content and order within specific loci and the lack of obvious orthologs for O-antigen biosynthetic genes suggest this species may utilize novel pathways for its LPS biosynthesis.

Pertussis Toxin

Pertussis toxin is an adenosine diphosphate (ADP)-ribosylating toxin that, *in vitro*, exerts a wide variety of biological effects related to interference with cell signaling pathways (Masin et al. 2006). *In vivo*, it inhibits neutrophil recruitment, resulting in leukocytosis and delayed antibody-mediated clearance. The toxin structural genes (*ptxA–ptxE*) and those required for its transport (*ptlA–ptlH*) are found in all members of the *B. bronchiseptica* cluster, but toxin expression has been observed only in *B. pertussis*.

Type III Secretion System

The type III secretion system (TTSS), a virulence factor of many bacterial pathogens, consists of a needle-like macromolecular complex through which specific effector proteins that interfere with normal cellular functions are delivered directly to eukaryotic cells. *B. bronchiseptica* and ovine strains of *B. parapertussis* possess a functional TTSS, primarily encoded by the *bsc* locus, that induces non-apoptotic cell death and alters the host immune response, the latter possibly contributing to persistence of infection (Abe et al. 2008). The *bsc* locus includes 30 genes that direct the synthesis of the secretion apparatus, potential effector proteins, translocons (which form the pores in eukaryotic cells through which effectors are transported), and other components of this highly structured system. The only *Bordetella* effector protein so far characterized is BopC or BteA, whose corresponding gene lies outside the *bsc* locus.

The TTSS genes are highly conserved but not expressed in the laboratory strains of *B. pertussis* and human-adapted *B. parapertussis* that have been investigated. However, the recent demonstration of TTSS activity in low-passage clinical isolates of *B. pertussis* (Fennelly et al. 2008) indicates this system may be more widely operative in *Bordetella* than originally believed. *B. avium* does not possess the *bsc* genes, and there is no evidence suggestive of a secretion system of this class (Sebaihia et al. 2006).

Iron Acquisition

Bordetella employ numerous mechanisms to overcome the severe iron limitation encountered in the host environment (Brickman et al. 2007). Members of the *B. bronchiseptica* cluster produce a dihydroxymate siderophore, alcaligen, that acquires iron from the eukaryotic iron-binding proteins lactoferrin and transferrin and is required for maximal virulence in *B. bronchiseptica* (Foster and Dyer 1993; Register et al. 2001). Species of the *B. bronchiseptica* cluster also utilize the catechol siderophore enterobactin, which they do not synthesize themselves, through a type of "siderophore piracy" wherein a *Bordetella* enterobactin receptor scavenges iron from enterobactin produced by other nearby bacteria. This receptor additionally sequesters iron from other structurally similar xenosiderophores, and its synthesis is up-regulated by certain host catecholamines, including epinephrine, norepinephrine, and dopamine, which may act as environmental cues to drive appropriate transcriptional responses. A homolog of the gene encoding the ferric enterobactin receptor has been identified and shown to be expressed in *B avium* (Sebaihia et al. 2006).

A system for heme utilization has also been characterized among species of the *B. bronchiseptica* cluster and in *B. avium*. Genome sequence analysis suggests that a number of additional iron-scavenging systems may be operative in *Bordetella*, with some unique to particular species (Parkhill et al. 2003; Sebaihia et al. 2006). The precise role of specific iron acquisition systems in the various *in vivo* and *ex vivo* environments encountered during the *Bordetella* life cycle currently remains unknown.

Biofilm Formation

Bacterial biofilms are surface-adherent communities of bacteria embedded within an extracellular matrix of bacterial proteins, DNA, and polysaccharides. Biofilms afford protection against host defenses and significantly increase resistance to antibiotics and environmental stresses. Growth of *Bordetella* within biofilms, characterized by production of a poly-β-1,6-GlcNAc encoded by the *bps* locus, has recently been demonstrated both *in vitro* and *in vivo* (Mishra et al. 2005; Irie and Yuk 2007; Sloan et al. 2007). The *bps* locus is required for biofilm formation and persistent colonization of the nasal cavity in mice by *B. bronchiseptica*. Few studies have so far addressed the complex process of biofilm development in *Bordetella*, but available data suggest that several adhesins, including FHA, fimbriae, and pertactin, also play a role in either formation or stability (Irie et al. 2006; Sloan et al. 2007; Serra et al. 2008). *B. bronchiseptica* growing in biofilms is highly tolerant of several antibiotics at levels toxic for planktonic cells (Mishra et al. 2005), and a biofilm mode of

existence may underlie the frequently chronic nature of *Bordetella* infection.

Genetic Regulation of Virulence Factors

Expression of all known protein toxins and adhesins of the classical *Bordetella* is positively regulated by a two-component sensory transduction system encoded by the *bvgAS* locus (Beier and Gross 2008). BvgS is a transmembrane protein with a periplasmic N-terminal domain responsive to environmental signals and a cytoplasmic C-terminal domain that interacts with the transcriptional activator BvgA. Typical *in vitro* growth conditions, including temperatures approximating 37°C and the relative absence of SO_4^- ions or nicotinic acid, activate a BvgS-dependent phosphorelay cascade, which ultimately leads to activation of BvgAS-inducible genes, which include *bvgA* and *bvgS* themselves, by phosphorylated BvgA (BvgA~P). Also among the BvgAS-inducible genes is *bvgR*, located immediately downstream of *bvgAS*, whose product acts to repress transcription from an alternate set of BvgAS-repressed genes. During growth at ~26°C or below or in the presence of high concentrations of SO_4^- ions or nicotinic acid, the activity of the phosphorelay cascade is greatly diminished, the level of BvgA~P falls, and transcription of BvgAS-induced genes ceases. Expression of BvgAS-repressed genes is simultaneously activated due to the absence of BvgR.

This process, termed phenotypic modulation, has until recently been envisioned as a reversible switch between two distinct phases: the Bvg^+ or virulent phase, characterized by maximal BvgAS-induced virulence gene expression, and the Bvg^- or avirulent phase, in which virulence gene expression is absent. Recent studies have led to the rediscovery of an additional, intermediate phase (Bvg^i; Cotter and Miller 1997) that was first described more than 50 years ago but largely forgotten until knowledge of the genetic basis for virulence gene expression in *Bordetella* began to emerge. The Bvg^i phase is triggered by semi-modulating conditions (e.g., low levels of nicotinic acid) and is characterized by expression of a unique set of genes not activated in either the Bvg^+ or Bvg^- phase as well as a subset of genes known to also be expressed in the Bvg^+ phase. It is now understood that promoters of BvgAS-regulated genes differ in their affinity for binding BvgA~P. Genes with high-affinity promoters, including those for adhesins such as FHA, are activated early in the Bvg^- to Bvg^+ transition by the relatively low levels of BvgA~P present during growth under semi-modulating conditions, and these genes are expressed in both the Bvg^i and Bvg^+ phases. Expression of "late" genes with low-affinity promoters, including those encoding many toxins, is triggered only by the high levels of BvgA~P that accumulate during growth in the fully modulated Bvg^+ phase. These and other observations have led to the current concept of phenotypic modulation in *Bordetella* as a rheostat rather than an on-off switch. Although only three phases have so far been documented (Bvg^+, Bvg^i, and Bvg^-), it is postulated that BvgAS controls a continuum of many different, possibly overlapping, expression states.

BvgAS controls expression of more than 500 genes, including those encoding known or putative virulence factors, metabolic enzymes, folding and transport proteins, and other functional categories. Maintenance of a system that precisely controls the transcription of such a large and diverse number of genes suggests phenotypic modulation is an important adaptive response in *Bordetella*, perhaps optimizing growth and survival in the various niches encountered within the host and during transmission. There is good evidence that the Bvg^- phase is neither expressed nor required for full virulence *in vivo* (Cotter and Miller 1994; Akerley et al. 1995), but it appears the switching mechanism itself must be operative for maximal colonization of the respiratory tract (Irie and Yuk 2007). Adhesins and other Bvg^i-phase gene products may assist in transmission and initial colonization of the host, while the late Bvg^+-phase virulence factors may be necessary for growth and immune evasion *in vivo*. Current data suggest that the Bvg^- phase, perhaps induced by low temperature, is optimal for the *ex vivo* environments encountered during transmission between hosts when there is no apparent need for toxins or other virulence factors. In *B. bronchiseptica*, motility is a prominent Bvg^--phase phenotype that may aid bacteria in the search for nutrients and environments more hospitable to growth. Interestingly, neither *B. pertussis* nor *B. parapertussis* expresses motility under any growth condition and, unlike *B. bronchiseptica*, neither appears able to survive for extended periods of time outside a host. However, the specific conditions that modulate gene expression in nature are unknown, and there is no definitive understanding of the role of modulation in the *Bordetella* life cycle.

The *bvgAS* locus is also subject to phase variation, as described for fimbriae, in which spontaneous deletions or frameshift mutations in the promoter irreversibly abolish expression of all *bvgAS*-activated genes. It is not known how, or whether, organisms in this state contribute to the success of *Bordetella* as pathogens.

PATHOGENESIS

Patterns of Disease

Bordetella of veterinary importance cause acute respiratory disease with high morbidity and low mortality but also circulate subclinically among susceptible populations (de Jong 2006; Ford 2006; Gaskell et al. 2006; Jackwood and Saif 2008). Infection occurs at any age, although young animals are at highest risk for acute disease, perhaps as a result of age-related alterations in the epithelium of the nasal mucosa (Larochelle and Martineau-Doize 1990) and/or immaturity of the immune system. *Bordetella* are frequently found in association with viral and other bacterial respiratory pathogens and predispose to secondary infection with certain of these agents. Such coinfections often result in increased severity of clinical signs; specific synergistic effects on colonization, pro-inflammatory cytokine production, and other aspects of disease have been demonstrated (Cook et al. 1991; Brockmeier et al. 2008). Temperature, humidity, air quality, animal density, and other environmental and management factors also affect the incidence and severity of disease. Common clinical signs include coughing, sneezing, dyspnea, oculonasal discharge, poor weight gain, and lethargy.

Despite its isolation from a wide range of host species, *B. bronchiseptica* is associated with clinically apparent disease in relatively few, including pigs (atrophic rhinitis and neonatal pneumonia), dogs (infectious tracheobronchitis), cats (tracheitis and bronchopneumonia), guinea pigs (bronchopneumonia), and rabbits (snuffles). A few genetically unique, closely related clones have been reported as the primary cause of death in several recent morbillivirus outbreaks in seals (Register et al. 2000). *B. avium* is a primary pathogen in domesticated turkeys (turkey coryza or bordetellosis) and an opportunistic disease agent in chickens. It is widespread among numerous wild avian species and causes clinically recognizable disease in at least a few. Until recently, there was no evidence that *B. hinzii* is capable of causing disease in hosts other than humans. It is now known that some strains induce mild to moderate respiratory disease in turkeys (Register and Kunkle 2009). *B. parapertussis* alone induces only mild respiratory disease in sheep, but nasal carriage is common, and infected lambs are predisposed to severe, acute *Mannheimia haemolytica* bronchopneumonia (Martin 1996).

Occasional episodes of zoonotic disease involving *B. bronchiseptica* or, more rarely, *B. hinzii* or *B. avium* occur, typically manifested as respiratory infection of young, elderly, or immunocompromised patients (Mattoo and Cherry 2005; Spilker et al. 2008; Harrington et al. 2009). Pleuritis, septicemia, meningitis, and wound infection have also been reported. In recent years, the frequency of reported *Bordetella* zoonoses has increased, including several cases involving immunocompetent individuals (Berkelman 2003; Rath et al. 2008). It is unclear whether this reflects a true rise in the number of cases or is the result of improved methods of identification.

The Disease Process

Bordetella are readily transmitted in natural host species through direct contact with infected animals or contaminated fomites. *B. bronchiseptica*, but not *B. avium*, is also spread via droplet aerosols (de Jong 2006; Jackwood and Saif 2008). The initial event in the disease process is colonization of the nasal cavity, which establishes a primary reservoir of infection for subsequent spread to the lower respiratory tract. The temperature at the surface of the nasal mucosa, particularly the anterior portion, is several degrees less than the internal body temperature, prompting the hypothesis that newly deposited *Bordetella* may exhibit temperature-induced "early" $BvgAS^+$- and Bvg^i-specific phenotypes, including production of FHA and fimbriae and FHA-dependent biofilm formation (Cotter and Miller 1997; Irie and Yuk 2007). Consistent with this are results of a recent *in vivo* study demonstrating FHA-dependent adherence of *B. bronchiseptica* to non-ciliated olfactory cells in the nasal cavity as well as formation of microcolony aggregates on the nasal mucosa (Irie and Yuk 2007). Nonetheless, definitive proof of the existence of Bvg^i-phase bacteria in the nasal cavity has yet to be presented.

Bordetella also readily attach to ciliated cells, and this is a prominent feature of infection in both the upper and lower respiratory tracts (fig. 21.1). The

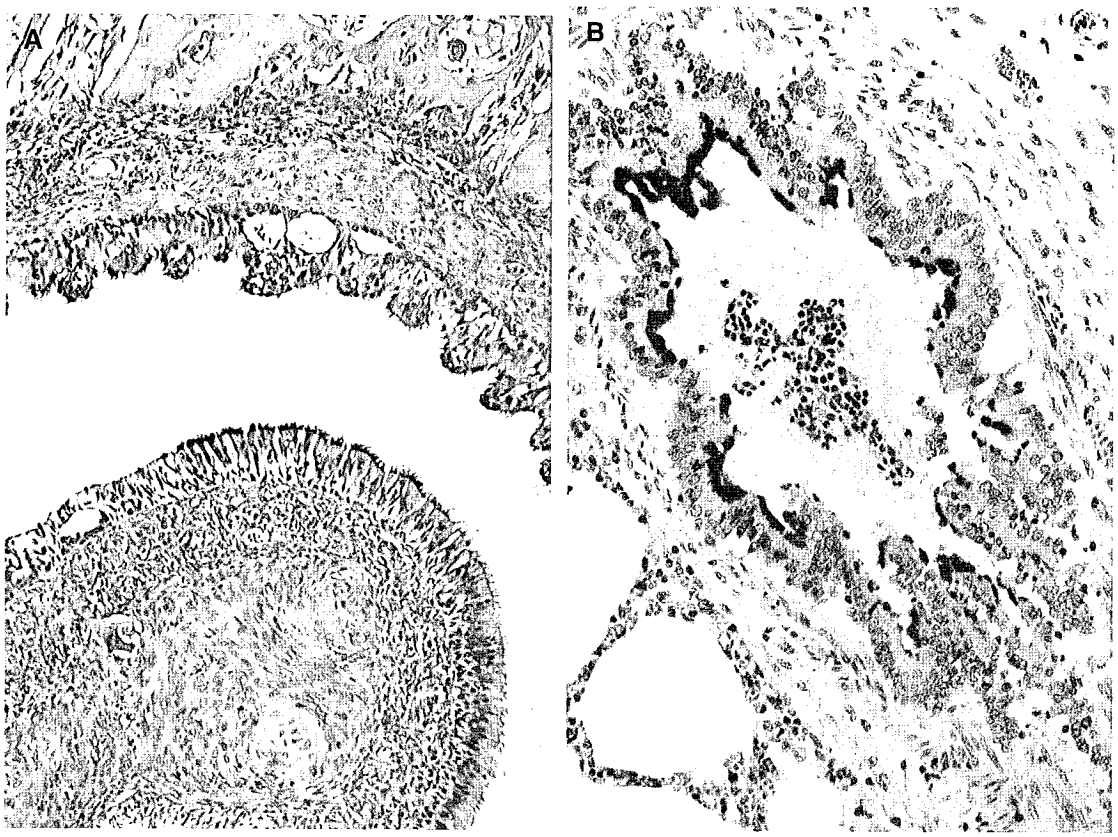

Figure 21.1. Colonization by *B. bronchiseptica* (red staining) of the ciliated epithelium of the conchae (A; 160×) or bronchus (B; 400×) of an infected pig. Areas denuded of cilia are apparent in the conchae. A neutrophilic inflammatory infiltrate is present in the lumen of the bronchus. (See color plate)

nature of this interaction in the nasal cavity is not understood, but it appears not to require FHA or fimbriae (Mattoo et al. 2000; Irie and Yuk 2007). In contrast, both *in vitro* and *in vivo* studies suggest a role for FHA, pertactin, fimbriae, and possibly additional adhesins in attachment to ciliated cells of the trachea (fig. 21.2; Cotter et al. 1998; Mattoo et al. 2000; Edwards et al. 2005). Replicating bacteria lead to production of TCT, likely responsible for initial impairment of the mucociliary clearance mechanism. Host cytokines and other inflammatory mediators elicited by the invading bacteria cause further tissue damage, thus opening the door to infection with other pathogens. As bacteria in the nasal cavity advance more deeply into the respiratory tract, the rise in temperature may promote a shift toward the Bvg^+-phase late gene expression, including synthesis of protein toxins that inhibit phagocytic cell function, further compromising microbial clearance. Although not true intracellular pathogens, *Bordetella* may invade and persist within phagocytic and other cell types (Brockmeier and Register 2000; Gueirard et al. 2005), which provide an environment protected from host immune responses.

Lesions

The severity of lesions in the nasal cavity in swine atrophic rhinitis varies widely depending on numerous factors, including the age of the animal, characteristics of the infecting strain, and housing and management (de Jong 2006). Atrophy of the ventral nasal turbinate bones is commonly seen at the height of infection but only rarely results in visible

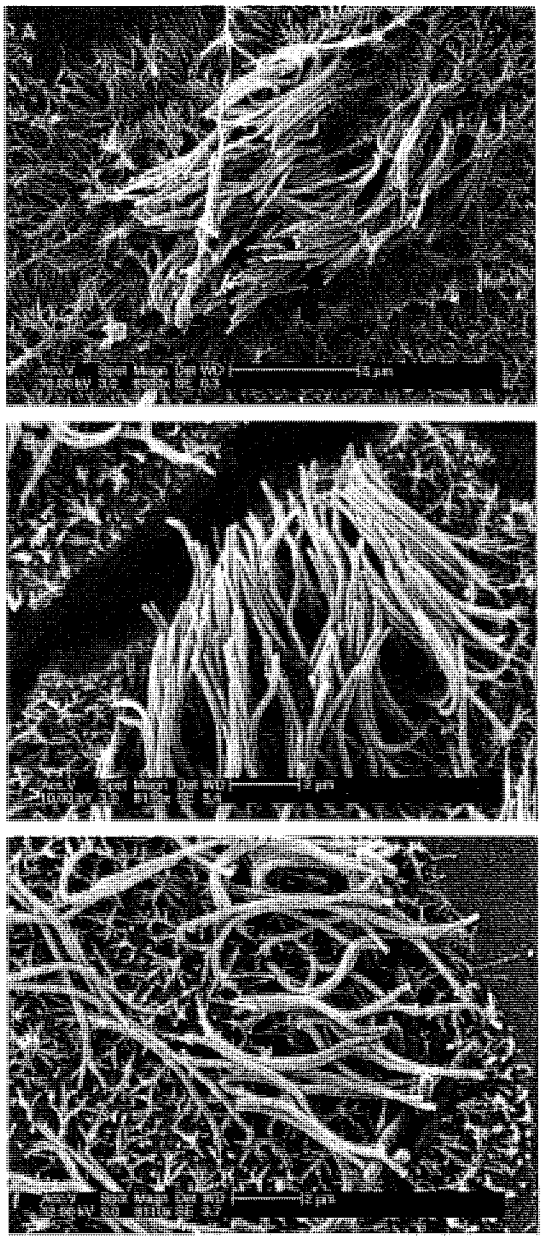

Figure 21.2. Binding of *B. bronchiseptica* to ciliated tracheal epithelial cells visualized by scanning electron microscopy. (A) Maximal binding, with preferential attachment to cilia, occurs when bacteria are in the fully modulated Bvg^+ phase. (B) A mutant strain locked permanently in the Bvg^- phase displays a greatly diminished capacity for adherence. (C) Ectopic expression of FHA by a phase-locked Bvg^- mutant restores ciliary attachment. (See color plate)

shortening or distortion of the snout unless toxigenic *P. multocida* is also present. In uncomplicated *B. bronchiseptica* infection, there is complete or nearly complete regeneration of the turbinate, such that lesions are mild or undetectable at slaughter. Microscopically, attenuation of the epithelium and loss of cilia are apparent, accompanied by inflammatory cell infiltration and fibroblastic proliferation in the lamina propria. Similar lesions are evident in rabbits and rodents; tracheitis is an additional frequent finding in dogs and cats (Ford 2006; Gaskell et al. 2006).

Pneumonic lesions typically have a monolobular or bilobular distribution and are characterized by necrosis, alveolar hemorrhage, neutrophil accumulation, and, eventually, fibrosis. Bronchiolar obstruction by inflammatory cell infiltrate and mucopurulent exudate also occur (fig. 21.1B). The most prominent lesion of bordetellosis in poultry is softening and distortion of the tracheal rings, ultimately leading to tracheal collapse (Jackwood and Saif 2008).

Ultrastructural changes include loss of ciliated tracheal epithelium, multifocal infiltrates in the tracheal lamina propria, depletion of mucus from goblet cells, and multifocal lymphoid nodules in the submucosa. The bronchial mucosa is largely unaffected, although changes in the bronchus-associated lymphoid tissue and occasional heterophil infiltrates may be apparent.

Immunity

The immune response to *Bordetella* has been assessed in limited ways in naturally infected animals. Antibody titers are easily detectable, but cross-reactivity with other bacteria confounds interpretation. Substantial species-specific differences due to the diversity of strains and hosts, the complexities of other flora of the respiratory tract, and other parameters make it difficult to define the immune response in natural infections.

Experimental infections have been performed in a wide variety of animals, including pigs, rabbits, guinea pigs, rats, and mice. Rabbit infections with *B. bronchiseptica* strain RB50 showed that this isolate from a healthy rabbit has an infectious dose of less than 10 organisms (Mattoo and Cherry 2005). While bacteria were often observed in the lower respiratory tract, this was variable at different time points. However, all rabbits remained healthy but consistently infected in the upper respiratory tract for at least 6 months, suggesting that the sub-

stantial antibody response measured was not sufficient to completely clear infection. Guinea pigs and rats similarly infected with RB50 and other isolates from healthy rabbits followed a similar course as that described in rabbits; bacteria grew rapidly in the nasal cavity during the first week of infection, spread sporadically and probably transiently to the lower respiratory tract, and persisted indefinitely in the upper respiratory tract, usually without overt signs. Limited data with other *Bordetella* species are roughly consistent.

The immune response to infection has been examined primarily in mice because of the availability of immunological tools and genetically manipulated animals, as well as space and cost issues. The time course of infection was substantially the same in mice as in rabbits, rats, and other animals. Low-dose inoculations with *B. bronchiseptica*, mimicking natural infections, resulted in slow and sporadic infection of the lower respiratory tract, making it difficult to determine at a given time point whether bacteria had not yet spread to the lungs or had already been cleared. High doses, typically 10^5–10^6 CFU, can synchronously colonize all respiratory tract organs and challenge the immune response to reveal the critical aspects involved in protection from severe disease. The caveat is that some effects observed in these animals may be more reflective of relatively rare severe disease than the more common subclinical infections. However, a high dose inoculum induced substantial lung pathology, which allowed the contributions of various virulence factors to be directly measured. Additionally, since the animals remained apparently healthy, recovered fully, and cleared the infection from the lungs, the immune components involved in bacterial control and clearance could be dissected using the powerful tools of molecular immunology of the mouse model.

Innate Immunity

Bordetella bronchiseptica induces a robust highly inflammatory response to infection. *In vitro*, macrophages respond to *B. bronchiseptica* by increasing expression of many molecules that likely contribute to this inflammatory environment. A small subset of these molecules that are dependent on TLR4 for their expression, including TNFα, are apparently critical, since TLR4-deficient or TLR4-depleted mice are highly susceptible to infection with as few as 1000 CFU (Mann et al. 2004b). A very early peak in TNFα was also observed in the lungs of experimentally infected mice and is critical to early control of the infection; mice genetically deficient or depleted of TNFα with antibodies also rapidly succumbed to as few as 1000 CFU (Mann et al. 2004a). A host of other pro-inflammatory factors were induced and appeared to contribute to the highly inflammatory response to infection. However, certain cytokines, such as IL-10, appeared to modulate inflammation and the subsequent generation of adaptive immunity (Pilione and Harvill 2006).

Adaptive Immunity

Mice lacking B cells failed to clear *B. bronchiseptica* from the lower respiratory tract, although they contained it and limited numbers to approximately 10^6 CFU (Kirimanjeswara et al. 2003). The high titers of antibodies generated were sufficient, when adoptively transferred to naive hosts, for protection of the lower respiratory tract, although they had no effect in the nasal cavity. IgA, present in low quantities in serum, is required for reduction in bacterial numbers in the nasal cavity (Wolfe et al. 2007). Mice lacking T cells also failed to clear *B. bronchiseptica*. Although the simplest explanation for this effect is that T cells are required for efficient generation of protective antibodies, it is likely that the interactions are more complex. As documented with *B. pertussis*, different T cell subsets are likely to contribute to clearance, or interfere with it, in different ways. *B. bronchiseptica* appears to take advantage of the ability of dendritic cells to skew the immune response. Mice lacking IFN-γ are more susceptible to infection and clear bacteria from the lower respiratory tract more slowly than do wild type (Skinner et al. 2005). By a mechanism involving the TTSS, *B. bronchiseptica* augments the production of IL-10, decreases IFN-γ, and delays clearance. Mice genetically deficient in IL-10 produced higher levels of IFN-γ earlier in the infection and eliminated *B. bronchiseptica* from the lungs and trachea more rapidly than did wild-type mice. Together, these data provide an example of how *B. bronchiseptica* manipulates host immunity through the TTSS. It appears likely that some of the many other factors that *B. bronchiseptica* induces and secretes similarly manipulate immunity to contribute to infection, persistence, and transmission between a wide range of different animal hosts.

Vaccines

B. bronchiseptica vaccines include live attenuated strains or antigen extracts of killed bacteria, alone and in combination with those of other pathogens. Intranasal vaccines generally induce higher antibody titers but can cause nasal discharge in some animals and are of limited efficacy in those previously exposed, probably because an attenuated vaccine strain cannot outcompete a resident wild-type strain. Newly described engineered avirulent strains may have improved safety and may prevent infection by wild-type strains, providing herd immunity (Mann et al. 2007). In contrast, parenteral vaccines have been shown to increase serum antibody levels even in exposed animals but can cause localized irritation and may induce lower levels of secretory antibody. Vaccines have been shown to protect against *B. bronchiseptica* tracheobronchitis in puppies and neonatal bronchopneumonia in pigs. However, experimental infections suggest that, similar to *B. pertussis* vaccines in humans, parenteral *B. bronchiseptica* vaccines are likely to prevent severe disease but have little effect on infection rates, and therefore fail to provide herd immunity.

CONCLUSION

Bordetella are exquisitely adapted to colonization of the respiratory tract mucosa, often in the absence of overt disease. The apparent evolution of host-restricted species from a broad host range *B. bronchiseptica* progenitor, coupled with the availability of species-specific genome sequences and global transcriptional profiling, provides a highly informed framework for investigation of the basis for host specificity. Such studies have already identified novel potential colonization factors and vaccine immunogens currently being targeted for further analysis. Although rarely used to date, multidisciplinary approaches that include comparative genomics, experimental microbiology in natural host model systems, and mathematical modeling could additionally facilitate an understanding of the complex host–pathogen interactions that occur during various stages of infection and disease.

Continued development of faster and cheaper methods for large-scale DNA sequencing increasingly permit both inter- and intraspecies genome-level comparisons that expose fascinating aspects of the population structure and evolution of the *Bordetella*. The recent establishment of a publicly available *Bordetella* MLST database, funded by Wellcome Trust, will further aid such studies and also assist in revealing the frequency with which novel lineages emerge as well as the potential importance of immune competition and other factors.

An appreciation of the finely tuned, dynamic transcriptional response mediated by the BvgAS signal transduction system in *Bordetella* as a continuum, as opposed to the previously held notion of an "on-off switch," is an important conceptual advance that will undoubtedly lead to a more complete understanding of the *Bordetella* life cycle. Of special interest is the possible role in transmission played by bacterial products specific for the Bvg^{i} phase and perhaps other, yet-to-be identified functional phases along the the Bvg^{+}/Bvg^{-} continuum.

Despite many recent advances, much remains to be discovered as to how the multiplicity of adhesins, toxins, and other products employed by *Bordetella* species in their respective hosts function cooperatively in the establishment and maintenance of infection and the development of disease.

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Pathogenesis of Bacterial Infections in Animals

Fourth Edition

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Prevalence and antigenic differences observed between *Bovine viral diarrhea virus* subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States

Julia F. Ridpath,[1] Robert W. Fulton, Peter D. Kirkland, John D. Neill

Abstract. *Bovine viral diarrhea virus* (BVDV) is divided into 2 different species within the *Pestivirus* genus, BVDV type 1 (BVDV-1) and BVDV type 2 (BVDV-2). Further phylogenetic analysis has revealed subgenotype groupings within the 2 types. Thus far, 12 BVDV-1 subgenotypes (a–l) and 2 BVDV-2 subgenotypes (a and b) have been identified. The purpose of the current study was to determine the prevalence of BVDV subgenotypes in the United States and Australia and to determine if there are detectable antigenic differences between the prevalent subgenotypes. To determine prevalence, phylogenetic analysis was performed on 2 blinded panels of isolates consisting of 351 viral isolates provided by the Elizabeth Macarthur Laboratory, New South Wales, and 514 viral isolates provided by Oklahoma State University. Differences were observed in the prevalence of BVDV subgenotypes between the United States (BVDV-1b most prevalent subgenotype) and Australia (BVDV-1c most prevalent subgenotype). To examine antigenic differences between the subgenotypes identified in samples from the United States and Australia, polyclonal antisera was produced in goats by exposing them at 3-week intervals to 2 noncytopathic and 1 cytopathic strain of either BVDV-1a, BVDV-1b, BVDV-1c, BVDV-2a, or *Border disease virus* (BDV). Virus neutralization (VN) assays were then performed against 3 viruses from each of the 5 subgenotypes. Comparison of VN results suggests that there are antigenic differences between BVDV strains belonging to different subgenotypes. The present study establishes a foundation for further studies examining whether vaccine protection can be improved by basing vaccines on the BVDV subgenotypes prevalent in the region in which the vaccine is to be used.

Key words: Antigenic diversity; *Bovine viral diarrhea virus*; pestivirus; prevalence; subgenotype.

Introduction

Bovine viral diarrhea virus (BVDV; family *Flaviviridae*, genus *Pestivirus*) is segregated into 2 different species, type 1 (BVDV-1) and type 2 (BVDV-2).[44] Although this segregation was first based on phylogenetic analysis,[34,37] subsequent characterization of viral strains from the 2 species has demonstrated antigenic differences.[39] The clinical significance of the observed antigenic differences was evidenced by the failure of vaccines and diagnostics based on BVDV-1 strains to control and detect, respectively, BVDV-2 strains.[13] The recognition of genomic and, particularly, antigenic difference between the 2 BVDV species led to the redesign of vaccines and diagnostics.[36]

Further phylogenetic analysis has revealed subgenotype groupings within the BVDV-1 and BVDV-2 species. Thus far, 12 BVDV-1 subgenotypes (a–l)[48] and 2 BVDV-2 subgenotypes (a and b)[17] have been identified. Novel subgenotypes of BVDV-1 have been identified in isolations of BVDV from cattle in South Africa and Switzerland but have not yet been assigned names.[5,28,29] Different BVDV subgenotypes predominate in different geographic locations.[4,5,14,15,21,23,25–28,30–35,42,43,45–47] The clinical significance of segregation into subgenotypes is still a matter of discussion and is not officially recognized by the International Committee on Taxonomy of Viruses. In addition to genomic differences, studies have shown antigenic differences between subgenotypes as demonstrated by differences in cross-neutralization,[5,35] monoclonal antibody binding,[11] and response of persistently infected animals to vaccination.[22] It is not known if variations among subgenotypes is significant enough to impact detection or the protection afforded by vaccination. It is possible that regional control programs would be improved by taking into

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Table 1. Viral isolates used in generation of polyclonal antisera.*

Goat	Isolate	Genotype	Biotype	Country of origin
1	7443	BVDV-1a	Noncytopathic	United States
	Singer	BVDV-1a	Cytopathic	United States
	CSU 454nc	BVDV-1a	Noncytopathic	United States
2	NY-1	BVDV-1b	Noncytopathic	United States
	TGAC	BVDV-1b	Cytopathic	United States
	NE	BVDV-1b	Noncytopathic	United States
3	AUS B619	BVDV-1c	Noncytopathic	Australia
	AUS B826c	BVDV-1c	Cytopathic	Australia
	AUS B522	BVDV-1c	Noncytopathic	Australia
4	RS886	BVDV-2	Noncytopathic	United States
	296c	BVDV-2	Cytopathic	United States
	125nc	BVDV-2	Noncytopathic	United States
5	BD31	BDV	Noncytopathic	United States
	CB5c	BDV	Cytopathic	United States
	WA 85-3295	BDV	Noncytopathic	United States

* Goats were sequentially inoculated with viral isolates in the order listed. BVDV = *Bovine viral diarrhea virus*; BDV = *Border disease virus*.

consideration the BVDV subgenotypes present in the design of diagnostics and vaccines.

Materials and methods

Subgenotype prevalence survey

Phylogenetic analysis was performed on sequences derived from 2 blinded panels consisting of 351 viral isolates provided by the Elizabeth Macarthur Laboratory (EMAI; New South Wales, Australia) and 514 viral isolates provided by Oklahoma State University (OSU; Stillwater, OK). The EMAI panel was collected over a 25-year period from cattle in Australia and was selected to represent a diversity of clinical presentations and geographical locations. The majority of these isolates were made from samples collected from persistently infected (PI) animals. The OSU panel was collected between June 2007 and June 2008 from PI animals identified in southwestern U.S. feedlots.

The basis of the phylogenetic analysis was a comparison of 5′-untranslated region sequences generated by cycle sequencing of polymerase chain reaction amplicons as described previously.[41] Segregation into subgenotypes was based on comparisons to BDDV-1[47,48] and BVDV-2[17] subgenotype type sequences previously reported.

Virus propagation for use in inoculum

Viruses were propagated in either bovine turbinate (BT) cells (BVDV-1a–c, BVDV-2a) or ovine fetal turbinate (OFT) cells (*Border disease virus* [BDV]). Bovine turbinate and OFT cells were grown in complete cell culture medium that was composed of minimal essential medium (F15 Eagle medium[a] supplemented with 10% fetal bovine serum, l-glutamine [final concentration, 1.4 mM], and gentamicin [final concentration, 50 mg/l]). The fetal bovine serum contained no BVDV and no antibodies against BVDV.[7] Viral titers were determined via limiting dilution in BT cells (BVDV-1a–c, BVDV-2a) or OFT cells (BDV).[38] Endpoints were based on cytopathic effect (cytopathic strains) or binding of monoclonal antibodies to the E2 structural protein (noncytopathic strains) as detected by immunoperoxidase staining as described below.[6]

Production of caprine polyclonal antisera

Handling and treatment of goats used to generate polyclonal antisera complied with the Animal Welfare Act.[2] Polyclonal antisera was produced in goats that tested free of BVDV based on virus isolation and free of BVDV antibodies based on serum neutralization, using the strains BVDV1a-Singer and BVDV2a-296c, as described previously.[38] Goats were housed in individual, climate-controlled pens under biosafety laboratory 2 containment. Goats were inoculated at 3-week intervals with 1 cytopathic and 2 noncytopathic strains of either BVDV-1a, BVDV-1b, BVDV-1c, BVDV-2a, or BDV (Table 1). Because pestivirus strains cannot be purified,[44] the inoculum consisted of 5 ml of freeze-thawed lysate of infected cells (1.0×10^6 tissue culture infective dose [TCID]/ml) delivered by the nasal route.[40] Polyclonal antisera were harvested 3 weeks after the third inoculation.

Virus neutralization tests

Titers of viral neutralizing antibodies in serum were determined against 3 noncytopathic isolates from each of the 5 genotypic groups (BVDV-1a–c, BVDV-2, and BDV; Table 2). Prior to comparing cross-neutralizing titers, all sera were initially diluted to a virus-neutralizing titer of 1/256 against the cytopathic virus used to generate the antisera. A microtiter plate assay was then used to determine titers with endpoints based on the binding of monoclonal antibodies to the E2 structural protein.[6] Briefly, following that initial dilution, serial 2-fold dilutions of sera in complete cell culture medium were prepared. A 100-µl aliquot of diluted serum and a 50-µl aliquot of virus containing 1,000 TCID were added to each well and incubated for 1 hr at 37°C. At the end of this incubation period, 20,000 BT cells (BVDV-1a–c and BVDV-2a isolates) or OFT cells (BDV isolates) in a 100-µl aliquot

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Table 2. Genotypes of isolates used in viral neutralization tests.*

Isolate	Genotype	Country of origin	Used in inoculation?
VM	BVDV-1a	United States	No
7443	BVDV-1a	United States	Yes
C24v	BVDV-1a	United States	No
NY-1	BVDV-1b	United States	Yes
NE	BVDV-1b	United States	Yes
TGAN	BVDV-1b	United States	No
AUS B675	BVDV-1c	Australia	No
AUS B843	BVDV-1c	Australia	No
AUS B666	BVDV-1c	Australia	No
890	BVDV-2a	United States	No
1373	BVDV-2a	United States	No
296nc	BVDV-2a	United States	No
BD31	BDV	United States	Yes
WA 85-3295	BDV	United States	Yes
Idaho 207	BDV	United States	No

* BVDV = *Bovine viral diarrhea virus*; BDV = *Border disease virus*.

of complete cell culture medium were added to each well. Inoculated cultures were incubated for 4 days at 37°C. Cell monolayers were rinsed in deionized, double-distilled water and fixed by adding 50 µl per well of fixation buffer (60% w/v bovine serum albumin, 40% v/v acetone in phosphate buffered saline [PBS]) and incubating for 10 min at room temperature (RMT). The fixation buffer was then removed, and plates were dried at 37°C for 1 hr. After drying, a 50-µl aliquot of primary monoclonal antibody (N2 hybridoma supernatant[37]) in PBSTN binding buffer (0.05% v/v Tween-20, 2.95% w/v sodium chloride in PBS) was added followed by incubation at RMT for 1 hr. The primary monoclonal antibody solution was removed, and wells were washed with 50 µl of PBST wash buffer (0.05% v/v Tween-20 in PBS). A 50-µl aliquot of goat antimouse immunoglobulin G (IgG)[b] (secondary antibody) prepared in PBSTN binding buffer was then added followed by incubation at RMT for 1 hr. After incubation, the secondary antibody was removed, cells were washed with PBST, and a 50-µl aliquot of protein G conjugated to horseradish peroxidase[c] prepared in PBSTN was added. After a 1-hr incubation at RMT, substrate in the form of 3-amino-9-ethylcarbazole was added, as described previously,[1] and color allowed to develop for 10 min. Reaction was stopped by removing substrate solution and adding water to each well. Stained cells were observed with a microscope.

Each neutralization assay was done in a replicate of 5, and titers were calculated using the Spearman–Kärber method.[16] The endpoint dilutions reflected the highest dilution of serum that inhibited the growth of virus. The serologic relatedness was expressed by 2 different methods. The simpler method involved calculating the ratio (P, stated as percentage) of the heterologous virus neutralization (VN) value as compared with the homologous VN value using the following formula:

$$P = 100 \times (BA/AA),$$

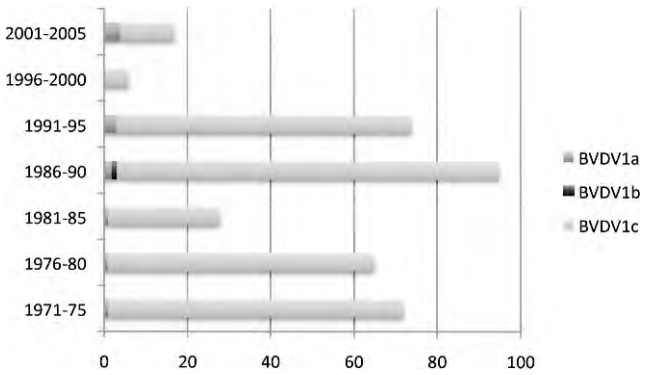

Figure 1. Temporal distribution of Australian *Bovine viral diarrhea virus* (BVDV) subgenotypes collected over a 25-year time span. The numbers of isolates characterized as BVDV-1a, BVDV-1b, or BVDV-1c are listed for 5-year time blocks.

where BA is the VN titer against strain B using antiserum A, and AA is the VN titer against strain A using antiserum A.

A second method determined the coefficient of antigenic similarity (R) between each subgenotype and between each subgenotype and BDV by a method previously described[3,5] using the following formula

$$R = 100 \times \sqrt{\frac{AB \times BA}{AA \times BB}},$$

where R is the coefficient of antigenic similarity, AB is the VN titer against strain A using antiserum B, and BB is the VN titer against strain B using antiserum B.

Results

Three subgenotypes of BVDV-1 and 1 subgenotype of BVDV-2 were identified among the BVDV isolates included in the 2 panels. Among the 351 isolates of the EMAI panel were 11 (3.1%) BVDV-1a strains, 1 (0.3%) BVDV-1b strain, 338 (96.3%) BVDV-1c strains, 4 (1.1%) BVDV-2a strains, and 1 (0.3%) BDV isolate. The temporal distribution of subgenotypes over the 25-year span is shown in Figure 1. The subgenotype of the BDV isolate was not determined for the current study. In contrast, phylogenetic analysis of the 514 isolates in the OSU panel revealed 62 (12.1%) BVDV-1a strains, 387 (75.3%) BVDV-1b strains, no BVDV-1c strains, 65 (12.6%) BVDV-2a strains, and no BDV strains.

The average VN value for each serum against strains from each of the 4 subgenotypes plus BDV is shown in Figure 2. Error bars represent the standard error of the mean. In all cases, the highest average VN titers against each subgenotype were observed when strains from 1 subgenotype were neutralized using antiserum produced in goats against strains from the same subgenotype (homologous neutralization). Virus neutralization titers were higher against

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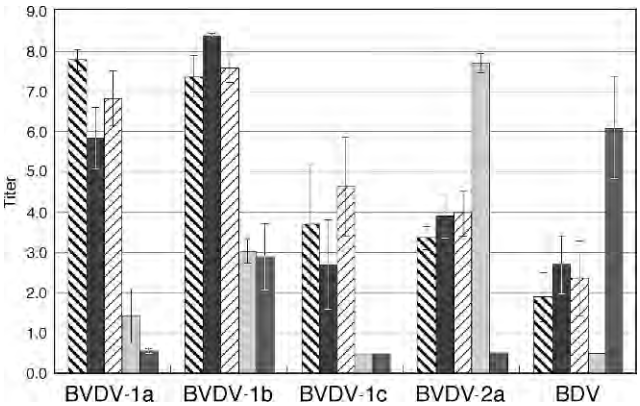

Figure 2. Virus neutralization (VN) titers of goat polyclonal sera produced against different pestivirus species and subgenotypes. Average VN titers of goat polyclonal sera produced against *Bovine viral diarrhea virus* (BVDV)-1a, BVDV-1b, BVDV-1c, BVDV-2a, and *Border disease virus* (BDV) strains when used to neutralize BVDV-1a, BVDV-1b, BVDV-1c, BVDV-2a, and BDV strains. The Y-axis is the log base 2 of the reciprocal of the endpoint dilution. Error bars represent the standard error of the mean.

viruses from different subgenotypes within the same species as those strains used to produce the antisera than to viruses from other species (Table 3). The VN values were between 36% and 52% of homologous neutralization for the 3 BVDV-1 subgenotypes. In contrast, VN values were less than 10% of homologous neutralization for viruses from different species.

The coefficients of antigenic similarity (R-values) between BVDV-1 (all 3 genotypes), BVDV-2, and BDV were less than 10, indicating significant antigenic differences (Table 4). The R-values among the 3 BVDV-1 subgenotypes ranged between 36 and 52 (Table 4). The R-value of 36 between BVDV-1a and BVDV-1b strains was similar to the R-value of 35 between BVDV-1a and BVDV-1b determined in a previous study.[5]

Discussion

In the current study, a marked difference was observed in the prevalence of BVDV subgenotypes in panels generated from Australian and U.S. isolates. The predominant species in each panel was BVDV-1; however, in the EMAI panel, the predominant subgenotype was BVDV-1c (>96%), while the predominant subgenotype in the OSU panel was BVDV-1b (>75%). Previous studies have shown variations in the presence and prevalence of BVDV species and subgenotypes in different continents and countries. A summary of those BVDV subgenotypes surveys based on isolates from 18 different countries is shown in Table 5. The number of subgenotypes identified in each country varies from 2 to 9, and the number of isolates characterized ranged from 8 to 475. Similar to the results of the current study, the most frequently reported species was BVDV-1. The most frequently reported subgenotype was BVDV-1b with 16 countries isolating BVDV-1b among cattle populations. Of these 16 countries, 6 countries identified it as a predominant strain as was observed in the OSU panel characterized in the present report. Although the presence of BVDV-1c strains was reported in 6 countries, Australia is unique in having a predominance of BVDV-1c strains. Further, the high percentage of isolates in 1 subgenotype (>96%) is also unique to the Australian samples, despite the fact that they were obtained from a wide range of locations over a 20-year period. The lack of diversity may be related to the fact that there are no ungulates native to the continent and that BVDV may have been introduced to Australia with the importation of cattle, goats, or sheep. In contrast to the United States, modified live BVDV vaccines have never been used in Australia. Although it is tempting to speculate that this has limited the genetic diversity in Australia, the diversity of observed BVDV-1 subgenotypes in European countries does not correlate with the use of modified live vaccines (Table 5). The geographic isolation and import restrictions in Australia have also probably limited the genetic variability of BVDV isolates. The single isolate of the type 1b subgenotype is of interest. Although the isolate cannot be linked directly to the known importation of a PI animal, it is likely that this virus is linked to importations of genetic materials from North America or England, the sources of most imported bovine genetics, prior to

Table 3. Cross-neutralization as a percentage of homologous neutralization.*

	BVDV-1a strains	BVDV-1b strains	BVDV-1c strains	BVDV-2a strains	BDV strains
Anti-BVDV-1a	100	36	52	2	2
Anti-BVDV-1b	36	100	39	4	5
Anti-BVDV-1c	52	39	100	7	7
Anti-BVDV-2a	2	4	7	100	1
Anti-BDV	2	5	7	1	100

* BVDV = *Bovine viral diarrhea virus*; BDV = *Border disease virus*.

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Table 4. Coefficients of antigenic similarity (R) between different pestivirus species and subgenotypes.*

Pestivirus species or subgenotype	BVDV-1a	BVDV-1b	BVDV-1c	BVDV-2a	BDV
BVDV-1a	100	36	52	2	2
BVDV-1b		100	39	4	5
BVDV-1c			100	7	7
BVDV-2a				100	1
BDV					100

* BVDV = *Bovine viral diarrhea virus*; BDV = *Border disease virus*.

the introduction of more stringent import requirements (P.D. Kirkland, unpublished data, 2009).

The number of isolates characterized in the current study is larger than the majority of previously published surveys. Although there is no accepted definition of the number of isolates that must be examined to determine subgenotype prevalence, surveys based on less than 50 isolates are probably not as reliable for prevalence determination as larger surveys. This is particularly true if care is not taken to compensate for multiple isolates from the same outbreak.

In the present study, antigenic variation based on differences in calculated R-values was observed between pestivirus species (BDV, BVDV-1, and BVDV-2) and between BVDV-1 subgenotypes (BVDV-1a–c). The variation between species was greater than the variation between subgenotypes. The antigenic variation between the BVDV-1 and BVDV-2 species has led to the inclusion of BVDV-2 strains in some vaccines.[36] It is not known if antigenic variations among subgenotypes are great enough to influence detection or the protection afforded by vaccination. Previous studies have shown antigenic differences based on cross-neutralization,[5,35] monoclonal antibody binding,[11] and response of PI animals to vaccination.[22] In a study[5] comparing 7 different BVDV-1 subgenotypes isolated from cattle in Switzerland, a variation was noted in the coefficient of antigenic similarity among subgenotypes similar to that seen in the present study. In the Swiss study, antisera collected from animals vaccinated using a

Table 5. Summary of studies characterizing *Bovine viral diarrhea virus* (BVDV) subgenotypes isolated from 19 different countries.*

Continent	Country	No. of field isolates	BVDV-1 subgenotype													BVDV-2†
			a	b	c	d	e	f	g	h	i	j	k	l	Unassigned	
Europe	Austria[25]	310	X	X		X	X	X	X	M			X			X
	Germany[43]	61	X	M		M		X	X							X
	Italy[23]	88	X	M		X	X	X	X	X						X
	France[26]	48	X	X		X	M							X		X
	Switzerland[5]	169		X			M			M			X			
	Spain[4]	44		M	X					X						
	Slovenia[45]	43		X		M		M	X							
	Finland[47]	8				M		X				X				
	United Kingdom[46]	62	M	X							X					X[14]
South America	Argentina[27]	29	M	M	X											X
	Brazil[47]	10	M	X		X										X
	Chile[35,42]	33, 10	X	M	X											M
	Peru[42]	15		M												
North America	Canada[34]	15	X	X												M
	United States[15,21]	54, 131‡	X	M												X
Africa	South Africa[28]	25	X	X	X										X	
India	India[33]	13		X												X[32]
Asia	Japan[31]	475	X	M	X							X				X
Australia	Australia[30]	89	X		M											

* BVDV-2 strains were not identified in the original surveys for the United Kingdom and India but were subsequently identified from isolated outbreaks. M = the most frequently isolated subgenotype(s) in the survey; X = subgenotype has been identified in isolates from that country but at a lower frequency

† Most of the studies did not differentiate between subgenotypes of BVDV-2a and BVDV-2b. Where data were available, BVDV-2a strains outnumbered BVDV-2b strains.

‡ Data from 2 surveys were combined.

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Table 6. Neutralizing antibodies observed following vaccination with commercial *Bovine viral diarrhea virus* (BVDV) vaccines.

Vaccine	No. of animals	Time postvaccination*	Titer			Reference no.
			BVDV-1a	BVDV-1b	BVDV-2	
BVDV-1a MLV	3	21 days	43.0†	34.5‡	Not done	11
	3	21 days	94.2†	25.1‡	Not done	11
BVDV-1a Killed	5	6 weeks	31.2†	27.9‡	Not done	10
	5	6 weeks	23.6†	34.4‡	Not done	10
	5	6 weeks	43.7†	18.7‡	Not done	10
BVDV-1a MLV	18	1.5 months	630.3	26.0	11.3§	12
	18	3.0 months	181.0	90.5	18.4§	12
	18	18 months	147.0	29.9	26.0§	12
	10	42 days	189.3‖	34.4¶	7.0	20
	6	42 days	733.4‖	322.5	50.8	20
BVDV-1a, BVDV2a MLV	12	42 days	95.9	40.3	215.3	20
BVDV-1a, BVDV2a Killed	51	116 days	42.5	17.6	29.3	18

* In the case of killed vaccines, time postvaccination refers to the period of time after the second dose of killed vaccine.
† Average of virus neutralization (VN) values for 6 BVDV-1a strains genotyped subsequent to publication of the study.
‡ Average of VN values for 12 BVDV-1b strains genotyped subsequent to publication of the study.
§ Average of VN values for 9 BVDV-2 strains.
‖ Average of VN values for 3 BVDV-1a strains.
¶ Average of VN values for 2 BVDV-1b strains.

BVDV-1a killed vaccine neutralized all 7 subgenotypes. However, neutralization of viruses from BVDV-1 subgenotypes different from the BVDV-1 subgenotype of the vaccine virus (heterologous neutralization) varied from 34% to 51% of the VN values of homologous neutralization. A summary of reports detailing antibody responses observed in cattle following vaccination with commercial vaccines is shown in Table 6. Although the practical significance of these antigenic differences among subgenotypes is undetermined, lower VN values for BVDV-1b strains were observed following vaccination with both killed and modified live vaccines containing only BVDV-1a strains or both BVDV-1a and BVDV-2a strains for 11 of the 12 vaccine studies listed in Table 6. It is interesting to note that inclusion of both a BVDV-1a strain and a BVDV-2a strain, which theoretically should increase the antigenic range of the vaccine protection, did not alleviate the lower VN values for BVDV-1b strains. Is the reduced neutralization of BVDV-1b strains of practical significance? Although there is currently no definitive answer to this question, there are published studies regarding the level of neutralizing antibody associated with prevention of viremia and clinical disease and the transmission of virus by PI animals in the face of vaccination. In a study assessing protection afforded by passively acquired neutralizing antibodies,[8] it was found that fever and systemic spread of virus were detected in calves that had viral neutralizing titers of 256 or lower following experimental exposure to a highly virulent BVDV-2 strain. Calves that had viral neutralizing titers lower than 16 developed clinically severe disease. In a study of transmission of virus to vaccinated calves exposed to cattle persistently infected with BVDV-1b,[19] it was shown that titers up to 64 did not prevent BVDV-1b from being isolated after exposure. In a study assessing fetal protection in cattle,[24] both nonvaccinated and vaccinated pregnant cattle were exposed to 4 PI animals of which 2 carried a BVDV-1b strain and 2 carried a BVDV-2a strain. Vaccinated animals received 2 doses of a killed vaccine containing a BVDV-1a strain and a BVDV-2a strain. Titers in vaccinated animals averaged 724 against a BVDV-1a strain and 351 against a BVDV-2a strain prior to exposure to PI animals. Fetuses were harvested at approximately 150 days gestation. BVDV could be isolated from brain tissue of 14 out of 14 fetuses of nonvaccinated animals and from 4 out of 14 fetuses of vaccinated animals. Eight of the fetuses, from nonvaccinated cattle, carried the BVDV-2 strain (57%) and 6 carried the BVDV-1b strain (43%), based on virus isolation from brain tissue. In contrast, the BVDV-2 strain could be isolated from brain tissue of only 1 of the 4 infected fetuses of vaccinated animals (25%), while the BVDV-1b strain was isolated from brain tissue of all 4 (100%). These studies suggest that the cross-neutralization between BVDV-1a or BVDV-2a strains may not be great enough to prevent infection with BVDV-1b strains.

In the last decade, there has been a consolidation of veterinary biologics companies accompanied by a reduction in the range of strains used in BVDV vaccines. In the United States, these strains belong predominately to the BVDV-1a and BVDV-2a geno-

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types.[20,36] In particular, the assimilation of regional companies has resulted in a removal from the market of BVDV vaccines based on strains endemic to the region. Large international companies offer BVDV vaccines based on a limited number of strains. Although further research is necessary, variation in prevalence of BVDV subgenotypes and variation in cross-protection between BVDV subgenotypes suggest vaccine protection may be improved by basing vaccines on the BVDV subgenotypes prevalent in the region in which the vaccine is to be used.

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Sources and manufacturers

a. GIBCO®, Life Technologies Corp., Carlsbad, CA.
b. Cappel Laboratories Inc., Cochranville, PA.
c. Zymed Laboratories Inc., San Francisco, CA.

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ORIGINAL ARTICLE

Evaluation of three experimental bovine viral diarrhea virus killed vaccines adjuvanted with combinations of Quil A cholesterol and dimethyldioctadecylammonium (DDA) bromide

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Abstract Bovine viral diarrhea virus (BVDV) infections cause respiratory, reproductive, and enteric disease in cattle. Vaccination raises herd resistance and limits the spread of BVDV among cattle. Both killed and modified live vaccines against BVDV are available. While modified live vaccines elicit an immune response with a broader range and a longer duration of immunity, killed vaccines are considered to be safer. One way to improve the performance of killed vaccines is to develop new adjuvants. The goal of this research was evaluate new adjuvants, consisting of combinations of Quil A cholesterol and dimethyldioctadecylammonium (DDA) bromide, for use in killed vaccines. Responses to three novel killed vaccines, using combinations of Quil A and DDA as adjuvants, were compared to responses to a commercial modified live and a commercial killed vaccine. Vaccination response was monitored by measuring viral neutralizing antibodies (VN) levels and by response to challenge. All three novel vaccines were efficacious based on reduction in virus isolation, pyrexia, and depression. Compared to a commercial killed vaccine, the three novel vaccines elicited higher VN levels and reduced injection site inflammation.

Keywords Bovine viral diarrhea virus, adjuvant · Quil A cholesterol · Dimethyldioctadecylammonium bromide · Killed vaccine

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Introduction

Reproductive and respiratory disease associated with infection with bovine viral diarrhea viruses (BVDV) result in significant economic losses for dairy and beef producers. The term BVDV refers to two distinct groups of viruses, BVDV1 and BVDV2, which are classified as two different species within the *Pestivirus* genus of the *Flavivirus* family (Ridpath 2008) Acute infections result in disease of varying severity, depending on the viral strain, the immune and reproductive status of the host, and the presence of secondary pathogens (Callan and Garry 2002; Evermann and Barrington 2005). Acute infections with BVDV, regardless of clinical presentation, are always accompanied by immune suppression due, at least in part, to the death of immune cells within lymph nodes and gut associated lymphoid tissue and reduction of numbers of circulating white blood cells (Liebler-Tenorio et al. 2002, 2004; Liebler-Tenorio et al. 2003a, b). The suppression of the immune system leaves infected animals vulnerable to secondary infections. In addition to acute infections, noncytopathic BVDV strains may establish life-long persistent infections. These persistent infections are the result of fetal exposure to BVDV before the development of the immune system (Brock et al. 2005). The PI animal is thought to be the major vector for introduction of BVDV into herds. There are two different goals for BVDV vaccination, one is to limit clinical disease (including immunosuppression) and viral shed following acute infections. The other is to prevent the fetal infection that leads to persistent infection.

Both modified-live virus (MLV) and killed vaccines are available for control of BVDV infections (Fulton 2005). MLV vaccines elicit a faster, longer lasting and broader response. However, post-vaccinal disease, particularly mucosal disease in PI animals, has been observed following vaccination with MLV vaccines. Killed vaccines are more stable and are safe for use in all animals. Killed vaccines are comprised of viral antigen (Ag) that has been inactivated by various methods, such as heat, chemicals or physical disruption, and can no longer replicate in vivo. Most commonly in inactivated BVDV vaccines, the Ag is enhanced with an adjuvant component, because the viral Ag is typically poorly immunogenic (Kwissa et al. 2007). Although considered to be the safer, killed vaccines may have incomplete or short-lived immune responses compared to MLV vaccines. Booster vaccinations are usually required to achieve protective immune responses. In addition, use of adjuvant may result in injection site reactions.

Adjuvant combinations (coadjuvantation) can potentially overcome some of the problems observed with killed vaccines (Fraser et al. 2007). The Q-series of adjuvants, used in this study, are comprised of multiple interchangeable molecules, including Quil A, cholesterol, and dimethyldioctadecylammonium (DDA) bromide. Quil A is an enriched fraction of a tri-terpenoid saponin derived from the bark of the South American tree *Quillaja saponaria* commonly used in veterinary vaccines (Kirk et al. 2004). When Quil A is combined with cholesterol (QAC) it forms helical, nanometer scale worm-like micelles (Mitra and Dungan 2001). DDA is a cationic, micelle-forming surfactant (Lincopan et al. 2007). In this study, three experimental vaccines, adjuvanted with Q-series adjuvants were compared to commercially available vaccines. Vaccination response was determined by measuring viral neutralizing antibodies (VN) levels before and after vaccination and by response to exposure to live BVDV following vaccination (challenge). The response to challenge was monitored by measuring changes in circulating white blood cells, basal temperature, and virus isolation.

Materials and methods

Experimental design

Cattle were assigned to one of six vaccination protocols (Table 1). In protocol 1 (control protocol), cattle were injected subcutaneously, on days 0 and 21, with a sterile saline solution. In protocol 2 cattle were injected subcutaneously with a modified live virus vaccine on day 0 of the study (prepared by the protocol used for a commercial vaccine). In protocol 3 (killed vaccine prepared by protocol used for another commercial vaccine), cattle were injected subcutaneously on days 0 and 21 of the study. In protocols 4, 5, and 6, cattle were injected subcutaneously, on days 0 and 21 of the study, with experimental vaccines that were adjuvanted with different formulations of the Q-series adjuvants (see below).

Screening, housing and allotment of animals

Healthy, non-vaccinated beef calves (of mixed breed and gender), approximately 7 to 12 months of age (body weight range 272 to 442 kg) were tested and confirmed negative for BVDV antigen based on immunohistochemistry performed on skin biopsies (University of Nebraska Veterinary Diagnostic Center, Lincoln, NE) (Brodersen 2004). Animals were screened for serum antibodies against BVDV by virus neutralization assay and only animals with VN titers <1:2 were enrolled in the study (Ridpath et al. 2008).

Processing at arrival included administration of an injectable parasiticide (doramectin, Pfizer Animal Health, New York, NY) and a metaphylactic anti-microbial for BRD (tulathromycin, Pfizer Animal Health, New York, NY). Acclimatization began approximately 1 week before Day 0. The animals had ad libitum access to commercial feed and to water from a municipal source.

This 56-day study used a generalized, randomized, incomplete block study design, with animals assigned to blocks of five animals each by body weight. The animal was the experimental unit. Within each block, treatments were randomly assigned to animals using a computer-generated plan. Animals were housed by vaccination protocol group from Day 0 to Day 21. On Day 35, the calves were randomly assigned to commingled pens and

Table 1 Vaccination protocols for 6 experimental groups

Group	1 Negative control	2 MLV vaccine	3 Killed vaccine	4 Experimental vaccine	5 Experimental vaccine	6 Experimental vaccine
Virus	None	Live virus	Killed virus	Killed virus	Killed virus	Detergent extracted virus
Adjuvant	None	None	PreZent-A	QAC DDA Carbopol	QAC DDA Carbopol R1005	QAC DDA Carbopol
Inoculation	Day 0 Day 21	Day 0	Day 0 Day 21	Day 0 Day 21	Day 0 Day 21	Day 0[a] Day 21[b]

[a] High titer extract

[b] Low titer extract

moved into isolation rooms before challenge with BVDV-2 on Day 42. Animals were housed by block with two blocks in each pen, six pens total. Blocks were randomly assigned to pens in each phase such that the weight variability within each pen was minimized. This design was balanced, 10 animals in each treatment group.

Vaccination protocols

Animals in group 1 received 0.9% sodium chloride sterile solution, on days 0 and 21, and served as the control group. Animals in group 2 received a MLV BVDV-2 vaccine. The antigen concentration and formulation of this vaccine was the same virus and titer as that used in the production of the BoviShield Gold® vaccine line (Pfizer Animal Health, Kalamazo, MI). Briefly, this vaccine was a freeze-dried preparation of modified-live BVDV2 (propagated on an established bovine cell line) and was rehydrated with 0.9% sodium chloride sterile solution. This vaccine contained no adjuvant and was only administered once to this group on Day 0. Animals in group 3 received a killed virus BVDV-2 [inactivated with 5% binary ethyleneimine (BEI); 44–72 h inactivation at 34–38° C] vaccine adjuvanted with Amphigen and QAC. This adjuvant is the same as the adjuvant in CattleMaster® Gold™, (PreZent-A™, Pfizer Animal Health). The BVDV2 antigen concentration and formulation of this vaccine was also the same as that of the CattleMaster Gold vaccine line (Pfizer Animal Health). Animals in groups 4, 5, and 6 received killed-virus vaccines containing novel adjuvants as listed in Table 1. The concentration of BVDV Ag in the vaccines used for groups 4 and 5 was the same as the concentration present in the killed vaccine used for group 3 (2,750 RU/mL). The animals in group 6 received an experimental vaccine that differed in the method of antigen preparation but had the same adjuvant as the vaccine used for group 4. A detergent extraction method was used to prepare Ag enriched for BVDV proteins. The BVDV2 virus was extracted with sodium deoxycholate (1.5 h with mixing, 25°C), clarified by centrifugation (3,000×*g* for 30 min at 25°C), concentrated by dialysis using a 10 kD-cutoff ultrafiltration membrane, and sterilized using a 0.2-micron filter. Two different antigen concentrations were used in the two doses. The Ag concentration in the vaccine used for the first vaccine dose in group 6 was at a higher concentration (>12,000 RU/mL). The concentration in the second dose was 2,750 RU/mL.

All treatments were administered subcutaneously in a single 2-mL dose on Days 0 and 21, with the exception of Group 2 as noted above.

Formulation of vaccines

The following stock solutions were used in the formulation of the adjuvants used for protocols 4, 5, and 6. Quil A was dissolved in water to prepare a 50 mg/mL stock solution. Cholesterol (FabriChem Inc., Trumball, CT) was dissolved in ethanol to make a 17 mg/mL stock solution and the DDA was dissolved in ethanol to make an 18 mg/mL stock solution. Both of these stocks were filter sterilized using a 0.2-μ filter. Carbopol 974 P NF, a synthetic anionic polymer used as an adjuvant in commercial vaccines, was dissolved in water to prepare a 1.5% (*w*/*v*) stock solution and sterilized by autoclaving at 121°C for 20 min. The stock for Bay-R1005 was dissolved in phosphate buffer at a stock solution of 4.57 mg/mL and filter sterilized using a 0.2-μ filter. Bay-R1005 [N-(2-Deoxy-2-L-leucyclamino-b-D-glucopyranosyl)-N-octadecylodecanoylamide hydroacetate] is an amphiphilic molecule that forms micelle in aqueous solution. It is a common adjuvant component in commercial vaccines.

The adjuvant used for protocols 4 and 6 was identical. These protocols differed only in the viral antigen. The Group 4 and 6 formulations contained buffer, 50-μg/mL Quil A, 50-μg/mL cholesterol, 25-μg/mL DDA, and 0.075% *w/v* of Carbopol. Antigen was combined with (add 80% *v/v*) buffer followed by addition of Quil A, cholesterol-ethanol, and DDA-ethanol using constant stirring. An appropriate volume of Carbopol was added to attain a final concentration of 0.075%. The pH was adjusted to pH 7±0.2 with 4N NaOH. The viral antigen for protocols 4 and 5 were identical. The adjuvant for protocol 5 differed from that of protocols 4 and 6 in that R1005 also was added to a final concentration of 50 μg/mL for this formulation and the pH was not adjusted.

Antigen concentrations in vaccines were determined by ELISA. Readings were compared to a standard curve, which was prepared using an USDA accepted manufacturing reference standard. Antigen concentrations are stated in relative units (RU). The virus strain used in all vaccines was strain 53637. This strain belongs to the BVDV2a subgenotype.

BVDV-2 challenge

On Day 42 all the animals were challenged with a noncytopathic BVDV-2a (strain 24515) at a concentration 5.4 $\log^{10}$ $TCID_{50}$ per 5-mL dose. The challenge inoculum was delivered intranasally, approximately 2.5 mL per nostril, using a compressed gas nebulizer. Dose titration assays were conducted on pre- and post-challenge samples as described previously. (Cortese et al. 1998)

Please note that two different BVDV2a strains were used in vaccine formulations (strain 53637) and challenge studies (strain 224515). Based on phylogenetic analysis performed using sequences from the 5′ untranslated region, these two strains belong to the same subgenotype, but are not identical.

Blood testing

Blood samples were collected on days 0, 21, 41, and 56. Serum from blood samples was harvested, divided into aliquots, and submitted for testing or stored frozen at <−20°C. Viral neutralizing titers in collected sera were determined against three BVDV1a strains (BVDV1a-Singer, BVDV1a-5960, and BVDV1a-NADL), three BVDV1b strains (BVDV1b-TGAC, BVDV1b-Illc, and BVDV1b-2110), and three BVDV2a strains (BVDV2a-296c, BVDV2a-ND 8799, and BVDV2a-MsSt T-4529) as described previously (Ridpath et al. 2010). Buffy coat samples were harvested from whole blood collected on days 40 through 56. These samples were assayed for replicating BVDV by tissue culture isolation followed by Ab staining (Cortese et al. 1998). Total circulating white blood cell counts (WBC) and platelet counts were determined as described previously (Ridpath et al. 2007).

Health observations

On Days -1 to 39, animals were observed daily for general health. After the BVDV-2 challenge, the animals were monitored each morning for clinical signs of BVDV disease (depression, nasal discharge, and respiratory distress) and each afternoon for general health. For 1 h after each vaccination animals were observed for systemic reactions, such as anaphylaxis, depression, reduced coordination, increased respiration, diarrhea, inappetence, or trembling. Injection-site reactions were observed on Days 0, 1, 2, 3, 7, and 21 for the first vaccination, and on Days 21, 22, 23, 24, 28, and 42 for the second vaccination. All palpable injection-site reactions were measured (length × depth × width) and the size

recorded. Rectal temperatures were recorded from day -1 to day 56. Fever was defined as a rectal temperature >40.3°C (104.5°F). Leukopenia was defined as a 40% decrease in WBC counts compared to pre-challenge WBC count means.

Data summary and analyses

In the challenge phase, rectal temperatures, serology data, viremia data, WBC counts, and platelet counts were analyzed with a general linear repeated measures mixed model with fixed effects (treatment, time-point, and treatment by time-point interaction) and random effects (pen, animal within treatment and pen, which was the animal term, and the residual). Linear combinations of the parameter estimates were used in a priori contrasts after testing for a significant ($P \leq 0.05$) treatment or treatment by time-point effect. Comparisons were made between treatments at each time-point. Back transformed least squares means (from serology), their standard errors and their 90% confidence intervals were calculated from least squares parameter estimates obtained from the analyses.

Results

Serum neutralizing titers prior to challenge

All animals were seronegative to BVDV-1 and BVDV 2 before vaccination (Day -1). Figure 1 presents SN titers on Day 41 (41 days past first vaccination, 20 days after the second vaccination, and 1 day before challenge). On day 41, group 1 (control animals) were seronegative to BVDV-1 and BVDV-2. All animals in Groups 3 through 6, and eight of 10 animals in Group 2, had BVDV-2 titers of at least 1:8 on Day 41. The mean BVDV-1 and

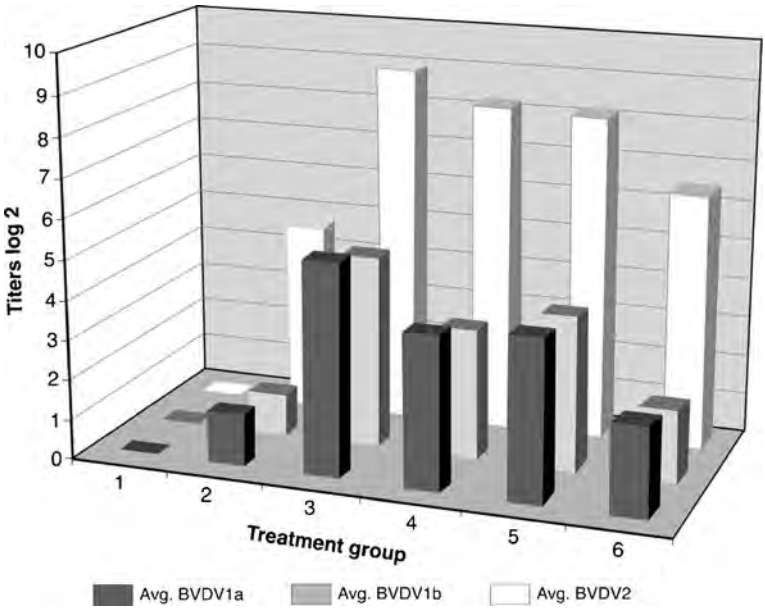

Fig. 1 Average neutralizing titers of animals from different treatment groups against strains of BVDV1a, BVDV1b, and BVDV2

BVDV-2 titers of Group 2 was statistically significantly ($P \leq 0.0015$) smaller than those of Groups 3, 4, 5, and 6. The mean BVDV-2 titer of Group 6 was statistically significantly ($P \leq 0.0335$) smaller than those of Groups 3, 4, and 5. The mean BVDV-1 titer of Group 4 was statistically significantly ($P \leq 0.0357$) smaller than the mean of Group 3.

Response to challenge

Virus isolation was performed on samples collected between Days 41 and 56 (Table 2). Virus positive samples were detected longer and more frequently from animals in group 1 than animals from groups 2 through 6. A statistically significant difference ($P \leq 0.0031$) was found between the number of positive animals in Group 1 (10 of 10) compared to the numbers of positive animals (one of 10 to three of 10) for all other treatment groups. Results for Groups 2 through 6 did not differ significantly from each other.

Figure 2 graphs rectal temperatures throughout the study. Slight increases in mean rectal temperatures of generally <0.56°C (1°F) from pre-vaccination levels were observed following the primary and booster vaccinations in all treatment groups. Following challenge, the mean rectal temperature of Group 1 first rose to >39.4°C (103.0°F) on Day 50 and peaked on Day 52 at 40.1°C (104.2°F). The mean rectal temperatures for Groups 2, 3, and 5 never exceeded 39.4°C (102.9°F) on any day. For Group 4, a mean temperature greater than 39.4°C (103.0°F) was only recorded on 1 day (day 51). The mean temperature for Group 6 peaked at 39.6°C (103.3°F) on Day 52. Using the pre-determined, post-challenge fever criterion listed in 9 CFR §113.215, eight of 10 animals in Group 1, no animals in Groups 2 and 3, one of 10 animals in Groups 4 and 5, and two of 10 animals in Group 6 were febrile. The number of febrile animals in Group 1 was statistically significantly larger ($P \leq 0.0190$) than those of all other groups. The differences observed between mean daily temperatures for groups 2 through 6 were not statistically significant.

Figure 3 plots the number of animals with leukopenia by study day. For Group 1, there was at least one animal with leukopenia (> 40% decrease in WBC from baseline) every day from Days 46 through 56. In contrast, there were never any leukopenic animals in Group 2. For Groups 3, 4, 5, and 6, leukopenic animals were present on Days 47 through 50, 47 through 49, 47 through 50, and 47 through 51, respectively. All animals in Group 1, eight of 10 in Group 6, one of 10 in Group 3, and three of 10 in both Groups 4 and 5 experienced leukopenia, none (0 of 10) of the animals in Group 2 were leukopenic. There were no significant differences ($P > 0.05$) in platelet counts among any of the treatment groups on any study day.

Table 2 Results of virus isolation

Group	# of animals with at least 1 VI pos sample	# of days VI pos samples detected in group	Greatest # of animals VI pos on any one sampling date	Day of first VI pos sample	Day of last VI pos
1	10	11	10	46	56
2	3	4	1	47	51
3	1	2	1	47	48
4	2	3	1	47	50
5	2	2	1	47	49
6	2	4	2	49	52

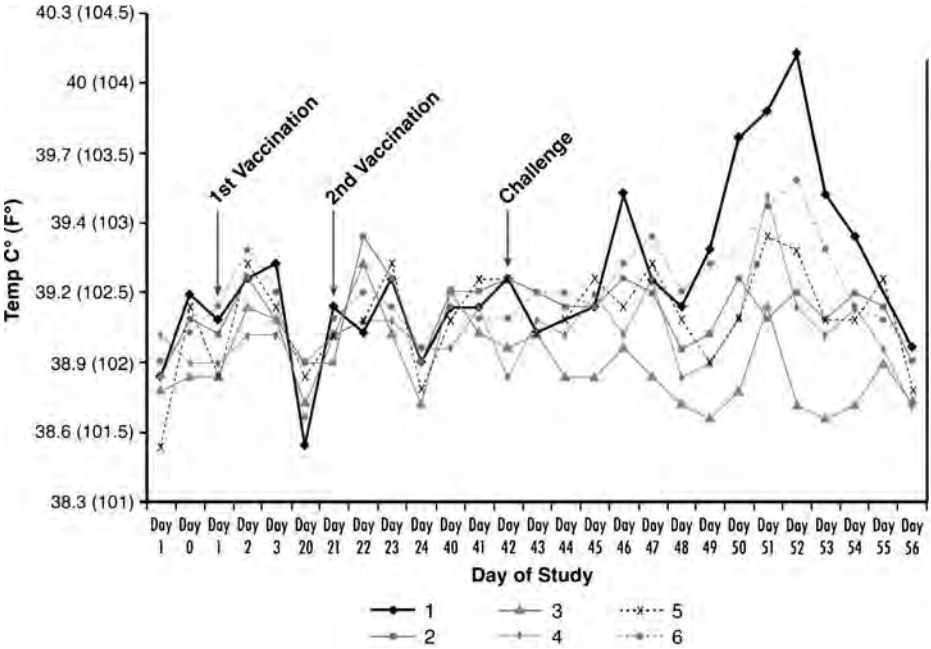

Fig. 2 Average daily temperature of animals from each treatment group

There were no statistically significant differences ($P>0.05$) among groups in the occurrence of depression, nasal discharge, or increased respiratory effort. One animal in Group 4 was removed from the study on Day 53 due to a severely swollen left shoulder that became infected. The injury was not related to vaccination.

Injection-site reactions

The number of animals with observable infection site reactions and the size of the largest reaction are detailed in Table 3. The largest reactions occurred in Group 3 on the days following vaccinations, with reaction sites measuring from 0 to 133.52 mL on Day 1 and 0 to 27.44 mL on Day 22. Small residual reactions were still palpable in Groups 1, 2, 3, and 6 on Day 42. Reactions in all groups resolved rapidly within 48 h of vaccination. The volumes of reactions for Group 3 were statistically significantly ($P\leq0.0497$) larger than those of Group 1 on Days 22, 23, and 24, and Group 2 on Days 22 through 24 and 28, and larger than those of Groups 4, 5, and 6 on Days 22 through 24.

Discussion

Serological response as determined by development of neutralizing antibodies and clinical presentation following challenge exposure are the most commonly used methods to gauge the protective response induced by BVDV vaccines (Kelling 2004). In this study, two doses of any of the killed vaccines resulted in higher neutralizing titers than one dose of the MLV vaccine. The highest titers were observed after use of the conventional (prepared according to protocol used for a commercially available vaccine) killed vaccine. Titers in all animals

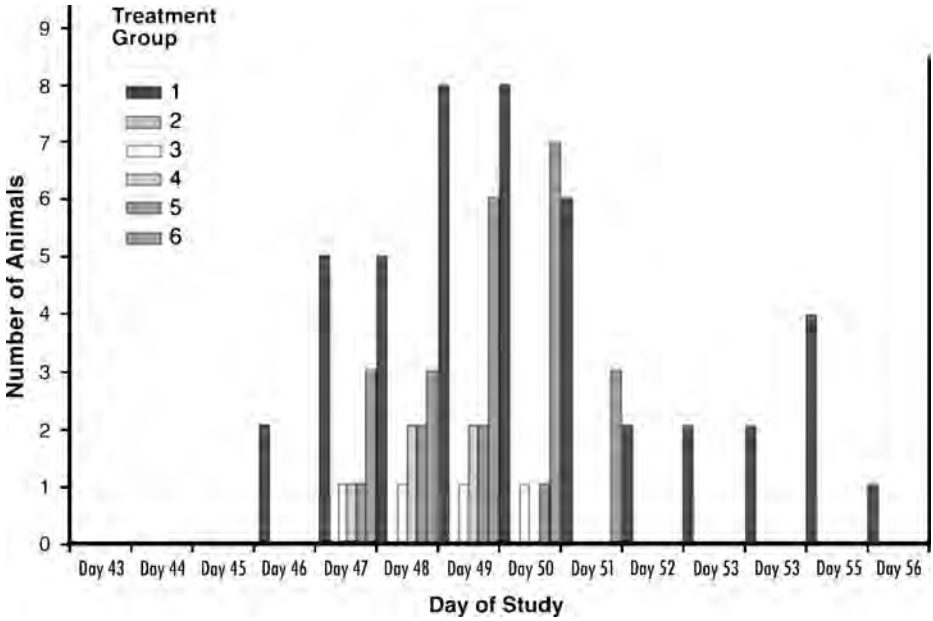

Fig. 3 Number of leukopenic animals from each treatment group following challenge on day 42

were higher against BVDV2 strains compared to BVDV1a or BVDV1b strains reflecting the inclusion of BVDV2, but not BVDV1a or BVDV1b, antigen in the vaccines.

Animal receiving mock vaccinations did not develop measurable titers of neutralizing antibodies prior to challenge and were not protected from viral replication or clinical disease following challenge exposure as determined by virus isolation, pyrexia, and leukopenia. Virus was isolated from at least one animal in all vaccinated groups but less frequently than from animals in the non-vaccinated group and for a shorter duration of time. Among the vaccinated groups, virus was isolated from more animals in Group 2 (MLV vaccine, 3 animals) compared to Groups 3, 4, 5, and 6 (killed virus vaccines, 1–2 animals). However, there was no significant difference in the length of viral shed between animals vaccinated with MLV vaccine and animals vaccinated with killed vaccines.

More animals, vaccinated with the three experimental killed vaccines, experienced pyrexia compared to animals vaccinated with the conventional killed and MLV vaccines. However, only 1–2 animals were affected and the pyrexia was low grade and of short duration compared to non-vaccinated animals. Similarly more animals, vaccinated with the three experimental killed vaccines, experienced leukopenia compared to the animals vaccinated with the conventional killed and MLV vaccines. The number of leukopenic animals was significantly higher in group 6 compared to the other vaccinated groups and was only slightly lower than the non-vaccinated group. The duration of leukopenia was similar in groups 3, 4, 5, and 6 (both conventional and experimental killed vaccines) and was significantly shorter than the duration of leukopenia in the non-vaccinated group.

Injection site reactions were more pronounced in animals vaccinated with the conventional killed vaccine. The function of adjuvants is to augment the effects of a vaccine by stimulating the immune system to respond more strongly to the vaccine antigen. While the exact mode of action of most adjuvants is unknown it is thought that they work by mimicking specific sets of evolutionarily conserved pathogen-associated molecular

Table 3 Injection site reactions

Days post 1st vaccination	Group 1		Group 2		Group 3		Group 4		Group 5		Group 6	
	# Animals	Largest reaction[a]	# Animals	Largest reaction[a]	# Animals	Largest reaction[a]	# Animals	Largest reaction[a]	# Animals	Largest reaction[a]	# Animals	Largest reaction[a]
1	1/10	1.31	0/10		1/10	133.52	2/10	4.12	0/10		4/10	2.75
2	0/10		0/10		9/10	15.71	1/10	2.62	0/10		2/10	15.71
3	0/10		0/10		4/10	5.24	1/10	1.57	0/10		1/10	1.43
7	0/10		0/10		6/10	3.53	2/10	2.45	1/10	2.45	0/10	
21	0/10		0/10		1/10	0.26	1/10	0.03	0/10		0/10	
22	0/10		0/10		8/10	37.44	2/10	2.95	3/10	6.28	4/10	39.27
23	0/10		0/10		5/10	9.42	2/10	2.75	1/10	2.45	2/10	4.58
24	0/10		0/10		4/10	4.19	0/10		2/10	2.45	0/10	
28	1/10	2.95	1/10	1.18	4/10	23.04	5/10	4.91	4/10	17.02	3/10	5.04
42	1/10	0.65	1/10	0.79	2/10	1.57	1/10	.09	0/10		2/10	1.05
Total measureable observations	3		2		44		17		11		18	

[a] Volume in mL

patterns (PAMPs). The immune system recognizes PAMPs as non self molecules and activates the innate immune system. Activation of the innate immune system by adjuvants results in a greater immune response. However, over stimulation or stimulation that elicits an inappropriate response results in adverse reactions that expend physiological resources but do not contribute to enhanced protection. An example of an inappropriate response is injection site reactions. Swelling at the injection site increases animal discomfort and may reduce carcass quality. These reactions result from a nonspecific irritation of the immune system and do not contribute to improved protection against specific vaccine antigen. The perfect adjuvant would increase the strength of the vaccine antigen specific response (as reflected by development of neutralizing antibodies) and have very limited nonspecific immune irritation response (as reflected by little or no injection site reactions).

While eliciting the lowest titers of serum neutralizing antibodies, the MLV vaccine exhibited the highest level of protection based on prevention of pyrexia and leukopenia. Higher protection in spite of lower levels of neutralizing antibodies may be related to the stimulation of T cell responses in addition to B cell responses. It is interesting to note that while preventing clinical presentation of disease, the MLV vaccine was no more effective than killed vaccines in preventing viremia. The experimental vaccine used in Group 6, while giving some protection, was the least effective of the vaccines. This suggests that the process used for enriching for viral proteins, may have eliminated or reduced antigens that are important to the development of protective immunity.

Protection elicited by the experimental killed vaccines used in groups 4 and 5 was similar or only slightly reduced from that of the conventional killed vaccine. These two experimental vaccines demonstrated an advantage over the conventional killed vaccine in that they had significantly reduced injection site reactions. This suggests that these adjuvants maintained the protective immune response while ameliorating non-protective inflammatory responses. Vaccination always carries with it a physiological cost. Production of an immune response requires energy and can result in animal discomfort. It is desirable to produce the greatest protective response at the lowest physiological expense. One way to decrease the physiological cost is to reduce non-protective responses. Non-protective inflammatory responses in particular contribute to physiological cost, increase animal discomfort and may decrease carcass value. The advantage of these vaccines would lie in their ability to produce a protective immune response as a reduced physiological cost.

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Bovine Viral Diarrhea Virus: Global Status

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KEYWORDS

• Bovine viral diarrhea viruses • BVDV1 • BVDV2 • Pestivirus

The term, *bovine viral diarrhea viruses (BVDVs)*, refers to a diverse group of single-stranded RNA viruses currently classified as two different species, BVDV1 and BVDV2, within the pestivirus genus. One or both species have been isolated from ruminants in all continents with the exception of Antarctica. The global status of BVDVs is in transition. Even as control programs in Scandinavia result in near eradication of BVDVs in those regions,[1] there is a growing recognition of BVDV–associated diseases in species other than cattle in North America, Southeast Asia, and Africa.[2–6] In 2007, the Office International des Epizooties added BVDV to its list of reportable diseases. It is only listed as a reportable disease of cattle, however, not as a reportable disease of multiple species. This may lead to confusion as BVDV also replicates in and causes reproductive disease in domestic species, such as pigs, goats, and sheep and in several wildlife species.

In the past decade there has been an assimilation of regional veterinary biologics companies by large international companies. This assimilation has been accompanied by removal from the market of BVDV vaccines based on strains endemic to the region. Although further research is necessary, regional variation in prevalence of BVDV subtypes (species and subgenotypes) and variation in cross-protection between BVDV subtypes suggest vaccine protection may be improved by basing vaccines on the BVDV strains prevalent in the region in which the vaccine is to be used.

In addition, a putative new member of the pestivirus genus has been identified that causes disease syndromes in cattle that are indistinguishable from clinical presentations associated with BVDV infection.[7,8] One research group has proposed that this emerging virus be called BVDV3.[9] Differentiation of this virus from BVDV1 and BVDV2 strains and restricting the expansion of this virus will be important challenges in the design of future BVDV eradication and control programs.

DISCOVERY AND CLASSIFICATION

The first recorded observation of the disease, bovine viral diarrhea (BVD), dates back to 1946 when a novel transmissible disease in cattle was observed in the state of New

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York.[10] This disease was characterized by leukopenia, pyrexia, depression, diarrhea, anorexia, gastrointestinal erosions, and hemorrhages. The causative viral agent was isolated in 1957 and named BVDV.[11] A similar but more severe disease, termed *disease X*, was reported several years later in Canada.[12] Although similar to BVD, disease X tended to affect fewer animals per herd but had a higher mortality rate. Unlike BVD, disease X (later termed, *mucosal disease*) could not be transmitted experimentally.[13] A disease with the same clinical presentation was described by several groups in the late 1950s in Germany and termed, *Schleimhautkrankheit*.[14] Virus neutralization was used to demonstrate that the viral agents isolated from BVD and mucosal disease cases in North America and Schleimhautkrankheit in Germany and the United Kingdom were the same.[15,16] Subsequently it was demonstrated that BVDV was antigenically related to hog cholera virus (since renamed classical swine fever virus [CSFV]).[17] A decade later, immunologic similarities were also noted between BVDV, CSFV, and the causative agent of border disease in sheep (termed, *border disease virus*).[18] In 1973, the term, *pestivirus*, was coined for this antigenically related group of viruses[19] and they were classified as a genus within the family Togaviridae.[20] At that time flaviviruses were also classified as a genus within the togavirus family. Advances in the molecular characterization of flaviviruses, however, revealed fundamental differences in molecular structure, replication strategy, and genetic organization between flaviviruses and other members of the Togaviridae. These findings led to the reclassification of the flaviviruses, into a new virus family, Flaviviridae, which initially contained one genus, flavivirus.[21] The publication of the genomic sequence of the BVDV strain, NADL, the first pestivirus to be sequenced, revealed that pestiviruses had greater similarities in genomic organization, gene expression, and replication strategy to the Flaviviridae than the Togaviridae.[22] This led to the reclassification of pestiviruses, in 1991, as a new genus within the Flaviviridae family.[23] Subsequent phylogenetic analysis of isolates identified as BVDV, based on host of origin and clinical presentation, revealed two distinct genetic groups, BVDV1 and BVDV2,[24,25] which were later classified as two different species within the pestivirus genus.[26] In addition to the four recognized pestivirus species, BVDV1, BVDV2, border disease virus, and CSFV, additional species have been proposed (**Table 1**).[9,27–30] In 2004, a novel pestivirus was isolated from fetal bovine serum originating from Brazil.[31] Subsequently, other isolates from South America and Southeast Asia were identified that grouped with this virus based on phylogenetic analysis.[7,32] Some of the isolations were from fetal bovine serum, one was from a buffalo, and, significantly, some were isolated from apparently persistently infected (PI) cattle. Similarity in clinical presentation to BVDV1 and BVDV2 and the ability to establish PIs in cattle has led some researchers to suggest that this group of viruses be referred to as BVDV3.[9]

CHARACTERISTIC TRAITS

Physical Characteristics of the Viral Particle

BVDV is included as a member of the Flaviviridae because it shares many characteristics with other viruses within this family.[26,33] All Flaviviridae are spherical viruses 40 to 50 nm in diameter. The viral particle consists of an outer lipid envelope surrounding an inner protein shell or capsid that contains the viral genome. The lipid envelope, which is derived from the membranes of the infected host cell, makes these viruses susceptible to inactivation by solvents and detergents. The molecular weight of the BVDV virion has been estimated as 6.0×10^7 and the buoyant density in sucrose is 1.10 to 1.15 g/cm^3. BVDV, however, as do the other members of the pestivirus genus, differs from the rest of the Flaviviridae in that the lipid envelope that surrounds the virion is pleomorphic. The

Table 1
Species in the pestivirus genus

Recognized Species	Geographic Distribution	Host
BVDV1	Worldwide; nearing eradication in some European regions	Wild and domestic ruminants, pigs, and rabbits
BVDV2	Worldwide, although more prevalent in North and South America; nearing eradication in some European regions	Wild and domestic ruminants, pigs, and rabbits
CSFV	Eradicated in United States and Canada	Pigs, sheep, and cattle
Border disease virus	Worldwide	Sheep, goats, pigs, cattle, and wild ruminants
Putative species[a]		
Giraffe	Kenya	Giraffe
HoBi-like (BVDV3)	South America and Southeast Asia	Cattle
Pronghorn	United States	Pronghorn antelope
Bungowannah	Australia	Pigs

[a] Listed in order of increasing phylogenetic divergence from recognized pestivirus species.

resulting variation in size of enveloped particles impedes purification by banding in sucrose gradients, accurate estimation of size, and identification by electron microscopy. In addition, pestiviruses can be differentiated from other viruses within the Flaviviridae by their resistance to inactivation by low pH. Although most flaviviruses and hepaciviruses are inactivated by low pH, pestiviruses are stable over a broad pH range. Infectivity is not affected by freezing but decreases at temperatures above 40°C. As discussed previously, BVDVs are inactivated by organic solvents and detergents. Other methods of inactivation include trypsin treatment (0.5 mg/mL at 37°C for 60 minutes), ethylenimine (reduction of 5 log10 units using 10 mmol/L at 37°C for 2 hours), electron beam irradiation (4.9 and 2.5 kGy needed to reduce virus infectivity 1 log10 unit for frozen and liquid samples, respectively), and gamma irradiation (20–30 kGy).

The Viral Genome and Gene Products

The genomes of all Flaviviridae consist of a single strand of positive-sense RNA, between 9 and 12.3 kilobases long, that codes for a single open reading frame (ORF). There is no subgenomic mRNA associated with Flaviviridae infections. The viral structural proteins are encoded at the 5′ end and the nonstructural proteins are encoded in the remaining 3′ end. The ORF is translated as one long polyprotein that is cleaved after translation into individual proteins. The nonstructural proteins assist in this cleavage and in replication of positive and negative-strand RNA.

The genome of a pestiviruses, in the absence of insertions, is approximately 12.3 kilobases long with an ORF that contains approximately 4000 codons. The ORF is preceded and followed by 5′ and 3′ untranslated regions of 360 to 390 nucleotides and 200 to 240 nucleotides, respectively. The 5′ terminus does not contain a cap structure.[34] Although there is no poly(A) tract present at the 3′ end, pestiviruses genomes terminate with a short poly(C) tract. Although the genome is made up of single-stranded RNA, there is considerable secondary structure inherent as evidenced by its ability to bind CF-11 cellulose in the presence of 15% ethanol,[35] solubility in 2 mol/L LiCl,[22] and resistance to hydrolysis by low concentrations of RNase A.[36] It is

thought that stem and loop structures found in the 5′ untranslated region are integral to the formation of an internal ribosomal entry site that functions to direct the attachment of assembly of the ribosome at the first codon of the ORF (for review, see Neill[37]). The order of the individual viral proteins within the polyprotein is as follows: Npro-C-Erns-E1-E2-p7-NS2/3-NS4a-NS4b-NS5a-NS5b. The attributes and functions of each viral protein are listed in **Table 2**. In comparison to the other genera within Flaviviridae, pestiviruses encode two unique proteins. The first is the nonstructural protein, N^{pro}, which is encoded at the beginning of the ORF (preceding the region coding for the structural proteins). The N^{pro} is a proteinase, which cleaves itself from the viral polypeptide. This protein also functions in the suppression of the host's innate immune system. Studies suggest N^{pro} prevents the production of type I interferon by blocking the activity of interferon regulatory factor 3.[38,39] The second unique gene product is the envelope glycoprotein, E^{rns}, which possesses an intrinsic RNase activity. This protein is found in the infectious viral particle and secreted in soluble form. It is thought that the secreted E^{rns} prevents the induction of beta interferon by binding to and degrading double-stranded RNA.[40]

Variations in Genotype (Species), Subgenotype, Biotype, and Virulence

The viruses grouped under the heading of BVDV are highly heterogeneous and encompass viruses belonging to two different species. Viruses of either species may exist as two different biotypes, cytopathic and noncytopathic, and are of varying virulence from avirulent to highly virulent. The two recognized species of BVDV are BVDV1 and BVDV2. Although this segregation was first based on phylogenetic analysis,[24,25] subsequent characterization of viral strains from the two species demonstrated antigenic differences.[41] The practical significance of the observed antigenic differences was evidenced by the failure of vaccines and diagnostics based on BVDV1 strains to control and detect BVDV2 strains.[42] Although BVDV1 and BVDV2 viruses are cross-reactive, they can be differentiated based on antigenic differences. Neutralizing antibody titers found in convalescent sera are typically severalfold higher against viruses from the same species compared with viruses from the other species,[43,44] and monoclonal antibody binding patterns are distinct.[25,45]

Although antigenic differences may be used to differentiate the species, nucleotide sequence relatedness is the most reliable criteria. Differences between BVDV1 and BVDV2 genomes are not relegated to any one region and differences may be found throughout the entire genome.[46] Differences between BVDV1 and BVDV2 in some genomic regions, however, are more amenable to comparison or have greater biologic significance. The 5′ untranslated region is the most commonly used region for detection and characterization because of highly conserved motifs that are favorable to PCR amplification. Because the N^{pro} region is unique to pestiviruses, however, comparison of this region is gaining favor for characterization of putative pestivirus species.

Phylogenetic analysis has also revealed subgenotype groupings within the BVDV1 and BVDV2 species, 12 in BVDV1 viruses (BVDV1a, BVDV1b, BVDV1c, BVDV1d, BVDV1e, BVDV1f, BVDV1g, BVDV1h, BVDV1i, BVDV1j, BVDV1k, and BVDV1l)[47] and two in BVDV2 viruses (BVDV2a and BVDV2b).[48] Furthermore, additional subgenotypes of BVDV1 have been identified in isolations of BVDV from cattle in South Africa and Switzerland but have not yet been assigned names.[49–51] The clinical significance of segregation into subgenotypes is a matter of discussion. Different BVDV subgenotypes predominate in different geographic locations[24,49,51–69] and studies have shown antigenic differences between subgenotypes as demonstrated by differences in cross-neutralization,[49,61] monoclonal antibody binding,[45] and response of PI animals

Table 2
Bovine viral diarrhea viruses proteins

Protein	Size (kd)	Structural	Attributes	Function
N^{pro}	20	N	Unique to pestivirus genus Highly conserved Sequence frequently used in phylogenetic comparisons	Autoprotease Suppresses host immune response
C (Capsid)	14	Y	Conserved	Forms nucleocapsid of virion
E^{rns}	48	Y	Unique to pestivirus genus Glycosylated Found in the infectious viral particle and secreted in soluble form Detection target for antigen-based tests	Integral viral membrane protein Ribonuclease activity of soluble form suppresses host immune response Possesses minor neutralizing epitope
E1	25	Y	Glycosylated	Integral viral membrane protein
E2	53	Y	Glycosylated Immune system–generated antibodies against E2 after vaccination with killed or modified live vaccine Sequence displays most variability of all viral proteins	Integral viral membrane protein Possesses dominant neutralizing epitopes
p7	7	N	Required for production of infectious virus but not RNA replication	Unknown
NS2/3	80	N	Highly conserved Detection target for antigen-based tests Immunodominant nonstructural protein Antibodies generated against protein after modified live vaccination but not killed vaccination Cytopathic viruses cleave to NS2 and NS3	RNA helicase Serine protease Cleaves itself and downstream nonstructural proteins from viral polyprotein
NS4a	7.2	N	Hydrophobic	Serine protease cofactor
NS4b	38	N	Hydrophobic	Replicase component
NS5a	55	N	Phosphorylated	Replicase component
NS5b	81	N		RNA-dependent RNA polymerase

to vaccination.[70] It is not known, however, if variations in subgenotypes are significant enough to have an impact on the detection or the protection afforded by vaccination.

BVDV strains may exist as one of two biotypes, cytopathic and noncytopathic. Segregation into biotype is based on activity in cultured epithelial cells.[11,71] Noncytopathic viruses predominate in nature. Cytopathic viruses are rare and usually found in association with outbreaks of mucosal disease, a rare highly fatal form of BVDV infection. It is believed that cytopathic strains arise from noncytopathic strains through a mutational event that results in the cleavage of the NS2/3 viral protein to the NS2 and NS3 proteins. The most frequently observed mutational event resulting in BVDV biotype change is recombination, in which short pieces of genetic code are inserted into the genome of a noncytopathic BVDV strain.[72] The inserted code may originate from another BVDV or from the infected host cell. Not all recombination events are associated with biotype change, however, as some noncytopathic viruses have insertions[46,73] and some cytopathic viruses do not have insertions.[74] Cytopathology in vitro does not correlate with virulence in vivo. The most clinically severe form of acute BVDV infection is associated with noncytopathic viruses.[24,73,75–77] The significance of BVDV biotypes to control programs lies in that noncytopathic BVDV, but not cytopathic BVDV, may establish a PI in a fetus that persists throughout its lifetime. For this reason most modified live BVDV vaccines use cytopathic BVDV.

High and low virulence BVDV2 strains have been characterized.[41,75,78–82] In the United States, low virulence BVDV2 strains have been selected for use in modified live vaccines[43] and high virulence BVDV2 strains have been used as challenge strains to assess the efficacy of protection induced by vaccination.[83,84] Although there is variation in virulence in BVDV1 strains[85] there is little information available in the literature. To date, the most virulent BVDV strains characterized belong to the BVDV2 species.

Clinical Presentation

Clinical presentation varies depending on strain of virus, species of host, immune status of host, reproductive status of host, age of host, and concurrent infections with other pathogens. Although the term, *diarrhea*, is prominent in the name, respiratory and reproductive disease associated with BVDV infection are more commonly reported.[86,87] The clinical presentation of reproductive disease is due to the direct infection of the fetus and the outcome depends on the stage of gestation in which the fetal infection occurs.[88] Although abortions and weak calves have been attributed to BVDV infection in late gestation,[89] infections occurring earlier in gestation generally have greater impact on reproduction. Fetal infections in cattle, occurring between 42 and 125 days of gestation, result in fetal reabsorption, mummification, abortion, congenital malformations, or the establishment of PI animals. PI animals shed BVDV throughout their lifetimes and are important carriers in the introduction of BVDV into naive populations. If a PI animal becomes infected with a cytopathic BVDV it may succumb to a relatively rare but invariably fatal form of BVD, called mucosal disease.[90]

Although the PI animal is frequently the source of BVDV entering a herd, subsequent spread of the virus and observed clinical disease are the result of acute infection. The severity of acute infections is dependent on the viral strain, immune status of host, and presence of secondary pathogens. BVDVs are lymphotrophic and acute infections result in the reduction of circulating lymphocytes[41] and the suppression of innate immune functions.[91–93] The resulting immunosuppression results in reduced resistance to secondary pathogens and increased severity of clinical disease after secondary infections.[94] The interaction of BVDV with secondary pathogens is thought to be one of the contributing factors in bovine respiratory disease complex (BRD).

Emerging Recognition of Bovine Viral Diarrhea Virus–Associated Disease in Nonbovine Hosts

Although BVDV infections are most commonly associated with cattle, there is evidence, based on virus isolation and serology, that BVDVs replicate in a wide variety of domesticated and wild ruminants. Losses to food and fiber producers due to infection of nonbovine domesticated species are usually associated with reproductive disease. It has been demonstrated that BVDVs are the causative agents of reproductive disease in goats,[95] sheep,[96,97] and pigs.[98–102] It seems that infection of pregnant animals belonging to these species results in clinical presentation similar to that seen in cattle. The primary consequences are due to the direct infection of the fetus and the outcome of infection depends on the stage of gestation in which the fetal infection occurs. One of the resulting outcomes is the birth of PI animals. Recently the emergence of BVDV-associated abortions and congenital defects (including PI) in New World camelids has led to renewed interest in BVDV in nonbovine hosts. Before 2002, prevalence in New World camelid populations was reported to be low and no clinical disease was associated with exposure.[103] The conventional wisdom was that although infections in New World camelids might occasionally occur, they were rare and resulted from contact with cattle. These assumptions were called into question by the isolation of BVDV from a stillborn alpaca[104] and the subsequent identification of crias PI with BVDV.[105–107] In a 2008 survey conducted among members of the (US-based) Alpaca Owner and Breeder Association, 25.4% of herds had been exposed to BVDV, based on serology, and 6.3% had PI crias in residence.[108] The PI crias may be unthrifty and suffer from congenital defects, such as stunting or abnormal coat properties, or they may seem normal. Just as PI cattle are considered the main vector for introduction of BVDV to naive herds, it seems that PI alpacas shed virus that readily infects naive cohorts. In contrast to BVDV infections in cattle in which there is extensive heterogeneity in infecting BVDV strains, BVDV isolated from PI alpaca belong to one subgroup of one species and seem to be relatively similar based on phylogenetic analysis.[3,107] These reports indicate that a limited number of BVDV strains have adapted to improved replication in alpaca, that these strains circulate between alpaca populations in the absence of contact with cattle, and that PI alpacas are a source for the introduction of BVDV into naive alpaca herds. The comparatively low incidence of exposure, homogeneity of BVDV strains, and limited use of vaccines suggest that eradication of BVDV from commercial alpaca herds in the United States is achievable via testing and elimination of PI animals followed by surveillance via serology to rapidly detect and control any reintroduction of BVDV into alpaca herds.

Although there is evidence of BVDV replication in wild ruminants based on serology and virus isolation, including antelope, giraffe, African buffalo, bison (European and American), big horn sheep, mountain goats, and various *Cervidae* (reindeer, kudu, roe deer, red deer, fallow deer, mule deer, white-tailed deer, and caribou),[2,5,6,28,50,109–113] the level of exposure and prevalence of PI animals is largely unknown. Regional surveillance based on serology varies significantly depending on method of sampling. In serologic surveys of white-tailed deer populations residing in the United States, sampled deer collected by net gunning[114–117] reported higher BVDV exposure rates than surveys that sampled deer harvested by hunters.[118] The difference in exposure rate may be related to differences in populations sampled. Net gunning results in sampling of animals of both genders and all age groups whereas hunter-harvested animals tend to be predominantly adult males.

Recently, several studies have focused on BVDV infections in white-tailed deer. PI white-tailed deer have been harvested from the field[6,118] and generated under

experimental conditions.[119,120] The clinical progression of acute disease after inoculation of fawns was similar to that seen in BVDV infections in cattle and included fever and reduction of circulating lymphocytes.[121] Similarly, the clinical presentation of BVDV-associated reproductive disease in white-tailed deer is similar to that observed in cattle and includes readsorption, abortion, mummification, and birth of PI fawns.[122] The presence of neutralizing antibody titers in pregnant does seem to protect against BVDV infection in the fawns they carried. Transmission of BVDV between PI cattle and pregnant deer resulting in the birth of PI fawns has been confirmed under experimental conditions.[123]

Because free-ranging wild ruminant populations are frequently in contact with domestic cattle, possible transfer of BVDV between cattle and wild ruminants has significant implication for proposed BVDV control programs. Furthermore, reintroduction of endangered species to ecosystems that include domestic cattle may fail due to BVDV transmission from cattle to the newly introduced, BVDV naive species.

DETECTION AND CONTROL

BVDV diagnostics have focused on the detection of PI animals. Historically, virus isolation in cultured bovine cells has been the standard against which other tests were evaluated. Because of ease and lower expense, however, antigen detection by immunohistochemistry or antigen capture ELISA (ACE) and nucleic acid detection by reverse transcriptase–polymerase chain reaction are gaining favor. In contrast to virus isolation, these methods do not differentiate between deposited nonreplicating viral remnants and biologically active virus. No one-time test can reliably differentiate between acute and PIs. For that reason, two positive tests from samples collected 3 weeks apart are necessary to diagnose PI. Antigen-based testing methods, such as immunohistochemistry and ACE, are robust and results are highly reproducible between laboratories. These tests frequently depend, however, on one or two monoclonal antibodies, which target viral proteins. A viral variant has been identified that escapes detection by immunohistochemistry and ACE tests based on the binding of a monoclonal antibody against the viral protein E^{rns}.[124] Prevalance of such BVDV variants is unstudied. Multiple testing strategies, including polyclonal or pooled monoclonal antibodies that detect more than one viral glycoprotein, may be necessary to detect all PI calves and facilitate eradication of BVDV.

In addition to tests that recognize all BVDV species and subgenotypes in circulation, there is a need for tests that differentiate between BVDV1 and BVDV2 and other species of pestiviruses. Until the early 1990s, there were only three recognized species in the pestivirus genus, BVDV, border disease virus, and CSFV. Subsequently, BVDV were divided into two different species, BVDV1 and BVDV2.[24,25] Four additional putative pestivirus species have been identified, based on phylogenetic analysis. In chronologic order they are Giraffe[125] (isolated from one of several giraffes in the Nanyuki District of Kenya suffering from mucosal disease-like symptoms),[126] HoBi (first isolated from fetal bovine serum originating in Brazil and later from samples originating in Southeast Asia),[31] Pronghorn (isolated from an emaciated blind pronghorn antelope in the United States),[29] and Bungowannah (isolated after an outbreak in pigs, resulting in stillbirth and neonatal death, in Australia).[127] Three of these putative new species have been isolated from only one geographic region. In contrast, infection with the HoBi species has been isolated from cattle samples originating in South America and Southeast Asia.[31,128] The clinical presentation (reproductive disease and PI) in dairies in Thailand mimics that of BVDV1 or BVDV2 and has led to the suggestion that this group of viruses be called BVDV3.[9] Based on phylogenetic and

antigenic differences it is probable that diagnostics and vaccines designed to detect and prevent BVDV1 and BVDV2 infection will not be effective against the HoBi species.[31] Introduction of the HoBi species into new geographic regions would have serious consequences for BVDV control programs.

Vaccination is effective in reducing the spread of BVDV but cannot on its own eliminate BVDV from populations. This is because control by vaccination is compromised by the heterogeneity observed in BVDV strains, by the lack of complete fetal protection afforded by vaccination, and, most significantly, by the failure to remove PI animals from cattle populations. Killed and modified live vaccines are available for the prevention of BVD. There are two different goals for BVDV vaccination that are reflected in two different label claims that may be pursued in licensing US BVDV vaccines.[129–131] One goal is to prevent clinical disease after exposure to BVDV. The corresponding label claim for licensure would be, "Aids in prevention and/or reduction of BVD disease." The "BVD disease" referred to in this label claim is not defined as respiratory, enteric, or reproductive disease. In practice, clinical presentation (pyrexia, diarrhea, and nasal discharge) or factors associated with immunosuppression (leukopenia) or hemorrhaging (thrombocytopenia) are used as criteria for reduction in animal health after BVDV exposure.[84,132] Another goal is to prevent the fetal infection that leads to PI. The label claim for this goal would be, "Aids in prevention of fetal infection including PI calves." The level of protection that is required to prevent acute disease or fetal infection is largely unknown. It has been shown that a titer of 16 is required for reduction of clinical disease whereas a titer greater than 256 is required to prevent systemic shed.[133] The level of neutralizing titer required for fetal protection is unknown. Although protective B-cell immune responses have been studied more frequently, it has been demonstrated that T-cell–based immune responses are protective on their own.[134–136] Licensing requirements for validation of BVDV vaccine efficacy are currently based on clinical trials involving the challenge of vaccinates with a single intranasal inoculation of a BVDV field strain. Under field conditions, however, animals are more likely to be exposed over an extended period of time by cohousing with a PI animal. The importance of including prolonged viral exposure in evaluating the efficacy of vaccines is suggested by studies demonstrating fetal exposure to BVDV after contact between pregnant vaccinated cattle and PI cohorts.[137]

Design of vaccination programs need to take into account stressors that reduce an animal's ability to respond to vaccination, differences in immune response related to age, and pregnancy status and periods of greatest vulnerability to infection and negative outcomes of infection. Thus, different strategies may need to be devised for establishing programs for neonates, breeding herds, stocker calves, replacement heifers, and dairy or feedlot production.

BOVINE VIRAL DIARRHEA VIRUSES ERADICATION PROGRAMS

The success of control efforts in Scandinavia[138–140] demonstrates BVDV eradication is possible and has led to the development of other regional control programs.[141–145] It is not possible, however, to take a one-size-fits-all approach to the design of eradication programs for different regions.[145] Program design will vary based on the incidence of BVDV, density of animal populations, animal movement, contact with wildlife populations, level of producer compliance, variation in circulating BVDV strains, and prevalent type of production unit or industry. Although the details of program design may differ by region, to be most effective, a control program must provide for biosecurity aimed at the development of management practices that prevent BVDV from being

introduced into a herd, surveillance (to detect and remove PI animals), and means, such as vaccination, to limit BVDV spread if it is introduced into a herd.

SUMMARY

Despite eradication efforts, BVDV infections remain a source of economic loss for producers worldwide. The clinical presentation, pathogenesis, and basic biology of BVDV are complex. There are two states of infection (acute infection and PI), a continuum of virulence, two biotypes (cytopathic and noncytopathic), two species (BVDV1 and BVDV2), and many subgenotypes. The prevalence of BVDV species and subgenotypes varies by geographic region. Although infections are most commonly associated with cattle, they also occur in a wide variety of domesticated and wild ruminants. The circulation of BVDV in nonbovine species has economic, ecologic, and control implications. Although the economic impact of BVDV is largely due to the effects of acute infections, PI animals are the most common sources of virus and the most frequent vectors for introduction of virus into naive herds. Vaccines are effective in limiting the spread of BVDV in populations but are insufficient as a stand-alone tool for eradication. Recent advances in BVDV research and diagnostics have led to the development of improved and expanded testing aimed at the detection and removal of PI animals. This in turn has contributed to rapidly increasing number of regional eradication and control programs. The most efficacious of these are built around a three-pronged attack, consisting of biosecurity, surveillance, and control.

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The Contribution of Infections with Bovine Viral Diarrhea Viruses to Bovine Respiratory Disease

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KEYWORDS

• Bovine viral diarrhea viruses • Respiratory disease
• Persistent infection • Immunosuppression • Pathogen synergy

Although bovine viral diarrhea viruses are arguably the most researched of all viral pathogens associated with bovine respiratory disease (BRD), the impact of bovine viral diarrhea viruses (BVDV) on BRD is still unknown. This uncertainty is because the determination of total impact must factor in the contribution of acute, uncomplicated BVDV infections, impact of high incidence of respiratory disease in animals persistently infected with BVDV, impact of the immunosuppression that accompanies acute BVDV infections and predisposes animals to secondary infections, and impact of increased virulence of pathogens caused by synergy in coinfections. Adding to the complexity is the heterogeneity observed among BVDV strains that results in differences in genotype (species), biotype, and virulence. Because variation among BVDV strains underlies the complicated nature of these infections this article begins with a review of the factors that give rise to BVDV heterogeneity and then proceeds to the different ways in which BVDV infections contribute to BRD.

BASIS OF HETEROGENEITY OBSERVED AMONG BOVINE VIRAL DIARRHEA VIRUSES STRAINS

Size of Lipid Envelope Surrounding Virion is Variable

The term BVDV refers to a heterogeneous group of viruses that belong to two different species, BVDV1 and BVDV2, within the *pestivirus* genus of the Flavivirus family.[1,2] Similar to other Flaviviridae, the infectious viral particle consists of an outer lipid envelope surrounding an inner protein shell or capsid that contains the viral genome.

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Pestiviruses differ from other members of the Flaviviridae in that the lipid envelope that surrounds the virion is pleomorphic. The practical significance of the resulting variation in size of enveloped particles is that it precludes identification by electron microscopy in clinical samples. Further, this variation in size prevents the purification of the virus by standard techniques used in virology. This lack of purification means that the BVDV component of most killed and modified-live vaccines is not pure BVDV but a mixture of viral proteins and proteins derived from the cells in which the virus was propagated.

The Genome of Bovine Viral Diarrhea Viruses is a Single-Stranded RNA Prone to Mutation

The BVDV genome consists of a single strand of RNA about 12.3 Kb long. The genomic organization is the same as that of all pestiviruses. In the absence of insertions, the genome codes for one open reading frame (ORF) approximately 4000 codons long. The ORF is preceded and followed by 5′ and 3′ untranslated regions (UTR) of 360 to 390 nucleotides and 200 to 240 nucleotides, respectively. Much of the heterogeneity observed among BVDV is caused by the variability inherent in having a single-stranded RNA genome. Point mutations in this type of genome are introduced with each round of virus replication because there is no proofreading function associated with replication. The impact of this heterogeneity is observed as differences in genotype, biotype, and virulence. It is thought that the accumulation of point mutations over time resulted in the branching of BVDV into two genetically distinct species, now known as BVDV1 and BVDV2.[3,4] Although the first studies, segregating BVDV strains into two species, were based on comparison of the 5′ UTR, differences between BVDV1 and BVDV2 strains are found throughout the genome.[5,6] Antigenic differences, between the two species, are revealed in the laboratory by serum neutralization studies using polyclonal sera[7–11] and by monoclonal antibody binding studies.[4,12] These antigenic differences are also shown in nature by the birth of BVDV2 persistently infected animals to dams that had been vaccinated against BVDV1 strains.[4] The fact that cattle persistently infected with and immunotolerant of one species have a serologic response to vaccination with another BVDV species further demonstrates antigenic differences between the two species.[13] Although modified-live BVDV1 vaccines may induce antibodies against BVDV2 strains, the titers average one log less than titers against heterologous BVDV1 strains.[7–11] These observations led to the inclusion of both BVDV species in many BVDV vaccines.

Genomic Recombination that Result in Changes in Biotype

BVDV1 and BVDV2 strains may exist as one of two biotypes (cytopathic and non-cytopathic) based on their activity in cultured epithelial cells, with non-cytopathic BVDV predominating in nature.[14,15] Studies of proteins associated with BVDV replication in cultured cells reveal that cytopathic BVDV could be distinguished from non-cytopathic BVDV by the production of an extra nonstructural protein known as NS3.[16,17] This protein is the result of the cleavage of another nonstructural protein, NS2/3. Comparison of NS2/3 coding region of cytopathic and non-cytopathic BVDV revealed that genomes of most cytopathic viruses had under gone a recombinational event resulting in the introduction of a cleavage site into the NS2/3 protein.[18] The most commonly observed means of introducing this cleavage site was by insertion of host cell genetic sequences or duplicated BVDV genetic sequences inserted into the NS2-3 coding region.[19]

Exposure of cattle to viruses from either biotype may result in acute infection. In addition, infection of a bovine fetus before 125 days gestation to non-cytopathic, but not cytopathic, viruses may result in the establishment of a lifelong persistent infection.[20,21]

The prerequisite for such persistent BVDV infections appears to arise from specific B- and T-lymphocyte immunotolerance.[22,23] It is theorized that there is negative selection or downregulation of BVDV-specific B and T lymphocytes during ontogeny resulting in an absence of neutralizing and non-neutralizing antibodies and cell mediated immunity to the persistent BVDV.[24] The result is that viral proteins are accepted as self-antigens. Persistently infected animals (PIs) may appear normal or suffer from an array of congenital anomalies, including defects of the nervous system, eyes, skeletal system, and skin/hair (**Table 1**). In addition to these more noticeable defects, PIs are more likely than normal cattle to require treatment for respiratory tract disease and either become chronically ill or die.[25] The PI animal also has a high risk for succumbing to a highly fatal form of BVD called mucosal disease (BVD-MD). BVD-MD is the sequela of a persistent infection with non-cytopathic BVDV strain, followed by an acute concurrent infection with a cytopathic BVDV strain. The cytopathic BVDV inducing BVD-MD is most frequently derived from the non-cytopathic BVDV with which the PI animal is persistently infected.[26–29] Other sources for cytopathic BVDV include other cattle suffering from BVD-MD[30] and modified-live virus vaccines.[31–33] BVD-MD is invariably fatal. However, because the number of PI animals within a herd tends to be low, morbidity for BVD-MD also tends to be low. The major economic impact of PIs is not death caused by BVD-MD, but that these animals continually shed the virus and are the principal vector for introduction and maintenance of BVDV infections within cattle populations.[34]

Fetal exposure to BVDV after 125 days gestation, although not resulting in persistent infections, may give rise to many of the same congenital anomalies as persistent infection and there are reports that non-persistently infected cattle that were exposed to BVDV in utero have higher morbidity and mortality rates[35,36] and lower fertility rates[37] than cattle not exposed in utero.

BOVINE VIRAL DIARRHEA VIRUS INFECTIONS AND RESPIRATORY DISEASE

The contribution of BVDV infections to the development of clinical respiratory disease depends on several factors, including the virulence of the infecting BVDV strain, type of infection (acute or persistent), time of exposure (fetal or postnatal), and the

Table 1
Defects resulting from in utero exposure to BVDV

System/Tissue	Defect
Central nervous system	Cerebellar hypoplasia[74–81] Microencephaly, hydrocephalus, hydranencephaly[82] Hypomyelination[81,83–85]
Skeletal	Abnormal osteogenesis[86] Brachygnathism[87] Failure of limb development[87]
Eye	Optic neuritis[77,88] Cataracts[89] Retinal degeneration[77,90] Microphthalmia[91,92]
Endocrine	Thymic hypoplasia[93]
Skin/hair	Curly coat[94] Hypotrichosis/alopecia[95]

presence of secondary pathogens. It is not always possible to determine if clinical reports and surveys are describing outbreaks of respiratory disease in PIs or the contribution of BVDV to BRD in a herd as the result of acute infections. On the other hand, it is difficult to design studies of BVDV infections under controlled conditions that reproduce the numerous factors that contribute to the development of BRD.

IMPACT OF RESPIRATORY DISEASE IN PERSISTENTLY INFECTED ANIMALS

PIs are more likely to succumb to disease than their normal counterparts. Surveys of cattle entering feedlots in the United States indicate that although PIs represent less than 0.5% of the general population, they represent 2.5% to 5% of the cattle succumbing to fatal illnesses.[25,38] Because PIs have a higher rate of morbidity and mortality than the general population, a portion of the observed association of BVDV with BRD may be the result of PIs vulnerability to respiratory disease. In fact, in utero exposure to BVDV may predispose even non-PIs to BRD. A study by Barber and colleagues[35] detailing calf loss caused by disease in a dairy herd over a 3-year time span demonstrated that in utero exposure to BVDV results in a higher susceptibility to respiratory disease. In the first year of this study an outbreak of diarrhea among adult milking cows was traced to BVDV. Of 121 calves born that year, five died with mucosal disease-like symptoms and 21 (17%) died of pneumonia. Calf losses remained high the following 2 years with suppurative or fibrinous pneumonia as the leading cause of death. Testing results were not available for all animals dying of pneumonia, and data regarding animals that developed pneumonia but recovered was not included. BVDV was isolated from 36 animals examined with 12 being identified as persistently infected. In addition, the investigators looked at the relationship between the BVDV serologic status of cows and calf survival. It was observed that the death rate among calves born to BVDV seropositive cows was 22% (of these deaths 15/35 or 43% were caused by pneumonia), whereas the death rate among calves born to cows infected during pregnancy, based on serology, was 50% (of these 18/24 or 75% were caused by pneumonia). Based on this comparison, the investigators concluded that fetal exposure to BVDV, regardless of whether exposure resulted in persistent infection, reduced a calf's chance of survival and increased its risk for developing pneumonia. Further evidence that fetal exposure predisposes cattle to disease is provided by a study showing that calves born with titers against BVDV (indicating a fetal exposure) were twice as likely to develop serious illness.[36] Thus studies of association of pathogens with respiratory lesions, based on isolation or detection of pathogens currently present, may underestimate the contribution of BVDV fetal exposure to the development of BRD.

BOVINE VIRAL DIARRHEA VIRUS INFECTIONS AND THE DEVELOPMENT OF BOVINE RESPIRATORY DISEASE IN NORMAL CATTLE (CATTLE NOT EXPOSED IN UTERO)

Although PI and animals exposed in utero may make up a portion of respiratory disease cases, there are three lines of evidence showing that current acute-BVDV infections in cattle that were not exposed to BVDV in utero contribute to respiratory disease. One line of evidence is the detection of BVDV in lung lesions of acutely infected cattle. In a study of fatal BRD cases in Western Canada, 90 feedlot calves diagnosed at necropsy with BRD and nine control calves without BRD were examined for the presence of seven pathogens (BVDV, bovine herpesvirus-1, bovine respiratory syncytial virus [BRSV], parinfluenza-3 virus, *Mannheimia haemolytica* [MH], *Mycoplasma bovis*, and *Histophilus somni*) based on immunohistochemical (IHC) staining of lung tissue.[39] In addition, a distinction was made between persistent or acute

infection with BVDV based on IHC staining of skin biopsy samples.[40] BVDV was detected in the lungs of 26 calves succumbing to BRD but not in the lungs of any control animals. Of the 26 BVDV-positive calves, three were determined to be PI indicating that acute BVDV infections were more prevalent than persistent infections in these BRD cases.

A second line of evidence is based on serologic data collected from cattle in feedlots. Several studies have reported that higher serum antibody titers against BVDV in cattle on arrival to feedlots reduced the risk for subsequent BRD,[41–44] and conversely, increases in BVDV titer after arrival were associated with an increased risk for BRD.[41,42,44]

The third line of evidence is illustrated by a study comparing calves exposed to BVDV after receiving colostrum from seronegative dams to calves exposed to BVDV after receiving colostrum from seropositive dams.[45] Calves exposed to BVDV soon after birth that received passive antibodies against BVDV via ingestion of colostrum were 28% less likely to develop respiratory disease than exposed calves that did not receive passive antibodies.

Thus it appears that fetal exposure to BVDV and acute postnatal BVDV infections contribute to BRD. The mechanism of this contribution is variably dependent on the type of infection (acute or persistent), virulence of viral strain, impact of immune suppression, and interaction with secondary pathogens as discussed later.

IMPACT OF UNCOMPLICATED ACUTE INFECTIONS

Acute BVDV infection is the term used to describe clinical or subclinical disease that occurs in non-persistently infected immunocompetent cattle following exposure to BVDV.[46] Exposure is typically via short range, large droplet aerosols, or direct contact with infected animals. Exposure results in the infection of the nasal mucosa. Although there can be transmission from acutely infected cattle, the most frequent vector is a PI animal. Infection spreads from the nasal mucosa to the draining lymph nodes and from there is transmitted to other tissues via circulating lymphoid cells. The incubation period is 5 to 7 days and viremia is typically less than 15 days[46] but may be longer depending on viral strain, stress levels, and presence of secondary pathogens. Although not the primary site of replication, controlled studies and field reports demonstrate that BVDV can establish infections in the respiratory tract of cattle.[47–50] Although these infections result in damage to the epithelial surfaces of the respiratory system[51] and depletion of lymphoid tissue associated with the respiratory tract,[50,52–54] it is estimated that 70% to 90% are subclinical.[55] Although some studies have demonstrated clinical respiratory disease resulting from acute, uncomplicated BVDV infections under controlled conditions,[56,57] a model system that reproducibly replicates BVDV-associated pneumonia has yet to be developed. This is problematic for the study of BVDV infections and for demonstrating the efficacy of vaccines in preventing BVDV infections of the respiratory system.[58]

Differences in virulence[53,59–65] and pneumopathogenicity[57] exist among BVDV strains. The length of viremia is longer and the amount of viral shed is greater following infection with highly virulent BVDV compared with infections with low virulence strains.[59,61,66] Transmission by acutely infected animals is significant and the sources of virus increase as the number of infected animals increases.[60] Thus management to control outbreaks of severe, acute BVD requires a different approach than that used for subclinical BVD.

Although acute BVDV infections may, in some situations, directly result in respiratory disease, the majority of evidence suggests that BVDV infections contribute to

BRD mainly as potentiators for secondary infections (**Table 2**). It is theorized that BVDV potentiation of BRD occurs by two different mechanisms, immunosuppression[67,68] and synergism,[69] as discussed later.

IMMUNOSUPPRESSION ASSOCIATED WITH BOVINE VIRAL DIARRHEA VIRUS INFECTION

The immunosuppression that accompanies acute BVDV infections results from a combination of outright lymphoid cell death[2,63,64] and reduced function in remaining lymphoid cells.[67] Although there are several reports in the literature detailing in vitro studies of cell death or immune cell dysfunction resulting from infection with cytopathic BVDV strains, cytopathology in cultured epithelial cells is not analogous to cell death resulting in immunosuppression in vivo. The cytopathic effects observed following the infection of epithelial cells in vitro with a cytopathic BVDV is not equivalent to lymphoid depletion observed following BVDV infection in vivo for several reasons. For one, nearly all field strains of BVDV are non-cytopathic. Cytopathic strains of BVDV are only rarely isolated and usually from cases of BVD-MD. Secondly, the cell death observed in vivo is in lymphoid cell populations, not epithelial cell populations. Further, it appears that cell death caused by cytopathic BVDV in vitro may occur by a different mechanism than cell death that occurs following infection with non-cytopathic BVDV.[70,71] Finally, the lymphoid depletion that is associated with BVDV infections in vivo results from a complex and intertwined response of the innate and acquired immune system that cannot be replicated in a culture flask.

Infection, with either high- and low-virulence BVDV, results in the reduction of circulating lymphocytes[59,63,64] and the depletion of lymphoid tissue.[50,54] The mechanism behind lymphoid depletion following acute BVDV infection is largely undefined. Comparison of high- and low-virulence BVDV strains has yielded some intriguing results. The difference in pathology between high- and low-virulence BVDV strains is in the extent of cell death or loss, with reduction of circulating lymphocytes and lymphoid depletion being significantly higher following infection with highly virulent BVDV. The mechanisms involved in the destruction of lymphoid tissue by

Table 2
Reported interaction of BVDV with secondary pathogens in the development of BRD

Pathogen type	Viral Pathogen	Interaction
Viral	BRSV[68,96–98]	Synergism resulting in increased pathogenesis/dissemination
	Infectious bovine rhinotracheitis virus[72,99–102]	Synergism resulting in increased pathogenesis/dissemination
	Parainfluenza-3 virus[102]	Synergism resulting in increased pathogenesis/dissemination
Bacterial	*Mycoplasma bovis*[103,104]	Synergism resulting in increased pathogenesis, immunosuppression resulting in increased opportunist infections and chronic infections
	Mannheimia haemolytica[39,56,57]	Synergism resulting in increased pathogenesis, immunosuppression resulting in increased opportunist infections

non-cytopathic BVDV strains are poorly understood. Decreased numbers of circulating lymphocytes may be the result of trafficking from blood into tissue, a reduction in leukogenesis, or outright death of cells. The high percentage of apoptotic or necrotic lymphocytes observed in animals infected with a high-virulence BVDV strain suggest that the reduction may be caused by cell death.[50,53] This in vivo observation is supported by the in vitro observation that infection of a cultured lymphoid cell line, with a high-virulence virus leads to cell death.[70] Although this mechanism of cell death has yet to be defined, it appears to be different than the mechanism that induces apoptosis in cells infected with cytopathic BVDV.[71]

In addition to reducing lymphoid cell numbers, BVDV infections impair the function of cells associated with both the acquired and innate immune systems (**Table 3**).[67] The interactions resulting in immune suppression are complex and it is difficult to design experimental models that reflect natural conditions. Stages in the innate immune response that are reported to be suppressed in response to BVDV infection include interferon production, phagocytosis, chemotaxis, and microbicidal killing. On the acquired immune side, changes, such as downregulation of MHC II and IL-2, suppress the T-helper cell response.

SYNERGISTIC EFFECTS

Synergy occurs when coinfection of two or more pathogens results in an enhanced pathogenesis or change in character of the pathogenesis. Synergistic effects have been reported between BVDV and several viral and bacterial pathogens that are associated with BRD (see **Table 3**). Once again, when reading through the literature it is necessary to differentiate between enhanced pathogenesis observed following infection of PIs and synergy observed following acute coinfection with BVDV and a secondary pathogen. Synergism may occur by several different routes depending on coinfecting pathogens and target tissues. One possible result of synergy is increased dissemination of pathogens in tissues. Viral dissemination of BVDV and BHV-1 were higher in concurrent infections than in single infections.[72] Dual infections

Table 3
Effects of BVDV that result in immunosuppression

Change	Effect	Impact
Decreased chemotaxis Decreased Fe, CD14, and complement receptor expression Decreased phagocytosis	Decreased neutrophil function	Decreased ability to fight off bacterial infections
Decrease in TNF-α production and increase in IL-1 inhibitors	Decreased inflammatory and T-cell cytokine production	Decreased innate and adaptive immune response
Decrease in IFN production	Decreased antiviral response	Decreased ability to fight off viral infections
Decrease in MHC II production	Decrease in antigen presentation	Reduction in capacity of adaptive immune response
Decrease in MHC I expression	Decrease in cytotoxic T-lymphocyte response	Increase in immune evasion and viral infection

Abbreviations: IFN, interferon; IL-1, interleukin-1; MHC, major histocompatibility complex; TNF, tumor necrosis factor.

may also combine to reduce immune cell function. Liu and Lehmkuhl demonstrated that coinfections of bovine alveolar macrophage (AM) with BVDV and BRSV produced a synergistic depression on AM functions. Although the mechanism of synergy may not be defined, indirect evidence may point to synergistic interaction. BVDV is frequently detected in diseased respiratory tract tissues in association with MH.[39,73] As it is difficult to reliably reproduce respiratory disease under controlled conditions in infections with BVDV or MH alone, a synergistic relationship (albeit currently undefined) is indicated.

SUMMARY

Although it is difficult to confirm by direct experimental evidence, several lines of research indicate that BVDV may be a pivotal component in BRD. This research includes the following examples. Some strains of BVDV may cause BRD following acute uncomplicated infections. Calves exposed to BVDV in utero have increased susceptibilities to BRD. Acute BVDV infections result in a broad spectrum immunosuppression that predisposes cattle to opportunistic infections of secondary respiratory pathogens. Finally, synergistic interaction of BVDV and other respiratory pathogens during coinfections results in more clinically severe respiratory disease.

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Hypomyelination Associated With Bovine Viral Diarrhea Virus Type 2 Infection in a Longhorn Calf

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Abstract

A newborn Longhorn heifer calf presented with generalized tremors, muscle fasciculations, ataxia, and nystagmus. At necropsy, no gross central nervous system lesions were observed. Histologically, the brain and spinal cord had mild to moderate diffuse microgliosis and astrocytosis, minimal nonsuppurative encephalitis, and decreased myelin staining. Ultrastructural examination revealed thinning and absence of myelin sheaths. Various cell types were immunohistochemically positive for bovine viral diarrhea virus (BVDV). Noncytopathogenic BVDV was isolated from the brain and identified as BVDV type 2 by phylogenetic analysis. BVDV-induced hypomyelination is rare and analogous to lesions in neonates infected with border disease and classical swine fever viruses. This is the first documented case of hypomyelination in a calf specifically attributed to BVDV type 2 and the first description of the ultrastructural appearance of BVDV-induced hypomyelination.

Keywords

Bovine viral diarrhea virus, cattle, electron microscopy, hypomyelination, immunohistochemistry

Bovine viral diarrhea virus (BVDV) is a single-stranded, enveloped, positive-sense RNA virus grouped in the family Flaviviridae, genus *Pestivirus*, along with border disease virus (BDV) and classical swine fever virus (CSFV). BVDV strains are categorized into noncytopathic or cytopathic biotypes based on properties in cell culture, and 2 species have been identified: BVDV1 and BVDV2.[21] These 2 species may be further divided into subgenotypes; the predominant subgenotypes circulating in US cattle are BVDV1a, BVDV1b, and BVDV2a.[12] BVDV readily crosses the placenta, and the result of fetal infection depends on the viral strain, the immune status of the dam, and the timing of fetal exposure. Potential outcomes include perinatal death or weakness, early fetal death, persistent infection, and a variety of teratogenic lesions. Types of BVDV-induced central nervous system (CNS) defects include cerebellar hypoplasia, spinal cord hypoplasia, microencephaly, porencephaly, hydranencephaly, internal hydrocephalus, and a variety of ocular changes.[2,10,14] Hypomyelination has been reported in calves congenitally infected with BVDV;[5,10,20,23] however, hypomyelination is a relatively uncommon outcome compared with intrauterine infections with BDV and CSFV strains in sheep and pigs, respectively. Here we describe the histologic, immunohistochemical, and ultrastructural findings in a calf with severe hypomyelination presumably induced by naturally acquired intrauterine infection with a virus specifically genotyped as BVDV type 2.

History and Clinical Findings

A newborn female Longhorn calf was presented to the Texas A&M Veterinary Teaching Hospital with a history of congenital neurologic signs. The calf was unable to rise, and it exhibited generalized tremors and muscle fasciculations, grade 4 generalized ataxia,[9] and horizontal and vertical nystagmus. Spinal and cranial nerve reflexes, the pupillary light reflex, muscle strength, and mentation were normal. Because of a poor prognosis, the owner elected euthanasia, and the calf was euthanized at 8 days of age.

Necropsy Findings

The brain and spinal cord were grossly normal at necropsy. Gross lesions in other tissues were limited to mild cranioventral

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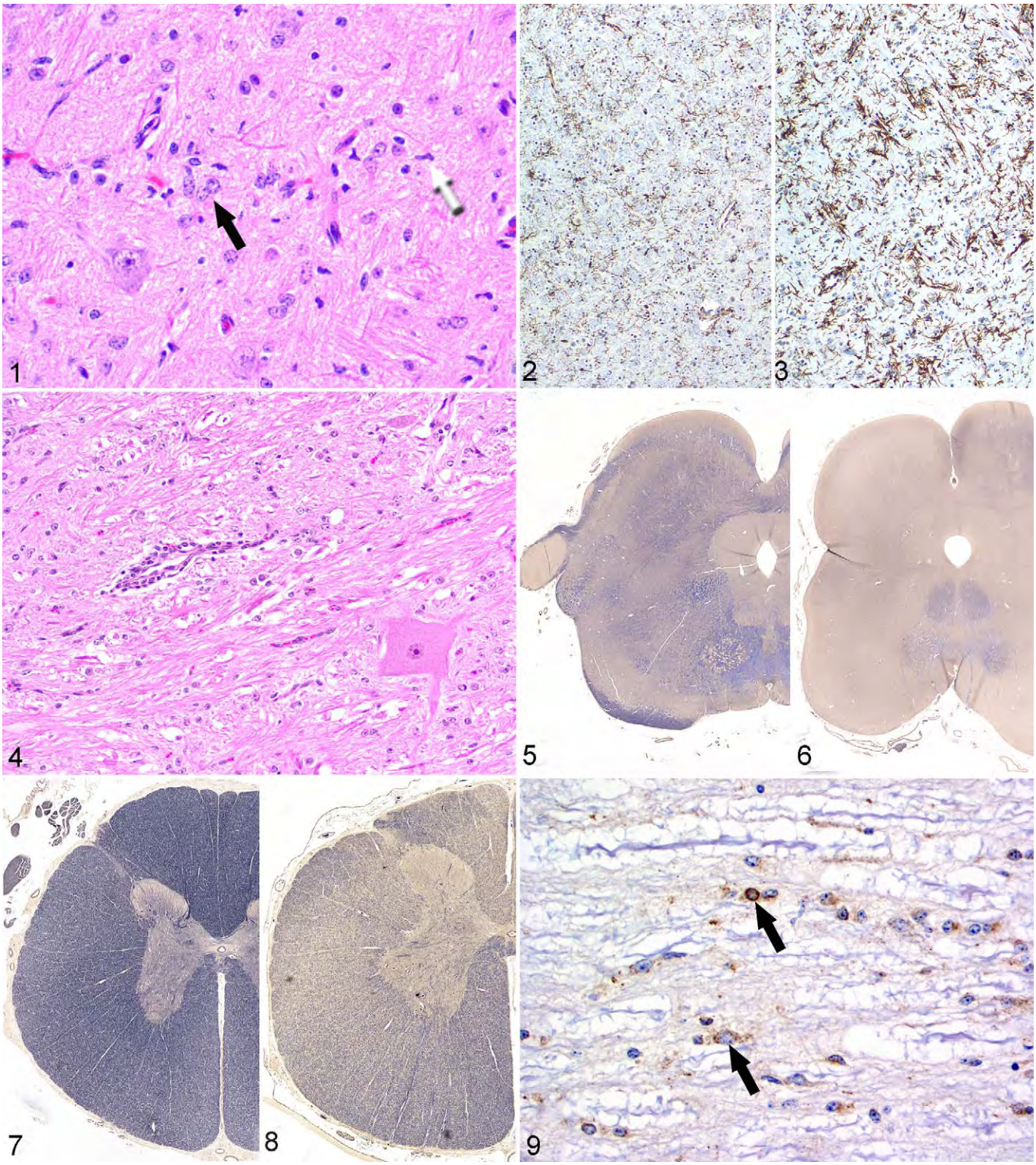

Figure 1. Midbrain; Longhorn calf. An increased number of astrocytes (black arrow) and microglia (white arrow) are evident. HE. **Figure 2.** Midbrain; control calf. Immunohistochemistry for microglia (Iba1). Streptavidin–biotin–peroxidase method, Gill's hematoxylin counterstain. **Figure 3.** Midbrain; Longhorn calf. Compared with Figure 2, there is increased immunoreactivity for microglia (Iba1) in the affected calf. Streptavidin–biotin–peroxidase method, Gill's hematoxylin counterstain. **Figure 4.** Medulla oblongata; Longhorn calf. A cuff of lymphocytes surrounds a small blood vessel. Gliosis is also evident. HE. **Figure 5.** Midbrain; control calf. Myelin staining is relatively strong in the control calf. Weil. **Figure 6.** Midbrain; Longhorn calf. Compared with Figure 5, myelin staining is greatly reduced in the affected calf. Weil. **Figure 7.** Spinal cord; control calf. The spinal white matter stains strongly for myelin in the control calf. Weil. **Figure 8.** Spinal cord; Longhorn calf. The

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consolidation of the lungs and a small amount of purulent exudate within the thickened umbilical cord. Tissue samples were fixed in 10% neutral buffered formalin, processed routinely for light microscopy, and stained with hematoxylin and eosin (HE). Selected tissue sections were stained with the Weil and Luxol fast blue methods; others were stained with the streptavidin–biotin–immunoperoxidase method and the following primary antibodies: microglia (Iba1; 1:000 dilution; Biocare Medical, Concord, CA), glial fibrillary acidic protein (1:1000 dilution; DakoCytomation, Carpinteria, CA), and BVDV (undiluted; New York State Animal Health Diagnostic Center, Ithaca, NY). Fresh samples of brain and spinal cord were immersed overnight in fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1M sodium cacodylate buffer), then washed and processed for electron microscopy. Briefly, after washing in buffer, the tissue was en bloc stained with 1% osmium tetroxide and 0.5% potassium ferrocyanide for 1.5 hours, then dehydrated in an ascending alcohol series followed by propylene oxide. The tissue was then embedded in epoxy resin. Control brain and spinal cord tissue for immunohistochemistry and electron microscopy were obtained from a 2-day-old female Charolais calf that presented for bilateral rear limb fractures and did not have clinical signs or lesions consistent with neurologic disease.

Histologically, a mild to moderate gliosis was evident throughout the brain and spinal cord, most notably in the cerebral white matter, cerebellar white matter, and brain stem (Fig. 1). The increased glial cell population included astrocytes and microglia. The astrocytes had large pale nuclei, often formed clusters, but generally did not have an increased amount of visible cytoplasm on HE staining. However, immunohistochemical expression of glial fibrillary acidic protein by astrocytes was increased in comparison with the control. Microglial cells were larger than normal and often had irregularly shaped nuclei. Compared with that of the control calf, microglial immunohistochemical reactivity was increased in intensity in the midbrain and spinal cord (Figs. 2, 3). A few swollen axons were evident in the medulla oblongata. Rare parenchymal blood vessels were surrounded by a small number of lymphocytes, generally not more than one cell layer in thickness (Fig. 4), and endothelial cells were often swollen. Weil and Luxol fast blue stains revealed decreased myelin staining in the cerebral white matter, midbrain, and spinal cord white matter, when compared with that of the control calf (Figs. 5–8). Other histologic findings included necrosuppurative omphalophlebitis and arteritis with intralesional bacteria and suppurative bronchopneumonia with intralesional plant material. Minimal multifocal neutrophilic inflammation was in the heart, liver, esophagus, omasum, and colon. Mild lymphocytic inflammation was evident in the dermis. The following tissues were unremarkable: kidney, spleen, adrenal gland, thyroid gland, eye, trachea, radial nerve, common peroneal nerve, tibial nerve, spinal nerve roots, dorsal root ganglia, skeletal muscle, urinary bladder, abomasum, rumen, small intestine, thymus, and bone marrow.

Immunohistochemistry for BVDV antigen in sections of spinal cord, medulla oblongata, cerebellum, and cerebrum revealed positive reaction in a variety of cell types, including astrocytes, oligodendrocytes, microglia, pericytes, ependymal cells, and endothelial cells (Fig. 9) but only rarely in neurons. Immunoreactivity was strongest in the spinal cord and medulla oblongata. In the cerebrum, BVDV immunoreactivity was relatively weak and confined to pericytes and occasional endothelial cells and glial cells within the white matter. In the skin, weak multifocal BVDV immunoreactivity was evident in the epidermis and within follicular epithelial cells. Strong BVDV immunoreactivity was evident in thyroid follicular epithelial cells. Immunohistochemistry of the control calf brain for BVDV was negative.

In semithin sections of the spinal and cerebral white matter prepared for ultrastructural examination, myelin sheaths were absent or markedly thinner in comparison with those in tissue from the control calf (Figs. 10, 11). Ultrastructurally, the thinning of myelin sheaths was accompanied by an increase in astrocytic processes, which were identified by the presence of cytoplasmic fibril bundles (Fig. 12). Astrocytic processes were often closely apposed to myelin sheaths or bare axons. An increase in microglial processes was noted, as identified by the presence of long endoplasmic reticulum cisternae; these processes were also seen in close apposition to myelin sheaths and axons. Amorphous accumulations of electron-dense material, interpreted as myelin debris (Fig. 13), were infrequently observed in the cytoplasm of microglia. Small, round, clear vacuoles were rarely seen within microglial cytoplasm.

BVDV was isolated from the brain with standard methods. The virus was of the noncytopathic biotype based on serial passage in cultured epithelial cells.[13] Phylogenetic analysis (Fig. 14), based on sequences generated from the 5′ untranslated region, was performed as described.[22] Segregation of BVDV strains based on comparison of sequences from the 5′ untranslated region is an accepted method for determining BVDV genotypes.[25]

Discussion

Hypomyelination is an uncommon outcome of transplacental BVDV infection. Most fetal infections with BVDV result in persistent subclinical infection, fetal death, or defects such as cerebellar hypoplasia, hydranencephaly, internal hydrocephalus, microencephaly, and porencephaly.[5,10,14] Neuropathologic descriptions of natural in utero BVDV infection resulting in hypomyelination are limited. In a dairy farm in the Netherlands,

Figure (continued) affected calf has much weaker myelin staining than the control calf in Figure 7. Weil. **Figure 9.** Cerebrum; Longhorn calf. Glial cells, including astrocytes and oligodendrocytes, are immunopositive for bovine viral diarrhea virus antigen in the spinal white matter (arrows). Streptavidin–biotin–peroxidase method, Mayer's hematoxylin counterstain.

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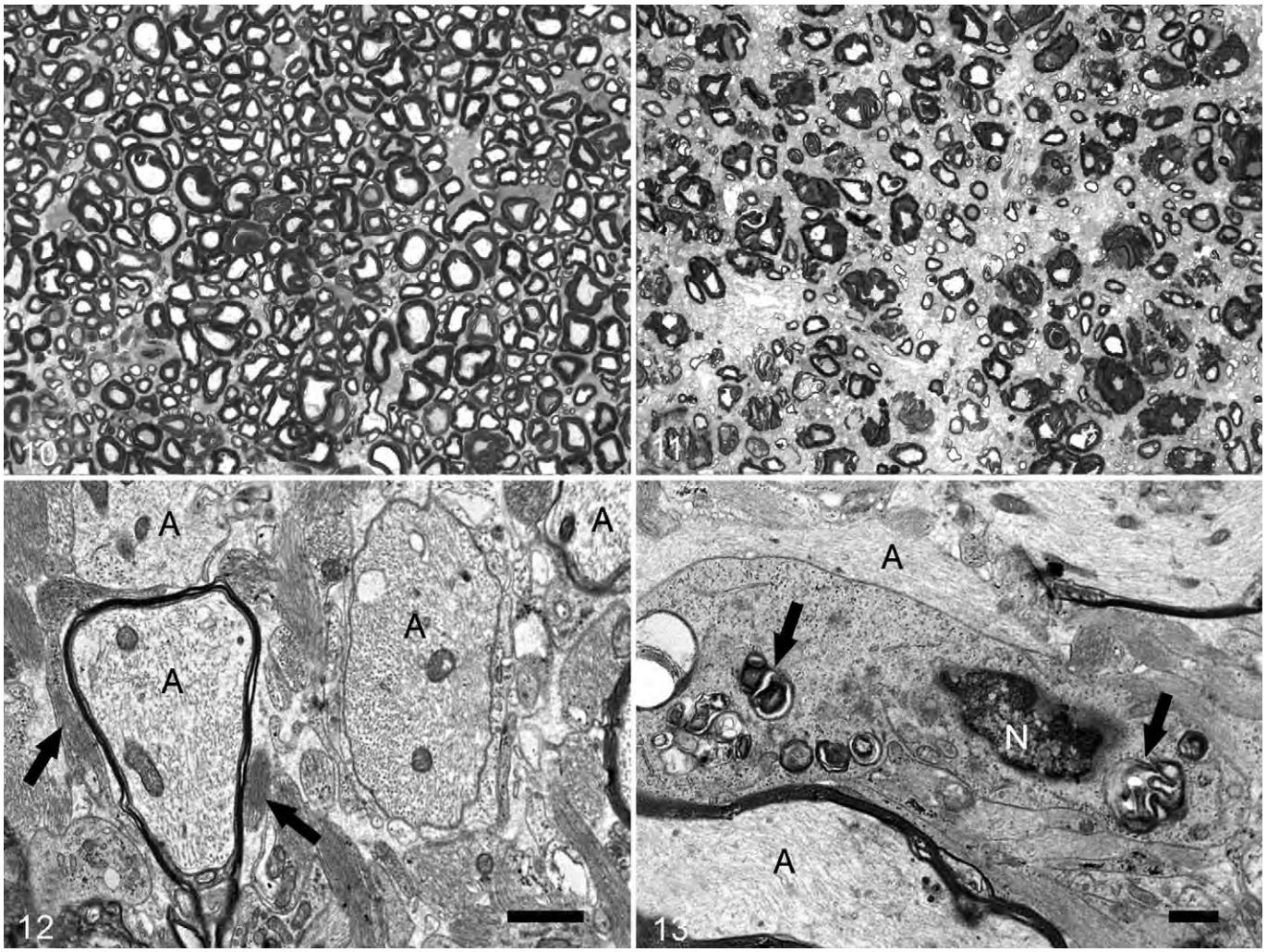

Figure 10. Spinal cord; control calf. Myelin sheaths are relatively well developed in the spinal white matter of the control calf. Toluidine blue. **Figure 11**. Spinal cord; Longhorn calf. Compared with Figure 10, myelin sheaths are less prominent in the affected calf. The swelling and irregularity of well-myelinated axons is presumably fixation artifact. Toluidine blue. **Figure 12**. Spinal cord white matter; Longhorn calf. Axons (A) have thin myelin sheaths and are surrounded by astrocytic processes containing fibril bundles (arrows). Electron micrograph. Bar = 1 μm. **Figure 13**. Spinal cord white matter; Longhorn calf. A microglial cell (N = nucleus) is closely apposed to axons (A) and contains accumulations of amorphous electron-dense material (presumed myelin debris) in the cytoplasm (arrows). Electron micrograph. Bar = 1 μm.

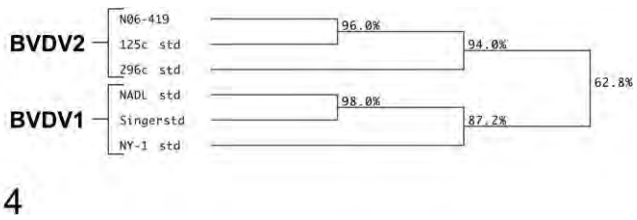

Figure 14. This dendrogram shows the phylogenetic location of the bovine viral diarrhea virus isolate in this case (strain No. N06-419) based on the comparison of sequences from the 5′ untranslated region.

an outbreak of BVD resulted in multiple calves being born with neurologic signs similar to those described here, and histologic findings included widespread loss of myelin staining, an increased number of microglia and astrocytes with enlarged vesicular nuclei, and mild mononuclear cell cuffing of blood vessels.[5] Another report describes a single case of BVDV in a newborn Holstein calf that resulted in ataxia, hypermetria, and intention tremors of the head.[23] Hypomyelination was reported in that case but not accompanied by gliosis or inflammation. A recent comprehensive British study suggests that hypomyelination is a relatively common outcome of BVDV infection in some herds;[20] it documented 23 herds over a 17-year period in which hypomyelination was the primary lesion in calves with fetal BVDV infection, resulting in neurologic signs. The pathologic findings were similar to those reported here. The reason for the apparent higher incidence of hypomyelination in some herds in unclear. Hypomyelination can occur in conjunction with other CNS defects in BVDV-infected calves. Cerebellar hypoplasia was the most common lesion in a study of natural transplacental BVDV

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infection in Germany; in fact, 5 of 25 animals showed evidence of concurrent hypomyelination.[14]

Experimental in utero BVDV infection has only rarely produced hypomyelination. In a study by Done et al,[10] pregnant cows injected with BVDV either aborted or gave birth to calves with morphologic defects in the CNS. The defects ranged from structural brain malformations to hypomyelination, and some calves had both. More recent studies involving in utero infection with BVDV produced varying lesions but no histologic evidence of reduced myelin.[4,19] In contrast to congenital BVDV infections, hypomyelination is relatively more common in congenital border disease and classical swine fever infections. Histologic lesions in both diseases typically consist of severe hypomyelination, gliosis, and a lack of inflammatory cell infiltration.[3,7,18]

The pathogenesis of hypomyelination resulting from in utero pestivirus infection is poorly understood. A deleterious effect on oligodendrocyte development or function is possible, although studies of experimental border disease indicate that BDV does not have a selective tropism for oligodendrocytes. Cell types infected by BDV include neurons, astrocytes, oligodendrocytes, endothelial cells, ependymal cells, and meningeal fibroblasts,[11,15] and studies with BVDV have produced comparable results.[4,19] Similarly, a variety of cell types were infected with BVDV in the case reported here. BDV-induced interference with thyroid function may play a role in pathogenesis. In one study, lambs with border disease had significantly lowered levels of thyroid hormones and the myelin-associated thyroid hormone–dependent enzyme CNP (2′,3′-cyclic nucleotide-3′-phosphodiesterase) in comparison with normal lambs.[1] Like this calf, the lambs in that study had viral antigen within thyroid follicular cells without histologic lesions. The role of CNP in myelinogenesis is questionable given that *Cnp1*-knockout mice develop structurally normal myelin sheaths.[16]

The variation in the nervous system defects in fetuses infected transplacentally by BVDV is intriguing. The outcome of BVDV in utero infection varies with the timing of the infection. Infections before immunocompetency at 150 days can result in abortion, congenital defects, or persistent infection, whereas those occurring after 150 days are typically cleared.[17] Few experimental transplacental BVDV infections have resulted in hypomyelination; thus, determining why it occurs in some cases is difficult. In the previously noted study, the viral inoculum was a mixture of BVDV strains, and all cows were inoculated at 100 days of gestation.[10] The variety of resulting lesions in that study suggests that the virus strain plays more of a role in determining the type of defect than the timing of infection. As a single-stranded RNA virus, BVDV lacks proofreading ability and is prone to a high mutation rate; the resulting viral strains have varying levels of virulence.[21] The tropism and effects of different viral strains on the fetal nervous system have only begun to be examined. Experiments suggest that type 2 BVDV strains infect the fetal brain more readily than do type 1 strains, although both types have similar tissue tropism.[4] In a previously reported case of BVDV2-induced neurologic disease in a 15-month-old heifer, the lesions were limited to meningoencephalitis, neuronal necrosis, and gliosis.[6] In the cited British study, isolated viruses classified by molecular techniques were either BVDV1a or BVDV1b.[20]

The ultrastructural appearance of the CNS changes in BDV- and CSFV-infected neonates has been described. As with the calf reported here, an increased number of astrocytic processes in close proximity to axons has been noted in BDV-infected lambs.[3] Cancilla and Barlow[8] suggested that astrocytic processes may retard myelin formation by inhibiting contact between axons and oligodendrocytes; but whether astrocytes truly play a role in the pathogenesis or if their proliferation is simply a response to injury remains to be determined. Microglial processes adjacent to thinned myelin sheaths containing myelin-like debris and clear vacuoles have been observed in BDV and classical swine fever infection.[3,7,8] Although rare, this finding suggests that destruction of preformed myelin (demyelination) or abnormally formed myelin (dysmyelination) may play a role in the pathogenesis and that the most appropriate term for the lesion may not be *hypomyelination*. Other reported changes include dilation of portions of the myelin sheath, lack of compaction of myelin lamellae, and intramyelinic vacuoles. Some of these changes were observed in this case, but they could not be definitively differentiated from tissue artifact.

Most previously described cases of BVDV-induced hypomyelination were not linked to a specific viral genotype. Phylogenetic analysis verified that the virus in this case was a strain of type 2 BVDV. Further study is required to determine the specific viral and host factors required for the development of hypomyelination in calves infected with BVDV in utero.

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Transboundary and Emerging Diseases

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REVIEW

Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication

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Summary

Existing strategies for long-term bovine tuberculosis (bTB) control/eradication campaigns are being reconsidered in many countries because of the development of new testing technologies, increased global trade, continued struggle with wildlife reservoirs of bTB, redistribution of international trading partners/agreements, and emerging financial and animal welfare constraints on herd depopulation. Changes under consideration or newly implemented include additional control measures to limit risks with imported animals, enhanced programs to mitigate wildlife reservoir risks, re-evaluation of options to manage bTB-affected herds/regions, modernization of regulatory framework(s) to re-focus control efforts, and consideration of emerging testing technologies (i.e. improved or new tests) for use in bTB control/eradication programs. Traditional slaughter surveillance and test/removal strategies will likely be augmented by incorporation of new technologies and more targeted control efforts. The present review provides an overview of current and emerging bTB testing strategies/tools and a vision for incorporation of emerging technologies into the current control/eradication programs.

Introduction

Bovine tuberculosis (bTB), caused primarily by *Mycobacterium bovis*, is endemic in many countries. This zoonotic disease constitutes a significant economic burden to the agricultural industries (Krebs, 1997). Economic losses worldwide are estimated to account for over $ 3 billion annually (Steele, 1995). In the United States of America (USA) and Great Britain, greater than $ 40 and £ 100 million annually, respectively, have been spent for the eradication of bTB in 2008/09. The USA total includes appropriated and emergency funding (K. Orloski, personal communication, 2010; Anon, 2010).

The control and eradication of bTB is mainly based on a test and slaughter policy and/or abattoir surveillance. Despite intensive eradication efforts over decades, bTB continues to be a problem with global perspective. Various factors have been identified as major constraints to eradication. In some countries, wildlife reservoirs constitute a continuous source for re-infection of cattle (Corner, 1994; Wilson et al., 2009; Table 1). Additional factors contributing to the persistence of bTB are limitations of

Table 1. Brief overview of bTB status and diagnostic strategies in the USA, in various European countries and in New Zealand

Country	Prevalence[1]	Wildlife reservoir[2] Maintenance (spillover/sentinel) hosts	Primary screening TST	Ancillary screening IFN-γ, proportion serial:parallel[3]	Compulsory removal of positives	Criteria for whole herd depopulation	Pre-movement testing
USA	<0.001	Deer	CFT	75:25	Yes (CCT and/or IFN-γ)	Decision made by state, federal, and animal health officials using a veterinary risk assessment and in co-ordination with the herd owner	Yes (CFT, positives confirmed by CCT or IFN-γ)[4]
Ireland	4.37	Badger (deer)	CCT	2:98	Yes (CCT and/or IFN-γ)	Decision of the local veterinarian[5]	Yes (CCT)[6]
United Kingdom	3.27					Decision made by Animal Health in consultation with	
Great Britain[8]	3.45	Badger (deer)	CCT	2:98	Yes (CCT and/or IFN-γ)	bTB policy officials and according to a local veterinary	Yes (CCT)[9]
Northern Ireland	2.67	Badger (deer)	CCT	0.1:99.9	No	risk assessment[7]	Yes (CCT)[9]
Spain	1.17	Wild boar, deer	CIT, CCT[10]	0:100	Yes (CCT and/or IFN-γ)	Decision of the local veterinarian[11]	Yes (CIT)[12]
Italy	0.58	(Wild boar)	CIT, CCT[10]	0:100	Yes (CIT and/or IFN-γ)	Decision of the local veterinarian[13]	Yes (CIT)[12]
France (OTF) overall	0.05	Wild boar, deer			Yes (CCT and/or IFN-γ)	At least 1 confirmed positive animal in a herd	Yes (CIT, IFN-γ)
Dordogne	0.8		CIT, CCT	9:91	Yes (CCT and/or IFN-γ)	At least 1 confirmed positive animal in a herd	Yes (CIT, IFN-γ)
Camargue	10.4		CIT, CCT	0:100	Yes (CCT and/or IFN-γ)	>10% positives in a herd	Yes (CCT, IFN-γ)
Germany (OTF)	0.01	Deer				Decision of the local veterinarian	
Austria (OTF)	<0.1	Deer				>40% positives in a herd	
New Zealand	0.32	Possum (ferrets, feral pigs, deer)	CFT	85:15	Yes (CFT and/or IFN-γ)[14]	Decision of the local veterinarian[15]	Yes (CFT,IFN-γ)[16]

[1]Herd prevalence numbers of bTB in 2007 (Anon, 2009c,d; Wilson et al., 2009; P. Livingstone, Animal Health Board, personal communication, 2009).
[2]Maintenance hosts, ''in brackets'' spillover or sentinel hosts (Corner, 2006).
[3]A serial IFN-γ test is used for re-testing TST-positive cattle, and a parallel test is used for testing TST-negative animals.
[4]Pre-movement testing is required for breeding animals moving out of states or zones that have not a TB-free status.
[5]Any herd or infected group of cattle where the total number of animals removed as reactor approaches 30% should be reviewed with a view to deciding the future strategy, e.g. confining of group to a specific location or possible removal of the whole group. IFN-γ assay and/or antibody-detection assay should be used prior to consideration of depopulation. These tests are of particular relevance in larger herds or those with valuable stock. Where the suitability of these tests has been ruled out or where they have failed to resolve the problem, then depopulation of the herd should be considered (E. Gormley, personal communication, 2009).
[6]Recommended for breeding stock. Compulsory for export.
[7]Whole herd slaughters are carried out occasionally, subject to a risk assessment by Animal Health veterinarians taking into account a range of herd and local factors. Mandatory conditions are culture confirmation of *M. bovis* infection and a high prevalence of test reactors (≥ 25%). Further factors taken into account are the local bTB incidence (breakdowns in low incidence areas, to prevent the development of new hotspots of bTB), the proportion of test reactors showing gross TB pathology at post-mortem examination and the severity of those lesions, the risk of re-infection if the premises are restocked with cattle, the affected herd's size, the risks posed to the local cattle herds and wildlife populations, the bTB history and herd incidence in the locality, the evidence that repeat TST and ancillary IFN-γ (and sometimes antibody) testing have failed to resolve the bTB incident (Great Britain: R. De la Rua-Domenech, personal communication, 2010; Northern Ireland: J. McNair, personal communication, 2010).
[8]Scotland has been an OTF region of GB since October 2009 (Anon, 2009f).
[9]Testing performed for movements from a 1 or 2 yearly tested herd, 60 days prior to movement.
[10]CIT in high-prevalence areas and CCT in low-prevalence areas.
[11]Herd with high number of positives in low-prevalence areas (exception: genetically high value animals).
[12]Testing performed for movements from a 1 or 2 yearly tested herd, 30 days prior to movement.
[13]Herd with high number of positives in low-prevalence areas.
[14]Serial testing: Cattle that are positive to both the CFT and IFN-γ are slaughtered. Parallel testing: Cattle that are negative to the CFT but positive to the IFN-γ are slaughtered.
[15]High number of reactors with extended infection observed at slaughterhouse. Depopulation of age cohorts with high prevalence of infection (>50%).
[16]CFT and in parallel IFN-γ in cases with increased risk of infection.

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diagnostic tests (concerning both sensitivity and specificity), larger herd sizes, increase in animal movements and trade, and changing options for control, such as limitations on whole herd depopulation. Countries with successful eradication of bTB, like Australia, focussed their national eradication programs on the herd rather than on the individual animal given the limitations of test accuracy (More, 2009). The tuberculin skin test (TST), which constitutes the primary screening test for bTB, is recognized to be a good herd test but a poor test for identifying individual infected animals (Dawson and Trapp, 2004). As herd size continues to increase in many countries, it becomes ever more difficult to justify whole herd depopulation for both economic and animal welfare concerns. Globally, live animal trade is increasing. In addition, trade agreements increasingly include concepts like regionalization, zoning, and compartmentalization as principles of disease control, as proposed by international organizations (Anon, 2008d). In Europe, the improvement of competitiveness assuring proportionate animal movement is a cornerstone of the European Union (EU) Animal Health Strategy 2007–2013 (Anon, 2006). Similarly, a transition of the bTB program from a State classification system to a science-based zoning approach addressing disease risk is proposed in the USA (Anon, 2009c). In consequence, traditional control/eradication strategies will likely be reinforced with more targeted control efforts requiring additional tools (e.g. extended pre-movement testing and cost-efficient diagnostic tests with high accuracy in individual animals).

The objective of this review is to provide an overview of current and emerging bTB testing tools/strategies and a vision for incorporation of emerging technologies into the current control/eradication programs.

Current Testing Strategies

Brief description of current bTB status and diagnostic strategies in various countries

In the EU, the overall prevalence of bTB is currently slightly increasing. In 2007, 0.53% of cattle herds were bTB positive compared to 0.48% in 2006 (Anon, 2009c). Both officially bTB-free (OTF) and non-OTF countries reported an increase in the proportion of bTB positive cattle herds. Ireland and the United Kingdom are currently facing the highest herd prevalences in Europe (4.37 and 3.27%, respectively, Anon, 2009c). In Great Britain, the incidence of bTB is increasing (Anon, 2009e). Eradication of bTB is expected to take at least 20 years (Anon, 2009b). In face of a reservoir of infection in badgers, vaccination will likely become a major component of Great Britain‘s bTB eradication strategy. Recently, a field trial evaluating the practicalities of delivering an injectable *M. bovis* BCG vaccine for badgers has been initiated in England, with licensing expected in mid-2010 and that of an oral vaccine probably in 2014 (Anon, 2009b). Vaccine strategies for cattle in the United Kingdom are also under consideration (Anon, 2007a). Complete eradication is not only difficult to achieve in high prevalence countries but also in countries with low proportions of bTB-infected herds (Anon, 2009c). Even in some OTF countries bTB prevalences are currently slightly rising. Trade and wildlife reservoirs both in non-OTF and OTF countries are major factors for re-infections of and spillover to livestock.

New Zealand undertook a great effort over the last 15 years to eradicate bTB, and the herd point prevalence was reduced from 2.4% in 1993 to 0.35% in 2004 (Ryan et al., 2006). Complete eradication, however, has failed so far, most likely because of the presence of wildlife reservoirs. Attempts to halt disease transmission from wildlife to livestock by oral vaccination of possums with lipid-formulated *M. bovis* BCG are currently under evaluation (Ramsey et al., 2009). A recent field trial demonstrated that oral vaccination with BCG significantly protected possums against natural exposure to *M. bovis* (Tompkins et al., 2009). Vaccination is being considered as a possible adjunct to the current practice of reducing the population of possums (annual costs of possum control accounting for US$ 30 million; Anon, 2005) to hasten the eradication of the disease from wildlife reservoirs.

In the USA, the bTB eradication campaign has been highly successful. The prevalence of bTB has decreased from 5% in 1917 to <0.001% in 2009. Eradication, however, remains elusive and new strategies are proposed to specifically address the continued challenges of imported cases of bTB from Mexico and the presence of a wildlife reservoir (i.e. white-tailed deer) in Michigan and potentially Minnesota. These strategies include more stringent testing of cattle entering from Mexico, enhanced efforts to mitigate risks from wildlife, accelerating diagnostic test development to support increased surveillance, transition from a state-based to a regional or zoning classification system for designation of bTB status, and increasing the options for managing bTB-affected herds. These changes will most likely result in several opportunities for application of new testing strategies, particularly with pre-movement testing – both at the USA/Mexico border and for interstate shipment. Additionally, increased numbers of bTB-affected herds will be managed via a test and slaughter as opposed to a whole herd depopulation strategy.

In Canada, eradication efforts are ongoing based upon slaughter surveillance and test/removal strategies, similar to the USA. As with other countries, spillover from bTB-infected wildlife (e.g. elk in Riding Mountain National Park and bison in Wood Buffalo National Park) hinders eradication efforts (Nishi et al., 2006).

In Mexico, procedures equivalent to the USA bTB program were implemented in 2001 to enhance the capacity for export of beef cattle to the USA; however, focal areas of high prevalence still exist especially in dairy cattle. Additionally, bTB program activity varies greatly in Mexican states.

The current status of control efforts in Central and South America, Indonesia, Africa, and Eastern European countries is not clearly defined, yet bTB is a prominent concern for the livestock industries of many of these countries.

Primary screening for bTB in live cattle is performed using one of the variants of the TST: the caudal fold test (CFT), the (mid) cervical intradermal test (CIT), or the comparative cervical test (CCT). In addition, the interferon gamma (IFN-γ) assay is applied either as a confirmatory test of reactors to the CFT or CIT (serial testing), or in alongside TSTs to increase diagnostic sensitivity (parallel testing). Diagnostic strategies including requirements for removal of individual animals testing positive, whole herd depopulation, and pre-movement testing differ largely between countries and regions and are summarized in Table 1. In OTF countries, control programs are usually limited to passive surveillance by post-mortem examination of all slaughtered cattle. The following section gives an overview of current ante- and post-mortem testing tools and indicates modifications which could contribute to optimize disease control and eradication.

Tuberculin skin test (TST)

The TST represents the OIE prescribed test for international trade and constitutes a delayed type hypersensitivity test (Anon, 2008a). It measures dermal swelling primarily because of a cell-mediated immune response (CMI) three days after intradermal injection of purified protein derivative (PPD) in the skin of the caudal fold (CFT) or neck (CIT), respectively. The skin of the neck is regarded to be more sensitive to a tuberculin-related hypersensitivity reaction than the skin of the caudal fold. To compensate for this difference, higher doses of PPD may be used in the caudal fold. Because animals are frequently exposed to or infected with various non-tuberculous mycobacteria, cross-reactive responses to PPD-B may occur as many antigens contained within PPD-B are shared between non-tuberculous and tuberculous mycobacteria. The CCT is used to differentiate between animals infected with *M. bovis* and those sensitized to PPD-B as a result of exposure to other mycobacteria. Responses to bovine and avian tuberculins are compared according to OIE guidelines or those developed by national eradication programs (e.g. use of a scattergram in the USA).

Historically, the TST has been the primary antemortem test available to support bTB eradication campaigns. Advantages of the TST and reasons for its wide use are low costs, high availability, long history of use and, for a long time, the lack of alternative methods to detect bTB. Still, this test has many known limitations including difficulties in administration and interpretation of results, need for a second-step visit, low degree of standardization, and imperfect test accuracy (De la Rua-Domenech et al., 2006).

Performance of TST techniques may vary because of differences in tuberculin doses, PPD preparations, site of application, and interpretation schemes. In fact, disparate performances have been found in many international studies (Monaghan et al., 1994): 68–96.8% sensitivity and 96–98.8% specificity for CFT, 80–91% sensitivity and 75.5–96.8% specificity for CIT, 55.1–93.5% sensitivity and 88.8–100% specificity for CCT. Recent data from some countries or regions indicate a markedly reduced sensitivity of TST in certain situations. A study in Northern Ireland showed that as few as 59% of animals with confirmed *M. bovis* infection were detected by CCT in some herds (Welsh, M., and McNair, J., unpublished results). Similarly, herd ($n = 42$) sensitivity of CCT in fighting bulls in Camargue, France, was 58% (10.6% sensitivity for individuals, $n \geq 9000$) with more than 80% of bTB cases detected upon subsequent slaughter inspection (Keck, N., unpublished results). Twenty-eight of the 42 herds contained at least one culture-positive animal and 142 animals had tuberculous lesions upon slaughter inspection. Immunosuppression caused by stress (handling of fighting bulls) or anergy because of an advanced stage of bTB possibly account for the low sensitivity of TST in some circumstances. These data may represent the lower end of the performance spectrum of TST and not average values. Still, a low sensitivity constitutes a serious problem for the sustainable success of control and eradication programs.

Worldwide, the *M. bovis* strain AN5 is used for bovine tuberculin production (details and also concerns are summarized in the supporting information). Tuberculin potency is estimated by biological methods (*in vivo* testing of tuberculin batches in sensitized guinea pigs or cattle against an international reference standard; Anon, 2008b). Standardization of tuberculins is critical for test accuracy. Variations in PPD production methods may lead to different antigenic profiles and therefore to qualitative differences between PPDs (Seibert and DuFour, 1940; Landi and McClure, 1969; Haagsma et al., 1982; Tameni et al., 1998). Moreover, the in vivo potency tests are inherently variable and of questionable precision, demonstrated by studies with repeated batch testing (Good et al., 2008). Importantly, a recent comparison of

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commercially available tuberculins has shown that if applied in a dose of 1 mg/ml, the majority of these tuberculins would not meet the required minimum dose of 2000 International Units (Bakker et al., 2009). In this study, potencies largely varied among the bovine tuberculins and, to a lesser degree, among the avian tuberculins. Marked variations were also found with regard to the relative potencies of tuberculin pairs (PPD-B and PPD-A) from the different manufacturers. As these tuberculins are used in current control and eradication programs, the marked variability of potencies may have direct implications for the diagnosis of bTB cases.

Improvements in the production and quality control of tuberculins would have the potential to enhance sensitivity and specificity of TST. In fact, improved and extended control of PPD activity after production by standardized methods resulting in higher reproducibility than *in vivo* guinea pig potency testing could markedly improve TST. Furthermore, more active and more specific tuberculins would be desirable. To date, limited progress has been achieved in this field, mainly because of the ill-defined nature of the antigens present in tuberculins as well as the complexity of PPD production.

Attempts to improve the TST by the use of defined antigens such as ESAT-6 (Pollock et al., 2003) have resulted in increased specificity compared to the PPD-based test (100 compared to 90%) but decreased sensitivity (82 compared to 86%). Only a high dose of the recombinant protein (400 μg) was able to induce these responses, which would make the test unaffordable. Co-administration of a synthetic lipopeptide resulted in induction of antigen-specific CMI using lower concentrations of ESAT-6. However, an adjuvant application harbours the risk of sensitization and constitutes, therefore, not a practical solution (Whelan et al., 2003). The combined use of ESAT-6, CFP-10, Rv3615c, and MPB83 in a cocktail of recombinant proteins with low protein doses (10 μg each) showed comparable sensitivity levels as the PPD-based TST in naturally infected cattle (Whelan et al., 2009). Encouragingly, a peptide cocktail containing 21 overlapping peptides of the same antigens (10 μg each) also enhanced sensitivity compared to recombinant proteins (Whelan et al., 2009). Additionally, an improved antigen cocktail (ESAT-6 and CFP-10 based) is currently being tested for use in humans (P. Anderson, personal communication) and application of these antigens to the TST for cattle is also currently under investigation (R. Waters, P. Anderson and K. Lyashchenko; research in progress). However, these promising results will need to be validated in large-scale field trials before such antigens can be used as part of control strategies.

Alternative tools for the read out of TST may improve its ease of use. Infrared thermography (IRT) is currently being evaluated in the USA to determine skin test reactivity without the need for direct measurement of skin thickness (Johnson and Dunbar, 2008). Preliminary data from 15 cattle sensitized with PPD-B, PPD-A, or nothing (control) indicate that 86% of cattle were correctly classified with infrared thermography compared to 80% with skin thickness measurements using callipers. In addition, infrared thermography may be performed 24 h after PPD injection when compared to 72 h with traditional reading; and, importantly, this technique may represent a more objective method. However, further studies are required to determine the specificity of the 24-h vs 72-h readings of infrared thermography as early arthus-type reactions may be falsely interpreted as positive at 24 h.

Interferon gamma assay

The IFN-γ assay (Rothel et al., 1990) is OIE listed as an alternative test for international trade (Anon, 2008a) and approved as a complementary bTB test by the United States Animal Health Association as described in USDA, APHIS, Bovine Tuberculosis Eradication, Uniform Methods and Rules (Anon, 2004a), and by the European Union (Anon, 2002). This in vitro assay is a laboratory-based test detecting specific cell-mediated immune responses by circulating lymphocytes. Briefly, the assay consists of two stages. First, heparinized whole blood is incubated with antigens (i.e., PPDs, specific antigens) for ~18–24 h. Antigenic stimulation induces production and release of IFN-γ by predominantly T lymphocytes. Second, IFN-γ present in the plasma supernatants is quantified in a sandwich ELISA. With PPDs, a differential optical density (OD) value is determined by subtracting the OD value achieved with PPD-A stimulation from that of PPD-B, thereby, analogous to an *in vitro* comparative skin test. In general, most laboratories also include a no stimulation negative control (e.g. PBS or media) and a mitogen or superantigen positive control [e.g. pokeweed mitogen (PWM) or staphylococcal enterotoxin B (SEB)].

The IFN-γ assay (Bovigam®, Prionics, Switzerland) is being incorporated into bTB eradication programs in many countries (reviewed by De la Rua-Domenech et al., 2006; Vordermeier et al., 2008), either in a serial testing regime as confirmatory test after CFT to enhance specificity or in a parallel testing regime to enhance sensitivity of TSTs (Table 1). Advantages of the IFN-γ assay are its increased sensitivity, the possibility of more rapid repeat testing, no need for a second visit to the farm and more objective test procedures and interpretation in comparison to the TST. Limitations comprise a reduced specificity, high logistical demands (culture start is required within 24 h after blood sampling), an increased likelihood of non-specific response in young animals (owing to natural

killer (NK) cell activity) and its high costs (reviewed by De la Rua-Domenech et al., 2006; Vordermeier et al., 2006), as well as the difficulties in the standardization of tuberculins already discussed in relation to the TST.

The combined use of the in vivo and in vitro CMI assays raises the question whether the IFN-γ responses are influenced by injection of PPDs for TST. Briefly, no significant effect can be observed after CCT (Doherty et al., 1995; Gormley et al., 2004; Coad et al., 2010). In contrast, injection of PPD-B for CFT boosts IFN-γ responses in cattle experimentally infected with *M. bovis* (Palmer et al., 2006). The boost was shorter than previously observed with sensitized cattle (Whipple et al., 2001) and lasted only from 3 to 7 days after the CFT. Importantly, there was no effect on the differential response to tuberculin and therefore no interference with the final assay interpretation. In naturally infected cattle, however, CFT-related boosting selectively increased the in vitro *M. bovis* PPD (PPD-B) response 3 days after CFT, resulting in an increased PPD-B response relative to the response to PPD-A (Coad et al., 2010). Additional studies are warranted to evaluate the influence of CFT on the IFN-γ responses (i) with tuberculous cattle exhibiting weak IFN-γ responses, (ii) with uninfected cattle, and (iii) to evaluate the effect in view of a possible depression of IFN-γ production.

Sensitivity and specificity of the IFN-γ assay have been estimated in a great number of international studies (reviewed by De la Rua-Domenech et al., 2006). Estimates of test sensitivity range from 73.0 to 100%, with a median value of 87.6%, and specificity from 85.0 to 99.6%, with a median of 96.6%. Variations in assay protocols may have resulted in disparate results in test accuracy between studies. More standardized procedures would be needed to strengthen reliability of screening tools within TB programs. In recent studies, parameters of the IFN-γ assay have been analyzed in view of defining a range of possible conditions (summarized in the supporting information). Importantly, assay conditions which have been identified to be of significant influence on the IFN-γ test performance and which have already been defined to optimize the assay (e.g. sample and culture conditions; validity controls and their interpretations) should be integrated in the commercial assay. Thus, users could refer to consolidated, standardized procedures at the implementation of the assay in their laboratory quality system. This would also increase the likelihood that the assay will be used in a more standardized way and it would facilitate national approvals of the IFN-γ (as it would not be efficient to repeat the validation of all relevant assay parameters in each country).

Many studies have demonstrated that the sensitivity of the IFN-γ assay is superior to that of the skin test (data from most relevant published studies and additional unpublished data are summarized in the supporting information). Concerns, however, exist about Bovigam specificity (Lauzi et al., 2000; Pollock et al., 2000; Palmer et al., 2006). The replacement of PPDs by defined antigens offers the greatest potential for the improvement of specificity (Pollock et al., 2000; Buddle et al., 2001). ESAT-6 and CFP-10 are outstanding diagnostic target proteins in the whole blood IFN-γ assay (Pollock et al., 2000; Vordermeier et al., 2001; Aagaard et al., 2003; Buddle et al., 2003; Waters et al., 2004; Vordermeier et al., 2005; Aagaard et al., 2006; Cockle et al., 2006). Results from a recent field trial in Great Britain indicate that a peptide cocktail composed of peptides from ESAT-6 and CFP-10 (E/C) achieved only a slightly better specificity (97.0%; Vordermeier, H., unpublished results) than the conventional PPD-based IFN-γ assay (96.6%; Anon, 2008f); IFN-γ sensitivities in this field trial were 91.0% for PPDs and 81.0% for E/C. The gain in specificity by the use of E/C may be especially striking in individual animals and in regions with a low bTB prevalence and in OTF countries. A study performed in Switzerland (OTF) testing a selected herd ($n = 69$) with a high percentage of non-specific reactors (i.e. false positives) in the PPD-based assay (specificity of 66.7%) resulted in an E/C-specificity of 97.1% (Vordermeier, H., and Schiller, I.; unpublished results), again, demonstrating the potential for improved specificity of the test when specific antigens are used in place of PPDs.

Defined mycobacterial antigens have not only the potential to increase specificity but also sensitivity: in a PPD-based IFN-γ assay the diagnosis of bTB may be masked by a high response to avian tuberculin superseding that to PPD-B, as may occur early after infection (Schiller et al., 2009c) or in animals co-infected with non-tuberculous mycobacteria such as *M. avium* ssp. paratuberculosis (Aranaz et al., 2006). As indicated earlier, however, overall results of current studies with defined antigens using ESAT-6 and CFP-10 demonstrated a reduced sensitivity compared to tuberculins, reduced by approximately 10% (Pollock et al., 2000; Buddle et al., 2003; Vordermeier et al., 2006; Vordermeier, H., unpublished results). Therefore, a wider range of antigens in addition to ESAT-6 and CFP-10 is needed for improving the sensitivity of specific antigen-based IFN-γ assays. In recent studies, promising antigen combinations for the IFN-γ assay resulting in improved sensitivity and specificity have been described (Cockle et al., 2006; Sidders et al., 2008; Schiller et al., 2009b). Importantly, Rv3615c and OmpATb were found to detect cattle with confirmed bTB but not responding to E/C. Recent data indicate a sensitivity of 89.0% and specificity of 97.0% for E/C combined with Rv3615c (Sidders et al., 2008; Vordermeier et al.,

2009). These data have been achieved after 24-h storage of blood samples. A shortening of pre-culture time, however, has the potential to further increase sensitivity of a peptide-based IFN-γ assay. As shown with E/C in experimentally infected cattle, 24-h storage of blood samples resulted in slightly decreased OD values and reduced predictive outcome using E/C compared to 8 h storage (Whelan et al., 2004). This warrants further examination in field reactors and with additional defined antigens.

High logistical demands limit the use of the IFN-γ assay. Currently, blood samples need to be transported to a laboratory and to be processed within 24 h. An ''in-tube'' or ''in-plate'' stimulation device allowing rapid stimulation of lymphocytes after blood collection would possibly have a positive impact on diagnostic sensitivity using defined antigens for stimulation. Such a device would surely be beneficial to overcome logistical difficulties and to reduce costs now required for ''express transportation''.

Current costs of the IFN-γ assay constitute a major disadvantage in relation to TST. Regarding full cost calculations of TST (e.g. $7-10 in the USA (M. Dutcher, personal communication, 2007) and £7.5 in GB (R. De la Rua-Domenech, personal communication, 2007), the difference between in vitro and in vivo CMI assays might be smaller than anticipated, particularly considering rising costs associated with on-farm visits. Yet constraints in terms of costs should be mitigated to make the IFN-γ assay more widely available. Increased use of the assay might allow high scale production at a lower price. Importantly, additional cost reduction could be achieved by applying automation at production and application (i.e. in diagnostic laboratories). Modification of the ELISA as a rapid ELISA would allow further reduction of laboratory costs.

The current interpretation of the Bovigam assay relies exclusively on the OD values of specific (PPD-based) stimulation and non-stimulation (PBS), without considering standards correcting possible plate-to-plate variations. The kit positive control exhibits a strong positive control and may produce a wide range of OD readings (Jungersen et al., 2002; Robbe-Austerman et al., 2006). Therefore, a different control would be needed to calibrate plate-to-plate variations. Alternative read-outs (such as percentage positivity) based on a standardized positive control should be considered. An alternative calculation of the cut-off may contribute to improve test accuracy.

The IFN-γ assay is generally applied to animals of more than 6 months of age. The reason for this age restriction is a high likelihood of non-specific responses to mycobacterial antigens in young animals triggered mainly by natural killer (NK) cells (Olsen et al., 2005). This phenomenon may possibly be overcome by the depletion of NK cells, thus allowing application of the IFN-γ assay with samples from animals <6 months of age (H.M. Vordermeier and A. Storset, research in progress).

In summary, improving specificity by the replacement of PPDs by defined antigens for stimulation, their application as an in-tube/in-plate stimulation device, in combination with a modified interpretation/cut-off and cost reduction may represent useful developments for the IFN-γ assay. Thus, the assay may be adapted to provide a highly specific and sensitive screening test for use as a stand-alone test or in conjunction with other screening tests. In addition, a multispecies IFN-γ assay for non-bovine species, such as camelids, cervids, dogs, and cats, would be a welcome tool for bTB screening and control in those species and for overall bTB control.

Post-mortem diagnosis of bTB

Abattoir surveillance with lesion detection during commercial slaughter is used as cost-efficient method for passive surveillance of bTB both in OTF and in non-OTF countries, in the latter to supplement live cattle testing. The finding of a tuberculous animal at slaughter initiates an investigation through TST of the herd of origin and any other potentially exposed animals (Whipple et al., 1996; Olea-Popelka et al., 2008). The success of such investigations is highly variable. In the USA, because of the lack of uniform animal identification regulations combined with inconsistent record-keeping, only 50–70% of such investigations result in identification of the herd of origin and in finding all exposed animals (Kaneene et al., 2006). In Australia, which has successfully eradicated bTB, meticulous animal identification was credited as a major factor in successful eradication (Cousins and Roberts, 2001; Radunz, 2006). In the British Isles, national cattle tracing systems allow accurate back-tracing following identification by meat inspectors of any suspect granuloma in commercially slaughtered cattle. The herds of origin of such animals are placed under precautionary movement restrictions pending confirmation of bTB in slaughter lesions by histopathology, culture and/or PCR.

Generally speaking, lesion detection exhibits a major lack in sensitivity. The sensitivity of abattoir surveillance may be as low as 28.5%, as indicated by a recent study in the USA (Anon, 2009a). Possibilities to improve the sensitivity of post-mortem detection of bTB may be limited. Continuous education and training of slaughter inspectors are certainly of major importance. In addition, the combined use of liquid and solid culture media has been reported to improve culture sensitivity (Hines et al., 2006). The addition of assays such as PCR detection of *M. bovis* DNA from formalin-fixed specimens has further enhanced some surveillance programs (Miller et al.,

2002). PCR assays to detect MTB complex bacteria are currently less sensitive than culture techniques. Therefore, important further steps would be to improve PCR sensitivity and to standardize PCR methods.

Genotyping of bacterial isolates or PCR products is increasingly becoming a standard tool for epidemiological disease control and eradication. Distinguishing *M. bovis* strains on a molecular basis provides important insights into the sources of infection and identification of practices or environments which may aid the spread and maintenance of tuberculosis. Importantly, transmission routes between livestock and wildlife may be identified by strain typing. In addition, transmission routes of bTB within livestock via animal movements become evident, a prerequisite for targeted disease control aiming at testing all potentially exposed animals. More details for post-mortem diagnosis of bTB (lesion detection, culture, PCR, and strain typing) are included in the supporting information.

Emerging Testing Strategies

Antibody detection assays

Antibody-based bTB assays offer the possibility for convenient, flexible, and generally cost effective platforms for bTB surveillance. The development path for an accurate antibody-based bTB test has been particularly arduous and disappointing, most likely because of the inherent nature of the disease. *Mycobacterium bovis* infection of cattle elicits an early and robust CMI response and a peculiarly weak antibody response (Pollock et al., 2001; Welsh et al., 2005). In early studies, the use of crude mycobacterial preparations that provided generally satisfactory test sensitivity resulted in poor assay specificity because of broad cross-reactivity with non-TB mycobacteria, such as *M. avium* (O'Loan et al., 1994; Gaborick et al., 1996). Many attempts have been made to identify immunodominant proteins with improved specificity. Antigen discovery efforts have unveiled numerous seroreactive targets (Fifis et al., 1992; Cataldi et al., 1994; Lyashchenko et al., 1998; Amadori et al., 2002; Koo et al., 2005). So far, MPB70 and MPB83 proteins of *M. bovis* appear to be the major serodominant antigens for detection of tuberculous cattle (Wood et al., 1992; Lightbody et al., 1998; McNair et al., 2001; Liu et al., 2007; Wiker, 2009).

Recent advances in both antigen discovery and immunoassay technology have facilitated progress in developing novel antibody-based tests for bTB. Importantly, studies have demonstrated the benefits of multi-antigen approaches, relying primarily on MPB83 plus additional proteins (Greenwald et al., 2003; Waters et al., 2006; Whelan et al., 2008). Cocktails of carefully selected antigens or multi-epitope fusion proteins have been used to demonstrate improved test sensitivity (Waters et al., 2006; Lyashchenko et al., 2008; Whelan et al., 2008). Regardless of the antigen cocktail or single-antigen approach, improved detection technologies offer opportunities for serological assays not previously realized with standard technologies (Waters et al., 2007; Whelan et al., 2008; Green et al., 2009). Apart from the selection of *M. bovis* -specific antigens for antibody detection, the immunoassay format and detection technology are crucial for developing accurate serodiagnostics. Promising recent developments include MAPIA (Waters et al., 2006), a fluorescence polarization assay (FPA) (Jolley et al., 2007), a rapid immunochromatographic test (Lyashchenko et al., 2008), a 96-well plate multiplex system (Whelan et al., 2008), a dual path platform assay (Greenwald et al., 2009; K. Lyashchenko, personal communication), a chemiluminescent platform (Green et al., 2009), and an improved ELISA (John Lawrence, IDEXX, Portland, Maine, personal communication, 2009), which are listed in a chronological order of development. Some may be used as a quick animal-side test for field use (Waters et al., 2006; Lyashchenko et al., 2008), whereas others are laboratory-based for automated large-scale testing (Jolley et al., 2007; Whelan et al., 2008; Green et al., 2009). MAPIA (Chembio Diagnostic Systems, Inc., Medford, NY, USA) is an efficient tool for large-scale antigen screening as well as for characterization of serological responses in cattle and other host species infected with *M. bovis* (Lyashchenko et al., 2004; Waters et al., 2006; Lyashchenko et al., 2008). The multiplex immunoassay (Enfer Scientific, Naas, Ireland) simultaneously detects and analyzes antibody responses to multiple antigens within a single well of a 96-well plate array format (Whelan et al., 2008). With the multiplex chemiluminescence (Enfer Scientific), single antigen chemiluminescence (Seralyte-Mbv, Pritest, Redmond, Wa), rapid immunochromatographic and dual path platform (Chembio Diagnostic Systems) tests, antibody responses are detected as early as 2 weeks – 2 months after infection with samples from experimentally infected cattle (Waters et al., 2006; Whelan et al., 2008; Green et al., 2009; K. Lyashchenko, unpublished observations). Additionally, field studies with each of these assays are encouraging (Meyer and Orloski, 2007; Whelan et al., 2008; K. Lyashchenko, unpublished observations); however, exact estimates of test accuracy under varying herd prevalence rates is still pending. The FPA (Diachemix, Grayslake, IL, USA) detects antibody responses to a small polypeptide of MPB70 using a convenient and automated platform. The FPA is highly specific (>99%), however, lacks sensitivity (26%) for use as an individual animal test (Meyer and Orloski, 2007; Ngandolo et al., 2009). The low sensitivity of the test may be because of the nature of the bovine immune response

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to MPB70 (i.e. generally occurs later during the course of disease), the limited antigenicity of the peptide used for the test and lower sensitivity of FPA compared to other technologies. Possibilities to include several antigens in the FPA platform would be very limited.

In summary, preliminary studies have indicated the potential for antibody-based tests primarily with positive samples from experimental infection trials, limited numbers of samples from naturally-infected cattle, and in certain instances, with un-blinded sample sets. Large-scale field trials are required to clearly define serological test accuracy, especially in direct comparison to CMI-based tests. Testing should include samples from appropriate populations of animals, as test accuracy may be impacted by environmental and host factors such as disease prevalence, nutrition, handling, stress, environmental mycobacterial exposure, and parasite burden. Effects of PPD administration for TSTs (e.g. CFT versus CCT, time after TST, tuberculin source and dose) on serological test results should also be considered. As with TST and IFN-γ -based assays, serological test performance will likely vary considerably between field test sites; thus, multiple studies at varying locales may be required. More details for antibody-detection assay are included in the supporting information.

Research Tests

Potential for new readouts of infection

New research tools are rapidly emerging for the characterization of bovine immune responses. Numerous primer sets are available for characterizing bovine cytokine/chemokine/transcription factor/etc. responses to bTB via real-time PCR techniques (Thacker et al., 2007) and bovine immune microarrays (Coussens and Nobis, 2002; Tao et al., 2004; McGuire and Glass, 2005; Almeida et al., 2007; Machugh et al., 2009). In addition, strategies for sequencing transcriptomes are being developed (Thacker, T., and Vordermeier, H.; unpublished results). The development of bovine cytokine and chemokine multiplex systems detecting several parameters in a single sample (Coad et al., 2010) raise the possibility to enhance sensitivity and/or specificity of antemortem cellular immunity diagnostic systems based on parameters like IL-10. The increased availability of monoclonal antibodies recognizing bovine cytokines also provides the opportunity to intervene in assays such as the IFN-γ assay to overcome modulating effects of cytokines such as IL-10 to increase sensitivity as has been demonstrated by Denis et al. (2007) for bTB and by Buza et al. (2004) in the case of Johne's disease. Such reagents and read-out systems may also be useful to counter the observed potential loss of sensitivity of TH1-cell-mediated diagnostic systems (like TST and IFN-γ assays) reported recently in the case of experimental Fasciola hepatica/*M. bovis* co-infections (Flynn et al., 2009). Thus, further cellular immunity-based tests may offer opportunities to improve test sensitivity in certain indications.

Options for Future Control Strategies

Incorporation of modified existing and emerging tests into traditional eradication campaign algorithms

Diagnostic tests may be used in different ways within control and eradication programs, depending on different epidemiological settings (for details please refer to the supporting information). Improved diagnostic tests will be a crucial factor for future eradication of bTB. There is a special need for individual animal tests, as high test accuracy represents a pre-condition for cost-efficient, targeted disease control. The positioning of modified existing and/or emerging tests as screening tests within bTB control/eradication programs will mainly depend on their performances and costs.

Changes required for the IFN-γ assay to overcome its current disadvantages and to allow its use as individual animal test comprise improvements of specificity, overall test accuracy and ease of use, in addition to cost reductions. New antigen combinations have been found which complement the current lead diagnostic antigens ESAT-6 and CFP-10 and result in superior sensitivity, specificity, and predictive values compared to PPD-based tests (Sidders et al., 2008; Schiller et al., 2009b; Vordermeier et al., 2009). Interestingly, recent data, using relatively small sample numbers that need to be confirmed in larger trials, indicate that even ESAT-6 and CFP-10 alone (without further antigens) could result in superior performance of the IFN-γ assay compared to that of CCT. Options how to utilize a new IFN-γ assay in bTB programs will largely depend on its costs. Currently, a major part of total IFN-γ test costs is attributed to logistics and laboratory costs. An automatable version of the IFN-γ assay, e.g. comprising in-tube/in-plate whole blood stimulation applicable in the field or in the laboratory, could significantly reduce these costs. Thus, an improved version of the IFN-γ assay may represent a powerful primary screening tool for bTB.

Antibody-based assays offer additional opportunities for bTB programs. Major advantages are ease of testing, ability to achieve quick results, and low costs. Possibilities for application of emerging antibody-based assays within programs will mainly depend on their performances. In countries with limited resources, where large-scale culling is not cost effective, serological tests are appealing as a cost-efficient means for removal of cattle with increased risk of bacterial shedding because of advanced disease (Pollock et al., 2005; Ngandolo et al., 2009). In countries

with established bTB eradication programs, serological tests could complement current CMI-based testing strategies and offer additional opportunities for herd, movement, sale barn, and abattoir testing. One important application of serological tests is the potential to detect animals anergic on CMI-based assays (Coad et al., 2008). As a pre-movement test, large numbers of animals could be rapidly tested at holding sites prior to movement. For slaughter surveillance, a serological assay would need to be highly specific (e.g. 99.9%) as false positive results would result in costly trace-back/epidemiological investigations. However, possibilities to use antibody-based assays to enhance slaughter surveillance may be limited for financial reasons. A rough cost/benefit calculation showed that costs per antibody-based test (including laboratory costs) must be very low (lower than $ 0.8) to achieve added value (increased overall sensitivity) at competitive costs compared with traditional slaughter surveillance (inspectors visual exams and follow-up of granulomas). This clearly limits the use of an antibody-based test as complimentary test for passive surveillance at slaughter. This conclusion is especially surprising, as we calculated a rather ''optimistic case'' in our cost/benefit analysis by assuming 90.0% sensitivity and 99.9% specificity for a potential serological assay (all other numbers for the cost/benefit analysis were derived from USA's slaughter surveillance costs for histopathology, PCR or culture and slaughter surveillance numbers from 2008; for details please refer to supplemental Table S1).

In countries lacking industry or government indemnity support for condemned animals, a test that only detects animals that are most likely shedding the organism may be used to diminish risks of transmission to other animals or humans. In this case, a high-specificity/low-sensitivity test at low cost may be applied even when disease prevalence is high. Herds may then be identified for follow-up testing with TST and IFN-γ assay. This procedure could also be applied as a complementary pre-movement test, especially when animals are moved from ''at risk'' regions. As with slaughter surveillance (i.e. inspectors visual exams), a lower sensitivity test would be used to identify herds for follow-up testing. This alternative strategy may be considered for countries with limited financial resources to significantly reduce (not necessarily to eradicate) bTB and its economic and zoonotic damage.

In summary, new screening tests are likely to be soon available and to offer further opportunities for control/eradication of bTB.

Conclusions

Considering current trends associated with bTB control/eradication programs (e.g. global increase in live animal trade, alternative strategies for whole herd depopulation, general financial limitations to fund programs) will be important to focus resources increasingly to targeted control strategies. Thereby, pre-movement testing on a national and international basis is becoming more important. Effective pre-movement testing, however, requires diagnostic tests with a high reliability as individual animal tests. Currently used screening tests (TST and IFN-γ assay) have originally been designed to meet demands as herd tests and their performances depict a broad range of sensitivity and specificity. A prerequisite to improve the accuracy of screening tests in individual animals would be to stipulate performances to the upper end of the performance spectrum. New or modified screening tests will be needed for cost-efficient, targeted disease control. Importantly, the replacement of tuberculins by defined antigens has the potential to significantly improve the performance of diagnostic tests. New testing technologies for disease screening are likely to be soon available. Authorities and other stakeholders that benefit from bTB programs should support the development and field validations needed to have improved diagnostic tools. Lacking a gold standard for the overall diagnosis of bTB (culture is regarded as gold standard for confirmatory diagnosis of bTB), the validation of new immunological assays requires further statistical tools (e.g. Bayesian analysis, calculation of Odds ratios).

Great progress has been achieved in the fields of culture technologies (e.g. liquid culture systems) and typing of mycobacterial strains. Molecular typing of mycobacteria isolated from domestic livestock and from wildlife allows establishing epidemiological links, necessary for the development of successful control strategies. Thereby, not only potential transmission routes between livestock and wildlife are identified but also the spread of bTB by trade. Epidemiological identification of infection sources and routes are a necessary part of targeted and effective disease control.

International trade inevitably represents a risk to introduce bTB. According to the current EU legislation, Member States or regions may achieve an OTF status if the percentage of infected bovine herds has not exceeded 0.1% per year and if at least 99.9% of herds have been OTF; both conditions are required for six consecutive years (Anon, 2004b). Individual herds are declared OTF if all cattle over six weeks of age reacted negatively to two TSTs (the first six months after the elimination of any infection and the second six months later). OTF countries or regions may be dispensed from tuberculin testing, meaning that surveillance is limited to post-mortem examination of all slaughtered cattle. For trade, cattle must originate from OTF countries or regions, or they must originate from an OTF herd and have been

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subjected to a TST with negative result within 30 days prior to shipment. Animals under six weeks of age are exempt from TST. There are four major possibilities how bTB may be spread by trade: First, re-infections or residual undisclosed infection of herds declared as OTF in non-OTF regions are not detected because of long periods between tests or false negative test results, especially at low disease prevalence (e.g. low sensitivity at early infection stage) or in cases of advanced infection with anergy. Second, infections of calves under six weeks of age are not detected as they exempt from testing. Third, new infections of herds in OTF regions (e.g. originating from wildlife, from extended animal contact on shared pastures, or from trade) may be undetected for a long period, as testing is optional in these regions. These issues warrant the question whether future approaches to manage the risk of introducing bTB should consider a lowering of the pre-export testing age, more sensitive pre-export testing of traded cattle (through parallel TST and blood testing and/or more severe interpretation of the TST), compulsory post-import testing, channeling imported cattle into approved quarantine units, and supplemental bTB surveillance in geographic areas that have an increased risk for exposure to wildlife TB. For economic reasons and competitiveness of agriculture, it is crucial to declare herds OTF as soon as possible to facilitate trade. More sensitive methods than TST, however, might be needed to prevent movement of animals infected with bTB, especially in the presence of wildlife reservoirs. Fourth, South American camelids form a special risk to transmit TB to cattle, as antemortem diagnosis of TB is problematic in camelids. Directive 92/65/EEC (Anon, 1992) states that the TST is the official method for certification of TB freedom in South American camelids intended for international trade in the EU. However, TST has not been fully validated in camelids, and recent studies indicate an extremely low sensitivity of this test in llamas. Dean et al. (2009) found that only 2 of 14 llamas with confirmed TB were positive by TST, as opposed to all of the 14 animals reacted positive by antibody-detection. Camelids are increasingly kept on pastures in close proximity to livestock cattle, illustrating that infected camelids represent a great risk to transmit TB to cattle. Therefore, diagnostic tests with reasonable sensitivity/specificity in camelids are highly needed for disease control and for international trade, and should be integrated into current legislation.

In the USA, requirements to achieve an OTF status are more stringent than in the EU. A state or zone must comply with the provisions of the Uniform Methods and Rules (Anon, 2004a) to qualify for OTF status. According to these rules, a state or zone must have zero percent apparent prevalence of infected cattle and bison herds and no findings of TB in cattle or bison in the state or zone for the previous 2–5 years, depending on the past recent history of TB in the state or zone. Points of testing for eradication/control campaigns include importation, within region movement, herd certification, and slaughter surveillance. For importation, OTF countries/states/zones may ban or restrict importation of cattle from non-OTF countries/states/zones. The USA imports cattle only from low prevalence states in Mexico; however, bTB-infected cattle still enter the USA from Mexico. While the vast majority of these cattle are targeted for short-term feedlots, occasionally they are housed near adult cattle and transmit the disease to USA stock. Although not possible for political reasons, banning importation of cattle from Mexico would diminish the number of cases of bTB within the USA. Likewise, states may consider denying access of cattle from bTB-affected regions/states within the USA. OTF regions may also impose stringent testing protocols to limit the risk of bTB-infected cattle from entering, especially when animals are from non-OTF or ''at-risk'' regions. Politically, defining regions that are ''at risk'' for bTB may provide leverage for policy makers and regulators to target populations for increased testing. Prior to entry into OTF regions, ''at risk'' cattle may then be subjected to additional testing and stricter interpretation(s). Additionally, regions may adopt bTB testing policies for within region movement and herd certification; thereby, providing additional assurance of bTB status that may benefit trade possibilities. Blood-based tests are particularly convenient for increased frequency and wide-scale testing, especially with improved tests offering high specificity.

In summary, new diagnostics with technological advances offer great promise and will be essential to fight bTB. Focus should be laid on the cost-effectiveness of diagnostic tests within bTB programs. A cost-effective test does not generally mean the cheapest test. Sustainable use of improved bTB diagnostics should go along with mid-term and long-term overall cost reductions spent for the eradication of bTB. Cost-effectiveness analysis including emerging tests, as soon as these are available, may help to identify testing strategies that provide the greatest impact with the lowest cost per unit of output.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Comparative performance of diagnostic techniques for bTB in different epidemiological settings.

Table S1. Numbers and assumptions used for cost/benefit evaluation of antibody-based tests integrated in slaughter surveillance

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Papers

Comparison of tuberculin activity using the interferon-γ assay for the diagnosis of bovine tuberculosis

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In this study, interferon-γ (IFN-γ) responses in whole blood cultures stimulated with tuberculins from different sources were compared with regard to their diagnostic reliability in cattle experimentally and naturally infected with *Mycobacterium bovis*. The IFN-γ responses to different concentrations of purified protein derivatives (PPDs) from *M bovis* and *Mycobacterium avium* were quantified. Significant differences (P<0.05) between sources and concentrations of PPDs used for stimulation were detected, indicating a need for standardisation of PPDs used in the IFN-γ assay. Additionally, a tool named 'relative potency 30' that allows rapid comparison of batches and sources of PPDs was defined.

BOVINE tuberculosis (TB) caused by *Mycobacterium bovis* continues to be a problem, even in countries with active control measures. There are many reasons for the failure to eradicate the disease. Limitations in the sensitivity and specificity of diagnostic tests contribute to the persistence of bovine TB. The interpretation and accuracy of test data are confounded by variations in the frequency of tuberculin testing and by the use of different tuberculin preparations (Gormley and others 2006).

The control of bovine TB has been based mainly on a test-and-slaughter approach and/or abattoir surveillance. The BOVIGAM (Prionics) interferon-γ (IFN-γ) assay (Wood and others 1990) is being incorporated into bovine TB eradication programmes in many countries (Vordermeier and others 2006). Compared with the tuberculin skin test, the IFN-γ assay has been shown to offer increased sensitivity, the possibility of more rapid repeat testing, the absence of any requirement for a second visit to the farm, and more objectivity as regards test procedures and interpretation. These advantages have been recognised by regulatory authorities, veterinarians and farmers (De la Rua-Domenech and others 2006, Vordermeier and others 2006). In recent follow-up studies of cattle that tested negative in skin tests for bovine TB, it was shown that animals that tested positive in the IFN-γ assay were more likely to have confirmed bovine TB than those that tested negative in the IFN-γ assay. This finding demonstrates the applicability of the assay as a confirmatory test for bovine TB (Coad and others 2008). In many international studies, the sensitivity of the IFN-γ assay has been shown to range from 80.9 to 100 per cent, and the specificity from 87.7 to 99.2 per cent (Vordermeier and others 2006). Apart from differences in the immune status and potentially confounding mycobacterial background infections or sensitisation of the animals, variations in the accuracy of the test may be partly related to differences in the technical parameters, such as the source and concentration of tuberculin and the interpretation criteria for positive test results. Variations in the performance of tuberculin may be attributable to differences in production methods between sources, or between different batches from a given manufacturer (Bakker and others 2005, Good and others 2008). In repeated skin test assays in guinea pigs and cattle, the potencies of tuberculin combinations including purified protein derivatives (PPDs) from *M bovis* and *Mycobacterium avium*, referred to as PPD-B and PPD-A, respectively, varied widely, thereby demonstrating the questionable precision of in vivo tests for determining PPD potency (Good and others 2008).

It was previously assumed that the source of tuberculin used for stimulation in the IFN-γ assay would not affect the diagnostic outcome. The studies of Whipple and others (2001) using cattle sensitised with heat-killed *M bovis* showed similar performances of PPDs prepared in the USA and in Australia. However, when testing bovine TB-free herds in Italy with the IFN-γ assay, Cagiola and others (2004) reported differences in specificity between various tuberculins, and a lower specificity of the comparative cervical skin test compared with the IFN-γ assay. Considering the lack of global standardisation of PPD sources and concentrations for use in IFN-γ assays, the aims of the present study were, first, to compare the activity levels of PPDs, sourced from different suppliers, in the BOVIGAM assay; secondly, to optimise tuberculins, in terms of the source and concentration, for the in vitro IFN-γ assay; and finally, to establish a reliable tool for determining the in vitro activity levels of different batches of PPD.

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Materials and methods

Cattle

Five male, TB-free, Holstein-Friesian calves were housed in accordance with institutional guidelines at the National Animal Disease Center (NADC), Ames, Iowa, USA, in a biosafety level 3 facility. All animal care and use procedures were reviewed and approved by the NADC Animal Care and Use Committee. All the calves received *M bovis* strain 95-1315 by aerosol administration at the age of six months, and infection was confirmed at post-mortem examination at the age of approximately one year using standard procedures as described previously by Waters and others (2003). Blood samples were taken repeatedly during the first month after inoculation.

Ten cattle that had been naturally infected with *M bovis* were obtained from herds with a history of bovine TB (as determined by Animal Health in the UK) and positive single intradermal comparative cervical tuberculin test results, and were housed in the animal unit at the Veterinary Laboratories Agency (VLA) – Weybridge, UK. Animal experiments at the VLA were undertaken under a licence granted by the UK Home Office after approval by the local ethical review committee. The animals ranged in age from five months to 2.5 years and were of the Holstein-Friesian breed. TB was confirmed in all 10 animals by postmortem analysis including culture.

TABLE 1: Evaluation of purified protein derivative (PPD) activity levels in five cattle experimentally infected with *Mycobacterium bovis*

	14 dpi Number of animals testing positive				29 dpi Number of animals testing positive *			
PPD source	20 µg/ml	10 µg/ml	1 µg/ml	0.1 µg/ml	20 µg/ml	10 µg/ml	1 µg/ml	0.1 µg/ml
1		1	2	2		2	4	4
2		2	1	1		4	4	4
3		0	3	2		2	4	3
4		2	1	1		2	4	4
5	0	1	1	0	1	4	3	3

* At 29 days postinoculation (dpi), samples from only four animals were valid for analysis, as one animal had a markedly elevated negative control (a sample incubated without PPD) value (optical density 2.5), indicating an increased level of circulating interferon-γ

TABLE 2: Evaluation of purified protein derivative (PPD) activity levels in 10 cattle naturally infected with *Mycobacterium bovis*

	Number of animals testing positive with a PPD concentration (µg/ml) of											
PPD source	20	10	5	2.5	1.25	0.625	0.25	0.125	0.05	0.025	0.01	0.005
1	9*	9*	9*	9*	9*	9*	9*	8	7	7	6	
2		9*	10*	10*	8	9*		10*		9*		4
3		9*	8	7	7	8		3		1		0
4		9*	9*	9*	9*	9*		9*		4		2
5	8	7	8	7	8		7		2		1	
6	9*	8	8	7	9		7		2		2	

* PPD concentrations at which 90 per cent or more of the animals were diagnosed as test-positive

Antigens

Tuberculin preparations from five manufacturers (sources 1 to 5) were used to stimulate in vitro IFN-γ responses in naturally and experimentally infected cattle. Samples from animals naturally infected with *M bovis* were additionally tested with a second batch of one tuberculin (source 6). All tuberculins were used at eight serial dilutions. Depending on their original concentration, twofold and then (at lower concentrations) fivefold dilutions were prepared (sources 1, 5, 6: 20, 10, 5, 2.5, 1.25, 0.25, 0.05, 0.01 µg/ml; source 2: 15, 7.5, 3.75, 1.875, 0.938, 0.188, 0.038, 0.008 µg/ml; sources 3, 4: 10, 5, 2.5, 1.25, 0.625, 0.125, 0.025, 0.005 µg/ml). The experimentally infected cattle were tested with three serial dilutions (10-fold: 10, 1, 0.1 µg/ml; source 5 was additionally used at 20 µg/ml). The manufacturers' suggested shelf-life for the tuberculins was generally 24 months; one source alone suggested a shelf-life of 36 months for its PPD-A. At the time of the study all the PPDs were within the official shelf-life of 24 months. In a separate study, seven additional batches from source 1 were tested, all of which were beyond their suggested expiry date (these batches were between 28 and 66 months old at the time of this separate study). The suppliers were Prionics, the Tuberculin Production Unit at the VLA – Weybridge, AsureQuality, Istituto Zooprofilatico Perugia, and Lelystad Biologicals.

Pokeweed mitogen (PWM; Sigma) and staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB; Sigma) were included as positive controls at 5 µg/ml (PWM) and at 1 µg/ml (SEB), to measure the viability of the IFN-γ-producing cells in the blood samples.

IFN-γ assay

Whole blood cultures were performed in 96-well plates by mixing 0.25 ml heparinised blood with 25 µl of antigen-containing solution. The supernatants were harvested after 24 hours of culture at 37°C in air plus 5 per cent carbon dioxide. IFN-γ concentrations were determined using the BOVIGAM ELISA kit. Optical density was determined at 450 nm (OD_{450}). A result was considered positive if the PPD-B OD_{450} minus PPD-A OD_{450} was at least 0.1, and the PPD-B OD_{450} minus the unstimulated OD_{450} was at least 0.1. Only samples with an unstimulated OD_{450} of less than 0.2 and PWM/SEB-stimulated OD_{450} of more than 0.5 were considered valid for analysis. For recombinant antigens, a stimulated OD_{450} minus unstimulated OD_{450} of at least 0.1 was considered positive.

Statistical analysis

Statistical analysis was performed using GraphPad Instat software version 3 (GraphPad). Differences in potency between tuberculins from different suppliers were assessed by parametric analysis of variance and unpaired two-tailed *t* tests, as the data were normally distributed. Associations between the age (shelf time) and potency of the tuberculins were assessed using non-parametric Spearman correlation. The significance level was set at $P<0.05$.

Results

Analysis of PPDs in experimentally infected animals

Tuberculins (PPD-B and PPD-A) produced by different manufacturers were used at different concentrations for the stimulation of whole blood taken from five experimentally infected cattle at days 0, 9, 14 and 29 after infection. The interpretation of the IFN-γ assay was determined according to standard criteria as described above. The results are summarised in Table 1. None of the animals tested positive at day 0 (not shown) or at nine days postinoculation (dpi). At 14 dpi, all of the animals showed high production of IFN-γ after PPD stimulation (as shown for source 2 in Fig 1). Despite this substantial IFN-γ production, most of the animals were diagnosed as (false) negative because PPD-A induced higher levels of IFN-γ than PPD-B did, and therefore the standard interpretation was negative. The effect of PPD concentration was most dramatic with the PPD from source 3: at 14 dpi all the animals were false negative with 10 µg/ml while three of five animals were correctly diagnosed at 1 µg/ml (Table 1). The IFN-γ production apparently levelled off when PPD-B was used at 1 and 10 µg/ml but increased when PPD-A was used at the higher concentration (results not shown).

With increasing time after infection, this effect decreased. At 29 dpi, the responses to PPD-B generally exceeded responses to PPD-A; however, exceptions to this were detected that appeared to relate to the source and concentration of the PPD being used for the assay. With PPDs from source 2, all the animals were correctly diagnosed as bovine TB reactors at each concentration evaluated. PPDs from

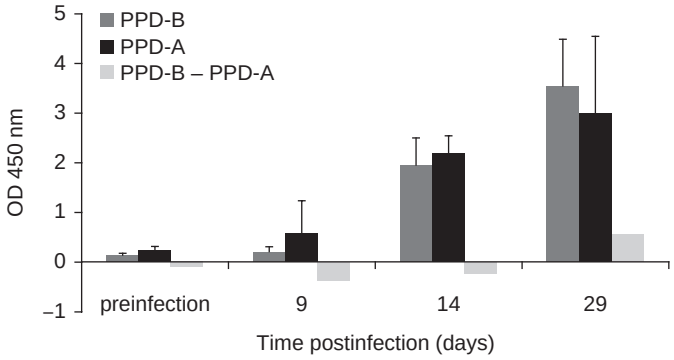

FIG 1: Interferon-γ responses to purified protein derivatives from source 2 (10 μg/ml) during the time course of infection, tested in five experimentally infected cattle. Duplicate samples from individual animals were analysed, and the data are presented as mean (se) optical density (OD) values

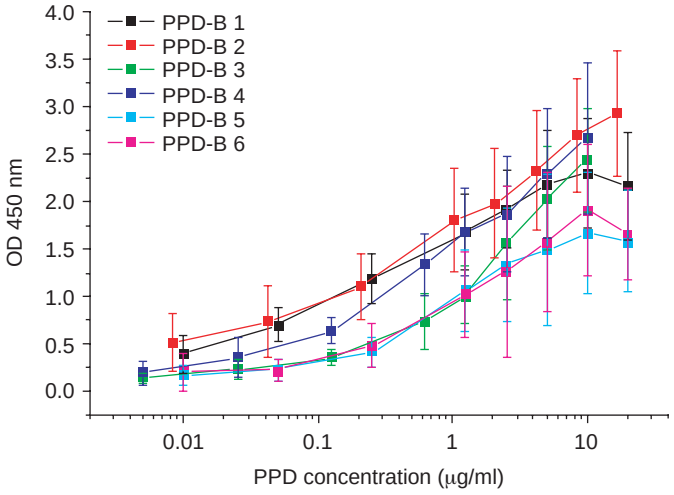

FIG 2: Tuberculin activity levels of six different *Mycobacterium bovis* purified protein derivatives (PPDs), tested at the indicated concentrations in five cattle naturally infected with *M bovis*. Duplicate samples from individual animals were analysed, and the data are presented as mean optical density (OD) values

sources 1, 3 and 4 gave false negative diagnoses at 10 μg/ml but the diagnoses were correct when 1 μg/ml was used, suggesting that the strong response to PPD-A is more concentration-dependent than the response to PPD-B. With PPDs from source 5, the test accuracy was dramatically diminished at a concentration of 20 μg/ml, again because the response to PPD-A exceeded that to PPD-B.

Analysis of PPDs in naturally infected animals

In order to extend these findings beyond experimentally infected animals, the various PPDs were assessed in 10 skin-test positive naturally infected cattle. The diagnoses (according to the standard criteria for interpretation of the IFN-γ assay) relative to PPD source and concentration are shown in Table 2. Responses after stimulation with PPDs from sources 1, 2 and 4 were comparable and strongest, and demonstrated a high sensitivity across a range of concentrations. Fewer infected animals were detected with PPDs from sources 5 and 6 as compared with those from sources 1, 2 and 4 (Table 2). Even at a dilution of 10-fold or more, the PPDs from sources 1, 2 and 4 did not lose diagnostic sensitivity, whereas PPDs from the other sources yielded correct diagnoses only at the highest concentration. These differences were evident when comparing the minimum concentrations necessary to detect at least 90 per cent of the animals as being infected, or, in other words, the concentration below which the test sensitivity wanes (Table 2).

Looking at average IFN-γ production at the various PPD-B concentrations (Fig 2), PPDs with a high diagnostic sensitivity were more efficient at inducing IFN-γ production even at low PPD-B concentrations (sources 1, 2 and 4), thereby suggesting that some of the variation in the diagnoses achieved may be related to the potencies of the different PPDs.

Quantitation of PPD activity

A potency index (named relative potency 30 [RP30]) was developed for further quantitative assessment of the observed differences in activity. The RP30 is defined as the protein concentration (μg/ml) or as activity (iu/ml) of a given PPD needed to obtain 30 per cent of the response (RP30) of the peak value of a reference PPD (OD_{max}) (Fig 3). Data from all 10 naturally infected animals were used to calculate individual RP30 values. The mean RP30 values are shown in Table 3, in terms of iu and also as compared on the basis of protein concentration. On the basis of protein concentration, the RP30 values of the PPDs ranged from 0.12 to 2.02 μg/ml (lower values indicate higher activity). Using the activity levels expressed in iu, as provided by the manufacturers, similar differences between the most active PPDs (sources 1 and 2) and the least active PPD (source 3) were evident. Differences between PPDs with a low RP30 (sources 1 and 2), medium RP30 (source 4) and high RP30 (sources 3, 5 and 6) were are also statistically significant (Fig 3) (*t* test, $P<0.05$). PPDs from sources 1, 2 and 4 also gave the best diagnostic results in naturally infected animals (Table 2), suggesting that the RP30 as defined here may be a useful indicator for standardisation and improvement of the IFN-γ assay. RP30 values had a direct relationship with the potency of the respective tuberculin for detecting infected animals. The RP30 values correlated positively with the minimum test concentration (Fig 4) (Spearman $r=0.8971$, $P<0.05$) before the test sensitivity waned (see Table 2), a value the authors termed 'minimum diagnostic concentration'.

Shelf-life of PPDs for the IFN-γ assay

Seven different batches of PPDs from source 1 that had been kept on the shelf for various time periods had passed their expiry dates by four to 42 months. The IFN-γ responses elicited by these batches were compared with the responses elicited by a valid batch in whole blood from 10 cattle naturally infected with *M bovis*. The RP30 values were determined for the different preparations and the values were analysed against the ages of the PPD-B batches (Fig 5a). As expected, there was a significant correlation (Spearman $r=0.9341$, $P<0.005$) between loss of activity (as defined by increased RP30 values) and increasing age of the PPD batch. Despite this up to fourfold reduction in RP30 over time, the effect was most evident at low PPD concentrations and could be overcome at higher concentrations (>5 μg/ml; data not shown). Age-related decreases in potency were also evident with PPD-A batches from source 1, especially at <1 μg/ml (data not shown). When PPD-A and PPD-B batches were used at the standard test concentration normally employed in the IFN-γ test (10 μg/ml), all batches were able to correctly classify the reactor animals as test-positive (Fig 5b).

Comparison of PPD activity levels using skin test and IFN-γ test

The potency of PPD used in the skin test is estimated by in vivo tests with guinea pigs and/or cattle. To assess whether the activity levels estimated by in vivo models correlate with in vitro activity levels, potency data obtained by both approaches were compared (Table 3). The different PPD-Bs tested were in a range of 25,000 to 50,000 iu/mg (in vivo potency range). PPD-Bs from sources 1 to 3 had similar in vivo potencies (from 25,000 to 35,000 iu/mg), whereas PPD-B from source 4 was significantly more potent (50,000 iu/mg). In vitro, however, sources 1 and 2 were the most active ones (RP30 values of 0.12 and 0.14 μg/ml, respectively), followed by source 4 (RP30 0.26 μg/ml). In contrast, the PPD-B from source 3 was substantially less active in vitro (RP30 2.02 μg/ml).

Discussion

Diagnostic accuracy is an inherent aspect of test-and-slaughter policies, and diagnostic tests must satisfy national and local requirements for both sensitivity and specificity. These are important factors for disease identification and economic considerations within a bovine TB control programme.

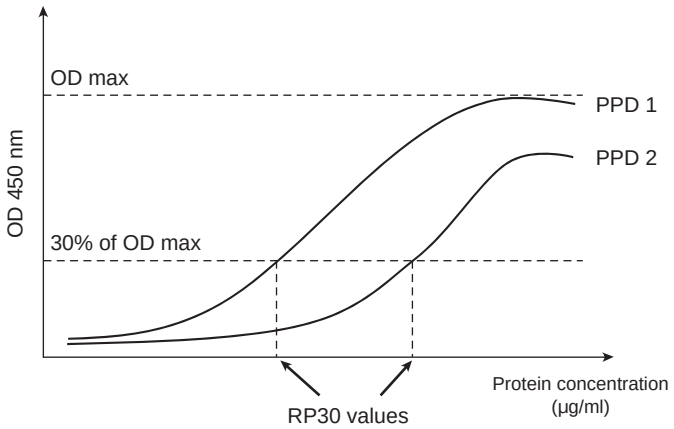

FIG 3: Schematic representation of potency testing of purified protein derivative (PPD) batches by in vitro interferon-γ assay. Relative potency 30 (RP30) is the tuberculin concentration required to induce 30 per cent of the maximum interferon-γ response. OD Optical density

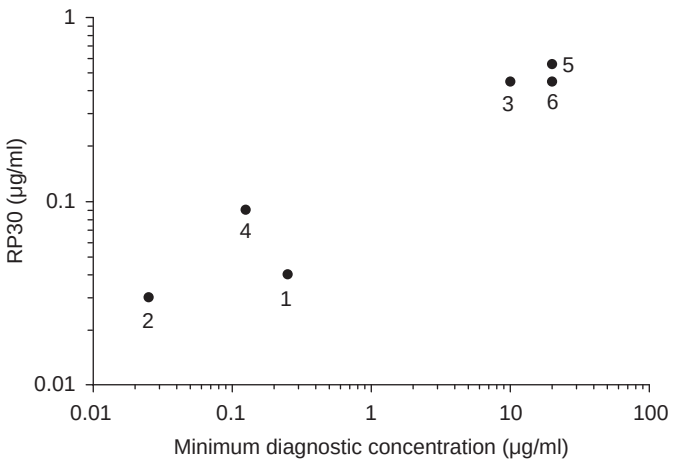

FIG 4: Correlation between relative potency 30 (RP30) and the potency of tuberculins to detect animals infected with *Mycobacterium bovis*. Minimum diagnostic concentrations were defined as the lowest concentrations that allowed the correct classification of infected cattle, as shown in Table 2. The statistical analysis is Spearman correlation (r=0.8971, P<0.05). Numbers indicate the purified protein derivative source

Accurate testing procedures, therefore, may considerably impact the outcome of a TB eradication or control programme. In this study, one of the crucial components was analysed using the IFN-γ assay, that is, PPDs, by introducing a novel tool, RP30, which determines the protein concentration at which a specific PPD preparation has 30 per cent of maximal activity. Analysing the RP30 values of tuberculins from different sources revealed differences of up to 17-fold between the most active and least active preparations. Further, it was possible to detect differences in potency in batches of different ages, even when they were from the same supplier. Most of the differences were more evident at lower protein concentrations, while at high protein concentrations the differences tended to disappear, probably because of saturation of the system. Nevertheless, some differences in sensitivity were also shown in samples from naturally infected animals (Table 2), indicating that the use of suboptimal PPDs may result in a loss of sensitivity, which is the most crucial parameter when testing for the purpose of disease eradication. Similarly, it is important to balance the specificity, because this may determine the economic feasibility of an eradication programme. When the same PPDs were tested in uninfected cattle from Switzerland and the UK, the authors found that the PPDs with the highest potency in TB-infected cattle (indicated by the lowest RP30 values) turned out to be the most specific PPDs in uninfected cattle, due to low cross-reactivity of bovine tuberculin and/or sufficiently high activity of avian tuberculin to detect non-specific IFN-γ production in response to non-tuberculous mycobacteria (data not shown). Careful balancing and standardisation of the assay will help to enhance both its sensitivity and specificity.

The differences that were found between PPDs cannot be explained easily. All PPDs had been produced according to World Organisation for Animal Health (OIE) standards and they had a phenol content of <0.5 per cent (w/v). International guidelines such as those of the OIE (Anon 2008) and European Pharmacopoeia (Anon 2002) regulate key steps of PPD production, giving a basis for standardised tuberculin production. However, some variations in production processes such as culture conditions (media, culture time) and the protein precipitation and filtration steps, may explain the differences in the antigenic profiles and variations in concentration, and may lead to qualitative differences between PPDs (Seibert and DuFour 1940, Landi and McClure 1969, Haagsma and others 1982, Tameni and others 1998). Standardised and reproducible tests are therefore needed in order to monitor the quality of PPD batches reliably. The authors suggest that the RP30 index may be used as a quality control measure for PPDs.

Furthermore, the RP30 offers a technique to optimise and balance the activity of tuberculins for the IFN-γ assay. Currently, the activity of a PPD is measured in iu using an in vivo potency test (which is known be quite variable) in guinea pigs (Good and others 2008). Bovine PPD from source 1 used in the present study failed the guinea pig potency test repeatedly, but was highly active in the cattle potency test (data not shown). In recognition of this discrepancy, a wide potency range is acceptable for skin test tuberculins (Anon 2004). The present study shows that optimising tuberculins and balancing them on RP30 may improve the IFN-γ assay. In this context, the authors emphasise the importance of the method used to determine the protein content during PPD production, given that different assays provide different estimates of protein content. The values estimated by the techniques of Lowry and Kjedahl (the latter technique is the methodology most commonly used by PPD manufacturers and acknowledged to be the most precise) may differ by as much as 25 per cent (Sapan and others 1999; C. Carter, personal observation).

Another variable in accurate diagnosis may be the time point at which the diagnostic test is performed after infection. As demonstrated in the experimentally infected animals in the present study, the responses to avian PPD may exceed those

TABLE 3: Evaluation of in vitro potencies of bovine purified protein derivative (PPD-B) batches using whole-blood samples derived from 10 naturally infected cattle

	In vivo potency*	In vitro potency†			
PPD-B source	(iu/mg)	RP30 (µg/ml)‡	95% CI	RP30 (iu/ml)§	95% CI
1	25,000	0.12	0.04-0.21	3.07	0.91-5.24
2	30,000	0.14	0.06-0.21	4.16	1.83-6.49
3	25,126	2.02¶**	0.14-3.92	50.67¶**	3.55-97.79
4	50,000	0.26¶**	0.11-0.42	13.18¶**	5.26-21.09
5	NA	1.91¶**	0.05-3.77	NA	NA
6	NA	1.73¶**	0.15-3.61	NA	NA

* Data from manufacturers (established in guinea pig and cattle bioassays)
† The in vitro potencies were estimated in terms of the relative potency 30 (RP30), that is, the tuberculin concentration (in µg/ml or iu/ml) required to induce 30 per cent of the maximal interferon-γ production in response to that PPD-B
‡ Parametric analysis of variance, P=0.032
§ Parametric analysis of variance, P=0.002
¶ Unpaired two-tailed *t* test, P<0.05 compared to PPD-B from source 1
** Unpaired, two-tailed *t* test, P<0.05 compared to PPD-B from source 2
NA Not available

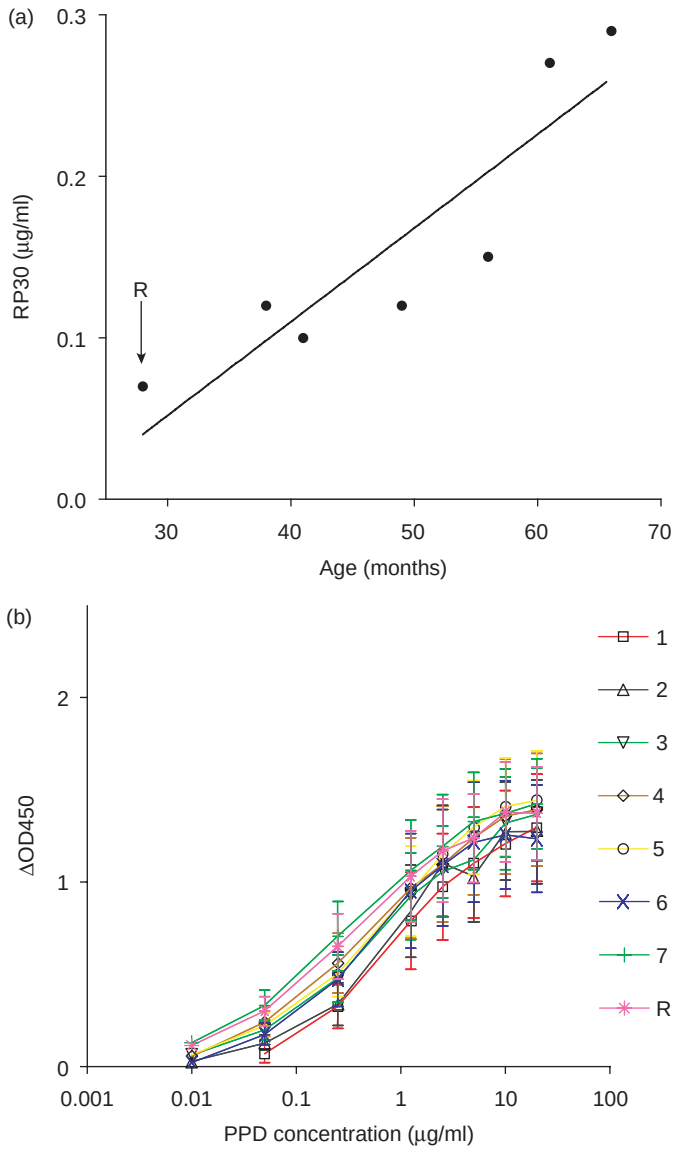

FIG 5: Relationship between the ages of eight different PPD-B batches from source 1 and activity level (RP30) in the interferon-γ assay. (a) Age-related increase in the RP30 of different PPD-B batches from source 1. Spearman correlation r=0.9341, P<0.005. (b) Dose-response titration of tuberculin batches PPD-B and PPD-A of different ages. The PPD-B–PPD-A differentials are shown. 1 Oldest expired PPD-B or PPD-A batches, 7 Most recent expired batches tested, R Unexpired batches. Results are expressed as mean (se) relative optical density (OD) values obtained after testing 10 naturally infected cattle

to bovine PPD at an early time point after infection. This effect has also been reported by Rhodes and others (2000). It is not totally clear whether the experimental infection truly mimics natural infection; in particular, the use of high doses of infectious agent in experimental infections might logically be expected have an effect on response times. Nevertheless, the dose-response curve of PPDs was found to be similar in naturally and experimentally infected animals (data not shown), thereby suggesting that the method of infection is not one of the main parameters.

Comparison of the sensitivity of the different PPDs at different concentrations in the 10 naturally infected animals showed that the most active PPDs work over a very broad range in the IFN-γ test. Such PPDs are therefore quite robust against small variations in protein concentration, while PPDs at the limit of their activity spectrum do not have much flexibility. The sensitivity values shown in Table 2 also indicate a tendency for PPDs with good RP30 values to be more sensitive. The authors would therefore predict that the RP30 indicates the performance of a PPD in the field; however, this assumption needs further evaluation.

In conclusion, not all PPDs are equal, but the selection of active PPDs at the appropriate concentration will result in accurate and reliable diagnosis. It will therefore be important to standardise PPDs in order to tailor the diagnostic system to the needs of an eradication or control programme.

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Mini review

Bovine tuberculosis: Effect of the tuberculin skin test on *in vitro* interferon gamma responses

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ABSTRACT

Bovine tuberculosis (bTB) is a disease of zoonotic and economic importance. In many countries, control is based on test and slaughter policies and/or abattoir surveillance. For testing, cell mediated immune- (CMI-) based assays (i.e., tuberculin skin test (TST) supplemented by the interferon gamma (IFN-γ) assay) are the primary surveillance and disease control tests for bTB. The combined use of the *in vivo* and *in vitro* CMI assays to increase overall sensitivity has raised the question of whether the IFN-γ response is influenced by injection of purified protein derivatives (PPDs) for TST. Published data on the influence of the TST, applied as the caudal fold test (CFT) or the comparative cervical test (CCT), on the IFN-γ assay are contradictory. Reviewing published data and including additional data, the following conclusions can be drawn: (1) in naturally infected cattle, PPD administration for the single or repeated short-interval CCT neither boosts nor depresses PPD-specific IFN-γ production. Disparate results have been concluded from some studies using experimental infections, emphasizing the importance of confirming initial experimental-based findings with studies using cattle naturally infected with *Mycobacterium bovis*. (2) In cattle experimentally infected with *M. bovis*, PPD administration for CFT boosts PPD-specific IFN-γ production for up to 7 days without any effect on test interpretation. Importantly, in naturally infected cattle, CFT-related boosting selectively increases the *in vitro M. bovis* PPD (PPD-B) response 3 days after CFT, resulting in an increased PPD-B response relative to the response to *Mycobacterium avium* PPD (PPD-A). In non-infected cattle, it cannot be excluded that the CFT induces a mild boost of the PPD-specific response, particularly in animals sensitized to environmental, non-tuberculous mycobacteria, thus decreasing the specificity of the IFN-γ assay. (3) In general, there is a lack of data clearly characterizing the effect of TSTs on the IFN-γ assay. Further studies are required to clearly describe the effects of both CFT and CCT in non-infected animals and in naturally infected cattle, especially in low reacting infected cattle.

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1. Introduction

Bovine tuberculosis (bTB) is a disease of zoonotic and economic importance. The causative agents are pathogens belonging to the *Mycobacterium tuberculosis* complex group, mainly *Mycobacterium bovis* but occasionally *Mycobacterium caprae* in some European regions (Prodinger et al., 2005) and *M. tuberculosis* in Africa (Berg et al., 2009). Despite intensive efforts over decades, bTB remains recalcitrant to eradication globally.

Where they are implemented, the major pillars of bTB eradication and control programs worldwide are based on test and slaughter policies and/or abattoir surveillance. The diagnosis of bTB in live animals is primarily based on the detection of specific cell mediated immune (CMI) responses. The primary screening test used for active surveillance is the tuberculin skin test (TST). In addition, the *in vitro* interferon gamma (IFN-γ) assay (Bovigam assay; Wood et al., 1990) is often used as an ancillary test to supplement the *in vivo* TST.

The combined use of the *in vivo* and *in vitro* CMI assays has raised questions as to whether *M. bovis* specific IFN-γ responses are influenced by injection of purified protein derivatives (PPD) for the TST. The caudal fold test (CFT) has been reported to boost PPD-specific IFN-γ responses (Rothel et al., 1992; Whipple et al., 2001), and this has formed the basis for the policy of applying the IFN-γ assay between 3 and 30 days after CFT in the USA (Anon, 2005a). In contrast, no significant boost has been attributed to the comparative cervical skin test (CCT; reviewed by De la Rua-Domenech et al., 2006). Published data on the influence of the TST on the IFN-γ assay are, however, contradictory. Our communication addresses this issue by reviewing published data and by drawing conclusions from these studies including new data.

2. Description of tuberculin skin tests and the interferon gamma assay

The TST can be carried out in a variety of ways and is applied differently in many countries (Anon, 2009). Factors that influence the performance of the test include: (1) the anatomic location of PPD injection: either intradermally in the caudal fold of the tail, or in the neck. (2) The choice of tuberculins: either as single intradermal test using tuberculin prepared from *M. bovis* (PPD-B) alone, or as comparative skin test, comparing responses to PPD-B and *Mycobacterium avium* PPD (PPD-A). (3) The frequency of testing: TST may be carried out as a single test, or animals may be retested in order to follow-up inconclusive reactors or to increase overall TST sensitivity. Retesting with TST often requires an interval of at least 42–60 days to allow desensitization to occur (Radunz and Lepper, 1985; Anon, 2004, 2005a).

The most common applications of the TST are the caudal fold test (CFT), single cervical test (CIT) and comparative cervical test (CCT). For clarity, in this review we address CCT as single CCT (analogous to the alternatively used abbreviation "SICCT").

With the CFT, PPD-B is injected into a fold of skin at the base of the tail. Skin measurements are not recorded; however, any palpable swelling or induration 72 h after injection is considered a positive reaction and the animal is considered a "reactor". Since animals are frequently exposed to or infected with various non-tuberculous mycobacteria (e.g., *M. avium* ssp. *avium*, *M. avium* ssp. *paratuberculosis* and many other environmental mycobacteria) that potentially cross-react with PPD-B, a proportion of animals are expected to react in the CFT. This proportion varies depending on the local levels and types of mycobacterial background exposure. The average proportion reported in the USA varies widely according to the region of the country ranging from <1 to 10% reactors (Kathy Orloski and Sharon Hietala, personal communication).

With the CIT, PPD-B is injected into the skin of the neck and the change in skin thickness due to swelling is measured after 72 h.

For the CCT, either as a follow-up test after a single intradermal test or as a stand-alone test, two sites are shaved on the lateral side of the neck and PPD-B and PPD-A are injected separately at adjacent sites. The relative change in skin thickness at the two sites 72 h post-injection is used to differentiate *M. bovis* infection from exposure to nontuberculous mycobacteria.

The primary clinical expression of TST reactivity (i.e., a delayed type hypersensitive (DTH) response) is characterized by the presence of oedema (Anon, 2009) and induration (Doherty et al., 1996). Histologically, a DTH reaction is characterized by endothelial activation (with associated oedema) and predominately mononuclear cell infiltrates at 48–72 h after administration of antigen (Abbas et al., 2000). The interpretation is based on the measurement of skin swelling, occurring with or without clinical symptoms (e.g., diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes). Depending on the prevalence and the risk of *M. bovis* infection in herds, different interpretation schemes may be applied (Anon, 2009). For the CCT in the US and in the UK, data representing changes in skin thickness responses to PPD-B and PPD-A are plotted on a scattergram for interpretation of the CCT and results are interpretated as negative, suspect, or reactor (Anon, 2005a). The skin of the neck is regarded to be more sensitive to tuberculin-related DTH reactions than the skin of the caudal fold. To compensate for this difference, higher doses of PPD can be used in the caudal fold (Anon, 2009). The variety of TST techniques including the use of different tuberculin doses, PPD preparations and interpretation schemes confounds objective comparison of these methods in different local settings. The disparate performances of TST in many international studies have recently been summarized by De la Rua-Domenech et al. (2006) and Vordermeier et al. (2006): 68–96.8% sensitivity and 96–98.8% specificity for CFT, 80–91% sensitivity and 75.5–96.8% specificity for CIT, 55.1–93.5% sensitivity and 88.8–100% specificity for CCT.

For the IFN-γ assay, a laboratory-based test measuring *in vitro* CMI responses, whole blood is cultured with PPD-B and PPD-A at 37–39 °C overnight. The IFN-γ produced by antigen specific lymphocytes is quantified in a sandwich ELISA. The relative IFN-γ amount is calculated by subtract-

Table 1
Summary of most relevant studies analyzing the influence of CFT on the IFN-γ assay.

Ref.[a]	Study design	PPD sources	Results	Comments and further conclusions
1	Experimental infection of cattle with *M. bovis* ($n = 4$) and *M. avium* ($n = 4$). Time period between infection and CFT: not specified. Period of IFN-γ analysis after CFT: 59 days.	*For CFT and IFN-γ assay*: CSL (Parkville, Victoria, Australia).	Temporary drop of IFN-γ responses (PPD-B, PPD-A) in *M. bovis*-infected cattle (2 out of 4) at day 3. Gradual increase of IFN-γ responses (PPD-B, PPD-A) between 7 and 59 days after CFT, with a peak at 28 days. No change of IFN-γ responses (PPD-B, PPD-A) in *M. avium* infected cattle.	The relative PPD response and final test result were not influenced by CFT.
	Uninfected cattle ($n = 50$) were tested weekly for 4 weeks after CFT.		Low number of false positive cattle at each test (from 0 to 2 cattle).	A lower cutoff was used compared to the current manufacturer's instructions of the Bovigam® assay (Prionics AG, Schlieren, Switzerland). Unfortunately, the data, especially separate PPD-B and PPD-A responses, are not shown in the publication. Therefore no further conclusions can be drawn.
2	Sensitization of cattle ($n = 20$) with a killed preparation from *M. bovis* AN5. Time period between sensitization and CFT: over 2 years. Period of IFN-γ analysis: 77 days. CFT at day 0, additional CCT at day 7 (group A, $n = 7$) or day 63 (group B, $n = 7$). No TST (group C, $n = 6$).	*For CFT*: Not specified. *For IFN-γ assay*: CSL (Parkville, Victoria, Australia) and USDA (National VeterinaryServices Laboratories, Ames, IA, USA).	Boost of the IFN-γ responses (PPD-B, PPD-A) between 3 and 28 days after CFT (groups A and B).	Despite the boost of IFN-γ responses after CFT and a temporary drop immediately after CCT (at day 10 in group A and day 66 in group B), the relative PPD response and final test result were not influenced by TST (neither by CFT nor CCT) during the course of the study.
3	Experimental infection of cattle ($n = 19$) with *M. bovis.* Time period between infection and CFT: 55 days. Period of IFN-γ analysis: 80 days. CFT at day 0, additional CCT at day 7 ($n = 10$) or day 55 ($n = 9$). Non-infected cattle ($n = 10$). CFT at day 0, additional CCT at day 7 ($n = 5$) or day 55 ($n = 5$).	*For CFT*: USDA (National Veterinary Services Laboratories, Ames, IA, USA). *For IFN-γ assay*: CSL (Parkville, Victoria, Australia).	Boost of the IFN-γ responses (PPD-B, PPD-A, ESAT-6:CFP-10) between 3 and 7 days after CFT in *M. bovis*-infected cattle. No effect of CCT. Non-infected cattle: mild boost (statistically not significant) of the IFN-γ responses to both PPD-B and PPD-A 3 days after CFT, but not in response to ESAT-6 and CFP-10 fusion protein (ESAT-6:CFP-10).	The relative PPD response and final test result were not influenced by CFT.
4	Experimental infection of cattle with *M. bovis* ($n = 9$) or *M. kansasii* ($n = 4$). Non-infected cattle ($n = 10$). Time period between infection and CFT: 55 days. Period of IFN-γ analysis: 70 days. CFT at day 55.	*For CFT*: USDA (National Veterinary Services Laboratories, Ames, IA, USA). *For IFN-γ assay*: CSL (Parkville, Victoria, Australia).	Boost of the IFN-γ responses (PPD-B, PPD-A, ESAT-6:CFP-10) 7 days after CFT in *M. bovis*-infected cattle. No effect in *M. kansasii*-infected cattle. No effect on IFN-γ response to PWM.	Boosting effect had no impact on test interpretation.
5	Naturally infected cattle ($n = 8$). Period of IFN-γ analysis: pre-CFT, days 3 and 10.	*For CFT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	Boost of the relative PPD responses (PPD-B minus PPD-A); they were significantly higher at day 3 compared to pre-CFT values.	Selective increase of PPD-B response. No increase of PPD-A response.

[a] References: (1) Rothel et al. (1992). (2) Whipple et al. (2001). (3) Palmer et al. (2006). (4) Waters et al. (unpublished data). (5) Coad et al. (2010).

ing the optical density (OD) value achieved with PPD-A from that of PPD-B. The interpretation of the results varies between countries and is usually based on the prevailing disease levels and the level of exposure to environmental mycobacteria.

The Bovigam® IFN-γ assay has been incorporated into bTB control programs in many countries (reviewed by De la Rua-Domenech et al., 2006; Vordermeier et al., 2008) and is primarily used as an ancillary test to the TST. The IFN-γ assay offers improved sensitivity, the possibility of more rapid repeat testing, no need for a second visit to the farm and more objective test procedures and interpretation in comparison to the TST. Yet, with a variety of assay protocols in use, this can result in divergent test accuracy, ranging from 80.9 to 100% for test sensitivity and 87.7–99.2% for test specificity.

More standardized procedures for bTB tests would be desirable to strengthen the reliability of these tools within bTB programs. In recent studies, different parameters influencing the performance of the IFN-γ assay have been analyzed to define an optimal range for test conditions including vessel geometry options, culture conditions, sample handling options, and antigen optimization. Moreover, a tool for the standardization of PPD activity in the IFN-γ assay has been proposed based upon dose titration to determine relative potency (Schiller et al., 2009a, in press).

Two basic testing strategies, incorporating TST and IFN-γ testing are applied for *ante mortem* diagnosis of bTB within control and eradication programs: (A) CFT as the primary herd screening test, combined with CCT or IFN-γ assay as confirmatory (or "serial") tests of individual animals reacting to a TST and (B) CIT or CCT alone or combined with IFN-γ assay, performed as a parallel testing regime. Strategy A, for example, forms the basis of USA (Anon, 2005a) and New Zealand policy (Anon, 2005b) where the aim is to enhance the low specificity of the CFT. In addition, New Zealand uses the IFN-γ assay in parallel with the CFT to increase diagnostic sensitivity to find residual infection. Strategy B is used in most European Union Member States with endemic bTB to boost the sensitivity of the TST in infected herds. The IFN-γ test is not yet officially approved for use as a serial test in the European Union (Anon, 2004).

3. Influence of the CFT on the IFN-γ assay

All documented data until 2008 describing the effect of CFT on the IFN-γ assay originate exclusively from experimentally infected cattle (summarized in Table 1). Still, these experimental studies formed the basis for the routine field application of the IFN-γ assay after CFT. There is a lack of field studies analyzing the effect of the CFT on the IFN-γ assay. Published field studies using both the CFT and the IFN-γ assay have been designed to focus solely on a general evaluation of the use of Bovigam® in cattle subjected to the caudal fold test, but not on the possible boosting or depression effects of CFT on the IFN-γ (Ryan et al., 2000). Yet, this study has formed the basis for the IFN-γ testing protocol in New Zealand, where blood sampling takes place between 13 and 33 days after CFT (Anon, 2005b). In one of the first studies where cattle were experimentally infected with *M. bovis*, Rothel et al. (1992) reported a gradual increase in the

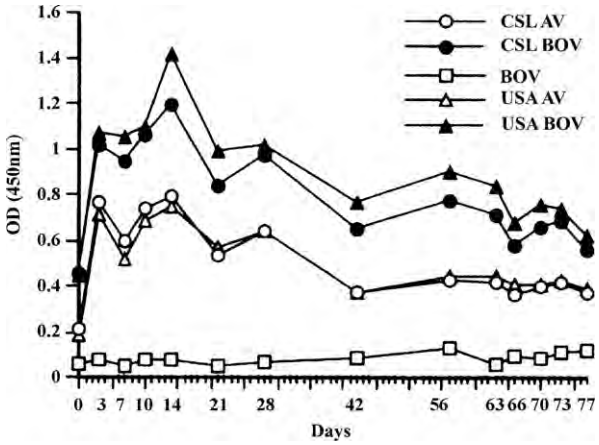


Fig. 1. Influence of CFT on IFN-γ responses in *M. bovis* sensitized cattle (taken from Whipple et al. (2001) with kind permission of the author and publisher). Comparison of OD values at each time point when blood samples were stimulated with PPD prepared in the USA and Australia or were mixed with PBS within 2 h of sample collection. The CFT was conducted on day 0, and the CCT was conducted on day 63. CSL Av, PPD pepared from *M. avium* in Australia; CSL Bov, PPD prepared from *M. bovis* in Australia; USA Av, PPD pepared from *M. avium* in the USA; USA Bov, PPD prepared from *M. bovis* in the USA.

OD values between 7 and 59 days after CFT, with a peak at 28 days. Re-analyzing these data we saw that PPD-B and PPD-A responses were affected similarly, the relative PPD response (PPD-B minus PPD-A) and therefore the final IFN-γ test outcome was not changed by CFT throughout the course of the study. Interestingly, in cattle experimentally infected with *M. avium*, IFN-γ responses to mycobacterial antigens were not affected by CFT (Rothel et al., 1992).

Whipple et al. (2001) analyzed samples from cattle sensitized with a killed preparation from *M. bovis* AN5 strain, and the IFN-γ responses were boosted within a period of 3–28 days after CFT (Fig. 1). When we extended the interpretation given in the study to the relative PPD response it became obvious that, again, the final IFN-γ test outcome was not changed by CFT. The results from Whipple et al. (2001) were used to define the application of Bovigam® in the USA (Anon, 2005a), where the assay is approved for use between 3 and 30 days after CFT. To our opinion, a study in sensitized cattle may be of limited relevance for natural situations, as responses elicited by sensitization of cattle with killed *M. bovis* may not accurately reflect responses to experimental or natural infection. Further studies in experimentally and naturally infected cattle, summarized in the following paragraphs, substantiate our concerns.

Palmer et al. (2006) confirmed a strong boosting effect between 3 and 7 days after CFT in cattle experimentally infected with *M. bovis*. After 7 days, however, OD values returned to levels before CFT. Interestingly, in non-infected cattle, a mild boost (although statistically not significant) was detected in the IFN-γ responses to both PPD-B and PPD-A 3 days after CFT, but not in response to stimulation with ESAT-6 and CFP-10 fusion protein (ESAT-6:CFP-10). Together, this study clearly shows that different results may be achieved in experimentally infected compared to sensitized cattle. When we extended the interpretation of

presented data to the comparative analysis of PPD-B and PPD-A responses, we saw that the relative PPD response and the final test result were not changed by CFT (Fig. 2).

Waters et al. (unpublished data) have compared the effect of CFT on the IFN-γ responses in cattle experimentally infected with *M. bovis*, *Mycobacterium kansasii* and in non-infected cattle. Effects on IFN-γ responses related to CFT varied depending on the animal group and on the stimulation antigen. Responses to PPD-A, PPD-B, and ESAT-6:CFP-10 fusion protein were boosted in *M. bovis*-infected cattle 7 days after injection of PPD-B for CFT; however, responses in samples from *M. kansasii*-infected cattle were not affected. While boosting effects were noted with *M. bovis*-infected cattle, test interpretation was not impacted (Fig. 3).

The only study analyzing the influence of CFT on the IFN-γ assay in naturally infected cattle has been performed very recently (Coad et al., 2010). Cattle with strong IFN-γ responses have been tested 3 and 10 days post-CFT, and the relative PPD responses were significantly higher at day 3 compared to pre-CFT values. Interestingly, PPD-A induced IFN-γ responses, were not increased in these cattle, as opposed to the before mentioned experimental studies (Palmer et al., 2006; Waters et al., unpublished data). The reason for this difference is not clear; it might indicate a difference concerning the infection stage. Cattle in the study with field reactors (Coad et al., 2010) could have been infected longer than those experimentally infected which tend to be monitored for shorter time periods. PPD-A responses, however, may especially be pronounced and even higher than PPD-B responses early after infection, as demonstrated in experimentally infected (Schiller et al., 2009b). Whether this phenomenon correlates to an increased likelihood of a PPD-A boosted IFN-γ response to PPD injection early during the infection course remains to be investigated.

In summary, the CFT boosted the IFN-γ responses in cattle experimentally infected with *M. bovis*. The boost in *M. bovis*-infected cattle was shorter than originally indicated with *M. bovis* sensitized cattle and lasted only until 7 days after the CFT. Importantly, extended analysis of published data showed that there was no effect on the relative PPD

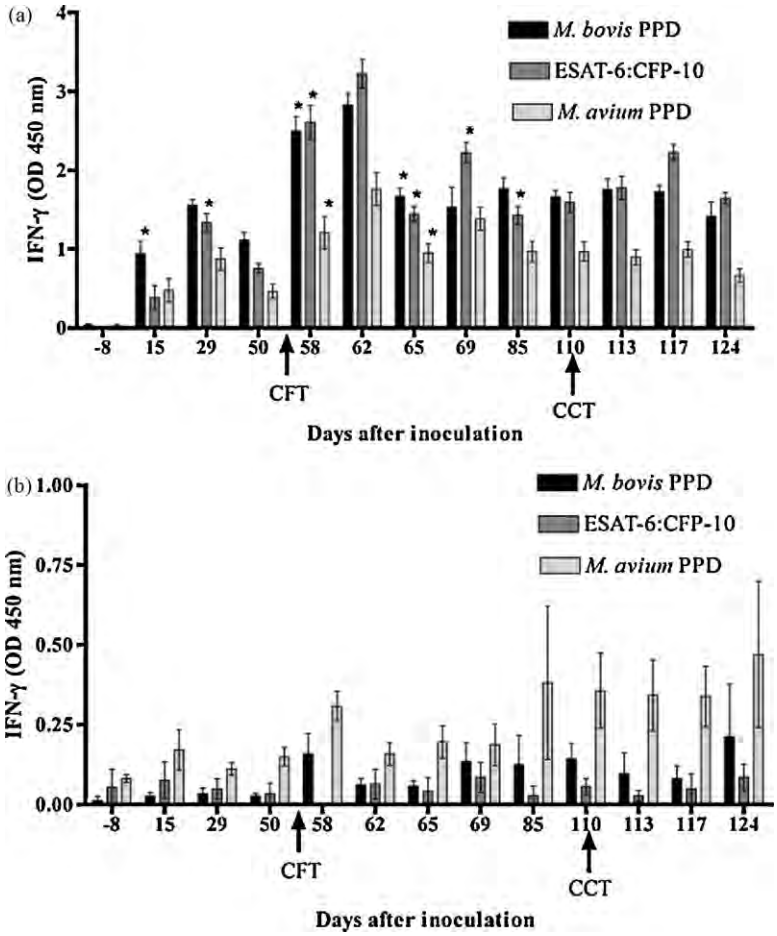
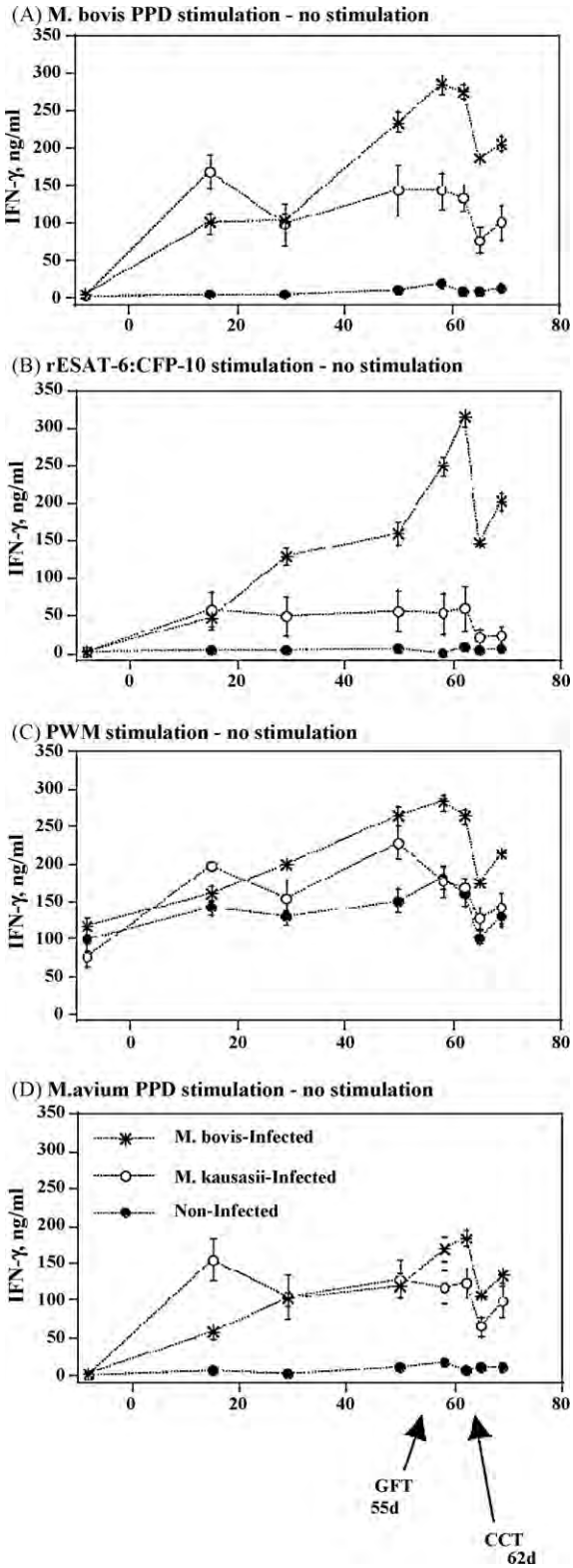


Fig. 2. Influence of CFT on IFN-γ responses in cattle experimentally infected with *M. bovis* (taken from Palmer et al. (2006) with kind permission of the author and publisher). Longitudinal IFN-γ response to *M. bovis* PPD, *M. avium* PPD, or recombinant ESAT-6 and CFP-10 (rESAT-6:CFP-10) fusion protein. Values represent the mean responses to each antigen minus the response to medium alone ±standard error of the mean (error bars) for eight to nine *M. bovis*-inoculated calves (A) or five noninoculated control calves (B). Day 0 represents the day of inoculation. Arrows represent time points when PPD was injected for tuberculin skin testing. *Significantly different than previous time point ($P < 0.05$).



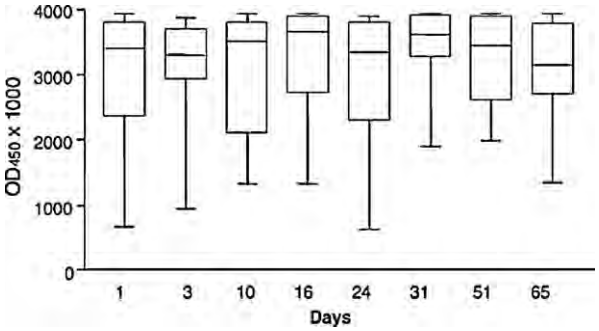


Fig. 4. Influence of CCT on IFN-γ responses in cattle naturally infected with *M. bovis* (taken from Gormley et al. (2004) with kind permission of the author and publisher). IFN-γ responses of 16 reactor cattle at time intervals (days) post-CCT. The day 1 blood sample was taken from all animals immediately prior to injection with tuberculin.

response on the final test interpretation. A limitation of all experimental studies was that responses were relatively strong; thereby, limiting the possibility for demonstration of subtle effects that may have been achieved with lower responses commonly observed in naturally infected field cases. Another major constraint is that hardly any data exist from naturally infected cattle, and that the few published reports are limited to only strong responders. Field studies taking into account different situations (e.g., epidemiology, stages of infections) are completely missing. In contrast to experimentally infected cattle, however, the relative PPD response was increased in these strong responders. Limited data from non-infected cattle indicate that a mild boost of the response to either bovine or avian tuberculin and thus a decreased specificity of the IFN-γ assay cannot be excluded shortly after applying the CFT.

4. Influence of the CCT on the IFN-γ assay

The interaction of CCT and *in vitro* IFN-γ production has been studied both in naturally and experimentally infected cattle (summarized in Table 2). All field studies indicate that CCT does not significantly boost or depress IFN-γ responses. Doherty et al. (1995) analyzed skin test reactor cattle up to 7 days, without finding evidence of an effect on IFN-γ production. Gormley et al. (2004) reached similar conclusions with naturally infected, culture confirmed cattle evaluated periodically up to 65 days post-skin test (Fig. 4).

Fig. 3. Effects of skin test on *in vitro* IFN-γ production to mycobacterial antigens by *M. kansasii*-, *M. bovis*-, and non-infected (control) cattle. Whole blood from control ($n = 10$), *M. kansasii*- ($n = 4$), and *M. bovis*-infected ($n = 9$) cattle at indicated time points after challenge (lower margin); cultured with media alone, 1 μg/ml pokeweed mitogen (PWM), 1 μg/ml rESAT-6:CFP-10, 5 μg/ml PPD-A, or 5 μg/ml PPD-B; and supernatants harvested for analysis of IFN-γ by ELISA. Values are presented as mean (±standard error) responses to *M. bovis* PPD (A), rESAT-6:CFP-10 (B), PWM (C) or *M. avium* PPD (D) stimulation minus the response to media alone. Arrows with CFT and CCT designation indicate time points of injection of PPD(s) for caudal fold and comparative cervical skin tests, respectively.

Table 2
Summary of most relevant studies analyzing the influence of CCT on the IFN-γ assay.

Ref.[a]	Study design	PPD sources	Results and conclusions
1	Naturally infected cattle (skin test reactors, $n = 18$). Period of IFN-γ analysis after CCT: pre-CCT.	*For CCT and IFN-γ assay*: Lelystad Biologicals (Lelystad, The Netherlands).	No significant effect on IFN-γ responses (PPD-B, PPD-A).
2	Naturally infected cattle (culture confirmed, $n = 16$). Period of IFN-γ analysis after CCT: 65 days.	*For CCT and IFN-γ assay*: Lelystad Biologicals (Lelystad, The Netherlands).	No significant effect on IFN-γ responses (PPD-B, PPD-A).
3	Experimental infection of cattle with *M. bovis* ($n = 35$). Non-infected cattle ($n = 5$). Time period between infection and CCT: 1–10 weeks. Period of IFN-γ analysis after CCT: 4–9 weeks.	*For CCT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	Transient increase of IFN-γ responses (PPD-B, PPD-A) 1 week after CCT in *M. bovis*-infected cattle. The PPD-A response increased to a greater extent than that to PPD-B, resulting partially in false negative test outcomes. *In vivo* and *in vitro* CMI responses may have been impacted by Tetanus toxoid administration prior to experimental infection with *M. bovis*.
4	Experimental infection of cattle with *M. bovis* ($n = 20$). Time period between infection and CCT: 147 days. Period of IFN-γ analysis after CCT: 10 days. Small increases of IFN-γ responses (PPD-B and PPD-A) at 10 days post-CCT compared to immediately prior to CCT	*For CCT*: MAF (Central Animal Health Laboratory, Upper Hutt, NZ).	
		For IFN-γ assay: CSL (Parkville, Victoria, Australia)	No significant effect in the differential response to PPD-B and PPD-A.
5	Experimental infection of cattle with *M. bovis* ($n = 10$). Time period between infection and CCT: 15 weeks. Period of IFN-γ analysis after CCT: 10 days.	*For CCT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	Reduced IFN-γ responses (PPD-B, PPD-A, ESAT-6 and CFP-10) 3 days after CCT. No effect on test interpretation in the PPD based assay, but decreased sensitivity for the ESAT-6 and CFP-10 based assays.
6	Naturally infected cattle (culture confirmed, $n = 10$). Period of IFN-γ analysis after CCT: 59 days.	*For CCT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	No significant effect on IFN-γ responses (PPD-B, PPD-A, ESAT-6 and CFP-10).
7	Repeat CCTs 5 times at 8-week intervals ($n = 6$) prior to infection. Controls without CCT ($n = 6$). Experimental infection with *M. bovis* 8 weeks after the 5th CCT. Period of IFN-γ analysis after CCT: 15 weeks post-infection.	*For CCT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	No significant effect of repeat CCTs on IFN-γ responses (PPD-B, PPD-A).
8	Naturally infected cattle (culture confirmed, $n = 15$). Repeat CCTs 3 times at 8-week intervals. IFN-γ analysis: pre-CCT, 3 and 10 days after each CCT.	*For CCT and IFN-γ assay*: Weybridge (Veterinary Laboratories Agency, Surrey, United Kingdom).	No significant effect of repeat CCTs on IFN-γ responses (PPD-B, PPD-A).

[a] References: (1) Doherty et al. (1995). (2) Gormley et al. (2004). (3) Thom et al. (2006). (4) Buddle et al. (1994). (5) Whelan et al. (2004). (6) Coad et al. (2007). (7) Thom et al. (2004). (8) Coad et al. (2010).

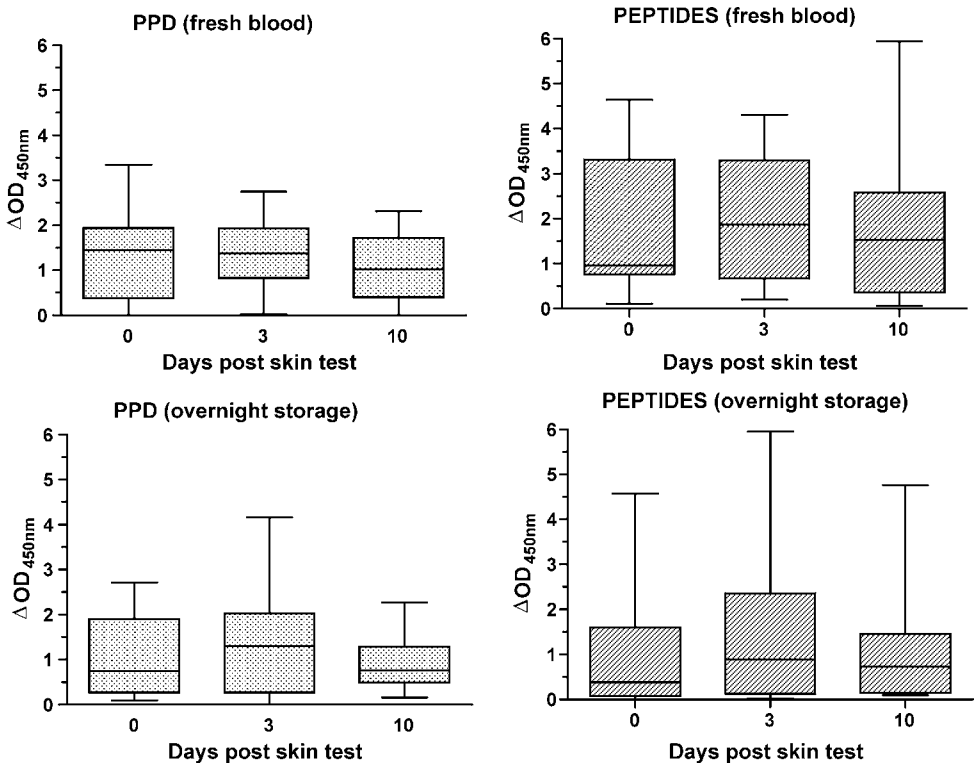


Fig. 5. Influence of CCT on IFN-γ responses in cattle naturally infected with *M. bovis* (taken from Coad et al. (2007) with kind permission of the author and publisher). Box and whisker plots showing the responses to the IFN-γ assay of 20 blood samples from 10 cattle. Results are displayed as OD at 450 nm for blood samples stimulated with either PPD from *M. bovis* or peptides from the *M. bovis* antigens ESAT-6 and CFP-10 minus the OD 450 nm for unstimulated control samples, for blood samples taken before and three and 10 days after the CCT, and cultured either on the same day (fresh blood) or after overnight storage.

In contrast, Thom et al. (2006) reported a transient increase of IFN-γ release in experimentally infected cattle 7 days after CCT both in response to PPD-A and PPD-B. In this study, however, *in vivo* and *in vitro CMI* responses may have been impacted by Tetanus toxoid administration prior to experimental infection with *M. bovis*. Small increases in IFN-γ release to PPD stimulation were observed in samples from experimentally infected cattle at 10 days post-CCT as compared to immediately prior to CCT (Buddle et al., 1994, 2002); again, there was no effect in the differential response to PPD-B and PPD-A. In another study using experimentally infected cattle (Whelan et al., 2004), IFN-γ responses were reduced 3 days after CCT, without effect on test interpretation in the PPD based assay. Still, reduced responses and decreased sensitivity were observed using ESAT-6 recombinant protein or a cocktail with peptides derived from ESAT-6 and CFP-10 for whole blood stimulation, which resulted in lower OD values than PPDs. The overnight storage of blood storage emphasized a reduction of OD values compared to 8 h blood storage.

These findings with experimentally infected cattle, however, could not be confirmed in naturally infected cattle (Coad et al., 2007). Detailed analysis of culture confirmed cattle during a follow-up period of 59 days after CCT indicated no decrease of IFN-γ responses, neither with PPDs nor with defined antigens (Fig. 5). The effects of repeat CCT on IFN-γ responses have been evaluated in several studies. Thom et al. (2004) have analyzed experimentally infected calves by repeating CCTs 5 times at 8-week intervals. IFN-γ responses did not significantly differ ($P > 0.05$) between the calves with repeated CCT and a control group without CCT. These observations were extended in a study using field reactor cattle that were repeat CCT-tested 3 times over a period of 6 months at 8-week intervals (Coad et al., 2010). Changes in IFN-γ responses were not detected

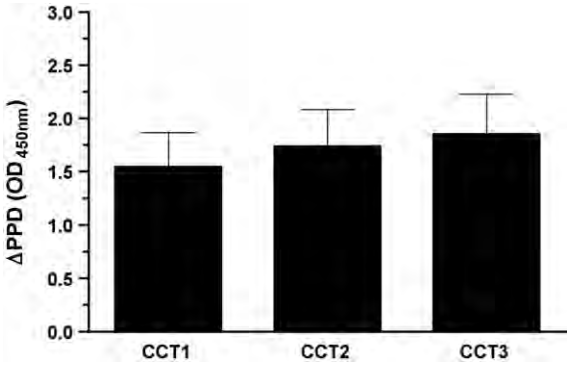


Fig. 6. . Influence of repeat CCT on IFN-γ responses in cattle naturally infected with *M. bovis* ($n = 15$). IFN-γ responses were measured in blood samples immediately prior to 3 consecutive CCT tests (CCT1–CCT3) over a period of 6 months at 8-week intervals. Each bar represents the mean IFN-γ values (OD 450 nm) of the PPD-B stimulated response minus the PPD-A response (±SEM). Coad et al. (2010).

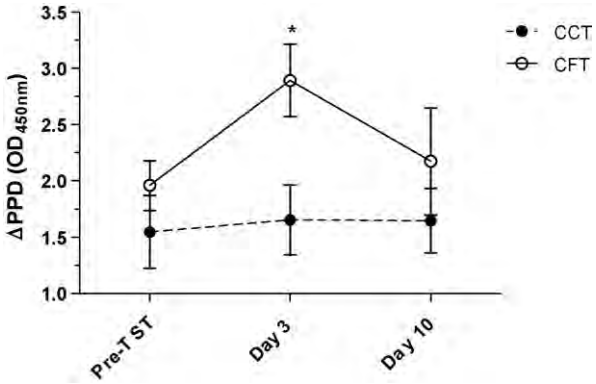


Fig. 7. Influence of CCT and CFT on IFN-γ responses in cattle naturally infected with *M. bovis*. Responses were measured prior to, three and 10 days post-TST. Mean comparative PPD response (PPD-B minus PPD-A, ±SEM) in cattle given the CFT ($n = 8$) or CCT ($n = 15$) are shown as open and closed circles, respectively. Response at day 3 was significantly different from the pre-CFT response, as indicated by an asterix (*$P < 0.05$, ANNOVA). Coad et al. (2010).

supporting the notion that repeat CCTs do not influence the performance of the IFN-γ test (Fig. 6). In this study, a direct comparison of the effects of CFT and CCT was also performed in a group of reactor animals. The data clearly demonstrate that, while the CCT does not impact subsequent IFN-γ responses immediately following the test, a significant selective PPD-B boosting effect (the PPD-A responses remained un-affected) is observed 3 days post-CFT, thus confirming the data described in the previous section (Fig. 7).

In summary, based on data from studies with naturally infected animals there is no evidence for the CCT to boost or depress mycobacterial-specific IFN-γ production. Furthermore, disparate results obtained in some studies with experimentally infected cattle could not be verified in naturally infected animals, thus emphasizing the importance to confirm findings in cattle naturally infected with bTB. Similarly to the CFT, there is a lack of data on the effect of CCT on the IFN-γ assay in weak responders or in non-infected cattle.

5. Conclusions

The influence of the TST on *in vitro* IFN-γ responses depends on whether it is performed as CCT or as CFT. While no effect of mycobacterial-specific IFN-γ production is obvious after CCT, there is a clear boost effect induced by CFT a few days after intradermal PPD application. Recent results from experimental infections (Palmer et al., 2006) and from naturally infected cattle (Coad et al., 2010), however, indicate a different time frame of boost responses (earlier and much shorter, about 3–7 days after CFT) than presumed from studies in sensitized cattle (3–28 days; Whipple et al., 2001). A major uncertainty is whether a boosting effect of CFT results in an increased comparative PPD response and, in consequence, an increased sensitivity of the IFN-γ assay (requiring a selective boost of PPD-B response), or whether the PPD-B boost is offset by a similar boost of the PPD-A response. Further studies in naturally infected cattle, especially weak responders, are needed. The second uncertainty is based on the fact that, in general, all data on the influence of TST on the IFN-γ assay originate from strong IFN-γ responders. The third uncertainty forms the limited knowledge available on the boost of PPD-A specific IFN-γ responses related to the time course of infection. The fourth lack of knowledge concerns non-infected cattle and the possibility of false positive IFN-γ results after CFT.

There is no obvious effect of the PPD source (used for TST and IFN-γ assay) on a possible boost effect. However, more data especially about exact PPD activities would be needed to conclude on this topic. In general, variable results from different studies are somewhat conflicting and highlight the complexity of the tuberculin response. The disparity in the various studies may result from the interaction of various factors, such as the specific immunologic status of animals including sensitization to or infection with a variety of non-tuberculous mycobacteria, the site of PPD injection, the time period between TST and blood sampling, the storage time of blood prior to addition of the antigens and the different PPD preparations used in the study. It is widely accepted that *in vivo* CMI responses vary and this notion is supported by the low reproducibility of guinea pig potency tests (Good et al., 2008). Moreover, PPD activity, as measured by *in vitro* IFN-γ responses, varies between batches and manufacturer (Bakker et al., 2005; Schiller et al., 2009b; Schiller et al., in press). An analysis method, named Relative Potency 30 (RP 30), has been shown to be useful for batch monitoring (Schiller et al., in press). The question remains as to whether *in vitro* potency of different PPDs correlates with *in vivo* activity. Regardless, extended batch control evaluation of PPDs (and emerging defined antigen reagents) by both *in vitro* IFN-γ and *in vivo* potency testing as well as routine biochemical measures could improve standardization of antigens for use in CMI assays.

In humans, the situation is similar to the veterinary field and contradictory effects have been reported. Recently, Vilaplana et al. (2008) monitored the evolution of TST responses on subsequent IFN-γ assays during a 4-week follow-up period. Their results showed an increase in post-TST responses, both in individuals with latent tuberculosis infection (LTBI) and in non-LTBI subjects, as measured by whole blood (Quantiferon, Cellestis) and isolated mononuclear cell ELISPOT IFN-γ assays. After a boost in the response upon TST, ELISPOT responses (measuring the number of IFN-γ positive cells) decreased towards pre-TST levels within 1 month, whereas the whole blood response (measuring IFN-γ release in plasma) did not. Van Zyl-Smit et al. (in press) demonstrated boosting 7 days post-TST both with ELISPOT and with whole blood IFN-γ assays; an increase of IFN-γ production, respectively of IFN-γ producing cells was noted in pre-TST IFN-γ release assay positive subjects (62.5%) and in pre-TST IFN-γ release assay negative subjects (12.5%). The boosting effect of TST seemed to reduce with time from TST (Baker et al., 2009). TST may not only induce boosting but also sensitization, demonstrated by increased IFN-γ to ESAT-6 upon a second injection of recombinant dimer protein ESAT-6 (Lillebaek et al., 2009).

In current bTB eradication programs, whole herd depopulation is becoming increasingly difficult to justify due to

economic necessity and animal welfare concerns. Therefore, effective disease eradication will require improved measures and tools. In this context, the accuracy of diagnostic tests plays a major role. The requirements for bTB tests have changed from herd tests to identifying infection at the level of the individual animal. High levels of standardization are desirable for TST and IFN-γ assays, as well as the possible interaction of the two tests. The IFN-γ assay, used in parallel to TST, has been shown to be more sensitive than TST (reviewed by Vordermeier et al., 2008), thus demonstrating that no boost by TST is needed to achieve sufficient *in vitro* IFN-γ production to reach a threshold level.

The serial use of TST and IFN-γ assays introduces a potential confounding variable for the IFN-γ assay. In summary, no impact can be verified after single or repeated short-interval CCT. CFT, however, is boosting to the subsequent *in vitro* IFN-γ response. Indeed, there is a lack of data clearly characterizing the effect of TSTs on the IFN-γ assay and further data would be needed to clearly describe this effect. We encourage further studies especially in animals with weak IFN-γ responses and in non-infected cattle, taking into account different background infections with environmental mycobacteria. Better knowledge of interactions in field situations could potentiate the effectiveness of the IFN-γ assay as a sensitive and specific tool for use in bTB eradication programs.

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ORIGINAL RESEARCH ARTICLE

Psychrotrophic Strain of *Janthinobacterium lividum* from a Cold Alaskan Soil Produces Prodigiosin

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We have explored the microbial community in a nonpermafrost, cold Alaskan soil using both culture-based and culture-independent approaches. In the present study, we cultured >1000 bacterial isolates from this soil and characterized the collection of isolates phylogenetically and functionally. A screen for antibiosis identified an atypical, red-pigmented strain of *Janthinobacterium lividum* (strain BR01) that produced prodigiosin when grown at cool temperatures as well as strains (e.g., strain BP01) that are more typical of *J. lividium*, which produce a purple pigment, violacein. Both purple- and red-pigmented strains exhibited high levels of resistance to β-lactam antibiotics. The prodigiosin pathway cloned from *J. lividium* BR01 was expressed in the heterologous host, *Escherichia coli*, and the responsible gene cluster differs from that of a well-studied prodigiosin producer, *Serratia* sp. *J. lividum* BR01 is the first example of a prodigiosin-producer among the β-Proteobacteria. The results show that characterization of cultured organisms from previously unexplored environments can expand the current portrait of the microbial world.

Introduction

Soil microbial communities are the most phylogenetically and functionally diverse communities on Earth. A single gram of soil may contain 10^9 to 10^{11} bacteria distributed among thousands of species, which play a significant role in biogeochemical cycling, bioremediation, and the production of antimicrobials and industrially useful small molecules and enzymes (Whitman *et al.*, 1998; Janssen, 2006). Despite estimates that fewer than 1% of soil bacteria are readily culturable (Amann *et al.*, 1990), culturing from soil continues to produce a new understanding of genetic and biochemical diversity, and more intensive efforts to culture from unexplored soils will accelerate the rate of discovery in microbiology. Combining traditional culturing with metagenomics and other culture-independent methods will be a powerful strategy to enhance our understanding of soil microbial communities.

As part of an effort to define the diversity of bacteria in undisturbed cold soils, we established the Alaskan Cold Microbial Observatory in the Bonanza Creek Long-Term Ecological Research site on an island in the Tanana River in interior Alaska where the soil temperature rarely exceeds 16°C (Viereck *et al.*, 1993). The bacteria in this habitat represent a reservoir of unsampled biodiversity. Although there has been intense interest in sampling from extreme, cold environments (Shi *et al.*, 1997; Zhou *et al.*, 1997; Priscu *et al.*, 1999; Christner *et al.*, 2001; Schadt *et al.*, 2003; Lavire *et al.*, 2006; Alekhina *et al.*, 2007; Hansen *et al.*, 2007), less attention has focused on cold, nonpermafrost soils.

Previous study of this Alaskan soil microbial community using culture-independent approaches revealed high species richness based on the sequences of 1033 16S rRNA genes from a single 0.5-g sample of soil (Schloss and Handelsman, 2006). The sequences were distributed among 19 bacterial phyla, 4 of which had no cultured representatives. The Alaskan soil contains an estimated 5000 different species per gram, using ≥97% identity in 16S rRNA gene sequence to define members of the same species (Schloss and Handelsman, 2006). Moreover, functional metagenomic analyses indicate diverse genes involved in LuxR-based quorum sensing (Williamson *et al.*, 2005a) and resistance to β-lactam antibiotics (Allen *et al.*, 2009).

The previous culture-independent analyses are complemented here by a culture-dependent analysis of bacterial isolates collected from the Alaskan Cold Microbial Observatory. Two pigmented isolates, one red and one purple, were identified as *Janthinobacterium lividum*. The characterizations of these strains are presented here, including the genetic analysis of pigment production and antibiotic resistance. We report that the red-pigmented isolate produces

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prodigiosin and the purple-pigmented isolate produces violacein, both in a temperature-dependent manner.

Methods

Culture conditions

A soil core was obtained from a site, designated BP-1, on an island in the Tanana River in the Bonanza Creek Long Term Ecological Research site approximately 30 km southwest of Fairbanks, Alaska, United States (64°40.24′ N, 148°14.46′ W) (Williamson *et al.*, 2005a; Schloss and Handelsman, 2006). The top 3 cm of the soil core was removed. Two soil horizons were visible: a silt layer that was deposited in a past flooding event topped by an organic layer resulting from years of biological activity after deposition of the silt. Each horizon was passed through a 5-mm sieve to remove large roots and organic debris. A 1-g soil sample from each horizon was suspended in 10 mL sterile deionized water and sonicated for 30 s in a bath sonicator (Model 2210 Branson, Danbury, CT). The sonicated soil suspension was serially diluted, and the dilutions were plated on 10%, 1%, and 0.1% tryptic soy agar (TSA) medium; nutrient broth gellan gum; VL55 gellan gum (Sait *et al.*, 2002); and 10% tryptic soy gellan gum (TSG). All culture plates were incubated at 6°C, 12°C, and 28°C for up to 2 months. At various intervals, colonies were picked, inoculated into the appropriate broth media, and stored in 96-well plates in 15% glycerol at −80°C.

An isolate of *J. lividum* that produced red colonies was isolated on 10% TSG at 6°C in the above collection, and this strain was designated BR01. This and other *J. lividum* isolates were cultured on 1/10th-strength TSA at temperatures no higher than 25°C, and were transferred to 4°C for several days to allow for pigment development. To improve pigment production, *J. lividum* isolates were also cultured on PGA medium (0.5% peptone, 1% glycerol, and 1.5% agar).

16S rRNA gene sequencing and analysis

Frozen stocks of 273 strains isolated on 1/10th-strength TSA were streaked for isolation and used to inoculate 1/10th-strength tryptic soy broth; broth cultures were grown until turbid. The cultures were lysed by freezing at −20°C followed by thawing. Genomic DNA from the lysed cells was used as the template for PCR with the primers 27f (5′-AGRGTTTGATYMTGGCTCAG) and 1492r (5′-GGYTACC TTGTTACGACTT) (Integrated DNA Technologies, Coralville, IA). Cycle conditions were an initial 5-min denaturation at 95°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, and a final extension of 10 min at 72°C. PCR products were purified using AmpPure (Agencourt Bioscience, Beverly, MA) and sequenced using the 27f primer and BigDye version 3.1 (Applied Biosystems, Foster City, CA). BLASTN (Altschul *et al.*, 1990, 1997) was used to determine the phylum-level designation of each isolate.

Genomic DNA was purified from cultures of *J. lividum* according to the manufacturer's instructions with the GenElute Bacterial Genomic Kit (Sigma-Aldrich, St. Louis, MO), and the 16S rRNA gene was amplified, purified, and sequenced as described above. Full-length sequences were obtained for the *J. lividum* 16S rRNA genes using primers 27f, 1492r, 787r (5′-CTACCAGGGTATCTAAT), and 787f (5′-ATTAGATA CCCYGGTAG) (Integrated DNA Technologies).

Screen for antibiotic production

To identify putative antibiotic-producing isolates, the Alaskan soil culture collection was replica printed from storage onto three types of media: 1/10th-strength TSA, VL55, and 1/10th-strength TSG. Duplicates were made of each plate to test the isolates for inhibition of *Bacillus subtilis* 168 and *Pseudomonas putida* F117. The plates were incubated at 6°C, 12°C, or 28°C until visible colonies formed. Colonies were fixed by spraying 0.5% water agar in a thin layer across the plate. A 100-μL overnight culture of each tester strain, *B. subtilis* 168, and *P. putida* F117, was mixed with 5 mL of soft Luria-Bertaini agar (LBA) (0.8% agar) and poured over each plate. After overnight incubation at 28°C, isolates that produced a zone of inhibition were then identified by 16S rRNA gene analysis as described above. *J. lividum* BR01 and BP01 were tested in the same manner for antibiosis against additional tester strains: *Salmonella enterica*, *Streptomyces griseus*, *Bacillus cereus* UW85, *Bacillus mycoides*, *E. coli* DH5α, *Erwinia herbicola*, and *Pseudomonas aeruginosa*.

In vivo *transposon mutagenesis*

The EZ-Tn5 <DHFR-1> transposome (Epicentre, Madison, WI) was introduced into electrocompetent *J. lividum* BR01 and BP01 according to the manufacturer's instructions. The mutant phenotype was confirmed and genomic DNA was isolated from each nonpigmented mutant (GenElute™ Bacterial Genomic DNA Kit; Sigma-Aldrich) and cloned into the *Pst*I site of pGEM-3zf(+) (Promega, Madison, WI). Ligation reaction products were electroporated into *E. coli* DH5α and plated onto LBA supplemented with 15 μg/mL of trimethoprim (to select for the transposon) and 100 μg/mL ampicillin (to select for the vector). Thousands of colonies from each mutagenesis reaction were screened. Plasmids were isolated from the resulting clones according to manufacturer's instructions with the ExpressMatrix (Bio101; Morgan, Irvine, CA). The manufacturer-supplied transposon-based sequencing primers were used to determine the sequence of the insertion site.

J. lividum *fosmid library construction and analysis*

Genomic DNA was isolated from *J. lividum* BR01 and BP01 (Table 1) using the GenElute Bacterial Genomic Kit (Sigma-Aldrich). A Hamilton syringe was used to shear the genomic DNA to fragments of 40 kb, which served as inserts for fosmid libraries using the CopyControl Fosmid Library Production Kit (Epicentre) (Table 1). Among 1000 colonies, clones harboring the pigmentation genes (pBRred and pBPpur) (Table 1) were identified by growing the two libraries for 40 h at room temperature and identifying red or purple colonies. Clones harboring genes conferring resistance to β-lactam antibiotics (pBRbeta and pBPbeta) (Table 1) were identified by growing the two libraries overnight at 37°C on LBA containing ampicillin (100 μg/mL).

Fosmid DNA was isolated from induced overnight cultures of pBRred, pBPpur, pBRbeta, and pBPbeta by alkaline lysis with buffers P1, P2, and N3 (Qiagen, Valencia, CA) followed by ethanol precipitation (Sambrook and Russell, 2001). To identify the genes responsible for the respective phenotypes, fosmid DNA was mutagenized with a transposon via the GPS-1 Genome Priming System (New England

Table 1. Strains and Plasmids Used in This Study

Strain or plasmid	*Relevant characteristics*[a]	*Reference*
Strains		
Janthinobacterium lividum strain BR01	Isolated from Alaskan soil; produces red pigment (prodigiosin) when grown at low temperatures	This study
J. lividum strain BP01	Isolated from Alaskan soil; produces purple pigment (violacein) when grown at low temperatures	This study
Escherichia coli strain EPI300	DH10B derivative with inducible promoter in front of *trfA*; induction increases copy number of pCC1FOS from *oriV*	Epicentre, Madison, WI
E. coli strain DH5α	General purpose lab strain	Hanahan (1983)
Plasmids		
pCC1FOS	Chl^R; fosmid cloning vector with inducible copy number when propagated in *E. coli* EPI300	Epicentre
pGEM-3zf(+)	Amp^R; standard cloning vector	Promega, Madison, WI
pBRred	Chl^R; subclone of *J. lividum* BR01 in pCC1FOS that turns *E. coli* EPI300 pink at cool temperatures	This study
pBPpur	Chl^R; subclone of *J. lividum* BP01 in pCC1FOS that turns *E. coli* EPI300 purple at cool temperatures	This study
pBRbeta	Chl^R; amp^R; subclone of *J. lividum* BR01 in pCC1FOS that confers resistance to β-lactam antibiotics on *E. coli* EPI300	This study
pBPbeta	Chl^R; amp^R; subclone of *J. lividum* BP01 in pCC1FOS that confers resistance to β-lactam antibiotics on *E. coli* EPI300	This study

[a]Antibiotic resistance phenotypes are abbreviated as follows: Chl^R, chloramphenicol resistant; Amp^R, ampicillin resistant.

Biolabs, Ipswich, MA). Each mutagenesis reaction was transformed into *E. coli* EPI300 and plated on LBA supplemented with antibiotics to select for the fosmid (20 μg/mL chloramphenicol) and transposon (20 μg/mL kanamycin). DNA was isolated from mutants that had lost the respective phenotype, and the transposon-based sequencing primers were used to sequence the genes of interest using BigDye version 3.1 (Applied Biosystems). Additional random insertion mutants were prepared to sequence the entire fosmid insert to at least threefold coverage. The region of pBRred containing *pigJK* was sequenced to ~15-fold coverage, and both the *pigBC* and *pigJK* junctions were PCR amplified and sequenced to confirm the annotation. Sequences were assembled using the STADEN software package (v. 1.4.0; http://staden.sourceforge.net/) and annotated using open reading frame (ORF) Finder (www.ncbi.nih.gov/gorf/gorf.html). ORF maps were drawn in SeqBuilder (Lasergene; DNASTAR, Madison, WI).

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) assays were performed according to Clinical Laboratory Standards Institute guidelines (NCCLS, 2004). Briefly, serial dilutions of antibiotics were made in a 96-well plate from 512 to 0.5 μg/mL, with no antibiotics in the last well. The final volume in each well was 90 μL in Mueller–Hinton broth (Becton, Dickinson and Company, Sparks, MD). Ten microliters containing $\sim 1\times10^5$ cfu of *J. lividum* BR01, *J. lividum* BP01, *E. coli* pBPpur, *E. coli* pBRbeta, and *E. coli* pBPbeta were added to the appropriate wells. MICs were tested with the following β-lactam antibiotics: ampicillin (Research Products International Corp., Mt. Prospect, IL), carbenicillin (Fisher Scientific, Fair Lawn, NJ), amoxicillin, penicillin G, piperacillin, cephalexin, cefamandole, and ceftazidime (Sigma, St. Louis, MO). Assays were performed in duplicate in three separate experiments using independent cultures with *E. coli* EPI300 carrying the pCC1FOS vector as the reference strain (Epicentre). Growth was evaluated within 24 h.

Purification of prodigiosin from J. lividum *BR01*

A 500 μL overnight culture of *J. lividum* BR01 grown in LB medium at 30°C was used to inoculate 500 mL of nutrient broth containing 1% glycerol in a 4 L Erlenmeyer flask, which was then incubated at 10°C with shaking at 250 rpm for 11 days, by which time the culture had become red-orange. Cells were removed by centrifugation, and the cell pellet, which retained most of the red color, was extracted three times by resuspending the cells in 50 mL of methanol, incubating on a rocker overnight, and centrifuging at 10,000 rpm for 15 min at 4°C. The final methanol extract was dried, and 486 mg of material was recovered. This extract was purified by column chromatography with a silica gel using a step gradient of dichloromethane and methanol. The active fractions were further purified by high performance liquid chromatography (HPLC) on a C-18 column using a water–methanol mixture acidified with 0.05% trifluoroacetic acid. The HPLC elution started with 4 min at 75% methanol and then increased to 100% methanol over 10 min followed by 5 min at 100% methanol (prodigiosin: $tR = 11.3$ min; heptylprodigiosin: $tR = 15.7$ min).

Purification of violacein from J. lividum *BP01*

Eleven 500 mL Erlenmeyer flasks each containing 50 mL of 0.5% (w/v) Bacto peptone and 1% (v/v) glycerol were inoculated with 50 μL of an overnight culture of *J. lividum*

BP01. Cultures were incubated at room temperature on a platform shaker at 200 rpm. After 13 days the cultures were dark purple. The cultures were combined, frozen, and lyophilized. Approximately 150 mL of methanol was added to the lyophilized culture and this mixture was incubated at 4°C for approximately 1 h on a rocker. The mixture was centrifuged and the methanol extract was decanted. This extraction process was repeated twice except that the incubation at 4°C was extended to 24 h. The methanol extracts were combined and dried to yield approximately 7.5 g of oil, which was purified according to the protocol outlined in Brady *et al.* (2001). Briefly, the oil was dissolved in 800 mL of 80% methanol and extracted three times with 800 mL of hexanes. The aqueous portion was diluted to 50% methanol and extracted twice with 1000 mL of chloroform followed by two more extractions with 500 mL of chloroform. The chloroform extract yielded 143 mg of purple oil. This extract was subjected to normal-phase column chromatography on a silica gel. Fractions were eluted from the column using a hexanes:ethyl acetate gradient acidified with 0.01% trifluoroacetic acid (TFA). The purple fractions from the silica column were combined and further purified using reverse-phase HPLC on a C-18 column (isocratic: 40% acetonitrile: 60% water acidified with 0.05% TFA; violacein: $tR = 9.2$ min).

GenBank accession numbers

pBRred and pBPpur sequences have been deposited in GenBank under accession numbers EF063590 and EF063591, and pBRbeta and pBPbeta sequences have been deposited under accession numbers EF063589 and EF063592, respectively. The 16S rRNA gene sequences for *J. lividum* BR01 and BP01 have been deposited in GenBank under accession numbers EU330448 and EU330449, respectively. GenBank accession numbers of each unique 16S rRNA gene sequence of the bacteria that were isolated on 10% TSA have been deposited under numbers HM113566-HM113684. These sequences are associated with the Alaskan soil metagenome project (NCBI GenomeProject database ID 28853).

Results

Biodiversity of Alaskan soil includes a red-pigmented J. lividum *strain*

To culture diverse bacteria, multiple media types, temperatures, and incubation lengths were employed. Dilute nutrients, such as those found in VL55 media, and long incubation times have been shown to increase the diversity of culturable isolates from soil (Janssen *et al.*, 2002; Sait *et al.*, 2002). Multiple cool temperatures were chosen for bacterial cultivation to capture a range of temperatures experienced in the Alaska soil habitat.

To assess the biodiversity of the Alaskan soil culture collection, the 16S rRNA genes of a subset of isolates were sequenced. Partial 16S rRNA sequences were obtained from 273 bacteria that were isolated on 10% TSA; the sequences represented 26 different genera distributed among the Proteobacteria (37.7%), Bacteroidetes (48%), Actinobacteria (12.8%), and Firmicutes (1.5%) (Fig. 1). The 16S rRNA gene

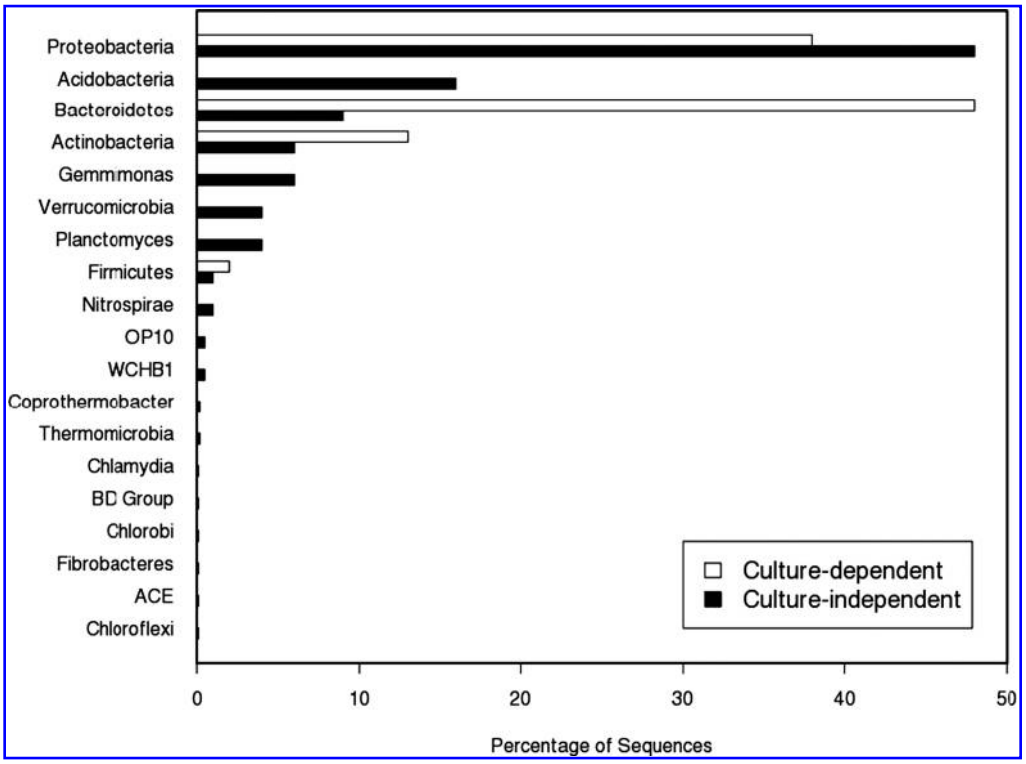

FIG. 1. The relative abundance of each phylum in the Alaskan soil culture collection isolated on 1/10th-tryptic soy agar ($n = 273$ 16S rRNA gene sequences) and in a culture-independent, PCR-derived clone library ($n = 1033$ 16S rRNA gene sequences) (Schloss and Handelsman, 2006). White bars represent sequences identified by culture-dependent methods, and black bars represent sequences identified by culture-independent methods. Candidate phyla OP10, WCHB1, BD group, and ACE have no sequenced representatives.

Table 2. Minimum Inhibitory Concentration (μg/mL) of Eight β-Lactam Antibiotics on *Janthinobacterium lividum* and Subclones After 24 h of Growth

	Minimum inhibitory concentration of strain or construct[a]				
β-Lactam antibiotic	J. lividum *BR01*	J. lividum *BP01*	E. coli *pBRbeta*	E. coli *pBPbeta*	E. coli *pCC1FOS (empty vector)*
Amoxicillin	256	128	>512	>512	4
Ampicillin	256	512	512	>512	8
Carbenicillin	128	256	128	512	4
Penicillin G	128	64	>512	>512	32
Piperacillin	>512	>512	>512	>512	4
Cephalexin	256	256	256	256	8
Cefamandole	256	256	256	512	0.5
Ceftazidime	8	16	8	4	0.5

[a]Names are defined in Table 1.

sequences of a different subset of 138 randomly selected isolates, representing the various media used in the initial isolation, were sequenced and revealed greater abundance of Proteobacteria (52.2%), Actinobacteria (20.3%), and Firmicutes (2.8%), and fewer Bacteriodetes (24.6%) (data not shown) than were detected among the 16S rRNA gene sequences from 10% TSA isolates alone. Overall, far fewer phyla were isolated by culturing than by a culture-independent analysis of the same soil (Fig. 1).

A screen of the Alaskan culture collection for antimicrobial activity identified six putative antibiotic producers. All inhibited the growth of *B. subtilis* 168 but not of *P. putida* F117. One isolate that produced a red pigment was further characterized.

The sequence of the 16S rRNA gene of the red antibiotic producer revealed that it was 100% identical to *J. lividum* (GenBank accession DQ640007). An additional 24 *J. lividum* isolates in the culture collection were identified with similar 16S rRNA gene sequences. Although the definition of *J. lividum* specifies that it produces a purple pigment (*Janthinobacterium* = purple bacterium, *lividum* = bluish), 9 of 24 *J. lividum* Alaskan soil isolates produced a red pigment (e.g., strain BR01) and the remaining 15 isolates produced the purple pigment typical of *J. lividum* (e.g., strain BP01) (Table 1). The full-length 16S rRNA gene sequences of a representative purple and red strain, *J. lividum* BP01 and *J. lividum* BR01, differed by two nucleotides over 1455 base pairs. Neither the red nor the purple pigments were visible in cultures grown above 20°C, although growth rate was not affected by temperature.

Given the difference in pigment production despite nearly identical 16S rRNA sequences, we examined other phenotypes of *J. lividum* BR01 and BP01. Except for the unique red pigmentation of nine *J. lividum* isolates and the temperature dependency of pigment production, the phenotypes of the Alaskan soil *J. lividum* isolates were similar to those of previously reported *J. lividum* isolates (De Ley *et al.*, 1978). Phenotypes tested include motility, proteolysis, lactose fermentation (data not shown), antibiosis, and antibiotic resistance. Pigmented *J. lividum* BR01 and BP01 inhibited the growth of *S. enterica*, *S. griseus*, *B. subtilis* 168, *B. cereus* UW85, and *B. mycoides*, but not *E. coli* DH5α, *E. herbicola*, or *P. aeruginosa*. No growth inhibition was observed for any tester strain when the *J. lividum* strains were grown at temperatures that prevented the appearance of pigmentation. Both strains of *J. lividum* (BR01 and BP01) exhibited high levels of resistance to seven of eight β-lactam antibiotics tested (Table 2).

Genetic characterization of β-lactam resistance in J. lividum *BR01 and BP01*

The genetic basis for β-lactam antibiotic resistance in *J. lividum* BR01 and BP01 was explored by selecting a fosmid clone library of each strain on ampicillin, followed by transposon mutagenesis and sequence analysis of the clones that conferred resistance on *E. coli.* We identified five clones from the *J. lividum* BR01 library and three from the BP01 library, and a restriction digest revealed overlap between ampicillin-resistant clones from the same library (data not shown). Therefore, a representative clone from each library was characterized and sequenced (clones designated pBRbeta and pBPbeta). Both pBRbeta and pBPbeta conferred high levels of β-lactam antibiotic resistance on *E. coli*, and in some cases (when tested on amoxicillin and penicillin G, e.g.) exhibited greater resistance than did the *J. lividum* strain from which the genes were isolated (Table 2). Sequencing revealed much congruency between pBRbeta and pBPbeta; the former has a 33.1-kb insert containing 32 ORFs, the latter has a 38.9-kb insert containing 33 ORFs, and they contain 22 ORFs in common (Fig. 2). Within the overlapping region, pBPbeta and pBRbeta are syntenous except for one additional ORF in the pBPbeta sequence that is not found in pBRbeta (Fig. 2). The 22 overlapping ORFs are, on average, 95.2% (SD = 3.9) identical between the two fosmids. Both encode a putative β-lactamase repressor protein, a putative carboxypeptidase, and a putative metallo-β-lactamase, and the amino acid sequences of the pairs of homologs have 99.2%, 95.4%, and 93.3% identity, respectively. The predicted protein sequence encoded by the two metallo-β-lactamase genes share 81% identity with a previously published metallo-β-lactamase (THIN-B) from *J. lividum* (Rossolini *et al.*, 2001). The genes encoding the metallo-β-lactamases from *J. lividum* BR01 and BP01 are designated *bla*LRA-20 and *bla*LRA-21, respectively (*bla*, β-lactamase; LRA, β-lactam resistance from Alaskan soil).

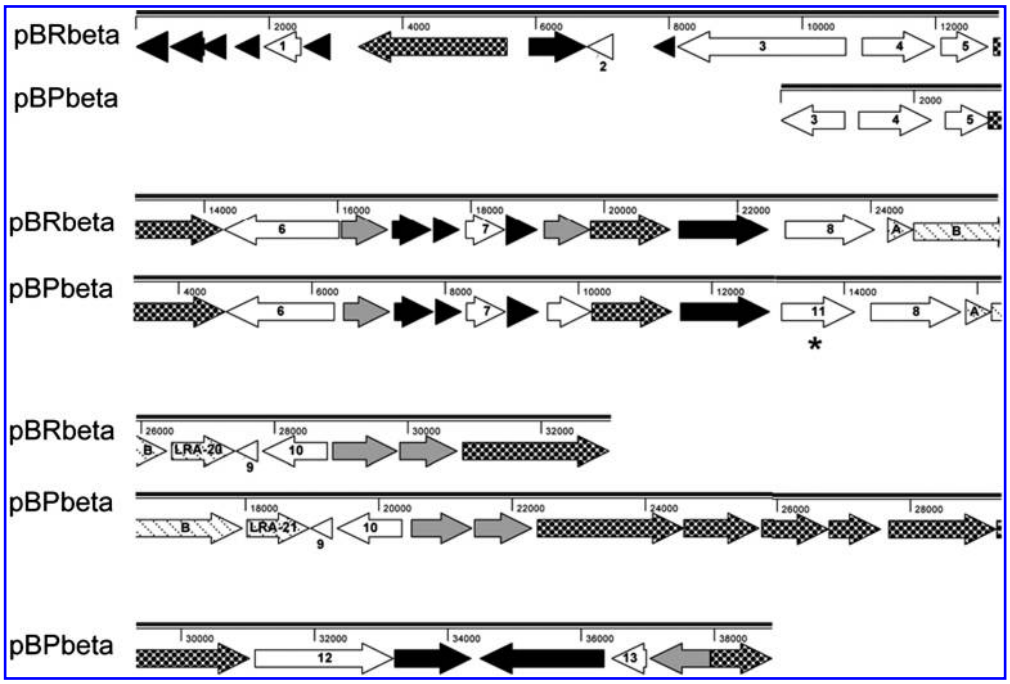

FIG. 2. Maps of open reading frames (ORFs) from pBRbeta (33,133 bp) and pBPbeta (38,875 bp), aligned across homologous regions. The ORFs with dotted diagonal lines in each map encode putative β-lactamase repressor proteins (A), carboxypeptidases (B), and metallo-β-lactamases (LRA-20 and LRA-21). The asterisk below the map of pBPbeta designates the ORF that is missing in pBRbeta. ORFs filled with black, gray, and black dots have homology to hypothetical ORFs, regulatory proteins, and transport proteins, respectively. Those ORFs lacking a color have homology to a methyltransferase (1), hemerythrin-like protein (2), oxidoreductase (3), branched-chain amino acid aminotransferase (4), ribonuclease I (5), AMP-dependent synthetase and ligase (6), electron chain protein (7), adventurous gliding motility protein (8, 11), TPR repeat protein (9), alcohol dehydrogenase (10), ferrichrome-iron receptor (12), and isochorismate hydrolase (13).

Isolation and characterization of J. lividum *BR01 pigment-producing gene cluster*

The red compound produced by *J. lividum* BR01 was purified, and a nuclear magnetic resonance (NMR) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis indicated that it was a mixture of prodigiosin and heptylprodigiosin (Fig. 3A, B). Transposon mutagenesis identified two genes, *smpB* and *pigE*, that were required for pigmentation and antibiosis. SmpB forms a complex with SsrA RNA that targets aberrant proteins and mRNAs for degradation (Keiler, 2008). This complex is ubiquitous among bacteria and is required for viability, response to stresses, and pathogenesis in many bacteria (Keiler, 2008). The other gene implicated in pigmentation, *pigE*, is part of the prodigiosin pathway in *Serratia* sp. (Williamson *et al.*, 2005b).

To obtain the entire complement of genes required for pigment production, the *J. lividum* BR01 fosmid library was screened in *E. coli* for red-pigmented clones. One pink clone,

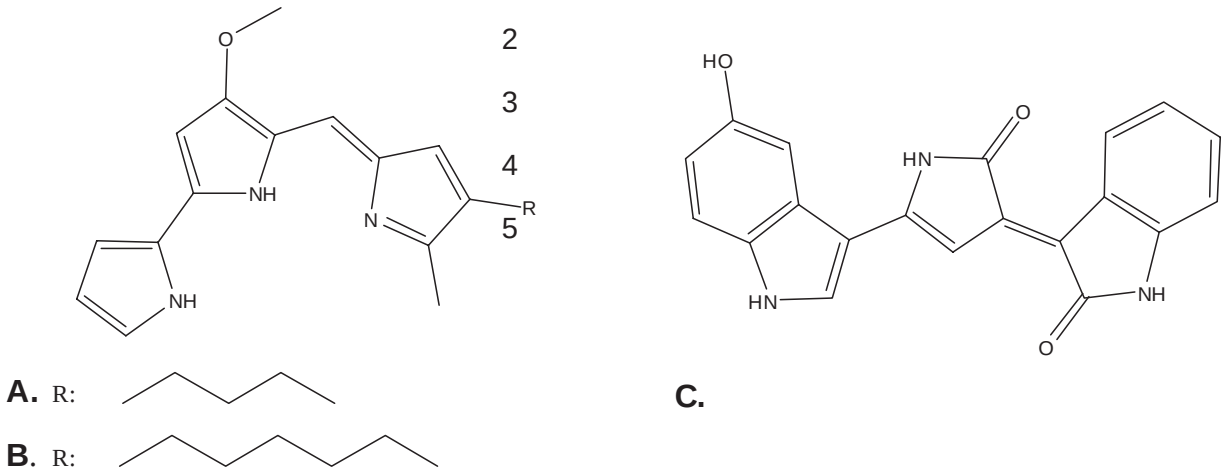

FIG. 3. Structures of compounds isolated in this study. Prodigiosin (**A**) and heptylprodigiosin (**B**) were purified from a culture of *Janthinobacterium lividum* BR01, and violacein (**C**) was purified from a culture of *J. lividum* BP01.

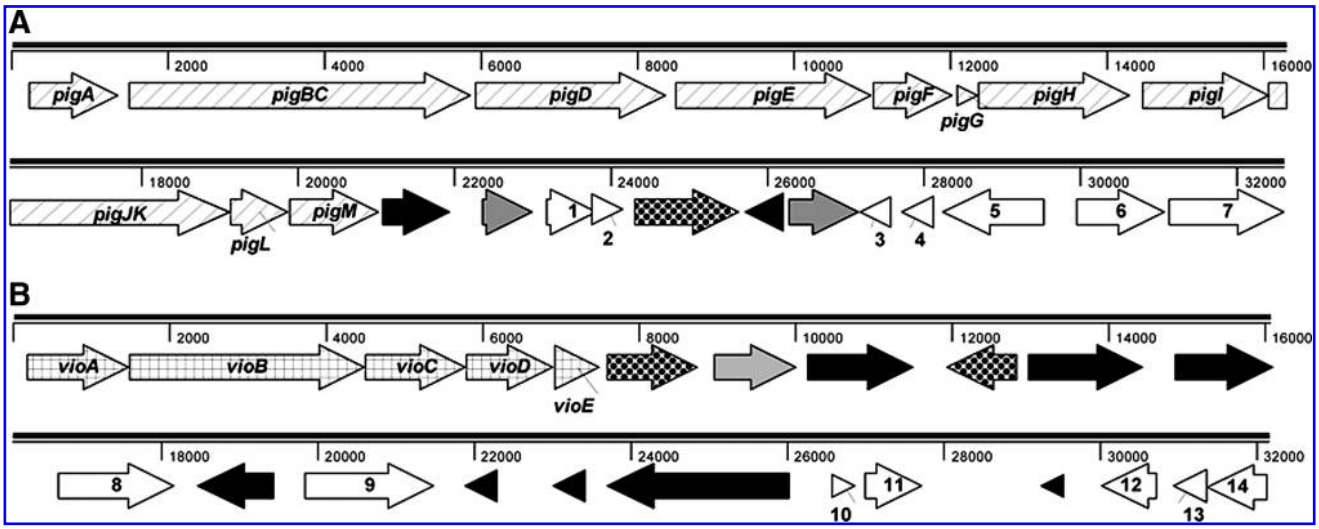

FIG. 4. Maps of ORFs from pBRred (32,611 bp) (**A**) and pBPpur (32,163 bp) (**B**). The ORFs with diagonal lines correspond to homologues of the *pig* pathway, which encodes prodigiosin biosynthetic genes, and the ORFs with grid lines correspond to homologues of the *vio* pathway, which encodes violacein biosynthetic genes. ORFs filled with black, gray, and black dots have homology to hypothetical ORFs, regulatory proteins, and transport proteins, respectively. Those ORFs lacking fill have homology to a D-aspartate O-methyltransferase (1), rhodanase (2), glyoxalase (3), osmotically inducible protein OsmC (4), L-sorbosone dehydrogenase (5), NAD(+) kinase (6), RecN (7), class C β-lactamase (8), α-amylase (9), transposase (10, 11), DNA polymerase III ε subunit (12), ribonuclease H (13), and methyltransferase (14).

pBRred, was identified and its insert (32.6 kb) sequenced, revealing a cluster of 11 genes that transposon mutagenesis indicated are required for prodigiosin biosynthesis (20.8 kb) (Fig. 4). The mean G+C content of the *pig* genes (61.7%) is consistent with the mean G+C content of the remaining ORFs on pBRred (62.8%) and the ORFs on the pBRbeta clone (63.4%). All of the deduced Pig proteins from *J. lividum* BR01 exhibit higher sequence identity to the proteins from the marine γ-Proteobacterium *Hahella chejuensis* (40–76%) (Kim *et al.*, 2006) than to the proteins from either *Serratia* sp. ATCC 39006 (32%–60%) or *S. marsescens* ATCC 274 (34%–60%) (Supplemental Table 1, available online at www.liebertonline.com).

Growth of the clone at 28°C or 37°C resulted in reduced pigment production, and optimal pigment production was observed when clones were grown at room temperature. It is possible that the reduced pigmentation of pBRred (pink) compared with *J. lividum* BR01 (red) was due to limitations of heterologous expression of the pathway.

Isolation and characterization of J. lividum *BP01 pigment-producing gene cluster*

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum and LC-MS/MS analysis of the purple compound produced by *J. lividum* BP01 matched that obtained with purified violacein (Fig. 3C). Random mutagenesis indicated that interruption of the *smpB* gene resulted in the loss of pigmentation, but no biosynthetic genes were identified by mutagenesis. When the fosmid library of *J. lividum* BP01 genome was then screened in *E. coli*, one clone (pBPpur) was identified that was dark purple when grown at room temperature but not when grown at 28 or 37°C. The complete sequence of the pBPpur fosmid clone (32.2 kb; Fig. 4) revealed the prototypical violacein biosynthetic operon (7.3 kb), which consists of the five genes *vioABCDE* (Sanchez *et al.*, 2006) and was originally identified in *Chromobacterium violaceum*. *In vitro* transposon mutagenesis of pBPpur resulted in nonpigmented mutants, and these insertions mapped to each of the genes (*vioABCDE*) in the violacein pathway. PCR amplification of the violacein cluster in *J. lividum* BR01 and of the prodigiosin cluster in *J. lividum* BP01 was negative (data not shown). Additionally, annotation of the DNA flanking the violacein gene cluster revealed a putative β-lactamase (Fig. 4). pBPpur was therefore tested for resistance to eight β-lactam antibiotics in an MIC assay, but the clone did not confer resistance on *E. coli* (data not shown).

Discussion

This is the first report of a red-pigmented *J. lividum* strain, revealed by the construction and analysis of a collection of bacteria cultured from nonpermafrost Alaskan soil. This study extends the known diversity of *J. lividum*, which is a member of the Burkholderiales in the β-Proteobacteria and has been found primarily in aquatic environments (Saeger and Hale, 1993) and soils (Gleave *et al.*, 1995; Rossolini *et al.*, 2001; Du *et al.*, 2007; Kawakami *et al.*, 2007). *J. lividum* has been reported to control amphibian fungal pathogens (Harris *et al.*, 2009), and can be toxic to other bacteria, viruses, and protozoa (Lichstein and Van De Sand, 1946; Andrighetti-Fröhner *et al.*, 2003; Matz *et al.*, 2004). Our results demonstrate the importance of culturing from novel environments and of challenging common assumptions; *J. lividum* BR01 and BP01 were nearly ignored because of the familiarity of the red and purple pigments. Our original predictions were that the purple isolate was *C. violaceum* and that the red isolate was *S. marcescens*. However, neither prediction was correct as 16S rRNA gene sequencing indicated that both isolates are *J. lividum*. The 16S rRNA gene sequences of BR01 and BP01 are virtually identical, both strains contain

metallo-β-lactamases, and the genes surrounding the β-lactamase genes are highly similar, yet the strains diverged in the acquisition of pathways for the production of different small molecules (presumably after the evolution of the β-lactamase genes). This highlights the point that neither colony morphology nor 16S rRNA gene sequence alone indicates the taxa present in a community or their characteristics (Jaspers and Overmann, 2004).

Pigment production in *J. lividum* BR01 and BP01 requires growth at temperatures below 20°C. *Serratia* sp. produce prodigiosin at temperatures below 30°C but not at 37°C (Williams *et al.*, 1971). *J. lividum* BP01 is the first example of cold-dependent pigment production among violacein producers. The temperature-dependent pigment and antibiosis by *J. lividum* BR01 and BP01 raise interesting questions about the ecological role of the pigment in these strains. The Alaskan soil rarely reaches temperatures above 16°C (Viereck *et al.*, 1993), so this habitat may have selected for *J. lividum* strains that produce the pigments at cool temperatures. Further studies are required to investigate the role of cold-activated pigment production and antibiosis in *J. lividum* survival *in situ*. The cloned pathways maintain the cold regulation in *E. coli* that they had in the native organisms, and the clones may therefore provide the basis for studying the mechanism of cold-specific gene regulation.

J. lividum BR01 is the first reported β-Proteobacterium that produces prodigiosin. Prodigiosins are of clinical interest because they are attractive immunosuppressive and anticancer therapeutics (Williamson *et al.*, 2006). Other prodigiosin-producing bacteria include *Streptomyces coelicolor*, *Hahella chejuensis*, *Pseudomonas magnesiorubra*, *Vibrio psychroerythreus*, and *Serratia* sp., which represent the Actinobacteria and γ-Proteobacteria (Williamson *et al.*, 2006). Prodigiosin is produced by the condensation of 2-methyl-3-amyl-pyrrole (MAP) and 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) (Williamson *et al.*, 2005b). The gene clusters for prodigiosin production in *Serratia* sp. (Harris *et al.*, 2004; Williamson *et al.*, 2005b) and *H. chejuensis* (Kim *et al.*, 2006) differ from the *J. lividum* BR01 cluster in several ways. First, the discrete *pigB* and *pigC* genes of *Serratia* sp., whose products sequentially produce MAP and execute the MAP-MBC condensation reaction (Williamson *et al.*, 2005b), exist as a fused gene in pBRred. Further, the *pigJ* and *pigK* genes, which are also separate genes in *Serratia* sp., are a fused gene in pBRred. Both PigJ and PigK are involved in the biosynthesis of MBC, although the role of PigK is uncertain. The third difference is that *pigN* is absent from the *J. lividum* BR01 prodigiosin cluster. In *Serratia* sp., *pigN* and *pigF* are thought to work together to direct the final step in the synthesis of MBC (a methylation reaction), and *pigN* mutants produce norprodigiosin as well as prodigiosin (Harris *et al.*, 2004; Williamson *et al.*, 2005b). A BLASTN analysis for *pigN* in the pBRred cluster yielded no regions of identity, so it remains unclear how the final step in synthesis of MBC is accomplished in strain BR01. Further studies are required to determine the effects of the genetic differences on the biochemical functions of the prodigiosin pathway in *J. lividum* BR01. Comparison of expression of the prodigiosin genes in *J. lividum* BR01 and *Serratia* sp. may aid in understanding how the gene cluster contributes to the ecophysiology of these organisms. The discovery of a prodigiosin-producing *J. lividum* strain expands the diversity of pathways for the biosynthesis of prodigiosin.

The goal of this study was to explore the possibility that previously unexplored Alaskan soil harbors novel biodiversity. The red-pigmented *J. lividum* strain indicates potential for discovery in the Alaskan soil culture collection. In this study of a cold soil, we found known organisms and natural products in new configurations and combinations. When examined in new ways, the culturable soil community may continue to be a fruitful source of biochemical and genetic diversity.

Disclosure Statement

No competing financial interests exist.

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Absorbed EVELISA: A Diagnostic Test with Improved Specificity for Johne's Disease in Cattle

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Abstract

The use of enzyme-linked immunosorbent assays (ELISAs) is recommended for Johne's disease (JD) control in dairy herds. In 2006, we developed a novel ELISA test for JD, named EVELISA (ELISA using ethanol extract of *Mycobacterium avium* subsp. *paratuberculosis*), which showed higher sensitivity than commercial ELISA tests. To further investigate the performance of EVELISA, we obtained 38 serum samples from cattle in a JD-free herd with suspected cases of serological false-positive reactions. When these samples were tested using the EVELISA and a commercial ELISA test, more than 70% of the samples were falsely identified as JD positive. Antibodies in the serum samples reacted strongly with antigens of various environmental mycobacteria, suggesting the presence of cross-reactive antibodies in the samples. The possible cross reactions in the EVELISA were inhibited markedly by the use of *Mycobacterium phlei* antigens for antibody absorption. When these samples were tested, 8 samples were classified as positive for JD by the EVELISA with the antibody absorption, whereas 27 samples were classified as positive for JD by the commercial ELISA. For an estimation of tentative sensitivity and specificity, the ELISA tests were performed on 38 serum samples from JD-negative herds with no suspected cases of serological false-positive reaction and 68 samples from cattle diagnosed as positive for *M. avium* subsp. *paratuberculosis* infection by fecal culture test. Sensitivity and specificity of the EVELISA with preabsorption of serum with *M. phlei* ("ethanol vortex absorbed-ELISA" or EVA-ELISA) were estimated to be 97.1% and 100%, respectively, whereas those of the commercial ELISA were 48.5% and 97.4%, respectively. Further, in 85 fecal culture-negative cattle in JD-positive herds, higher sensitivity of the EVA-ELISA than the commercial ELISA was demonstrated by a Bayesian analysis. This study indicates that the EVA-ELISA may form a basis for a sensitive diagnostic test with a higher level of specificity than that of the current commercial ELISA test.

Introduction

Johne's disease (JD) is a chronic, granulomatous enteritis of ruminants caused by an environmental mycobacterium, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Cocito *et al.*, 1994; Stabel, 1998). Mainly because of the reduction of milk production and premature culling of affected cattle, JD causes significant ongoing economic losses in the dairy industry (Hendrick *et al.*, 2005; Tiwari *et al.*, 2008). Complicating factors associated with JD control are the relatively low accuracy of current enzyme-linked immunosorbent assays (ELISAs) for the disease. For instance, a Bayesian statistical analysis of nongold standard data by Wells *et al.* (2006) determined that the estimated sensitivities of current ELISAs for JD were 26%–27%. In addition to the low sensitivity issue, several reports indicated that environmental mycobacteria caused false-positive reactions in current ELISA tests. For example, Roussel *et al.* (2007) reported a positive correlation between recovery of environmental, non-MAP mycobacteria from feces and false-positive ELISA results, which indicates that infection of cattle with environmental mycobacteria is linked to false-positive reactions in current ELISAs for JD.

We previously developed novel serological diagnostic tests for JD that showed higher sensitivities than commercial ELISA tests (Eda *et al.*, 2005, 2006; Speer *et al.*, 2006). As these tests did not cross-react with sera from artificially infected

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cattle with *Mycobacterium avium* subsp. *avium* (MAA) or *Mycobacterium bovis*, we hypothesized that the tests had higher specificity (lower false-positive rate) than commercial ELISA tests. In this study, one of those tests, named EVELISA (ELISA using ethanol extract of MAP) (Eda *et al.*, 2006), and a commercially available ELISA were tested for their specificity using bovine serum samples obtained from a JD-free herd with suspected cases of serological false-positive reactions. Also, serum samples from dairy herds with no reported suspected cases of false-positive reactions were tested to estimate and compare tentative sensitivities and specificities of the EVELSA and the commercial test.

Materials and Methods

ELISA procedures

Commercial ELISA. A commercial ELISA (IDEXX Laboratories, Westbrook, ME), termed ELISA-A in this study, was carried out according to the manufacturer's instructions. Also, according to the instructions, the ratio of optical densities of sample and positive control (S/P value) was determined and JD status was assigned using a cutoff S/P value of 0.25.

In-house ELISA. Another ELISA that we developed using ethanol extract of MAP, named EVELISA, was conducted as described previously (Eda *et al.*, 2006). MAP strains (Linda and K10) used for the EVELISA antigen preparation were obtained from the Agricultural Research Service, U.S. Department of Agriculture (Ames, Iowa). Other environmental mycobacteria were used for testing cross reactions of serum antibodies. The environmental mycobacteria included MAA, *Mycobacterium scrofulaceum*, *Mycobacterium szulgai*, *Mycobacterium gordonae*, *Mycobacterium kansasii*, *M. bovis* Bacillus Calmette-Guérin (BCG) and *Mycobacterium phlei* (provided by Dr. P. Small, Department of Microbiology, The University of Tennessee, Knoxville). Mycobacteria were cultured in Middlebrook's medium (Becton Dickinson, Cockeysville, MD) supplemented with 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ) at 37°C until used for experimentation. In the case of MAP, 2 mg/L Mycobactin J (Allied Monitor, Fayette, MO) was added to the culture medium. These *Mycobacterium* species were originally obtained from the American Type Culture Collection. Thus, they have been authenticated by the depositors as well as the curator. Mycobacteria were harvested from the liquid culture, suspended in 80% ethanol, and agitated by vortex to dislodge surface antigens. After removing the bacilli by centrifugation, dislodged antigen in the ethanol solution was immobilized on a 96-well plate by evaporating the solution, reacted with diluted serum samples followed by horseradish peroxidase-labeled goat anti-bovine immunoglobulin G (H + L) polyclonal antibody (Jackson ImmunoResearch Lab, Westgroup, PA). Binding of bovine serum antibody was detected by using a substrate of horseradish peroxidase (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) according to the manufacturer's instructions. For the EVELISA, S/P values were calculated and a cutoff S/P value of 0.20 was determined as described previously (Eda *et al.*, 2006). Preabsorption of cross-reactive antibodies in serum samples was conducted by diluting the samples 100× with buffer A (10 mM phosphate-buffered saline, pH 7.0, containing 0.05% [v/v] Tween 80 and 10% [v/v] SuperBlock [Pierce Biotechnology, Rockford, IL]) containing 0.5 mg/mL *M. phlei* Absorben (Allied Monitor) and incubating at room temperature for 30 min.

Serum samples

Group I. Serum samples were obtained from 38 Holstein cattle (aged 2–9 years [mean ± standard deviation = 5.18 ± 2.01]) in a Japanese dairy herd (total head count: 147) with no history of JD. JD-negative status of the herd was confirmed by a whole-herd testing procedure that involved repeated applications of fecal culture and real-time polymerase chain reaction (PCR) tests as follows. Prior to serum sampling, all animals in the herd were diagnosed as negative for MAP infection by the PCR test for three times with 2–4-month intervals. Also, all the animals in the herd were diagnosed as negative for MAP infection by the fecal culture test for six times with 5–15-month intervals. No JD-characteristic clinical sign was observed in animals in the herd. However, false-positive reactions in serum samples of several cattle were indicated by ELISA and complement fixation tests. The 38 cattle were selected to include most of the ELISA-positive animals and serum samples of the animals were collected at the same time of the last fecal culture test. Fecal culture tests were done using Herrold's egg yolk medium with Mycobactin at 2 mg/L after treatment of fecal samples with 0.75% hexadecylpyridinium chloride. Serum antibodies were detected by commercial ELISA and complement fixation tests with antigen produced at the biological products section of the National Institute of Animal Health. A real-time PCR test of fecal DNA samples was performed using the same methods described by Kawaji *et al.* (2007).

Group II. To evaluate the specificity of the ELISA tests under investigation here, 38 serum samples were obtained from cattle (female Holstein-Friesian cattle, aged 0.5–9 years [mean ± standard deviation = 3.4 ± 2.7]) in two Japanese herds; all 38 of these samples were negative for JD by fecal culture test. The fecal culture test was carried out once prior to the serum sample collection. No JD-characteristic clinical sign was observed in animals in the herds. Further, all the animals were diagnosed as negative for JD by a commercial ELISA (Kyoritsu Seiyaku, Tokyo, Japan).

Group III. To evaluate the sensitivity of the ELISA tests used in this study, 68 serum samples were obtained from female Holstein-Friesian cattle in three dairy herds in Pennsylvania and Minnesota (aged 1–8 years [mean ± standard deviation = 4.1 ± 2.0]). These cattle were diagnosed as positive for MAP infection by the fecal culture test. As these 68 cattle had repeatedly (at least twice) diagnosed as positive for MAP infection by fecal culture tests that were conducted at 6-month intervals in a longitudinal study (Whitlock *et al.*, 2000), it is unlikely that these are false positives due to "pass through" of bacilli (e.g., positives due to organisms passed through the digestive tract without causing infection). Of the 68 cattle, 36, 2, and 30 animals were categorized as low, medium, and high shedders, respectively, according to the criteria used in a previous study (Whitlock *et al.*, 2000).

Group IV. Eighty-five serum samples were collected from cattle on the same farms as Group III. These animals were

diagnosed as negative for MAP infection by the fecal culture test. These samples were used for Bayesian estimation of the probability sensitivity of a modified EVELISA.

In addition to these grouped samples, we used three serum samples collected from female Holstein-Friesian cattle in another Japanese herd. The animals were diagnosed as positive for MAP infection by PCR and fecal culture tests, and the serum samples were called "PCR- and fecal culture-positive (PF-P) serum samples" in this study. These samples were used to examine antibody binding to antigens of various mycobacteria and to evaluate the effect of different concentrations of *M. phlei* on antibody binding in EVELISA.

Statistical analysis

A Bayesian analysis of data from fecal culture-negative samples from JD-positive herds was conducted using a one-population binomial model (Branscum *et al.*, 2004). The model was applied to ELISA data (test positive or test negative) from cattle with uncertain MAP infection status. The probability of a positive test (apparent prevalence) was $AP = \pi Se + (1 - \pi)(1 - Sp)$. From this equation, the sensitivity of the ethanol vortex absorbed-ELISA (EVA-ELISA) test is given by the following equation: $Se = [AP - (1 - \pi)(1 - Sp)]/\pi$. The data were obtained from only fecal culture-negative cattle, so π Pr[JD positive | fecal culture negative]. Using Bayes' theorem, $\pi = (1 - Se_{FC})p/[(1 - Se_{FC})p + Sp_{FC}(1 - p)]$, where Se_{FC} and Sp_{FC} are the sensitivity and specificity of the fecal culture test, respectively, and p is the fraction of animals that have JD within a JD-positive source population. The posterior distribution of Se was approximated using a Monte Carlo sample of 50,000 iterates after a burn-in of 10,000 was discarded. The posterior probability that Se exceeds a value c is calculated using the following equation: $m^{-1}\sum_{j=1}^{m} I_{(c1)}(Se^j)$, where $m = 50{,}000$, Se^j denotes the jth simulated iterate from the posterior distribution of Se, and $I_A(x)$ denotes the indicator function, which equals 1 if $x \in A$ and 0 otherwise.

The nongold standard analysis contained parameters that were not estimable from the data alone, so additional input was needed to obtain inferences. This input manifested in the form of informative prior distributions, which were constructed using information from previously published research (Branscum *et al.*, 2004, 2005; Behr and Kapur, 2008; Mc *et al.*, 2008). WinBUGS version 1.4.3 (Lunn *et al.*, 2000) was used for Bayesian data analysis.

Results

Among the 38 Group I samples (sera from cattle in a JD-free herd with suspected cases of serological false-positive reactions), the ELISA-A and EVELISA incorrectly classified 72.9% and 75.7% of the samples as positive for JD, respectively.

Two Group I serum samples and two PF-P serum samples were tested for antibody binding to antigens of 10 different mycobacterial species. The levels of antibody binding to MAP extracts in the Group I samples were almost equivalent to those to other mycobacteria except *M. bovis* BCG and *M. szulgai* (Fig. 1A). In contrast, the levels of antibody binding to MAP extracts in the two PF-P serum samples were greater than those to extracts of other mycobacteria species (Fig. 1B). The antibody in the Group I samples reacted most strongly with *M. phlei* extract (Fig. 1A). Therefore, *M. phlei* was used in the following experiments as an absorbent.

Three Group I samples and three PF-P serum samples were preabsorbed with 0–2 mg/mL of *M. phlei* and tested for reactivity by the EVELISA. Antibody binding in the Group I samples was strongly inhibited by the *M. phlei* preabsorption, whereas that in the three PF-P samples was not decreased by preabsorption with *M. phlei* even at the highest concentration tested (Fig. 2). Further, we tested 33 Group I samples and 66 Group III samples with or without *M. phlei* (0.5 mg/mL) preabsorption. The percent of inhibition (average) caused by *M. phlei* preabsorption was 96.8% in Group I and 28.8% in Group III. The EVELISA with preabsorption

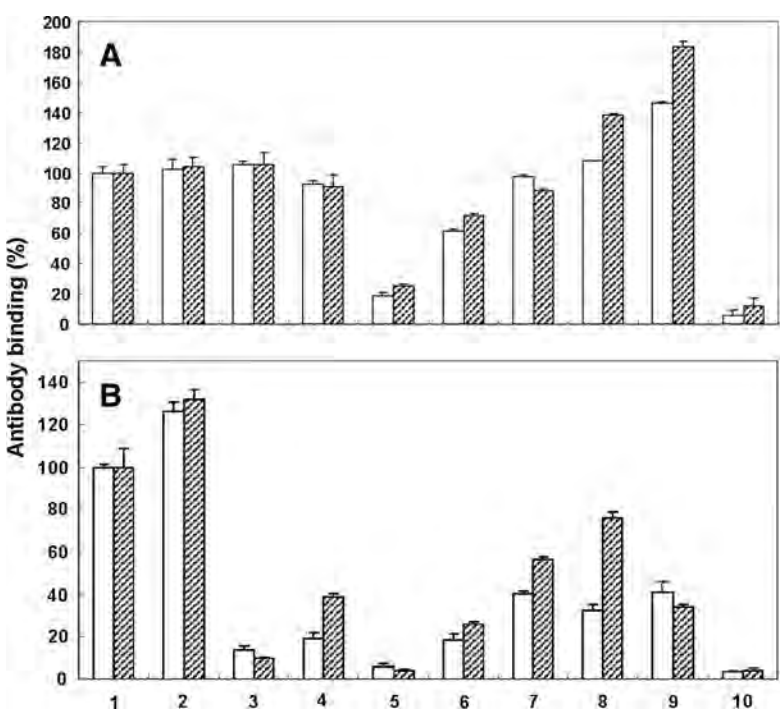

FIG. 1. Serum antibody reactions against various mycobacteria. Serum samples from two animals in Group I (animal 1 [open bar] and animal 2 [hatched bar]) (**A**) and samples from two polymerase chain reaction- and fecal culture-positive animals (animal 3 [open bar] and animal 4 [hatched bar]) (**B**) were reacted with antigens of various mycobacteria by the EVELISA procedure. Each bar represents mean ± standard deviation of antibody binding. *Mycobacterium* species used for the antigen preparation: 1, MAP Linda strain; 2, MAP 1974 strain; 3, *Mycobacterium avium* subsp. *avium*; 4, *M. scrofulaceum*; 5, *M. bovis* BCG; 6, *M. szulgai*; 7, *M. gordonae*; 8, *M. kansasii*; 9, *M. phlei*; 10, no antigen. EVELISA, ELISA using ethanol extract of MAP; MAP, *Mycobacterium avium* subsp. *paratuberculosis.*

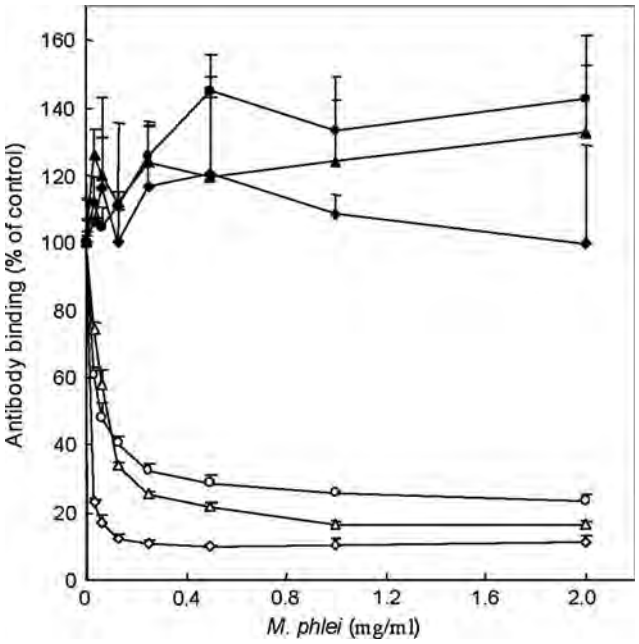

FIG. 2. Effect of absorption of cross-reactive antibodies with *M. phlei* on antibody binding to MAP antigens. Three polymerase chain reaction- and fecal culture-positive (closed symbols) and three Group I (open symbols) serum samples were treated with various concentrations of heat-killed *M. phlei* and then tested for antibody binding by the EVELISA procedure. The average of antibody binding levels (triplicate) obtained without *M. phlei* preabsorption was defined as 100%. Each symbol represents mean ± standard deviation of triplicate measurements.

of serum with *M. phlei* at a concentration of 0.5 mg/mL was named EVA-ELISA.

When 38 Group I samples were tested, only 8 (21.1%) were classified as positive for JD by EVA-ELISA, whereas 27 (71.1%) were classified as positive by ELISA-A (Fig. 3). Using 38 Group II (sera from cattle in JD-negative herds) and 68 Group III (sera from fecal culture-positive animals) samples, empirical sensitivity and specificity of the EVA-ELISA at a cutoff value of 0.20 were 97.1% and 100%, respectively (Fig. 3A), whereas those of ELISA-A were 48.5% and 97.4%, respectively (Fig. 3B).

Although the primary aim of the current study was to evaluate and compare false-positive rates, in a secondary analysis we tested whether the sensitivity of EVA-ELISA was higher than that of ELISA-A in a particular subgroup (Group IV, fecal culture-negative cattle). The sensitivities in Fig. 3A apply to fecal culture-positive cattle, which generally exclude newly and subclinically infected animals. Therefore, we also conducted an analysis of 85 Group IV samples, of which 14 were classified as positive for JD by EVA-ELISA. We set $Sp_{FC} = 1$ and used values of Se_{FC} and p that have appeared in previously published JD studies, that is, $Se_{FC} = 0.60$ (Messam *et al.*, 2008) and $p = 0.12$ (Branscum *et al.*, 2004). These inputs yield $\pi = 0.052$. On the basis of Group II data, which showed perfect specificity of EVA-ELISA, we set $Sp = 1$. The plug-in estimate of Se, which uses the unbiased estimate of AP of 0.165 (= 14/85), exceeds 1. A Bayesian analysis yields a range-respecting estimate of Se when a uniform (0, 1) prior distribution is used for this parameter. With this prior, we calculated the posterior probability that $Se > 0.485$, where 48.5% is the sensitivity of ELISA-A calculated using Group III data. As Group III involved fecal culture-positive animals only, we did

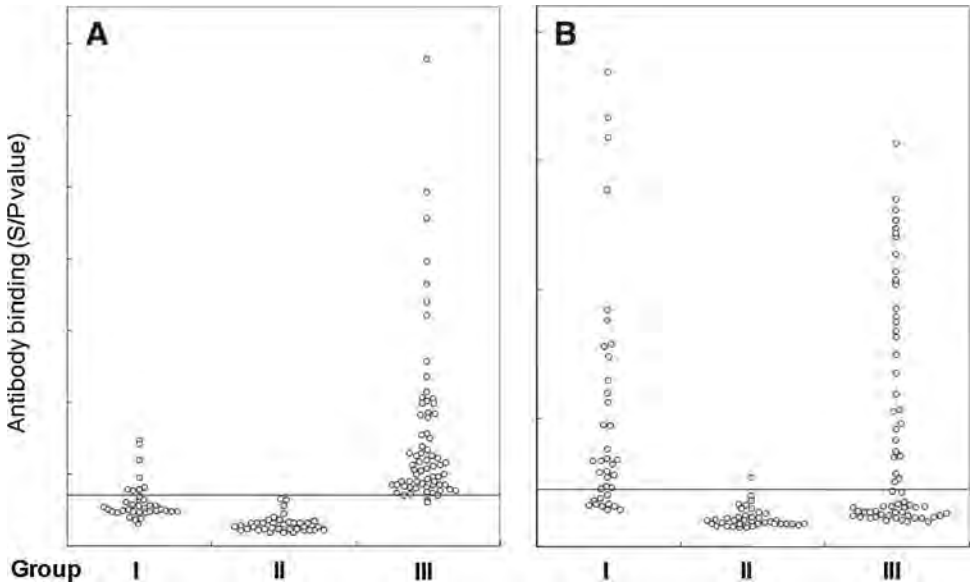

FIG. 3. Comparison of the EVA-ELISA (**A**) and ELISA-A (**B**) test results in Group I ($n = 38$), Group II ($n = 38$), and Group III ($n = 68$) samples. Serum samples in Groups I–III were tested for antibody binding using EVA-ELISA and ELISA-A. Each dot represents an average of duplicate measurements. To estimate tentative diagnostic sensitivity and specificity, cutoff (S/P) values of 0.2 and 0.25 (horizontal lines) were used for EVA-ELISA and ELISA-A, respectively. EVA-ELISA, ethanol vortex absorbed-ELISA; ELISA-A, commercial ELISA; S/P, the ratio of optical densities of sample and positive control.

not expect the sensitivity of ELISA-A to exceed 48.5% when applied to fecal culture-negative samples. Therefore, we calculated the posterior probability of the hypothesis H: Se 0.485 to investigate whether the Se of EVA-ELISA among fecal culture-negative cattle (Group IV) exceeded the expected maximum sensitivity of ELISA-A in this group. The posterior probability of H is 1.This result was virtually unchanged when we modeled π by placing a beta prior on p (instead of fixing the value of p at 0.12) that had a mode of 0.12 and 95% prior interval (0.04, 0.33). The exact prior is $p \sim$ beta (3.28, 17.74), which has been previously used as a prevalence prior for JD-positive herds (Branscum *et al.*, 2004). The same posterior probability also results from a prior on Se that is based on Group III data. To assess the impact of uncertainty about the empirical sensitivity of ELISA-A (48.5%), we calculated the posterior probability that Se exceeds the much higher value of 70%. The probability of 0.99 indicates a high degree of robustness to the presumed value of 48.5% for the sensitivity of ELISA-A.

Discussion

ELISA test continues to be used as one of the major diagnostic procedures to support the control of JD; however, the current commercially available ELISA tests suffer low sensitivity (Whitlock *et al.*, 2000; Nielsen and Toft, 2008) and are subject to low specificity (Osterstock *et al.*, 2007; Roussel *et al.*, 2007). Indeed, in this study, we found that a commercial ELISA (ELISA-A) and our in-house ELISA (EVELISA) had low specificity in sera of cattle from a JD-free dairy herd with suspected cases of serological false-positive reaction. However, the possible false-positive reactions in the EVELISA were subsequently reduced markedly with preabsorption of cross-reactive antibodies using *M. phlei* antigens (EVA-ELISA) without sacrificing the sensitivity of the EVELISA test. A large-scale, longitudinal prospective study is required to validate the EVA-ELISA's sensitivity and specificity estimated in this study.

Previous large-scale studies showed that specificities of commercially available ELISAs were high (e.g., 97%) (Nielsen and Toft, 2008), indicating that the false-positive reactions of ELISAs are not a widespread issue in dairy herds. We, therefore, did not use the data obtained from Group I samples for estimation of specificity. Roussel *et al.* (2007) reported that five beef herds in a southeastern region of Texas had high (22%–45%) seroprevalence of JD with low rates of MAP isolation (2%–7%), which could indicate a low specificity of the ELISA in the region (Roussel *et al.*, 2007). In this study, the specificity of EVA-ELISA was almost three times higher than ELISA-A in a dairy herd with suspected cases of serological false-positive reactions. Therefore, it will be of interest to examine how EVA-ELISA performs in the beef herds where current ELISAs showed low specificity.

Roussel *et al.* (2007) also showed that environmental mycobacteria were isolated from feces of 9%–43% of the cattle in the high-seroprevalence herds, whereas the rate was much lower (4%–6%) in cattle in geographically matched herds, suggesting that the possible low specificity of ELISA was due to the infection of cattle with environmental mycobacteria. Indeed, the commercial ELISA used in their study cross-reacted with antibodies in sera of calves experimentally infected with MAA, *Mycobacterium intracellulare*, *M. scrofulaceum*, or *Mycobacterium terrae* (Osterstock *et al.*, 2007). Therefore, the high proportion of antibody reactions in Group I samples in EVELISA may also have been due to infections of cattle with environmental mycobacteria. As MAA and *M. scrofulaceum* have been isolated from soil and water in cattle herds (Donoghue *et al.*, 1997; Norby *et al.*, 2007), these species may have been the cause of false-positive reactions in the EVELISA test.

Since Yokomizo *et al.* (1983, 1985) determined that heat-killed *M. phlei* was effective in reducing false-positive reactions in ELISA tests for JD, the bacteria has been routinely used for preabsorption of cross-reactive antibodies in commercial ELISAs. In this study, *M. phlei* antigens cross-reacted most strongly with two Group I samples tested. The ELISA-A test had strong reactions in Group I samples, despite that it uses *M. phlei* for preabsorption of cross-reactive antibodies. In contrast, antibody reactions of Group I samples in EVELISA were substantially decreased upon using *M. phlei*. Strong inhibition of antibody reaction by *M. phlei* preabsorption was observed with 44 serum samples from two other dairy herds with suspected serological false-positive reactions (data not shown), indicating that the observed antibody binding inhibition with *M. phlei* may not be a herd-specific phenomenon. The reason for this difference is presently unknown. The antigens used in EVELISA were prepared by gently extracting extracellular molecules using 80% ethanol (Eda *et al.*, 2006), suggesting that the antigen is composed of molecules associated loosely with the surface of MAP. Although the information about the antigens used in ELISA-A is not available, it is plausible that protoplasmic antigens (PPA) were used as described in previous papers (Sockett *et al.*, 1992; Klausen *et al.*, 2003; Paolicchii *et al.*, 2003). PPA is a crude antigen mixture prepared by thorough disruption of mycobacterial bacilli followed by removal of cell debris and cell wall components (Beam *et al.*, 1969). Considering the process of PPA preparation, it is likely that the preparation contains intracellular proteins that are well conserved among mycobacterial species (Bannantine *et al.*, 2002; Li *et al.*, 2005). Therefore, a possible explanation for the difference in the effect of *M. phlei* on EVA-ELISA and ELISA-A results is that antigens used in the ELISA-A contained conserved proteins that cross-reacted with the false-positive sera, whereas EVA-ELISA antigens did not.

Conclusions

Assuming the sensitivity of ELISA-A is at most 70% (in this study, its sensitivity in fecal culture-positive animals was much lower at 48.5%), the sensitivity of the EVA-ELISA when applied to fecal culture-negative animals was shown to be higher than that of the ELISA-A using a Bayesian analysis (Branscum *et al.*, 2005). This study also indicates that the specificity of ELISA test for JD may be improved by using the EVA-ELISA, especially in herds where current ELISA tests show low specificity.

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Disclosure Statement

No competing financial interests exist.

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Antibiotic Manipulation of Intestinal Microbiota To Identify Microbes Associated with *Campylobacter jejuni* Exclusion in Poultry▽†

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The ability of various subsets of poultry intestinal microbiota to protect turkeys from colonization by *Campylobacter jejuni* was investigated. Community subsets were generated *in vivo* by inoculation of day-old poults with the cecal contents of a *Campylobacter*-free adult turkey, followed by treatment with one antimicrobial, either virginiamycin, enrofloxacin, neomycin, or vancomycin. The *C. jejuni* loads of the enrofloxacin-, neomycin-, and vancomycin-derived communities were decreased by 1 log, 2 logs, and 4 logs, respectively. Examination of the constituents of the derived communities via the array-based method oligonucleotide fingerprinting of rRNA genes detected a subtype of *Megamonas hypermegale* specific to the *C. jejuni*-suppressive treatments.

Campylobacter jejuni, a spiral, flagellated epsilonproteobacterial commensal of poultry, is the predominant cause of bacterial food-borne illness in the United States, resulting in approximately 2 million cases per year. A role for endogenous poultry intestinal microbiota in competitive exclusion (CE) of *Campylobacter* was first investigated in 1982 (38). Since then, numerous studies have attempted to identify microbes associated with *Campylobacter* CE. Suspensions of intestinal bacteria, isolated from *Campylobacter*-free adult poultry and passaged under strict anaerobic conditions, were found to protect chicks from colonization by the pathogen (31). Bacteria derived from the scrapings of broiler intestinal mucosa were proven more effective than the earlier fecal culture, a result not surprising, as *Campylobacter* is known to preferentially colonize cecal crypts (4, 39). The CE function of the bacterial suspensions decreased with time in storage, however (39, 40). Evidence also indicates that CE may depend on the presence of strictly anaerobic bacteria (31). As an oxygen gradient likely occurs from the host epithelium into the luminal contents, a CE role for both mucosal and luminal microbes in concert is likely.

Attempts have been made to identify specific microbes antagonistic to *Campylobacter*, and initial attempts isolated mucin-dwelling organisms with *in vitro* antagonistic effects against the pathogen (35, 36). Recent experiments have identified numerous bacterial groups producing anti-*Campylobacter* bacteriocins (29, 41, 42, 44, 45). Direct treatment of market-weight birds with the therapeutic bacteriocin *Enterococcus faecium* E 50-52 is effective for removal of *Campylobacter* spp. immediately prior to slaughter (44).

Despite progress toward a solution to contamination of poultry products by *Campylobacter* species, incomplete or intermittent CE protection, combined with a lack of studies addressing long-term CE efficacy, indicates that the *Campylobacter* colonization problem is far from solved (35). In addition, risk factors for campylobacteriosis other than direct consumption of contaminated poultry include consumption of fresh vegetables and bottled water (14). *Campylobacter* has been found in poultry manure used to fertilize crops as well as in runoff from these farms (22, 24, 50). We believe that novel approaches for studying microbial ecology in the gut are necessary for development of intervention strategies, including competitive exclusion.

The work described here takes a functional approach to identify microbes associated with protection of the intestine from *Campylobacter jejuni* colonization, an approach we are calling antibiotic dissection. The cecal contents from a *Campylobacter*-free adult turkey were inoculated into day-old poults and the microbial communities in these poults modified by treatment with therapeutic levels of antibiotics. The resulting modified microbiota were then tested for the ability to outcompete a *C. jejuni* challenge, and a microbe potentially associated with *C. jejuni* exclusion was identified.

MATERIALS AND METHODS

Antibiotic dissection. Beltsville White turkey poults were hatched from the *Campylobacter*-free USDA flock housed on the National Animal Disease Center campus, with four poults per treatment. On day 1 after hatch, an adult was culled from the Beltsville White flock. The adult turkey was anesthetized to surgical depth by intramuscular injection with telazol (6 mg/kg of body weight), ketamine (8 mg/kg), and xylazine (4 mg/kg), followed by decapitation. Ceca were removed, and cecal contents were mixed 1:1 (vol/vol) with 1× phosphate-buffered saline (PBS). Poults were randomly assigned into six treatment groups, the uninoculated control (UC), inoculated control (IC), virginiamycin (VIR)-treated, enrofloxacin (ENR)-treated, neomycin (NEO)-treated, and vancomycin (VNC)-treated groups (Table 1). Antibiotics were chosen for both relevance to the poultry industry and variety of target molecule, such that the 50S and 30S ribosomal subunits, as well as RNA polymerase (RNAP) and bacterial cell walls, were targeted (Table 1). In addition, the chosen antibiotics are known to target various combinations of aerobes, anaerobes, and Gram-positive and -negative

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TABLE 1. Treatment groups of poults inoculated with *Campylobacter*- free adult turkey intestinal contents and the antibiotics used to derive the six treatment groups[a]

Treatment group	Adult inoculum	Antibiotic target molecule	Sensitivity			
			Anaerobes	Aerobes	Gram-positive bacteria	Gram-negative bacteria
UC	−					
IC	+					
VIR	+	50S	S	R	S	R
ENR	+	RNAP	R	S	S	S
NEO	+	30S	R	S	R	S
VNC	+	Cell wall	S	R	S	R

[a] UC, uninoculated control; IC, inoculated control; VIR, virginiamycin; ENR, enrofloxacin; NEO, neomycin; VNC, vancomycin. Antibiotic targets and sensitivities are derived from references 8, 19, and 43. S, sensitive; R, resistant.

bacteria, further suggesting that antibiotic treatment would generate varied microbial communities (Table 1). Roughly 0.3 to 0.5 ml diluted adult cecal contents per poult was administered to five of the groups by oral gavage directly into the crop. A remaining control group (UC) was sham inoculated with 1× PBS. Poults were housed in BSL-2 isolators and given UV-irradiated feed and filter sterilized water *ad libitum*. The UC and IC treatment groups received sterile water and food only. In an effort to achieve therapeutic treatment levels, the VIR treatment group received sterile food containing 0.3 mg/g virginiamycin, the ENR group received sterile water containing 0.05 mg/ml enrofloxacin, the NEO group received sterile water containing 0.3 mg/ml neomycin sulfate, and the VNC group received sterile water containing 0.8 mg/ml vancomycin. The therapeutic dose of enrofloxacin was determined from Baytril product inserts. Therapeutic doses of virginiamycin and neomycin were determined from primary literature describing treatment of poultry for necrotic enteritis (18) and colibacillosis (30), respectively. Vancomycin was used at the same dose as avoparcin used to treat necrotic enteritis (33). Avoparcin was not used, as it was never approved for use in the United States, having been linked to vancomycin cross-resistance (51). Individuals within each isolator were assumed to have consumed equivalent antibiotic amounts. To maintain isolation, the birds in these experiments were not weighed; however, weights taken from a separate group of poults suggest these poults were ≤80 g. Antibiotic treatment was maintained for 7 days posthatch. Days 8 through 14 posthatch constituted a resting period allowing antibiotics to be cleared from the intestinal tract. On day 15, poults were challenged via oral gavage with 5×10^3 CFU of chloramphenicol-resistant *Campylobacter jejuni* 11168 (28). *C. jejuni* 11168 was kindly donated by Qijing Zhang from the Department of Veterinary Microbiology & Preventive Medicine, Iowa State University. On day 22 posthatch, poults were killed as described above. One cecum from each poult was frozen on dry ice for storage until DNA could be isolated, and serial dilutions were made in 1× PBS from the contents of the other cecum. All animal experiments were conducted with approval by, and under the guidance of, the National Animal Disease Center institutional animal care and use committee.

***Campylobacter* culture.** Serial dilutions of cecal contents were plated on TSB agar supplemented with 10% defibrinated horse blood, 30 μg/ml cephalothin, and 5 μg/ml chloramphenicol. Plates were microaerobically incubated (5% O_2, 10% CO_2, and 85% N_2) at 42°C for 48 h and *C. jejuni* colonies counted.

DNA extraction. DNA was isolated from 200 mg cross sections of the frozen ceca such that luminal, mucosal, and intracellular microbes would be represented in the libraries. A modified QBiogene Fast Prep method was used to prepare DNA (QBiogene, Carlsbad, CA). Microbial cells were lysed in Boom L6 buffer by shaking with lysing matrix A at 5 m/s in a Fast Prep FP120 device for 30 s as described previously (6). DNA from the lysates was purified using phenol-chloroform extraction and ethanol precipitation.

ARISA. Automated ribosomal intergenic spacer analysis (ARISA) was performed as described previously, using primer set ITSF (5′-GTCGTAACAAGG TAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) (7, 9, 16). Primer ITSReub was 5′ end labeled with the 6-carboxyfluorescein (FAM) fluorochrome (Operon, Valencia, CA). Each 20-μl PCR mixture contained 4 ng template DNA. Similarity matrices were generated using the Dice coefficient, and cluster analysis was performed with the Mega 4.0 software program via the unweighted-pair group method using average linkages (UPGMA) (46).

OFRG analysis. Oligonucleotide fingerprinting of rRNA genes (OFRG) analysis was performed as previously described by Valinsky et al. (48). Briefly, bacterial 16S rRNA gene clone libraries were constructed in *Escherichia coli* DH5α using a USER Friendly cloning kit (New England Biolabs, Ipswich, MA) and PCR primers 27F (5′-GGGAAAGUAGRRTTTGATYHTGGYTCAG-3′) and 1492R (5′-GGAGACAUGBTACCTTGTTACGACTT-3′) (5, 27). PCR was performed in 20-μl reaction mixtures containing 50 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates (dNTPs), 0.5 mg/ml bovine serum albumin (BSA), 400 nM forward and reverse primers, 1 μl fecal DNA, and 1.75 U *Taq* DNA polymerase. Amplification was performed with an initial denaturation at 94°C for 5 min, cycling at 94°C for 30 s (denaturation), 48°C for 40 s (annealing), and 72°C for 60 s (extension), and a final elongation for 2 min at 72°C. The number of amplification cycles varied between 15 and 30 cycles for each DNA sample and was determined from the fewest cycles found to generate a PCR product barely visible when 5 μl was examined on an ethidium bromide-stained agarose gel. A 1,536-clone library was generated, with roughly 250 clones representing each of the treatments.

The 16S rRNA gene clone library was PCR amplified using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCCTTAATTAAGCTGA-3′) and UserOFRGRev2 (5′-GCCAAGCTTCCTGCAGGGTTTAAACGCTGA-3′) in reaction mixtures containing 50 mM Tris (pH 8.3), 0.5 mg/ml BSA, 2.5 mM $MgCl_2$, 250 μM dNTPs, 400 nM forward and reverse primers, 1 μl cells, and 1.75 U *Taq* DNA polymerase (5). Amplification was performed with an initial denaturation at 94°C for 10 min, 35 cycles of 94°C for 1 min and 72°C for 2 min, and a final elongation for 5 min at 72°C. Amplicons were arrayed onto nylon membranes with a multiblot replicator (V&P Scientific, Inc., San Diego, CA) as described previously (48). Membranes were hybridized overnight at 11°C with a set of 10-nucleotide (nt) bacterium-specific ^{33}P-labeled DNA probes (48). Two arrays were hybridized for each probe, stripped as described previously, and rehybridized with universal probe 27F (5′-AGRRTTTGATYBTGGYTCAG-3′). Hybridizations were visualized using a Typhoon variable mode imager (Amersham Biosciences, Pittsburgh, PA), and hybridization signals were analyzed with Image Quant TL image analysis software, version 2003 (Amersham Biosciences, Pittsburgh, PA). Fingerprints containing the designations N (neither positive nor negative hybridization event), 1 (positive hybridization event), and 0 (negative hybridization event) were generated based on control clone hybridization intensities and Bayesian classification (25). Clone fingerprints containing more than 10 uncertain (N) classifications were discarded from further analysis. OFRG fingerprints were clustered using greedy clique partitioning (GCPAT) (http://alglab1.cs.ucr.edu/OFRG/gcpw.php).

Sequence analysis. Clone sequencing was performed using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCCTTAATTAAGCTGA-3′), UserOFRGRev2 (5′-GCCAAGCTTCCTGCAGGGTTTAAACGCTGA-3′), 530F (5′-GTGCCAGCMGCCGCGG-3′), and 907R (5′-CCGTCAATTCMTTT RAGTTT-3′) (5, 27). Sequences were assembled and edited using Lasergene software (DNASTAR, Madison, WI). The sequences were compared to those in public databases by use of NCBI BLAST and Ribosomal Database Project II (RDP-II) (11). *Megamonas hypermegale* sequences were aligned using ClustalX, and phylogenetic analysis was performed using the neighbor-joining algorithm in Mega 4.0 with 1,000 bootstrap replications (46, 47). Sequence similarities were determined using PHYLIP DNAdist and distance-based operational taxonomic unit and richness determination (DOTUR) (15, 34).

Real-time PCR. Real-time SYBR PCR amplification was performed with an iCycler IQ5 optical system according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Reaction mixtures were composed of 1× iQ SYBR green supermix and 400 nM each primer. *Megamonas*-specific primers were designed for this study using the PRISE software program (17). The *Megamonas* type I (5′-ACTAAAGGAGGCCTAGTC-3′) and type II (5′-TCTAAAGGAG GCCTCTGAA-3′) forward primers were paired with the reverse primer MhypR2 (5′-CCCTAACAACAGAACTT-3′). Thermocycling required an initial

TABLE 2. Estimated amounts of antibiotic ingested per day per bird

Treatment group	Amt of antibiotic (mg) ingested per bird on:					Wk 1 daily avg (mg)
	Day 3	Day 4	Day 5	Day 6	Day 7	
VIR	0.32	4.6	1.8	0.6	2.3	1.9
ENR	0.13	0.3	1.3	1.6	1.8	1
NEO	NA[a]	7.5	7.5	11.3	9.8	9
VNC	6.7	13.2	15.0	34.7	25.3	19

[a] Water consumption was not measured.

denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final elongation for 5 min at 72°C.

Species-specific amplification signals were normalized against the universal bacterial 16S copy number quantified using SYBR universal bacterial primers 27F (5′-AGRRTTTGATYBTGGYTCAG-3′) and 342R (5′-CTGCTGCSYCC CGTAC-3′) (27). Real-time PCR with the universal primers was performed with the *M. hypermegale* cycling parameters mentioned above, except a 56°C annealing temperature was used.

Intestinal response to antibiotic treatment. An experimental repeat (no *C. jejuni* colonization was obtained) generated intestinal samples checked for overt intestinal morphological changes due to antibiotic treatment. At necropsy, 1- to 2-cm lengths of ceca were removed from the animals and placed directly into 10% formamide. Tissue samples were allowed to fix for 6 h and then transferred to 70% ethanol. Cross sections of approximately 4 μm were processed in low-melting-point paraffin and stained with hematoxylin and eosin. Images were captured using a Nikon Eclipse e400 microscope with an Optronics Magnafire camera (Galeta, CA). Morphometric measurements of villus height and villus section area were determined with the aid of Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Measurements were taken for the four longest villi per animal, and means of villus height and area were determined for each animal and treatment.

Statistics. Student's *t* test, analysis of variance (ANOVA), Tukey's pairwise comparison, and Spearman's *D* nonparametric rank order test were performed using the Paleontological Statistics (PAST) software package for education and data analysis (20).

Nucleotide sequence accession numbers. Sequences were submitted to GenBank under accession numbers FJ440020 to FJ440103 and FJ489243 to FJ489251.

RESULTS

Antibiotic intake. Therapeutic antibiotic doses were achieved or exceeded for the virginiamycin (therapeutic dose of 0.04 g antibiotic/kg live bird weight), neomycin (therapeutic dose of 0.07 g/kg of live bird weight), and vancomycin (therapeutic dose of 0.02 g/kg live bird weight) groups (Table 2). Birds in the enrofloxacin (therapeutic dose of 0.05 g antibiotic/kg live bird weight) group consumed roughly a quarter of the therapeutic dose.

***Campylobacter* quantification.** *Campylobacter jejuni* levels in the ceca of 21-day-old poults were quantified by plate counts (Fig. 1). Virginiamycin-derived microbiota resulted in an 18-fold *C. jejuni* colonization increase over the level for the control treatments, whereas enrofloxacin-, neomycin-, and vancomycin-derived microbiota reduced *C. jejuni* colonization 1 log, 2 logs, and 4 logs, respectively (Fig. 1). ANOVA and Tukey's least significant difference (LSD) analyses indicated that the virginiamycin increase was statistically significant ($P < 0.001$).

ARISA results. Principal coordinates analysis of the microbiota detected four clusters (Fig. 2). One cluster was composed of samples from the IC, VIR, ENR, and NEO treatments. The other clusters were each composed of samples from individual treatments. The primary axis separated the IC, VIR, ENR, and NEO samples from the VNC, UC, and day-old-poult samples, indicating the greatest difference between these two groups. The secondary and tertiary axes separated the VNC, UC, and day-old-poult samples into different quadrants, indicating significant separation between the microbiota in these three groups (see Fig. S1 in the supplemental material).

OFRG results. Oligonucleotide fingerprinting of rRNA genes was used to analyze 1,536 16S clones from antibiotic

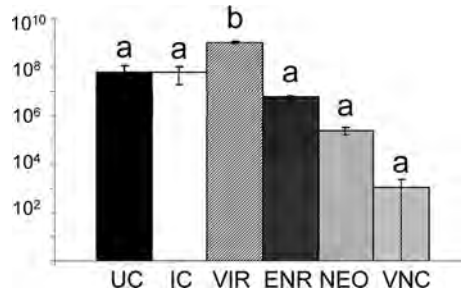

FIG. 1. *Campylobacter jejuni* plate counts from trial 2. Six treatments are represented: poults that received no adult cecal content (UC), poults that received adult cecal contents only (IC), and poults that received both cecal contents and therapeutic levels of one antibiotic, either virginiamycin (VIR), enrofloxacin (ENR), neomycin (NEO), or vancomycin (VNC). *C. jejuni* load was measured for four poults per treatment, with the exception of the VNC treatment (three poults). ANOVA generated an *F* statistic of 63.4 and a *P* value of <0.001. Tukey's least significant difference test identified two statistically significant groups (a and b), as noted above the data bars. Error bars indicate standard errors of the means.

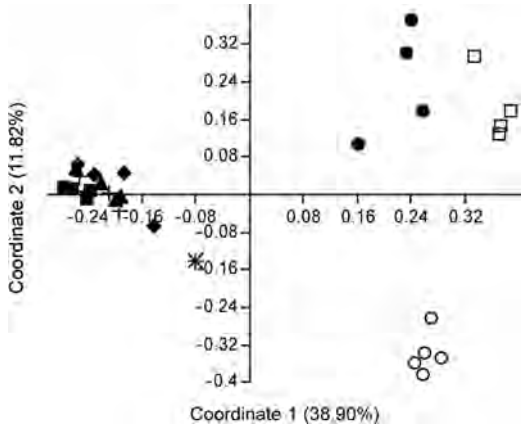

FIG. 2. ARISA fingerprint analyses of trials 1 and 2 combined. The Dice similarity index was used to estimate differences between fingerprints from 3-week-old poults. Treatments include the following: d1, poults killed at day 1 posthatch; UC, uninoculated controls; IC, controls inoculated with *Campylobacter*-free adult intestinal contents; ENR, inoculated and enrofloxacin-treated poults; VIR, inoculated and virginiamycin-treated poults; NEO, inoculated and neomycin-treated poults; and VNC, inoculated and vancomycin-treated poults. "Adult" indicates the *Campylobacter*-free adult intestinal inoculum. "Negative" indicates a no-template ARISA reaction. Principal component analysis was prepared from Dice similarity indices via the Paleontological Statistics software package. Microbiota from all 28 poults and the adult turkey inoculum are represented. ✳, adult; ○, day 1 posthatch; □, uninoculated control; +, inoculated control; ▲, virginiamycin derived; ■, enrofloxacin derived; ◆, neomycin derived; and ●, vancomycin derived. Alternate views of this graph are available in the Fig. S1 in the supplemental material.

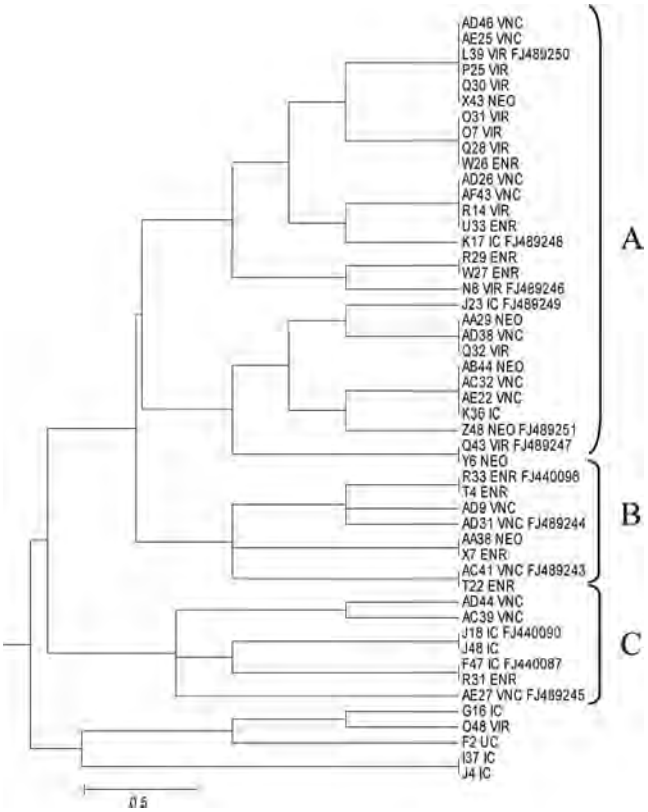

FIG. 3. Dendrogram generated from fingerprints of *Megamonas hypermegale* 16S genes cloned from six microbiota treatments. Fingerprints were clustered using the greedy clique partitioning algorithm. Labels consist of the clone identifier followed by the treatment designation. The sequenced clones display the appropriate GenBank accession number. Clusters B and C are composed of clones from *Campylobacter jejuni*-protective treatments. Cluster A includes *Megamonas* clones from all treatments. A full dendrogram of all 1,200 fingerprints is provided in Fig. S2 in the supplemental material.

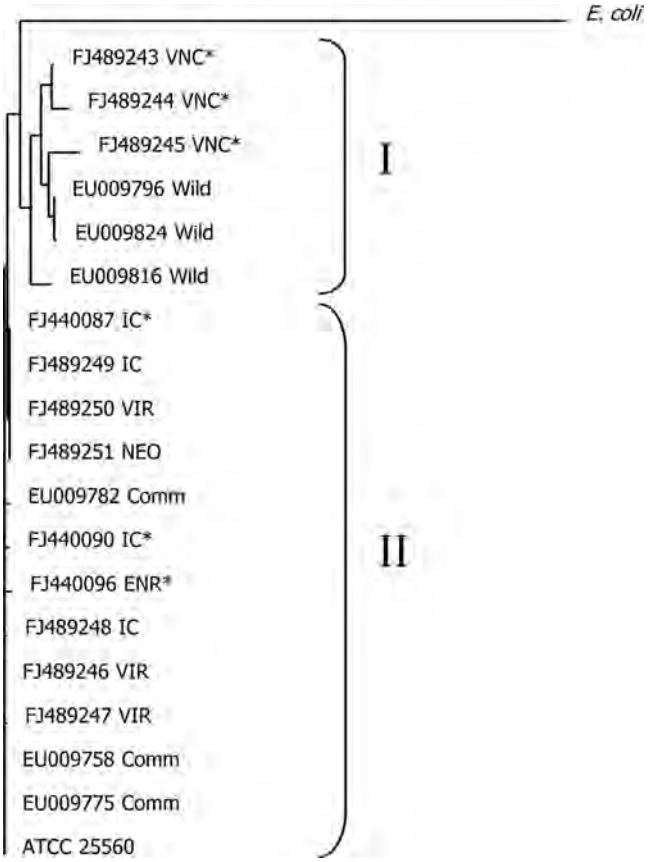

FIG. 4. *Megamonas hypermegale* sequence analysis. Type I, *Megamonas* 16S clones derived from the vancomycin treatment and isolated from wild turkeys in a previous publication. Type II, *Megamonas* clones derived from the inoculated control, virginiamycin, enrofloxacin, and neomycin treatments and isolated from 18-week-old commercially raised turkeys in a previous publication. Types I and II are different at the 97% sequence identity level, as determined by DOTUR analysis. Sequences denoted by an asterisk are derived from Fig. 3, clusters B and C.

dissection trial 2. Clones fell primarily into the *Clostridiales*, *Bacteroidetes*, and *Proteobacteria* taxa (see Fig. S2 in the supplemental material). Two small clusters were composed of clones from the *C. jejuni*-suppressive treatment but not the *C. jejuni*-conducive treatment. Sequence analysis indicated that the cluster was composed of *Megamonas hypermegale* 16S genes (Fig. 3, clusters B and C). An adjacent cluster (cluster A) was composed of 16S clones from both conducive and suppressive treatments. Sequence analysis of the *Megamonas* 16S clones identified two subtypes, type I, containing *Megamonas* clones from the VNC treatments, and type II, containing clones from the IC, VIR, ENR, and NEO treatments (Fig. 2 and 3). These two subtypes were distinct operational taxonomic units (OTUs) at the 97% sequence similarity level as determined by DOTUR analysis and had both previously been detected in turkey cecal microbiota (Fig. 4) (37).

***Megamonas hypermegale* quantification.** Real-time PCR was used to quantify *M. hypermegale* in the cecal contents (Table 3). *Megamonas* spp. were relatively abundant in all antibiotic dissection treatments that received the adult inoculum (Table 3). Type I and type II *Megamonas hypermegale* subtypes showed different responses to the antibiotic treatments, and Student's *t* test indicated significantly higher loads of *M. hypermegale* type II than of type I in all but the IC and VNC treatments (Fig. 4 and Table 3). Spearman *D* nonparametric rank order tests between *C. jejuni* and *Megamonas* type I ($P = 0.188$) and

TABLE 3. Quantification of *Megamonas hypermegale* types I and II in ceca of poults from six antibiotic dissection treatments

Treatment group	No. of real-time target genes/10,000 16S gene copies (SE)[a]		P^b
	M. hypermegale type I	*M. hypermegale* type II	
UC	0	2.2 (0.7) a	0.0300
IC	325 (188) a	5,493 (2,659) a	0.1006
VIR	13 (3) a	2,381 (542) a	0.0047
ENR	0 (0.01) a	2,282 (661) a	0.0136
NEO	32 (10) a	4,088 (1,212) a	0.0155
VNC	978 (164) b	93 (5.1) a	0.0058

[a] One-way ANOVA generated an *F* statistic of 13.55 and a *P* value of 0.0001 for *M. hypermegale* type I and an *F* statistic of 2.706 and a *P* value of 0.0563 for *M. hypermegale* type II. Tukey's least significant difference test identified two statistically significant groups (a and b).

[b] Student's *t* test.

between *C. jejuni* and *Megamonas* type II ($P = 0.323$) did not identify a correlation.

Morphometric measurements. Neither cecal villus height nor villus area differed significantly between treatments (data not shown).

DISCUSSION

In the current work, antibiotic treatment was used to generate subsets of the microbiota from a *Campylobacter*-free turkey flock, and these subsets were tested *in vivo* for competitive exclusion capability. The four antibiotics were chosen for their historic use in commercial poultry production and/or their various modes of action (Table 1). In addition, these antibiotics are controversial because of their potential influence on human health. Virginiamycin is a growth-promoting antibiotic commonly used in poultry production in the United States as well as a therapeutic treatment for necrotic enteritis and aflatoxosis (1, 2). However, virginiamycin also causes cross-resistance to quinupristin-dalfopristin, the drug of last resort that is used to treat patients with vancomycin-resistant *Enterococcus faecium* or methicillin-resistant *Staphylococcus aureus* infections (8, 12). Use of the antibiotic avoparcin for growth promotion was banned in Europe in 1997 because a statistically significant association exists between the use of the antibiotic in poultry production and the occurrence of vancomycin-resistant *Enterococcus* species (26). Subsequent to the ban, significant reductions have been observed in the poultry carriage rates of vancomycin-resistant enterococci (49). Enrofloxacin (Baytril) is a fluoroquinolone used to treat poultry for *Escherichia coli* and *Pasteurella* infections. Treatment of poultry with various fluoroquinolones, including enrofloxacin, induces ciprofloxacin cross-resistance in a variety of intestinal pathogens (23). Ciprofloxacin is the drug of choice for treatment of campylobacteriosis; however, recent increases in ciprofloxacin resistance are reducing the utility of this drug for efficacious treatment (13, 52).

C. jejuni colonization was inhibited to various levels by differently derived microbial subsets. Morphometric analysis of the gross structure of the cecal villi did not differ between treatments; however, fingerprint analysis of the antibiotic-derived consortia indicated that community composition was unique to each of the treatments (Fig. 2). Communities harbored by day-old poults, vancomycin-treated poults, and the 3-week-old uninoculated controls were all different from one another and very different from the other three antibiotic-derived communities (Fig. 2). As initial cecal communities are known to be dominated by lactobacilli, clostridia, and enterobacteria, principal component analysis (PCA) and clustering results are consistent with predicted vancomycin resistance shown by aerobes (53). In contrast, ARISA patterns from virginiamycin-derived communities clustered with the adult and inoculated control (IC) profiles, suggesting predominantly anaerobic communities (Fig. 2). This result was a bit unexpected, as virginiamycin and vancomycin are predicted to have similar ranges of activity (Table 1). However, the necessarily limited scope of antibiotic sensitivity testing for different bacterial species appears to have masked the true sensitivity ranges *in vivo*.

A repeat of the animal trial was performed to determine the repeatability of community selection using antibiotics *in vivo*. Fingerprint analysis of the antibiotic-derived consortia indicated that community composition was unique to each of the trials (see Fig. S3 in the supplemental material). It is not surprising to note that, independent of antibiotic treatment, ultimate microbiota composition is dependent on the initial community. This is demonstrated by the clustering of communities by experiment (e.g., clusters I and II).

In 1979, *M. hypermegale* was suggested as a competitive exclusion strain against *Salmonella enterica* (3). On the basis of 16S sequence, *M. hypermegale*, previously named *Bacteroides hypermegas*, was recently reassigned to the phylum *Firmicutes*, family *Acidaminococcaceae* (21, 32). *M. hypermegale* cells are large, up to 15 μm long, are obligately anaerobic, require fermentable sugars, and produce acetic and propionic acids (21). This analysis of the antibiotic-derived communities indicated two small clusters of *Megamonas hypermegale* 16S genes containing only clones from *C. jejuni*-suppressive treatments (Fig. 3, clusters B and C). DOTUR analysis of the *Megamonas* sequences indicated two subtypes at the 97% sequence identity level (Fig. 4). One type was composed of *M. hypermegale* sequences from the vancomycin-derived community, and the second type contained *M. hypermegale* sequences from the remaining treatments. The *Megamonas hypermegale* type strain, ATCC 25560, belongs to *Megamonas* type II (Fig. 4) (10). Examination of *Megamonas* sequences from a previous study indicated the presence of *Megamonas* type I in wild turkeys (GenBank accession no. EU009816, EU009796, and EU009824) and *Megamonas* type II in commercial birds (GenBank accession no. EU009775, EU009758, and EU009782) (Fig. 4).

To confirm whether *M. hypermegale* colonization correlated with *C. jejuni* suppression, real-time quantification was performed on the antibiotic-treated populations. *Megamonas* type I was significantly enriched in the VNC (*C. jejuni* suppressive) treatments compared to the levels for the other treatments, while *Megamonas* type II was (not significantly) suppressed (Table 3). Spearman *D* analysis of *C. jejuni* and *Megamonas* loads revealed no direct correlation. However, the analysis was performed across antibiotic treatments. If *Megamonas* is one of a group of organisms involved in CE, this correlation may have been obscured. Further perusal of the antibiotic dissection data, perhaps by combining trial results and metagenomic analysis, is needed to identify more microbes associated with *C. jejuni* exclusion. In addition, it will be necessary to isolate both *Megamonas* types and perform *in vivo* competition experiments against *C. jejuni*, as only the presence of *M. hypermegale* type I appears to correlate with *C. jejuni* suppression.

The preliminary results described here suggest a correlation between vancomycin-derived microbiota, including *M. hypermegale* type I, and *C. jejuni* suppression. In addition, a correlation is suggested between virginiamycin-derived cecal microbiota and the enhanced ability of *C. jejuni* to colonize (Fig. 1). In light of growth-promoting virginiamycin use on commercial farms in the United States, it will be interesting to determine whether type I *Megamonas* strains are virginiamycin sensitive.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Evaluation of the effects of *sdiA*, a *luxR* homologue, on adherence and motility of *Escherichia coli* O157 : H7

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Quorum-sensing (QS) signalling pathways are important regulatory networks for controlling the expression of genes promoting adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157 : H7 to epithelial cells. A recent study has shown that EHEC O157 : H7 encodes a *luxR* homologue, called *sdiA*, which upon overexpression reduces the expression of genes encoding flagellar and locus of enterocyte effacement (LEE) proteins, thus negatively impacting on the motility and intimate adherence phenotypes, respectively. Here, we show that the deletion of *sdiA* from EHEC O157 : H7 strain 86-24, and from a *hha* (a negative regulator of *ler*) mutant of this strain, enhanced bacterial adherence to HEp-2 epithelial cells of the *sdiA* mutant strains relative to the strains containing a wild-type copy of *sdiA*. Quantitative reverse transcription PCR showed that the expression of LEE-encoded genes *ler*, *espA* and *eae* in strains with the *sdiA* deletions was not significantly different from that of the strains wild-type for *sdiA*. Similarly, no additional increases in the expression of LEE genes were observed in a *sdiA hha* double mutant strain relative to that observed in the *hha* deletion mutant. While the expression of *fliC*, which encodes flagellin, was enhanced in the *sdiA* mutant strain, the expression of *fliC* was reduced by several fold in the *hha* mutant strain, irrespective of the presence or absence of *sdiA*, indicating that the genes *sdiA* and *hha* exert opposing effects on the expression of *fliC*. The strains with deletions in *sdiA* or *hha* showed enhanced expression of *csgA*, encoding curlin of the curli fimbriae, with the expression of *csgA* highest in the *sdiA hha* double mutant, suggesting an additive effect of these two gene deletions on the expression of *csgA*. No significant differences were observed in the expression of the genes *lpfA* and *fimA* of the operons encoding long polar and type 1 fimbriae in the *sdiA* mutant strain. These data indicate that SdiA has no significant effect on the expression of LEE genes, but that it appears to act as a strong repressor of genes encoding flagella and curli fimbriae, and the alleviation of the SdiA-mediated repression of these genes in an EHEC O157 : H7 *sdiA* mutant strain contributes to enhanced bacterial motility and increased adherence to HEp-2 epithelial cells.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157 : H7 is responsible for outbreaks of bloody diarrhoea, haemorrhagic colitis and haemolytic-uraemic syndrome (HUS) in humans (Nataro & Kaper, 1998). In addition to Shiga toxins that act on vascular endothelial cells to produce HUS (Karmali, 1989), EHEC O157 : H7 produces characteristic attaching and effacing (A/E) lesions on infected host epithelial cells (Francis *et al.*, 1986). The genes required for the formation of A/E lesions are encoded by the locus of enterocyte effacement (LEE), which contains 41 ORFs organized into operons *LEE1–LEE5* (Elliott *et al.*, 2000). The expression of the LEE operons is regulated by the LEE-encoded positive regulator Ler. Several positive and negative regulators have been shown to regulate *ler* expression. In previous studies, we have reported that Hha represses *ler* transcription, and that the deletion of *hha* increases expression of *ler* and *ler*-regulated *LEE1–LEE5* operons, thereby enhancing the adherence of the mutant strain to HEp-2 cells (Sharma & Zuerner, 2004; Sharma

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement; QRT-PCR, quantitative real-time polymerase chain reaction; QS, quorum sensing.

et al., 2005). In addition to LEE-mediated intimate adherence to epithelial cells, EHEC O157 : H7 presumably also uses surface appendages, such as flagella and fimbriae, in the initial stages of the adherence process. For example, recent studies have shown that flagella of EHEC O157 : H7 bind bovine intestinal mucos to promote intestinal colonization, and *fliC* deletion mutants exhibit reduced adherence to bovine intestinal tissues (Erdem *et al.*, 2007). Although EHEC O157 : H7 does not express type 1 fimbriae because of a 16 bp deletion in the *fim* switch (Roe *et al.*, 2001), it contains several other fimbrial operons, including the operons encoding curli and long polar fimbriae. Recent studies have implicated long polar fimbriae in adherence of EHEC O157 : H7 to epithelial cells, and intestinal colonization of ruminants (Torres *et al.*, 2007). Similarly, the expression of curli fimbriae has been shown to enhance adherence of EHEC O157 : H7 and O157 : NM strains to HEp-2 and Caco-2 cells, respectively (Rosser *et al.*, 2008; Uhlich *et al.*, 2002).

Quorum-sensing (QS) systems have emerged as important regulatory pathways in controlling the expression of multiple cellular functions in *E. coli* O157 : H7 such as flagellation, motility, and intimate adherence to epithelial cells (Reading *et al.*, 2007; Sperandio *et al.*, 2002, 2003). In some Gram-negative bacterial species, QS requires LuxR, a transcriptional factor that recognizes and binds to specific QS signalling molecules called autoinducers (Fuqua *et al.*, 1994). These autoinducers, which belong to a family of secreted molecules called acylhomoserine lactones, are synthesized via the pathways requiring LuxI-encoded autoinducer synthase (Fuqua *et al.*, 1994).

Like *E. coli* K-12 and *Salmonella*, EHEC O157 : H7 contains a LuxR homologue called SdiA, but lacks its cognate autoinducer-synthase-encoding gene (Henikoff *et al.*, 1990). Therefore, only a few studies have focused on the understanding of the biological significance of SdiA in controlling specific cellular functions in *E. coli* strains. For example, Kanamaru *et al.* (2000a) reported that over-production of SdiA from a high-copy-number plasmid reduces motility and the expression of some of the LEE-encoded genes of EHEC O157 : H7. Other studies have implicated *sdiA* as an important component of the regulatory cascade that governs the formation of biofilms by *E. coli* K-12 by regulating the expression of motility and chemotaxis genes in response to indole, which is a by-product of tryptophan metabolism (Lee *et al.*, 2007). However, types of regulatory controls exerted by the chromosomal copy of *sdiA* on the expression of genes, such as those encoded by the flagellar, fimbrial and LEE operons, which are essential for motility and adherence of EHEC O157 : H7 to epithelial cells, are not completely understood.

In this study, we describe the effects of *sdiA* deletion on the adherence of EHEC O157 : H7, with and without the gene *hha*, to HEp-2 cells, and correlate the degree of adherence of these mutants to the relative expression of LEE-encoded genes and the genes involved in the biosynthesis of flagella and fimbriae.

METHODS

Media and growth conditions. Bacterial strains were grown in either Luria–Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per litre of medium) or Dulbecco's modified Eagle's medium (DMEM), at 37 °C, with or without shaking (200 r.p.m.). Media were supplemented with appropriate antibiotics.

Construction of *sdiA* and *sdiA hha* mutants. EHEC O157 : H7 strain 86-24 Δ*stx2* Δ*lac* (Sharma & Zuerner, 2004) was used for construction of the mutants by using the lambda-Red-mediated recombineering method (Murphy & Campellone, 2003). Briefly, a 1.539 kb DNA fragment containing a 1.1 kb fragment encoding kanamycin resistance, flanked at its 5′ and 3′ ends with 254 bp and 175 bp sequences representing nucleotide regions immediately upstream and downstream of *sdiA*, respectively, was isolated from a recombinant plasmid using primers *sdiA*-F and *sdiA*-R (Table 1). This 1.539 kb fragment was purified from an agarose gel using a gel extraction kit, according to the manufacturer's instructions (Qiagen). The gel-purified fragment was electroporated into electrocompetent cells of EHEC O157 : H7 strain 86-24, and its *hha* mutant (Sharma & Zuerner, 2004) carrying plasmid pKM208; kanamycin-resistant colonies were isolated, and screened by PCR to confirm that *sdiA* had been deleted from the chromosome of the electroporated strain.

RNA isolation. An overnight culture of a bacterial strain grown in LB broth plus 100 μg streptomycin ml^{-1} was diluted 1 : 100 in DMEM, and incubated at 37 °C with shaking (200 r.p.m.). At an OD_{600} of approximately 1.0, the culture was mixed with 2 vols RNAprotect (Qiagen) by vortexing for 5 s, then incubated for 5 min at room temperature, and centrifuged at 5000 ***g*** for 10 min at 4 °C. Total RNA was isolated from cell pellets by using an RNeasy Mini kit, according to the manufacturer's instructions (Qiagen). RNA was electrophoresed in 1 % standard agarose gels using Tris/borate/EDTA buffer. Following electrophoresis, the gel was stained with ethidium bromide to make sure that the RNA appeared as a smear containing two prominent rRNA bands. Any contaminating DNA present in the purified RNA preparation was removed by DNase treatment using the TURBO RNase-free DNase kit, according to the manufacturer's instructions (Ambion). Purified RNA was checked for its concentration using a spectrophotometer (NanoDrop Technologies), and it was used as a template in a standard PCR (PE Biosystems) containing primers for *rpoA* amplification to verify that it was free of DNA contamination.

Quantification of gene expression by QRT-PCR. Quantitative real-time RT-PCR (QRT-PCR) was performed in 15 μl reaction volumes containing 2 μl (25 ng RNA) DNA-free RNA, 7.5 μl 2× QRT-PCR master mix, 0.6 μl RT/RNase block mixture (Stratagene), 0.225 μl 0.066 M reference dye ROX, 1.5 μl 1× SYBR Green (Molecular Probes), appropriate volumes of forward and reverse primers (Table 1), and RNase-free water. QRT-PCR was carried out in a single-step reaction format using Mx3005P (Stratagene) programmed to generate cDNA in one cycle of 30 min at 50 °C, followed by a single 10 min cycle at 95 °C, and 40 cycles of cDNA amplification (95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s). A comparative quantification option provided in the Mx3005P system set-up procedure was used for QRT-PCR data acquisition and analysis. The sample containing RNA from the parent strain was used as a calibrator to which all other RNA samples were compared, with respect to the expression of the target gene. To account for any variations in the amounts of RNA across samples, QRT-PCR

Table 1. Primers used in this study

Primer	Nucleotide sequence (5′–3′)	Location*
espA-F	ACGTCTTGAGGAAGTTTGGC	4662381–4662400
espA-R	ATCTAAAGCGTCAACCACGG	4662491–4662472
ler-F	GTAAACACCTTTCGATGAGTTCC	4688913–4688935
ler-R	GAGTCGATTCAGAAGCAGATTAC	4689001–4689023
rpoA-F	GGCTTGACGATTTCGACATC	4242887–4242906
rpoA-R	GGTGAGAGTTCAGGGCAAAG	4242997–4242978
fliC-F	TCGTCAAGTTGCCTGCATC	2700385–2700403
fliC-R	TTAGCTGCCACCCTTCATG	2700535–2700517
eae-F	TCTGTGTGGATGGTAATAAATTTTTG	4665647–4665671
eae-R	GTAAGTTACACTATAAAAGCACCGTCG	4665752–4665726
lpfA-F	CACCGTTAAGAGCGACCAGGG	4524871–4524891
lpfA-R	GAAGATTGCGATACCACCACG	4525036–4525016
csgA-F	GATGTTGGTCAGGGCTCAG	1548912–1548930
csgA-R	CCACCGAATTGTTTAACTGTC	1549033–1549013
fimA-F	TGTCCCTCAGTTCTACAGCG	5428049–5428068
fimA-R	TCCTAACTGAACGGTTTGATC	5428182–5428162
sdiA-F	CGCGTGATGTTAAAGATGGAAC	2693417–2693438
sdiA-R	CCTCGAGAAGTTTCTGCTGC	2694568–2694549
sdiA-F915	GTACGTCGACGACCAGGGGCGGATAGTC	2694628–2694646
sdiA-R864	GTACGTCGACTCAAATTAAGCCAGTAGCGG	2693592–2693611
lac-F824	GATCTCTAGAAGCATGATCTCGACTCGCTG	426579–426599
lac-R881	GATCTCTAGAGCGTTTATGCAGGGAATCGG	417736–417755

*The *E. coli* EDL933 nucleotide sequence (AE005174) was used for selection of these primer sequences.

amplification of each sample was normalized to samples containing primers for *rpoA* (Table 1). The passive reference dye ROX allowed compensation for non-PCR related variations in the fluorescence data acquisition. QRT-PCR data are presented as fold change in the expression level of the target gene relative to the expression level for the same gene in the calibrator, where the expression level of the calibrator is set to 1.0. Final data represent expression levels as means ($\pm$SEM) of the data collected from three independent experiments.

Determination of adherence to HEp-2 cells. Bacterial adherence to HEp-2 cells was performed by a previously described procedure (Sharma *et al.*, 2005). Briefly, fresh cultures (0.5 ml) of HEp-2 cells (5.0×10^4) were added to a well of a chamber slide (Nalge Nunc International), and incubated at 37 °C overnight in an atmosphere of 5 % CO_2 in air. The wells were washed three times in Dulbecco's PBS, followed by addition of 0.5 ml RPMI 1640 containing 1 % fetal bovine serum (Invitrogen), and 50 μl of an overnight bacterial culture prepared as follows: a few colonies of a bacterial strain grown overnight on LB agar were inoculated into LB broth, incubated at 37 °C on a shaker (200 r.p.m.) for 8 h, diluted 1 : 500 in fresh LB broth, and incubated overnight at 37 °C, without shaking.

The chamber slides were incubated at 37 °C for 3 h in an atmosphere of 5 % CO_2 in air. The chamber portion of chamber slide was removed, and the slide was washed four times in PBS, fixed for 1 min in 95 % ethanol, stained in toluidine blue for 15 s, washed in distilled H_2O, dipped in 95 % ethanol to fix the stain, washed briefly in water, and air-dried. A coverslip was mounted on the air-dried slide using VectaMount, and the slide was visualized under a microscope at ×100 magnification. Ten visual fields containing confluent HEp-2 cells were randomly selected for enumerating the number of bacterial cells adhered per HEp-2 cell. The adherence data are represented as the mean number of bacteria per HEp-2 cell in a given visual field obtained from three independent assays.

Determination of cellular morphology and motility. Bacterial wet mounts prepared from cultures grown in DMEM were examined, using a phase-contrast microscope at ×400 magnification, to determine any gross changes in cellular morphology, such as the appearance of filamentous cells as opposed to small rod-shaped bacterial cells typical of *E. coli* strains. For determination of bacterial motility, overnight cultures were centrifuged at 5000 ***g*** for 5 min, washed in PBS, and spotted (2 μl per spot) on motility agar plates, which were prepared by adding 0.32 % agarose to DMEM medium. The diameters of motility haloes were measured after incubation for 18 h at 37 °C.

Construction of *sdiA* complementation strains. The gene encoding *sdiA* was amplified by PCR from EHEC O157 : H7 strain 86-24 Δ*stx2* Δ*lac* using primers *sdiA*-F915 and *sdiA*-R864, which contain *Sal*I restriction sites at their 5′ ends (Table 1). These primers were used to amplify a 1.1 kb fragment containing the promoter and the ORF encoding SdiA. This 1.1 kb fragment was cloned into the *Sal*I site of a previously described temperature-sensitive plasmid (pSM80) used for deleting the *lac* operon in strain 86-24 (Sharma & Zuerner, 2004). The plasmid pSM80 containing the 1.1 kb *sdiA* gene fragment was introduced into *sdiA* and *sdiA hha* mutant strains by electroporation, and clones containing the *sdiA* gene in the *lac* region were selected by using a previously described allelic replacement procedure (Sharma & Zuerner, 2004). The insertion of the 1.1 kb fragment in the *lac* regions of *sdiA* and *sdiA hha* mutant strains was confirmed by PCR using primers *lac*-F824 and *lac*-R881 (Table 1).

Congo red binding and biofilm assays. The abilities of bacterial cells to bind the dye Congo red, and produce biofilms on plastic surfaces, were determined by the minor modifications of previously described procedures (Hammar *et al.*, 1996; Uhlich *et al.*, 2006a). For Congo red binding assays, Congo red and Coomassie brilliant blue dyes were used at 50 μg ml^{-1} and 6.25 μg ml^{-1}, respectively, in

YESCA agar medium. Bacterial strains grown overnight in LB broth were streaked onto this medium, and, following incubation at 28 °C for 48 h, the plates were checked for growth, and photographed under identical settings for exposure, background light and contrast to eliminate any digital biasing of the resulting images. For biofilm assays, 96-well polystyrene plates were inoculated with 200 µl of 1 : 100-fold diluted, overnight bacterial cultures grown in YESCA broth at 37 °C with shaking (175 r.p.m.). After incubation at 28 °C for 48 h, the plates were inverted on paper towels to drain the wells of the culture. The plates were heat-fixed, stained with 0.1 % crystal violet solution, and scanned at 590 nm to assess the amount of biofilm production. Biofilm production is given as the mean (±SEM) of three independent experiments.

Statistics. One-way ANOVA with Tukey–Kramer multiple comparisons test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software).

RESULTS

Deletion of *sdiA* enhances cellular adherence

As shown in Fig. 1, deletion of *sdiA* from EHEC O157 : H7 strain 86-24 enhanced adherence to HEp-2 cells twofold. Furthermore, deletion of *sdiA* from the *hha* mutant strain resulted in an additional twofold increase in bacterial adherence of the *sdiA hha* double mutant relative to the hyperadherent *hha* mutant strain, indicating that SdiA inhibits the adherence of EHEC O157 : H7 to epithelial cells.

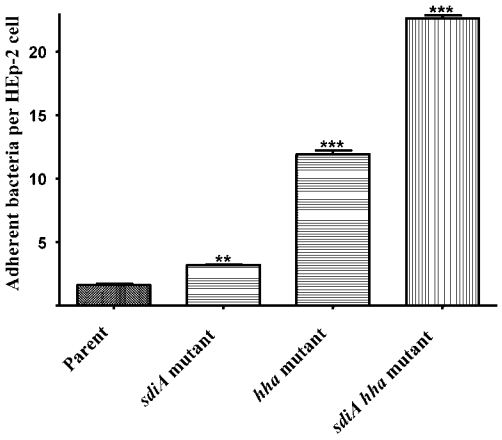

Fig. 1. Effect of *sdiA* on adherence of EHEC O157 : H7. Adherence of *sdiA* deletion mutants of EHEC O157 : H7 with or without *hha* was monitored by exposing HEp-2 cells to overnight bacterial cultures for 3 h at 37 °C. After washing and fixing, the HEp-2 cells were stained with toluidine blue and adherent bacteria were counted at ×100 magnification. Assays were performed in triplicate in which adherent bacteria were enumerated from 10 HEp-2 cells for each replicate. Error bars indicate SEM. The *P*-values, indicated as ** (<0.01) and *** (<0.001), are reported in relation to the adherence level exhibited by the parent strain.

Effect of *sdiA* on expression of LEE-encoded genes

In order to understand the genetic basis for the increased adherence of the *sdiA* deletion mutant strains to HEp-2 cells, we first analysed total RNA by QRT-PCR to determine expression levels for several LEE-encoded genes, as their increased expression has been shown to facilitate increased adherence to epithelial cells (Sharma & Zuerner, 2004; Sharma *et al.*, 2005). As shown in Table 2, *sdiA* deletion resulted in very low-level increases, which were determined to be statistically insignificant, in the expression of LEE-encoded *ler* (1.3-fold), *espA* (1.2-fold) and *eae* (1.6-fold), relative to the parent strain. However, in comparison, high levels of increase in expression observed for *ler* (15-fold), *espA* (22-fold), and *eae* (166-fold) in the *hha* mutant strain were independent of the presence or absence of the *sdiA* deletion, indicating that SdiA has either no or a very weak effect on the expression of LEE-encoded genes, in comparison with the stronger repression exerted by Hha on the same genes.

SdiA reduces flagellar and fimbrial gene expression

Several reports have described that the expression of flagellar and various fimbrial operons affects adherence of EHEC O157 : H7 to epithelial cells (Kim & Kim, 2004; Rosser *et al.*, 2008; Saldana *et al.*, 2009; Uhlich *et al.*, 2002). Therefore, we used QRT-PCR to examine the effects of *sdiA* deletion on the expression of *fliC*, *lpfA*, *csgA* and *fimA*, genes representing operons encoding flagella, long polar fimbriae, curli fimbriae and type 1 fimbriae, respectively. Since type 1 fimbriae are not expressed in EHEC O157 : H7 strains because of a 16 bp deletion in the *fimA* regulatory region (Roe *et al.*, 2001), the expression of *fimA* in *sdiA*, *hha* and *sdiA hha* mutant strains was expected to be identical to that of the parent strain. As shown in Table 2, *fliC* expression in the *sdiA* deletion mutant was significantly increased by 2.7-fold relative to the parent strain 86-24. On the other hand, *fliC* expression in the *hha* mutant strain was reduced by 5-fold compared with that in the parent strain. It was also apparent that the deletion of *sdiA* did not alter the expression of *fliC* in the *hha* deletion strain, suggesting that Hha is necessary for the expression of *fliC*, and the positive effect of *sdiA* deletion on *fliC* expression is evident in a hha^+ strain only. However, the expression of *lpfA*, the structural gene for the long polar fimbriae, in the *sdiA* (1.124-fold), *hha* (0.81-fold) and *sdiA hha* (0.934-fold) mutant strains was not significantly different from that of the parent (1.00-fold) strain. However, the expression of *csgA*, encoding the structural protein curlin of the curli fimbriae, was increased by approximately three- to fourfold in *sdiA* and *hha* deletion mutants relative to the parent strain. In the *sdiA hha* double mutant strain, the expression of *csgA* was increased by ninefold relative to the parent strain, and by two- to threefold relative to the *csgA* expression observed in either

Table 2. Effect of *sdiA* on the expression of adherence and motility genes

Strain	Relative change in gene expression*						
	LEE-encoded genes				Flagellar and fimbrial genes		
	ler	*espA*	*eae*	*fliC*	*lpfA*	*csgA*	*fimA*
Wild-type†	1.0 ± 0.06	1.0 ± 0.07	1.0 ± 0.11	1.0 ± 0.13	1.0 ± 0.08	1.0 ± 0.08	1.0 ± 0.15
sdiA mutant	1.3 ± 0.1	1.2 ± 0.07	1.6 ± 0.15	2.7 ± 0.29‡	1.12 ± 0.11	3.2 ± 0.33‡	0.98 ± 0.14
hha mutant	15.7 ± 0.9‡	21.8 ± 3.0‡	166.8 ± 12.8‡	0.14 ± 0.018‡	0.81 ± 0.16	4.5 ± 0.37‡	0.69 ± 0.13
sdiA hha mutant	16.0 ± 0.18‡	28.0 ± 2.3‡	120 ± 10.0‡	0.16 ± 0.02‡	0.93 ± 0.13	8.85 ± 0.67‡	0.86 ± 0.11

*The relative change in expression of each gene was computed from three independent experiments, and by testing each gene for its expression in duplicate. Values are means ± SEM.
†Expression values for each gene in the wild-type strain were set to 1 in order to determine the relative change in the expression of the same genes in the mutant strains.
‡Significant values ($P<0.05$).

sdiA or *hha* deletion mutants. The increased expression of *csgA* in *sdiA* and *hha* deletion mutants indicates that both SdiA and Hha exert a negative effect on the expression *csgA*. As expected, the expression of *fimA*, encoding the major structural protein FimA of type 1 fimbriae, was not significantly different between the parent strain (1.00-fold) and *sdiA* (0.98-fold), *hha* (0.68-fold) and *sdiA hha* (0.86-fold) deletion mutant strains.

sdiA deletion affects bacterial motility, but has no effect on cellular morphology

Kanamaru *et al.* (2000a) have shown that the expression of SdiA from a high-copy-number plasmid not only results in the appearance of elongated cells due to abnormal cell division, but also reduces the motility of EHEC O157 : H7. However, examination of the wet mounts of *sdiA*, *hha* and *sdiA hha* mutants under a phase-contrast microscope showed the presence of small, rod-shaped cells that are considered typical of *E. coli* strains (data not shown). On the other hand, visual examination of soft-agar motility plates revealed that motility of the *sdiA* mutant was increased by approximately 30 % relative to the parent strain, indicating that SdiA exerts a negative effect on the motility of EHEC O157 : H7 (Fig. 2). It was also apparent that the positive effect of *sdiA* deletion on motility was dependent on the presence of *hha*, since the motility of the *sdiA hha* double mutant was reduced to the levels that were observed for the *hha* mutant strain (approximately 35 % lower than the parent strain).

sdiA deletion increases Congo red binding by bacterial cells

It has been reported that the ability of *E. coli* strains to bind Congo red is affected by the levels of curli fibres expressed at the bacterial cell surface (Kim & Kim, 2004; Rosser *et al.*, 2008; Uhlich *et al.*, 2006b; Vidal *et al.*, 1998). Since the deletion of *sdiA* enhanced the expression of *csgA* (the gene encoding major structural component of curli fimbriae) in a *hha*-independent manner, we wanted to determine if an increased expression of *csgA* in strains deleted of *sdiA* would enable them to bind increased amounts of Congo red. In addition, we inserted a single copy of the wild-type *sdiA* gene in the *lac* region of the chromosome of *sdiA* and *sdiA hha* mutant strains to determine whether SdiA would complement the abilities of these strains to bind Congo red

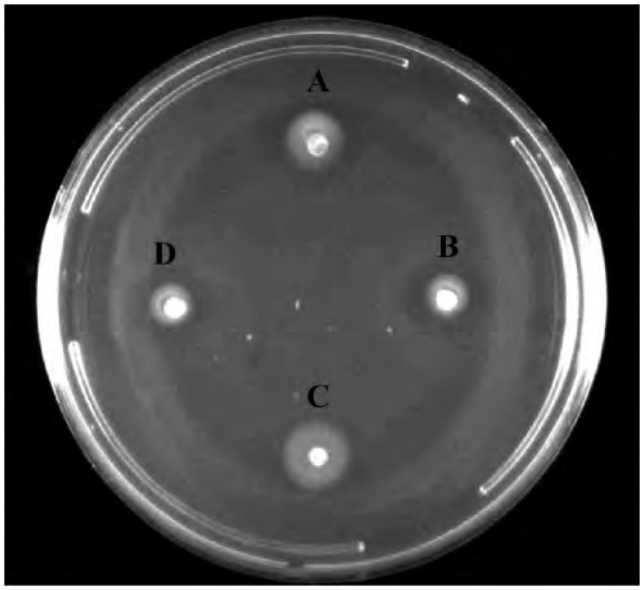

Fig. 2. Effect of *sdiA* deletion on the motility of EHEC O157 : H7. Each strain was spot inoculated onto soft DMEM agar. The diameters of the motility haloes (means ± SEM of three independent experiments) were as follows: A (parent), 9.38 ± 1.21 mm; B (*hha* mutant), 6.33 ± 0.73 mm; C (*sdiA* mutant), 12.28 ± 1.25 mm; D (*sdiA hha* mutant), 6.04 ± 0.82 mm. The difference in the motility diameter for each mutant strain was determined to be significant ($P<0.05$) relative to the parent strain.

in amounts comparable with those bound by the *sdiA*$^+$ or *sdiA*$^+$ *hha* strains, respectively. Visual examination, comparing growth of *sdiA* (Fig. 3a, panel C) and *sdiA hha* (Fig. 3b, panel C) mutants on Congo red medium, revealed the presence of dark-pink colonies with dark-red centres compared with the light-pink colonies with red centres (fish-eye appearance) produced by the *sdiA*$^+$ (Fig. 3a, panel A) and *sdiA*$^+$ *hha* (Fig. 3b, panel A) strains. Complementation of the *sdiA* (Fig. 3a, panel B) and *sdiA hha* (Fig. 3b, panel B) mutant strains with SdiA decreased Congo red binding to a level similar to that observed for colonies of the *sdiA*$^+$ (Fig. 3a, panel A) and *sdiA*$^+$ *hha* (Fig. 3b, panel A) strains.

Reduction of biofilm formation by *sdiA* in EHEC O157 : H7

The formation of biofilms in *E. coli* is influenced by motility and the expression of fimbrial structures, which are required for reversible and irreversible phases of adherence during the genesis of biofilms, respectively (Kim & Kim, 2004; Pratt & Kolter, 1998; Ryu & Beuchat, 2005; Wood *et al.*, 2006). Since we determined that the *sdiA* deletion caused increased expression of *fliC* and *csgA*, which correlated positively with enhanced motility and increased Congo red binding, we hypothesized that the *sdiA* deletion mutant might show increased biofilm production. As shown in Fig. 4, the amount of biofilm produced by the *sdiA* deletion mutant was only 1.56-fold higher than that produced by the *sdiA*$^+$ parent strain. On the other hand, the amount of biofilm produced by the *hha* mutant was 3.56-fold higher than the *sdiA*$^+$ parent and 2.3-fold higher than the *sdiA* deletion mutant strains. The deletion of *sdiA* in the *hha* deletion mutant caused only a small increase (1.22-fold) in biofilm formation over the *hha* mutant strain, suggesting that SdiA exerts effects of lower magnitude than Hha on the production of biofilms in EHEC O157 : H7. The fact that the *hha* deletion mutant displayed reduced expression of *fliC* (Table 2), reduced motility (Fig. 2), and increased *csgA* expression (Table 2) suggests that the repression of motility and increased expression of curli fimbriae are necessary for increased biofilm formation by EHEC O157 : H7. Thus, the increased motility caused by deletion of *sdiA* might be responsible for preventing enhanced biofilm formation by the *sdiA* mutant strain, despite its ability to express higher levels of *csgA* and increased Congo red binding compared with the *sdiA*$^+$ parent strain.

DISCUSSION

In this study, we have demonstrated that the deletion of a single chromosomal copy of *sdiA* significantly increases the

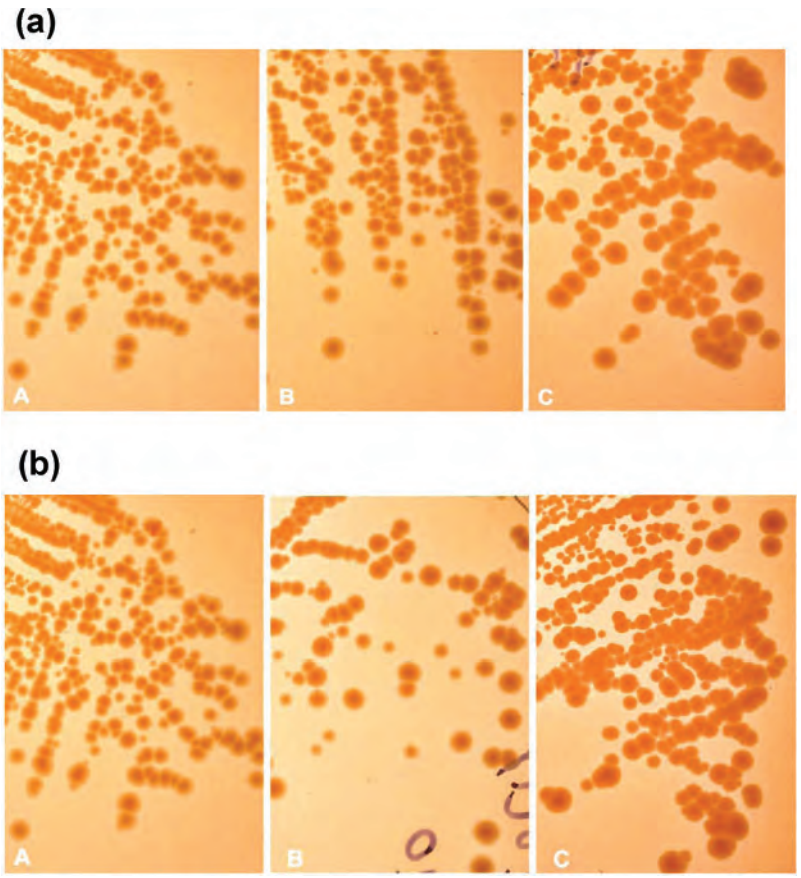

Fig. 3. Phenotypic production of curli on medium containing Congo red. Overnight bacterial cultures were streaked on YESCA agar containing Congo red. After incubation for 48 h at 28 °C, the plates were photographed to capture coloured phenotypes of colonies that grew on these plates. (a) Coloured phenotypes of the colonies produced by the parent strain (panel A), *sdiA* deletion mutant complemented with a wild-type copy of *sdiA* (panel B), and *sdiA* deletion mutant (panel C). (b) Coloured phenotypes expressed by the parent strain (panel A), *sdiA* complemented *sdiA hha* deletion mutant (panel B), and the *sdiA hha* deletion mutant (panel C).

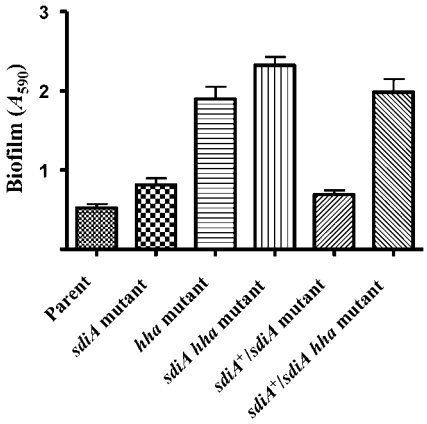

Fig. 4. Evaluation of biofilm formation in polystyrene plates. Overnight bacterial cultures were diluted 1 : 100 in YESCA broth and pipetted into the wells of a 96-well polystyrene plate. Following 48 h of incubation at 28 °C, the amount of biofilm produced was determined by crystal violet staining as described in Methods. Biofilm production is shown as the mean ± SEM of three independent experiments.

adherence of EHEC O157 : H7 and its isogenic *hha* mutant strain to HEp-2 cells, suggesting that SdiA exerts a negative effect on the adherence phenotype. These findings are in agreement with the results reported in a previous study showing that the expression of *sdiA* from a high-copy-number plasmid causes a dramatic reduction in the ability of EHEC O157 : H7 to adhere to epithelial cells (Kanamaru *et al.*, 2000a). In addition, Kanamaru *et al.* (2000a) demonstrated that an increased expression of *sdiA* from a high-copy-number plasmid results in abnormal cell division. However, deletion of *sdiA* from the EHEC O157 : H7 chromosome, as we have described in this study, had no affect on cellular morphology.

Unlike most of the known bacterial QS systems that activate virulence gene expression in EHEC O157 : H7 (Sperandio *et al.*, 1999, 2002), SdiA-mediated QS had been shown to repress transcription of the flagellar gene *fliC*, and the virulence genes *espD* and *eae* encoded by the *LEE4* and *LEE5* operons, respectively (Kanamaru *et al.*, 2000b). However, these negative effects of SdiA were demonstrated upon increased expression of *sdiA* from a high-copy-number plasmid. By using QRT-PCR, we demonstrated very low levels of increase in the expression of LEE-encoded genes *ler, espA* and *eae* in the *sdiA* deletion strain compared with the high levels of increase observed for these genes in the *hha* mutant strain. These low levels of increase in the expression of LEE-encoded genes observed in our study were statistically insignificant compared with the effect of SdiA reported for the repression of LEE-encoded genes in the study by Kanamaru *et al.* (2000a). Thus, the lack of a significant effect on the expression of LEE-encoded genes in the *sdiA* mutant in our study may be due to the reduced intracellular amounts of SdiA produced from a single chromosomal copy of *sdiA*. For example, it has been postulated that the majority of SdiA expressed from the chromosomal copy of *sdiA* is a monomer, and that expression of SdiA from a high-copy-number plasmid increases the levels of SdiA dimers, which are required for repressing transcriptional activities of the target gene promoters (Henikoff *et al.*, 1990; Michael *et al.*, 2001). The proposal of a repressor function of SdiA is supported by the presence of a helix–turn–helix DNA-binding motif, and, like most repressors, SdiA dimerizes or oligomerizes to affect the repression of their target gene promoters (Burz & Ackers, 1994; Burz *et al.*, 1994; Harrison & Aggarwal, 1990; Sharma *et al.*, 1998). Thus, the highly impaired adherence of EHEC O157 : H7 reported by Kanamaru *et al.*, (2000a) could very well have resulted from an increased intracellular availability of SdiA, so that concentrations of dimers reached levels that were high enough to inhibit transcriptional activities initiated from the affected promoters of the *LEE4* and *LEE5* operons.

Several studies have shown that the ability of EHEC O157 : H7 to produce flagella and certain types of fimbriae enhances its adherence to epithelial cells in animal models and in tissue culture assays (Erdem *et al.*, 2007; Torres *et al.*, 2007; Uhlich *et al.*, 2002). In the current study, we found that the strain deleted of *sdiA* showed increased transcriptional levels of *fliC* and enhanced motility compared with the parent strain. The overall smaller sizes of motility haloes produced in our studies could be attributed to the use of a minimal medium containing a higher percentage of agar (0.32 %). For example, strain 86-24 has been used by other investigators to examine the impact of QS signalling molecules (epinephrine and norepinephrine) on its motility (Sperandio *et al.*, 2003). In those studies, the sizes of motility haloes produced by strain 86-24 in the absence of epinephrine or norepinephrine were not substantially different from the ones observed in our studies, despite the fact that the motility haloes in those studies were determined on media containing a lower percentage of agar (0.25 %). Although the negative effects of SdiA on *fliC* expression and/or on motility were qualitatively similar to those reported for a strain of EHEC O157 : H7 producing high levels of SdiA (Kanamaru *et al.*, 2000a; Wei *et al.*, 2001), the negative effect of SdiA on *fliC* expression was only apparent in the strain wild-type for *hha*, because strains deleted of *hha* showed reduced expression of *fliC*, irrespective of the presence or absence of *sdiA*. These findings suggest that Hha plays a positive role, via an unknown mechanism, in the expression of *fliC*. Deletion of *hha* resulted in reduced *fliC* expression (and reduced bacterial motility), but enhanced expression of LEE-encoded genes, and bacterial adherence to HEp-2 cells; these findings are similar to those reported in a previous study (Sharma *et al.*, 2005), and suggest that the enhanced *in vitro* adherence of *hha* or *sdiA hha* mutants to HEp-2 cells may not require optimal expression of flagella. Since biosynthesis of both flagella and the type III secretion system requires investment of large amounts of cellular

resources, it is reasonable to assume that an increase in the expression of the type III secretion system in the *hha* mutant might compensate for the reduced production of flagella during bacterial adherence to HEp-2 cells.

Unlike the negative effect of SdiA on *fliC* gene expression that was apparent only in a *hha*-positive strain, the negative effect of SdiA on the expression of *csgA*, the gene encoding curlin of the curli fimbriae, was of a similar magnitude in strains with or without *hha*. However, the *sdiA hha* double mutant had the highest level of *csgA* expression, indicating an additive effect on *csgA* expression due to deletion of *sdiA* and *hha*. In light of a previous report demonstrating that a highly curliated strain of EHEC O157 : H7 displays a highly aggregated adherence pattern on HEp-2 cells, as opposed to the more localized adherence pattern observed for the lesser curliated strain (Kim & Kim, 2004), the increased expression of *csgA* might be one of the major factors, considering that the expression of LEE-encoded genes was not significantly affected by *sdiA*, responsible for the enhanced adherence of *sdiA* and *hha* mutant strains to HEp-2 cells. The enhanced expression of *csgA* contributing to an increased adherence of *sdiA* mutant strains to HEp-2 cells was corroborated by the ability of these strains to bind increased amounts of Congo red, and to produce slightly higher levels of biofilm than the strains with a wild-type *sdiA* gene. Since increases in Congo red binding and biofilm production are indicative of enhanced curli production (Kim & Kim, 2004; Uhlich *et al.*, 2006a), a reduction in the levels of Congo red binding and biofilm production by the *sdiA* mutant strains via complementation with a wild-type copy of *sdi*A provided additional evidence for the negative role of SdiA in *csgA* transcription and curli production. These results are corroborated by a study that utilized a whole-transcriptomic approach to show that SdiA represses curli formation in *E. coli* K-12 (Lee *et al.*, 2009).

Interestingly, *sdiA* deletion resulted in increased *fliC* expression and enhanced motility, but only a small increase in the amount of biofilm formation compared with larger increases in the levels of biofilm production in *hha* and *sdiA hha* deletion mutants. These results indicate that while the increased production of curli promotes adherence to HEp-2 cells, increased motility, on the other hand, reduces the ability of the *sdiA* mutant strains to produce biofilms. These findings suggest that in EHEC O157 : H7 strain 86-24, unlike other *E. coli* strains (Barrios *et al.*, 2006; Pratt & Kolter, 1998; Wood *et al.*, 2006), motility inhibits biofilm formation on abiotic surfaces, whereas increased production of curli allows increased adherence to both abiotic and biotic surfaces in motility-compromised *hha* mutant strains.

It is also apparent from the data presented in this report that SdiA serves as a strong repressor for the transcriptional regulation of *fliC* and *csgA*, and that it appears to have no effect on the expression of *lpfA* and *fimA*. Moreover, the additive effects of SdiA and Hha on the expression of *csgA* appears to suggest that these two repressor proteins either bind to independent sites in the *csgA* promoter region, or the binding of the one repressor protein precedes the binding of the other repressor to the same promoter-binding site to cause effective repression of the *csgA* promoter.

In conclusion, our study provides direct genetic evidence that expression of a single chromosomal copy of *sdiA* represses multiple phenotypes, such as flagellation, fimbriation, motility and adherence in EHEC O157 : H7. Based on the data provided in this report, SdiA exerts negative effects of varied magnitudes on different sets of genes. For example, the expression of LEE-encoded genes was not significantly affected compared with the expression of *fliC* and *csgA*, which were strongly repressed. Although *in vitro* gel-shift assays have shown that purified SdiA binds to its target promoters with the same affinity (Kanamaru *et al.*, 2000a), the lack of a consensus SdiA-binding sequence in the SdiA-regulated promoters might determine the relative affinities of these promoters for SdiA *in vivo*. Since EHEC O157 : H7, like *E. coli* K-12 and *Salmonella* spp., encodes *sdiA*, but lacks the ability to synthesize acylhomoserine lactones, which are the natural inducers of SdiA, understanding the nature of signals influencing expression or repressor activity of SdiA is important for clarifying the role of SdiA in virulence gene expression in these bacterial species. One potential signal could be indole, the metabolic by-product of tryptophan metabolism, which regulates biofilm formation by influencing the activity of SdiA (Lee *et al.*, 2007). Thus, correlating the expression of indole with SdiA effects on virulence gene expression might provide greater insight into the mechanism of SdiA-mediated regulation of gene expression in EHEC O157 : H7.

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Review

Molecular epidemiology of PRRSV: A phylogenetic perspective

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ABSTRACT

Since its first discovery two decades ago, porcine reproductive and respiratory syndrome virus (PRRSV) has been the subject of intensive research due to its huge impact on the worldwide swine industry. Thanks to the phylogenetic analyses, much has been learned concerning the genetic diversity and evolution history of the virus. In this review, we focused on the evolutionary and epidemiological aspects of PRRSV from a phylogenetic perspective. We first described the diversity and transmission dynamics of Type 1 and 2 PRRSV, respectively. Then, we focused on the more ancient evolutionary history of PRRSV: the time of onset of all existing PRRSV and an origin hypothesis were discussed. Finally, we summarized the results from previous recombination studies to assess the potential impact of recombination on the virus epidemiology.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes clinical syndromes typified as reproductive disorders in sows, and post-weaning pneumonia and growth reduction in piglets, namely the "porcine reproductive and respiratory syndrome" (PRRS). PRRS disease was first recognized during the almost concurrent epidemics in the United States (late 1980s) (Hill, 1990; Keffaber, 1989) and in Europe (early 1990s) (Wensvoort et al., 1991). Since then, the virus became endemic at the global level, with occasional outbreaks of more severe forms of PRRS that included sequelae of morbidity and mortality. Up until now, the virus still remains difficult to control despite the availability of several vaccines.

PRRSV is classified in the order *Nidovirales*, family *Arteriviridae* along with equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (Conzelmann et al., 1993; Meulenberg et al., 1993). It possesses a positive-sense RNA genome of approximately 15 kb in length, which encodes at least nine open reading frames (ORF), includes ORF1a, 1b, 2a, 2b, and 3–7 (Meulenberg et al., 1993; Wu et al., 2001). Among them, ORF5 encoding the major envelope protein is often used for phylogenetic analyses mainly because of its high variability. Regarding genetic diversity, PRRSV can be divided into two genotypes: Type 1, mainly comprised of viruses from Europe; and Type 2, mainly comprised of viruses from North America. Surprisingly, despite of their almost concurrent emergence and similar disease syndromes, the two genotypes differ greatly in terms of their genetic content (Mardassi et al., 1994; Murtaugh et al., 1995; Nelsen et al., 1999) and antigenic properties (Drew et al., 1995; Wensvoort et al., 1992).

Currently, there has been an accumulation of studies which utilized molecular sequence data to characterize various aspects of the virus, mostly focused on its epidemiology and evolution. As a result, our understanding of the genetic diversity and epidemiology of both types of PRRSV has been constantly changing. Many previous perspectives of PRRSV have been challenged. For example, the original idea of Type 1 PRRSV as a newly emerged homogeneous group has been challenged by the view that it is a highly diverse group whose history might be traced back to the post World War II period (Stadejek et al., 2006). Therefore, the majority of this review aims to provide the most updated knowledge of the genetic diversity and epidemiological characteristics of both types of PRRSV. Moreover, phylogenetic analyses using molecular clock models suggested that PRRSV might actually be an "ancient virus" in spite of its recent emergence. Thus we also examine the possible evolutionary history of the PRRSV prior to the emergence of Type 1 and 2 viruses. Lastly, the impact of recombination on PRRSV epidemiology is reviewed.

2. Diversity and evolution of Type 1 PRRSV

2.1. "Original" epidemic

The earliest record of a major Type 1 PRRSV epidemic was documented in the early 1990s in Western Europe (Wensvoort et al., 1991). During the epidemic, the first PRRSV (Lelystad virus) was isolated in The Netherlands and was regarded as the prototype of Type 1 PRRSV, which tended to form a highly homologous phylogenetic cluster (Lelystad-like) with contemporaneous samples from Belgium, France, Germany, Great Britain, Netherlands and Spain (Drew et al., 1997; Forsberg et al., 2002; Le Gall et al., 1998; Suarez et al., 1996). However, this Lelystad-like cluster could barely represent the diversity of Type 1 PRRSV, nor could it be viewed as the ancestral virus type from which most of the current Type 1 PRRSV derived until a broader survey was performed. A clearer picture of Type 1 PRRSV diversity was gradually uncovered as the sampling range expanded in Europe (Balka et al., 2008; Forsberg et al., 2002; Indik et al., 2005, 2000; Mateu et al., 2003; Oleksiewicz et al., 2000; Pesch et al., 2005; Stadejek et al., 2006, 2008, 2002). Although most of these samples were obtained after the isolation of Lelystad-like viruses, these samples formed well-supported clades in the phylogeny (e.g. Clades B–L, Subtype II and III; Fig. 1) that had diverged prior to the emergence of the epidemic clade (Clade A, Fig. 1), indicating they were not likely to be "progenies" of Lelystad-like PRRSV. In fact, the divergence of Lelystad-like clade (Clade A) from its neighboring European clades (Clades B–E) might be a relatively recent event, as suggested by the well-supported interior branching order in the phylogeny (Fig. 1). Therefore, the evidence suggested Type 1 PRRSV viruses had been present in the field long before the "original" epidemic in Western Europe. The lack of recorded data concerning the prevalence of PRRSV prior to the early 1990s epidemic is probably due to unawareness of this etiologic agent.

2.2. Phylogeography and transmission dynamics of Type 1 PRRSV in Western Europe

In Western Europe, Type 1 PRRSV was the cause of PRRS outbreaks. The monitoring of individual herds indicated both introduction of new variants and persistence of existing variants as important factors shaping the intra-herd diversity or causing a succession of disease outbreaks (Balka et al., 2008; Greiser-Wilke et al., 2009; Indik et al., 2000; Kiss et al., 2006; Pesente et al., 2006; Stadejek et al., 2008). A similar picture was suggested at the country level, where the large diversity seen was most likely caused by viral transmission from foreign countries through intensive porcine trade within the European Union, or the distribution of live-attenuated vaccines, as well as temporal evolution of local viruses (Balka et al., 2008; Greiser-Wilke et al., 2009; Indik et al., 2005; Mateu et al., 2006; Pesch et al., 2005; Prieto et al., 2009). Such a pattern is supported by the Type 1 PRRSV phylogeny, based on the entire collection of currently available Type 1 ORF5 sequences. Within each of the major clades, possible inter-country transmissions of viruses were observed, as evidenced by the heterogeneous geographical components of the clades (Fig. 1). However, an exception to this pattern was observed in several Italian clades (Clades K and L in Fig. 1) that diverged earlier from the rest of the Western European clades. The Italian clades are characterized by strong geographical structuring despite their relatively high diversity, which was also indicated by Forsberg et al. (2002). Nevertheless, in a phylogeny constructed by Pesch et al. (2005), several German, Polish and Dutch sequences were found to be grouped within the highly diverse Italian clade, implying its potentially complex geographical origin similar to the other Western European clades. Unfortunately, we were not able incorporate this information into our phylogeny due to unavailability of the sequences used in Pesch et al.'s study.

Comment on the transmission dynamics of Type 1 PRRSV in Western Europe is not possible at this time due to inadequate information. To generate insights valuable for disease control and prevention, it is important to further characterize the entire virus transmission network (e.g. compare the inter-country transmission frequencies) and examine its relationship with swine production and transportation routes in Europe. Such work should be based upon a more complete sequence collection representative of European diversity, and systematic investigation of import and export patterns of swine in the market.

2.3. Type 1 PRRSV in Eastern Europe

The sequencing and characterization of PRRSV isolated in countries to the east of Poland have provided ground-breaking information on the epidemiology and evolution of Type 1 PRRSV (Stadejek et al., 2006, 2008, 2002). In Belarus, Lithuania and Rus-

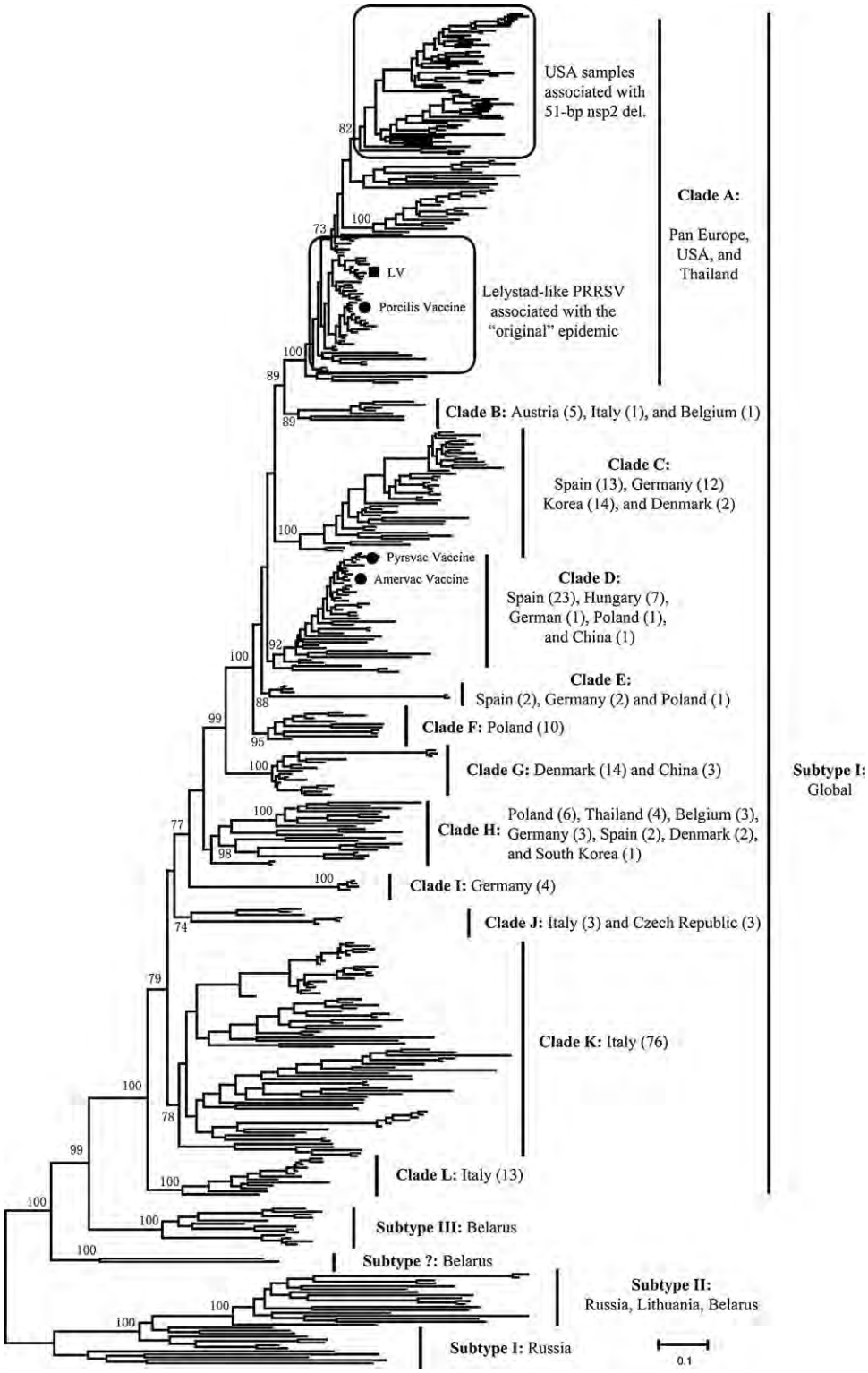

Fig. 1. Evolutionary history of Type 1 PRRSV based on a complete collection of ORF5 sequences from GenBank. The phylogenetic tree was constructed in MrBayes (Ronquist and Huelsenbeck, 2003) and was mid-point rooted. For clarity, only the bootstrap values of selected and well-supported nodes were shown. In the tree, we mapped the three subtypes defined by Stadejek et al. (2008). For Western European Subtype I, further classification into 12 clades with most inter-clade genetic distances >10% was completed. Indicated to the right of the figure is the geographical distribution for each clade, and the number in parentheses corresponds to the number of sequences that appeared within the clade.

sia, the virus diversity (measured by average pairwise comparison of percentage difference) had reached 18.2% and 12.0% for ORF5 and ORF7, respectively, which was significantly beyond the diversity in countries to the west (11.9% for ORF5 and 5.8% for ORF7) from current available sequences. This exceptionally large diversity indicated as long or longer establishment of the viruses in these countries than those to the west, which favored the hypothesis of Type 1 PRRSV emerging in Eastern Europe (including the Asian part of Russia) (Stadejek et al., 2006). Moreover, while there is frequent across-border transmission within Western Europe, a

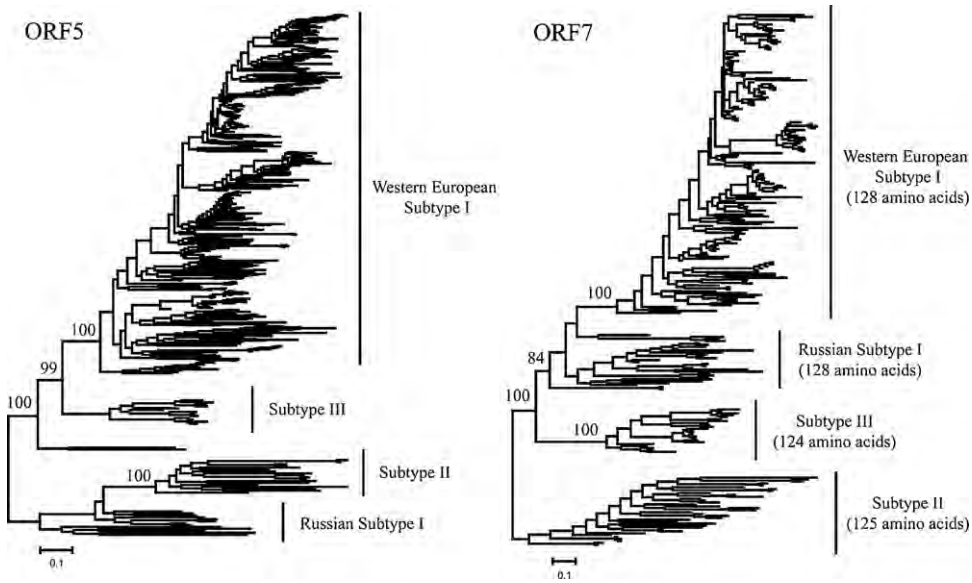

Fig. 2. Discordant ORF5 and ORF7 evolutionary history for Russian Subtype-I PRRSV. The phylogenetic trees were constructed in MrBayes and were mid-point rooted. For clarity, only the bootstrap values of selected and well-supported nodes were shown.

strong geographical demarcation was observed along the eastern Polish border (Stadejek et al., 2006, 2008). The highly diverse eastern viruses were seldom found in Western Europe based on current sampling, indicating that there was limited gene flow from the Eastern to the Western sides of the eastern Polish border. A plausible explanation for this geographical structuring would be less frequent livestock movement across the eastern Polish border compared to that among Western Europe countries. However, it is likely that Eastern European subtypes of PRRSV will eventually be circulating in Western Europe given the increasing communication between west and east in the swine industry.

2.4. Sub-typing and inconsistent evidence from ORF5 and ORF7 evolution

In the presence of extensive PRRSV Type 1 diversity, a classification system is needed to differentiate viruses with different genetic and antigenic properties, which can be beneficial to disease control and diagnostic practices (Stadejek et al., 2008). Originally, 4 subtypes were defined based on ORF5 sequence divergence and phylogenetic grouping (Stadejek et al., 2006). In a recent publication by Stadejek et al. (2008), ORF7 size polymorphism was recommended as a stable and independent indicator for sub-typing, and three previously identified subtypes were confirmed. In addition, isolates were enriched with additional samples from Eastern Europe countries including Russia. The three subtypes included a pan-Europe Subtype I, a Subtype II with samples from Belarus, Lithuania and Russia, and a Subtype III mainly constituted of Belarus samples. Their nucleoprotein sizes were 128, 125 and 124 amino acids, respectively (Stadejek et al., 2008). Nevertheless, care must be taken when differentiating viruses based on nucleoprotein size, because the same nucleoprotein size might be shared by viruses with distinct indels. For example, strain VL (an outlier to the any of the subtypes) has a nucleoprotein size of 125 amino acids, same as that of Subtype II viruses. But its insertion is between nucleotide (nt) 34 and 35 compared to ORF7 of prototype Lelystad, which is different from that of Subtype II (nt 140–141). Therefore, nucleoprotein size polymorphism alone is not robust enough for accurate sub-typing of Type 1 PRRSV. And it is preferable to compare the size polymorphism with the phylogenetic grouping before making any conclusions.

Moreover, the sub-typing scheme discussed above is not completely congruent with the evolutionary relationship depicted in ORF5 phylogeny. According to Stadejek et al. (2008), the two components of Subtype I—Russian Subtype I and Western European Subtype I—should be more closely related to each other than to the other subtypes as indicated by ORF7 polymorphism and phylogeny (Fig. 2, right panel). However, in the ORF5 phylogeny, the Western European Subtype I was more related to Subtype III PRRSV than to any of the Russian Subtype I sequences, and the latter formed a monophyletic lineage with Subtype II sequences (Fig. 2, left panel). In order to verify this was not an artifact in phylogenetic tree construction, we used a more straightforward approach by comparing pairwise inter-subtype genetic distances between Russian Subtype I, Western Europe Subtype I, and Subtype III for both complete ORF5 and ORF7 dataset. The results validated the phylogenetic incongruence (Fig. 3), which suggested that the evolutionary histories of ORF5 and ORF7 of the entire Russian Subtype I were very different from each other.

One explanation to this observation is recombination, which might have happened between two ancestral variants in Russia—one that was closely related to current Subtype I and another that was not. The resultant recombinant later became prevalent in the field, whereas the parental types went extinct or remain undetected. Alternatively, there could be convergent

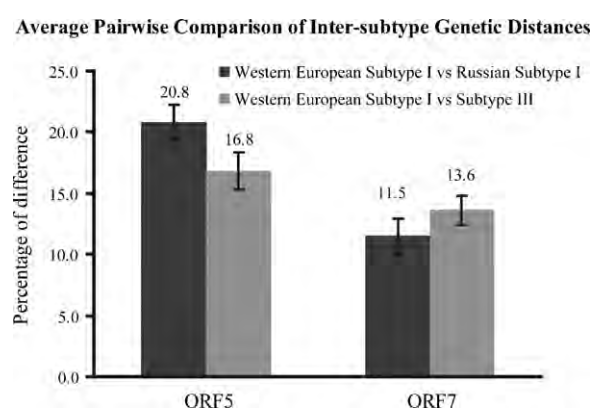

Fig. 3. Inter-subtype genetic distances comparison for Western European Subtype I versus Russian Subtype I or Subtype III PRRSV.

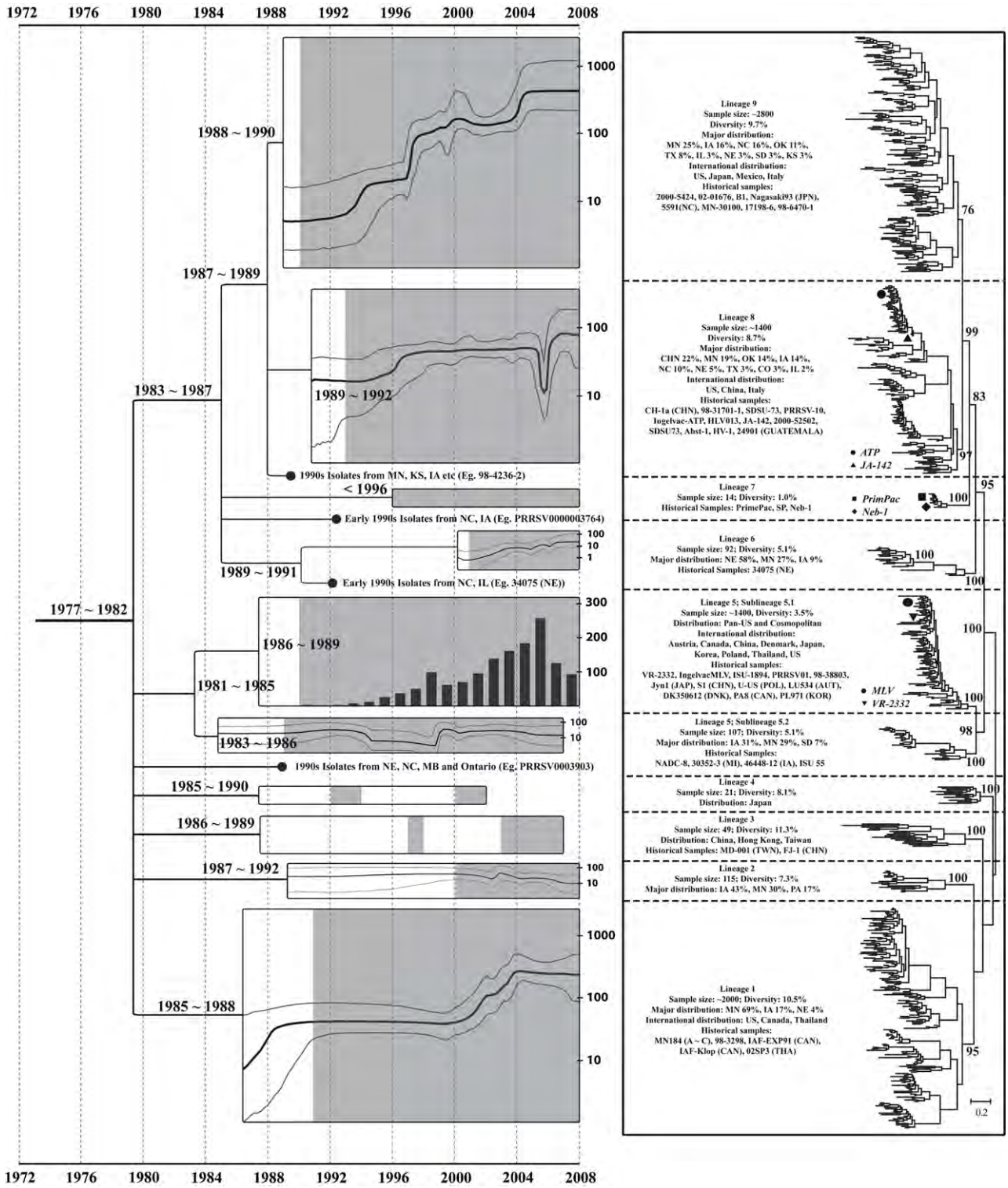

Fig. 4. Classification and evolutionary history of all Type 2 PRRSV lineages (modified from Shi et al., 2010). Right panel: overall phylogeny constructed using MrBayes, which was mid-point rooted and partitioned into lineages using dotted lines. Information for each lineage is indicated to the side of the phylogeny, including diversity of the lineage, its distribution within United States, its international distribution, as well as some well-known isolates. For clarity, only the bootstrap values of selected and well-supported nodes are shown. Left panel: a summary of the coalescence analyses of the overall and lineage datasets performed in BEAST (Drummond and Rambaut, 2007). It is based on the backbone structure of maximum clade credibility (MCC) trees reconstructed in BEAST, with unsupported nodes collapsed together to prevent uncertainty. Each box represented a well-supported lineage. The left boundary of the box was aligned to the emergence time of the lineage, whereas the right boundary of the box was aligned to the sampling time of the most recent sample. The shaded area inside the box corresponds to the periods when sequences were sampled. The black curve inside the box is a Bayesian skyline plot (BSP) and the grey curve represented upper and lower 95% Highest Posterior Density (HPD) intervals. The *y*-axis of the BSP is relative genetic diversity represented in $\log_{10}$ scale. For some of the lineages, BSP was not performed because of limited sample size or serious deviation from clock behavior (vaccine-associated lineages). In the cases of vaccine lineage, we used histograms instead, which revealed the sampling size during the course of its history. The *y*-axis of the histogram is the number of samples.

evolution for Russian Subtype I and Western Europe Subtype I in ORF7. The former explanation is more favorable given the fact that recombination does occur naturally for Type 1 PRRSV, with a potential breakpoint hotspot located within the conserved sequence stretches between ORF5 and ORF7 (Fang et al., 2007; Forsberg et al., 2002; Stadejek et al., 2006, 2008).

2.5. Distribution of Type 1 PRRSV outside Europe and Eastern Russia

Although the majority of its diversity is in Europe, Type 1 PRRSVs have been introduced to 5 non-European countries, including the USA (Fang et al., 2007; Ropp et al., 2004), Canada (Dewey et al., 2000), South Korea (Lee et al., 2010), China, and Thailand (Thanawongnuwech et al., 2004), based on currently available samplings. Each country, except for Canada (sequence not included in the phylogenetic tree), is associated with multiple introductions, evidenced by the observation that viruses from each country were found in more than one clade in the overall phylogeny (Fig. 1). Moreover, several Thai and Chinese strains were surprisingly closely related to two types of live-attenuated viruses in Europe. Since no record of applying European type MLV in these countries exists (Thanawongnuwech et al., 2004), the vaccine-related viruses were probably introduced through vaccinated swine breeders or through unauthorized use of these vaccines. Finally, it is necessary to address that some of the introductions from Europe have already caused huge impact on the local diversity. In the case of USA, there appears to have been a single introduction of Lelystad-like virus (Fang et al., 2007; Ropp et al., 2004). Progenies of this virus, identifiable by a genetic feature of 51-nt deletion in the non-structural protein 2 (nsp2) region of the replicase, have gained a remarkable genetic diversity (Fig. 1) and have been spread to more than 10 states within the past 10 years of evolution (Shi, personal communication).

3. Diversity and evolution of Type 2 PRRSV

3.1. Classification of Type 2 PRRSV

Two methods of Type 2 PRRSV differentiation have been used extensively to classify field Type 2 PRRSVs and to trace the origins of new infections. They are restriction fragment length polymorphism (RFLP) analysis (Wesley et al., 1998) and sequencing. The former employed three or more restriction enzymes to digest PCR amplified sequence (often ORF5). Based on the digestion pattern of each enzyme, an RFLP code was assigned to the sequence (Wesley et al., 1998). This method was originally designed to distinguish field samples from vaccine or vaccine related viruses (Wesley et al., 1998, 1999), but was later widely used in predicting the genetic relatedness and assessing the diversity of field samples (Cai et al., 2002; Cheon and Chae, 2000; Itou et al., 2001; Larochelle et al., 2003; Umthun and Mengeling, 1999; Wang et al., 2008). Although fast and relatively inexpensive, RFLP typing for field samples had been challenged for its reliability: in some cases closely related viruses could have distinct RFLP patterns (Cha et al., 2004), whereas in others the same pattern (e.g. RFLP 1-8-4, 1-4-2) was observed for highly divergent viruses (Han et al., 2006; Yoon et al., 2001). Therefore, the usefulness of RFLP analysis is quite limited for characterizing the molecular epidemiology of field PRRSV.

Compared to RFLP analysis, sequence analysis is more robust in revealing genetic relatedness. The comparison is usually carried out either by direct calculation of genetic distance between sequence pairs or by phylogenetic reconstruction. The former provides an intuitive measure of sequence similarity, whereas the latter demonstrates effectively the genetic relationships in general. The phylogenetic trees constructed in earlier studies were able to identify a VR-2332 associated clade, but there was no consensus on the size and constituents for the rest of the groupings, not to mention the relationship between them (Goldberg et al., 2000; Kapur et al., 1996; Larochelle et al., 2003; Meng et al., 1995; Umthun and Mengeling, 1999; Wesley et al., 1998). This was often due to inconsistency in the backbone structure and major groupings in the phylogenetic trees, both of which were poorly supported by bootstrap tests. Recently, in attempts to type the local viruses of Japan and Taiwan, respectively, Yoshii et al. (2005) and Wang et al. (2008) generated phylogenies with comparable structures that could be regarded as a relatively comprehensive representation of Type 2 diversity. In these trees, common groups were identified, including 3 clades constituted of mainly North American sequences (one clade contained PrimePac PRRS vaccine, one contained Canadian and Thai sequences, and one was associated with VR-2332 and Ingelvac PRRS MLV vaccine) and several highly diverse Asian clusters (Wang et al., 2008; Yoshii et al., 2005). This structure was later confirmed and replenished in the latest study using Bayesian phylogenies, which also established a high resolution typing system based on the entire collection ($n \cong 8500$) of currently available high quality ORF5 sequences (Shi et al., 2010). The study divided Type 2 PRRSV isolates into nine well-supported lineages (Fig. 4), including five large clusters ($n > 1000$) and four small groups, each separated by larger than 10% genetic distance from neighboring lineages. Furthermore, for each of the large lineages, diversity was characterized to a finer level using lineage and sublineage level trees (Shi et al., 2010). Nevertheless, even this construction is by no means the complete representation of Type 2 PRRSV diversity, because of a substantial lack of sequence information from Canada. Moreover, the fact that Canada has the earliest PRRSV positive serum so far detected (Carman et al., 1995) suggests the potential origin of Type 2 PRRSV. Therefore, characterization of virus sequences from Canada is essential to the understanding of the diversity and evolution of Type 2 PRRSV.

3.2. Distribution of Type 2 PRRSV

Type 2 PRRSV was also referred to as North American type PRRSV in view of its original identification and major distribution in North America. This is indeed the case. Of the nine lineages defined by Shi et al. (2010), seven have their diversity maintained by North American samples; while the remaining two are found only in Asian countries (Fig. 4). Within the seven North American lineages, introductions to Asian and European countries were frequently observed, some resulting in local outbreaks (An et al., 2007; Cha et al., 2006; Chen et al., 2006; Kang et al., 2004; Kim et al., 2009; Madsen et al., 1998; Shi et al., 2010; Thanawongnuwech et al., 2004). In the phylogenetic tree, these introduction events were represented as single branches or clusters of European or Asian sequences buried within the diversity of North American sequences.

Of all the North American lineages, Lineage 5 containing Ingelvac PRRS MLV vaccine is the most cosmopolitan (Fig. 4), with viruses introduced to more than 8 countries outside of the North American continent. Moreover, for countries which permitted the use of Ingelvac PRRS MLV vaccine, vaccine-like samples have been continuously detected in the field (An et al., 2007; Cha et al., 2006; Chen et al., 2006; Greiser-Wilke et al., 2009).

3.3. A re-examination of major epidemics and their evolutionary origin

3.3.1. 'Original' outbreak

Like Type 1 PRRSV, Type 2 viruses seemingly emerged long before the first recorded epidemic in the late 1980s (Hill, 1990;

Keffaber, 1989). The Type 2 prototype, strain VR-2332, was isolated from infected tissue samples collected in this epidemic (Collins et al., 1992). Moreover, within the period from 1989 to the early 1990s, a significant portion of US isolations were found to be closely related to VR-2332 (Kapur et al., 1996; Meng et al., 1995; Wesley et al., 1998), which was later typed as belonging to Lineage 5 (Fig. 4) (Shi et al., 2010), indicating that these viruses were associated with the outbreak. Following the epidemic, however, wild-type VR-2332-like isolates were believed to be no longer prevalent in North America, although a large number of vaccine derivatives have been continuously isolated from the field (Shi et al., 2010).

3.3.2. Outbreaks of 'acute PRRS'

Beginning in late summer 1996, there were increased numbers of outbreaks of severe PRRS in southeast Iowa as well as many other states of the USA. These outbreaks were characterized by an elevated abortion rate as well as sow mortality (Bush et al., 1999; Halbur and Bush, 1997). Surprisingly, a number of the herds experiencing this epidemic were vaccinated with either Ingelvac PRRS MLV alone or both Ingelvac PRRS MLV and PrimePac PRRS vaccines (Bush et al., 1999; Halbur and Bush, 1997; Key et al., 2001). The apparent protection failure by both live-attenuated vaccines might have been due to the fact that PRRSV isolates in this epidemic (Lineage 8 and 9, Fig. 4) were genetically distinct from available live-attenuated vaccines at that time (Lineage 5 and 7) (Key et al., 2001). In addition, sequence analysis showed these viruses themselves were very heterogenic and were not likely to be linked by a single emergence (Key et al., 2001; Shi et al., 2010). Therefore, the 'acute PRRS' outbreak was potentially due to multiple outbreaks in parallel. One follow-up question would be: what makes genetically distinct viruses cause a similar severe syndrome at the same time? The most plausible explanations are ones that include factors other than the PRRSV isolates themselves, such as changes in swine management practices or the presence of another pathogen (Bush et al., 1999), although these hypotheses have never been confirmed.

3.3.3. MN184 outbreak

Late in 2001, a virulent variant of PRRSV was identified in the US state of Minnesota (Han et al., 2006). These outbreak viruses all had the RFLP 1-8-4 pattern. Three isolates were sequenced in their entirety, MN184 A, B, and C, and their genomes were each characterized by three discontinuous deletions (11, 1, and 19 amino acids corresponding to VR-2332 positions 324–434, 486 and 505–523) in the nsp2 region of the replicase ORF (Han et al., 2006). The sudden emergence of the virus is still a mystery. Although extensive sequencing of the RFLP 1-8-4 viruses has been carried out in the USA, so far only distant relatives have been found (Fig. 4), which are not likely to be the immediate ancestral type to the outbreak causing variants. Nevertheless, past sequence surveillance had overly focused on US samples while ignoring the fact that Canada also detected a significant number of RFLP 1-8-4 viruses from the field (Larochelle et al., 2003). Given that there were substantial flows of weaned or feeder pigs from Canada into the US Corn Belt, particularly Minnesota, it is highly possible to find the origin of MN184 related variants in Eastern Canada.

3.3.4. Highly pathogenic PRRSV outbreak in China

Highly pathogenic PRRS, characterized by prolonged high fever, red colorations on the body, and high mortality rate in adult pigs (An et al., 2007; Tian et al., 2007), was first detected in the summer of 2006. In the following 12 months, the disease quickly spread to more than 20 provinces in China, causing huge losses to the country's swine industry. The causative agent was later shown in experimental infections of specific-pathogen free pigs to be only PRRSV (Tian et al., 2007; Zhou et al., 2008), although other unknown agents may have contributed to the disease severity. Moreover, phylogenetic reconstructions had indicated that the highly pathogenic PRRSV variant actually evolved from the local diversity of PRRSV in China (An et al., 2009; Hu et al., 2009; Zhou et al., 2008), which may be traced to a single introduction from North America (Shi et al., 2010). However, elucidating its evolutionary origin did not facilitate the identification of one or more virulence determinants for this highly pathogenic variant, which was originally suspected to be the discontinuous deletions of 1 and 29 amino acids in the nsp2 region of the genome. However, recent studies using chimeric infectious clone construction excluded the specific nsp2 deletions as potential determinants for the high virulence (Zhou et al., 2009). The genetic basis of this unusually highly virulent PRRSV remains unknown.

3.4. Transmission of PRRSV in North America

PRRSV can be transmitted via distance-dependent (area spread) and distance-independent routes. In area spread, the transmission from one herd to a nearby herd occurs even without active communication between the two farms. Various mechanisms have been proposed for area spread, including aerosol (Brockmeier and Lager, 2002; Torremorell et al., 1997), animal and insect vectors (Otake et al., 2003; Zimmerman et al., 1997) and contaminated fomites (Dee et al., 2003, 2002; Otake et al., 2002). In distance-independent routes, however, a communication between the two places is often required, and transmission can occur via transportation of pigs, introduction of semen (Christopher-Hennings et al., 1995; Woeste and Grosse Beilage, 2007), and even vaccine usage, if vaccine-related viruses are involved. In addition, indiscriminate needle use during drug and vaccine administration can facilitate iatrogenic transmission (Otake et al., 2002). Nowadays, the multiple site production system is widely practiced in the US swine industry. Since production sites are often located in different parts of the country, the chance of viral spread over long distance (distance-independent) could be significantly increased due to regular transportation of pigs between these sites.

To examine the relative importance of distance-independent routes versus distance-dependent routes in PRRSV transmission, a common approach is to test the correlation between sample genetic distances and geographical distances. The underlying principle is simple: if a virus is transmitted through a distance-dependent route, longer distance tends to separate virus populations more effectively and thus result in a more heterogeneous genetic content. This position no longer holds if the virus was transmitted through distance-independent routes. The test has been applied to two datasets, but with contradictory results. In the first analyses based on samples from 48 farms in Illinois, the test demonstrated no correlation between genetic similarity and geographical distances (Goldberg et al., 2000), which favored distance-independent routes. In a second study based on samples from 62 farms owned by the same company, the test indicated a significant negative correlation between genetic similarity and geographical distances (Mondaca-Fernandez et al., 2006), which favored area spread. In a third study, based on samples from Quebec, Canada, a different approach was used to assess the relative importance of possible routes of transmission. In this third study, homologous clusters were collected from the phylogenetic tree, and for each one, geographical components (herds of origin) and their potential relationships were investigated (Larochelle et al., 2003). The results indicated that both area spread and distance-independent routes (introduction of pigs) accounted for PRRSV transmission (Larochelle et al., 2003). In summary, different conclusions have been derived from the three studies above, which could be due to distinct local conditions on area spread as suggested by Mondaca-Fernandez et al. (2006). Alternatively, the transmission route might be linked to viral properties, such as contiguousness and population status. For example,

in the first study, Goldberg et al. used samples collected during 1997–1998, which was during the course of 'acute PRRS' outbreaks; whereas in the second study Mondaca-Fernandez et al. used a non-outbreak sampling. The actual influence of PRRSV outbreaks on viral transmission remains to be examined, but the observations on several PRRS outbreaks (e.g. European 'original' outbreak, Chinese highly pathogenic outbreak) suggest it is more efficient for the outbreak virus to be transmitted to a large geographic area.

Recently, Shi et al. (2010) characterized distance-independent type 2 PRRSV transmission on a nationwide scale. By using an ancestral reconstruction approach, they estimated the inter-state transmission frequencies among 10 pig-producing US states. The results indicated that distance-independent PRRSV transmission was a relatively frequent process. More importantly, the estimation of the frequencies of transmission among these states actually mirrors the asymmetric interstate pig flow in the United States, indicating the huge impact of modern swine production and transportation systems on virus transmission (Shi et al., 2010).

4. The pre-emergence evolution of PRRSV

4.1. Novel emergence, ancient history

Despite their almost concurrent emergences (i.e. original epidemics on the two continents, respectively) and similar clinical symptoms, Type 1 and 2 PRRSVs share only 55–70% nucleotide and 50–80% amino acid similarity in their various viral genes (Forsberg, 2005). This observation suggested the two genotypes of PRRSV have diverged significantly before their independent emergences. Two scenarios have been proposed for their pre-emergence evolution: (1) Type 1 and 2 PRRSVs diverged not long before emergence, which was followed by extremely high substitution rate (Hanada et al., 2005); (2) they diverged way ahead of the emergence (Forsberg, 2005), and evolved independently on the two continents for a long time.

A common approach to validate these scenarios is estimating the theoretical onset of the ancestral form of all PRRSV (i.e. the time of the most recent common ancestor, or tMRCA, of all PRRSV) by applying molecular clock model on serially sampled viral sequences. Hanada et al. (2005) estimated the tMRCA of all PRRSV to be around 1986 ± 1.8 and 1973 ± 1.3 years using distance-based and maximum likelihood-based methods, respectively. The results strongly favored the recent divergence of the two genotypes. However, as pointed out by Forsberg et al., Hanada et al. had chosen an uninformative dataset and inappropriate methodology, which leads to inaccurate estimation of the tMRCA (Forsberg, 2005). Based on an expanded dataset of ORF3 sequences, Forsberg (2005) re-estimated the tMRCA of the two PRRSV types to be around 1880 ± 15 years using codon model implemented in the maximum likelihood framework, which favored an early divergence of Type 1 and 2 PRRSV. Further evidence which supports the scenario of early divergence can be found from the estimation of tMRCA of Type 1 and Type 2 PRRSV, respectively. Currently available sequence information had placed the tMRCA of Type 1 PRRSV at 1946–1967 (Forsberg, unpublished data) and Type 2 PRRSV at 1977–1981 (Shi et al., 2010), respectively. Since the tMRCA of all PRRSV must be ahead of the tMRCAs of each genotype, it is highly unlikely that the divergence of Type 1 and 2 PRRSV occurred only a few decades ago.

4.2. The hypothesis of origin

Although it is plausible that PRRSV diverged from other arteriviruses more than 100 years ago, the pre-emergence evolutionary history of the virus remains a mystery. The epidemiological records of the virus are all recent compared to the estimated tMRCA. In fact, the earliest evidence of PRRSV infection in the domestic pig population (PRRSV antibody-positive serum) could only be identified back to mid 1980s in Europe (Grebennikova et al., 2004) and to 1979 in North America (Carman et al., 1995), leaving the majority of the PRRSV history untraceable. The question is: where was PRRSV at an earlier time? One possible scenario is that the virus remained unrecognized in the domestic pig population for a long period of time on two continents. Alternatively, the virus could have resided in another host before recent adaptation to the domestic swine. The latter scenario (i.e. species jump) rationalizes the total absence of earlier record of PRRS in the domestic pig population, and thus was more accepted by the current researchers. However, it does not account for the simultaneous emergence of PRRS, caused by two distinctly different genetic forms. The follow-up questions are: what host species was ancient PRRSV adapted to; and what caused the marked divergence between the viruses from two continents? To address these questions, Plagemann (2003) proposed a series of species jump events and migration events to the current diversity of PRRSV. Plagemann (2003) hypothesized that PRRSV had a LDV-like ancestor circulating in rodents based on the observation that LDV was the closest relative of PRRSV within the Arteriviridae family, which had genetic characters of a more "ancient" virus (in contrast to PRRSV which was typical of a new virus). The ancestral PRRSV in rodents was later adapted to Eurasian wild boars, which served as an intermediate host that brought the virus from rodents to the domestic swine population. To account for the divergence of the two types of PRRSV, Plagemann (2003) postulated that it started from introduction of wild boars from the Europe to the United States. The earliest record of such introduction was in 1912 when 14 wild boars from Europe were released in a game reserve in the southwestern part of North Carolina (Mayer and Brisbin, 1991). Following the introduction, PRRSV evolved separately in the wild boar populations in Eurasia and North American continents before their independent introduction and establishment to the domestic pig population (Plagemann, 2003).

Plagemann's hypothesis fills the knowledge gap of pre-emergence evolution of PRRSV, and is in congruence with the estimations of tMRCA of all PRRSVs. Nevertheless, there's a lack of virtual evidence (i.e. viral isolation) to validate rodents and wild boars as the hosts for ancestral PRRSVs. For example, if wild boars had a longer history of hosting PRRSV compared to domestic swine, we're expecting a larger diversity of the virus in wild boars. However, current surveillance in wild boars failed to detect such a pattern. In a recent study of PRRSV infection in Germany, viral sequences from wild boars were found to form two highly homologous groups that were clustered within the diversity of the Type 1 and Type 2 PRRSV, respectively (Reiner et al., 2009). The result indicated these PRRSV in wild boars were more likely to be spillover from the domestic pig viruses.

5. PRRSV recombination

Type 1 and Type 2 PRRSV have been shown to undergo relatively frequent intra-type recombination under *in vitro* conditions (van Vugt et al., 2001; Yuan et al., 1999). Nevertheless, the frequency of naturally occurring recombination was believed to be much lower, not to mention the ones that were circulating in the field (Shi et al., 2010; van Vugt et al., 2001; Yuan et al., 1999). In fact, most of the recombination events identified so far from the field were only represented by single sequences instead of having a group of circulating "recombinant progenies" (Fang et al., 2007; Forsberg et al., 2002; B. Li et al., 2009; Y. Li et al., 2009; Murtaugh et al., 2001; Shi et al., 2010; Stadejek et al., 2006, 2008; Yoshii et al., 2008; Yuan et al., 1999). This might be related to the biological fitness of the recombinants: the newly formed recombinants

need to gain selective advantages to compete with their parental type and gain dominance (Yuan et al., 1999). Alternatively, current studies might underestimate the recombination rate in the field due to surveillance limitations (focus on only a short stretch of genome from ORF5 to ORF7) and methodology failure. For example, as we mentioned in the previous context, the Russian Subtype I was potentially a recombinant group, but it was unlikely to be detected by regular recombination screening methods due to high sequence divergence.

Another interesting aspect in the recombination analyses is the location of breakpoints. In the *in vitro* study of recombination, there is no strong indication of crossover hotspots, although they seem to be located within regions flanked by high identity (van Vugt et al., 2001; Yuan et al., 1999). In the field, prior screening within the ORF5 to ORF7 genomic region indicated that most of the breakpoints were between ORF5 and ORF7 (Fang et al., 2007; Forsberg et al., 2002; Stadejek et al., 2006, 2008) or within ORF5 (Kapur et al., 1996; Y. Li et al., 2009; Murtaugh et al., 2001; Shi et al., 2010; Yuan et al., 1999), but none were detected in ORF7. Since the majority of genotyping and classification is based on ORF5, routine screening for recombination is required before sequence comparison or phylogenetic analyses.

6. Conclusions and future direction

PRRSV is highly dynamic in nature: on one hand the virus is continuously and rapidly evolving, generating "new variants" and expanding its diversity; on the other hand, modern food production systems act as a super-spreader that distributes viruses nationwide and even worldwide. Therefore, to control and prevent PRRSV, it is important to keep pace with virus dynamics. With the help of phylogenetic analyses, the diversity and the epidemiological and evolutionary history of PRRSV can be clearly visualized. However, sequence analysis methodology and updated reference datasets are only maintained by a few experts, and it is often difficult for veterinary and field personnel to collect information promptly. Therefore, it will be helpful to establish a web-based genotyping platform that maintains an up-to-date reference dataset and automatically performs phylogenetic analysis.

Conflict of interest statement

No conflicts declared.

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Detection of the Abnormal Isoform of the Prion Protein Associated With Chronic Wasting Disease in the Optic Pathways of the Brain and Retina of Rocky Mountain Elk (*Cervus elaphus nelsoni*)

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Abstract

Eyes and nuclei of the visual pathways in the brain were examined in 30 Rocky Mountain elk (*Cervus elaphus nelsoni*) representing 3 genotypes of the prion protein gene *PRNP* (codon 132: MM, ML, or LL). Tissues were examined for the presence of the abnormal isoform of the prion protein associated with chronic wasting disease (PrP^{CWD}). Nuclei and axonal tracts from a single section of brain stem at the level of the dorsal motor nucleus of the vagus nerve were scored for intensity and distribution of PrP^{CWD} immunoreactivity and degree of spongiform degeneration. This obex scoring ranged from 0 (elk with no PrP^{CWD} in the brain stem) to 10 (representing elk in terminal stage of disease). PrP^{CWD} was detected in the retina of 16 of 18 (89%) elk with an obex score of > 7. PrP^{CWD} was not detected in the retina of the 3 chronic wasting disease–negative elk and 9 elk with an obex score of < 6. PrP^{CWD} was found in the nuclei of the visual pathways in the brain before it was found in the retina. Within the retina, PrP^{CWD} was first found in the inner plexiform layer, followed by the outer plexiform layer. Intracytoplasmic accumulation of PrP^{CWD} was found in a few neurons in the ganglion cell layer in the *PRNP* 132ML elk but was a prominent feature in the *PRNP* 132LL elk. Small aggregates of PrP^{CWD} were present on the inner surface of the outer limiting membrane in *PRNP* 132LL elk but not in *PRNP* 132MM or 132ML elk. This study demonstrates PrP^{CWD} accumulation in nuclei of the visual pathways of the brain, followed by PrP^{CWD} in the retina.

Keywords

brain, chronic wasting disease, eyes, prion protein, retina, Rocky Mountain elk, visual pathways

Chronic wasting disease (CWD), a transmissible spongiform encephalopathy, has been reported in captive and free-ranging cervids.[3,32,37,38] An abnormal isoform of the prion protein has been found in cervids with CWD (PrP^{CWD}), and it can be detected with immunohistochemistry.[33] PrP^{CWD} has been reported in the brain, lymphoid tissues, and other organs (including the retina) in mule deer and white-tailed deer with CWD.[9,16,27,32] Misfolded prion proteins have been associated with other transmissible spongiform encephalopathies and have been reported in retinas of domestic sheep inoculated with the scrapie agent[7,14] and in humans with sporadic and variant Creutzfeldt–Jakob disease.[10] This communication documents the occurrence of PrP^{CWD} in the retina, optic nerve, and visual pathways of the brain in Rocky Mountain elk with natural occurring CWD. Disease progression in preclinical elk with natural exposure to CWD was estimated from the intensity and distribution of immunoreactivity and spongiform change in the brain stem at the level of the dorsal motor nucleus of the vagus nerve (obex score). Distribution of PrP^{CWD} in the retina and optic pathways was a late-stage event, observed only in elk with relatively high obex scores. Minor variation in the

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distribution pattern of PrP^{CWD} in the retina may be associated with a polymorphism at codon 132 of the *PRNP* gene, a site associated with incubation time in elk[8,23] and relative susceptibility and disease expression in humans (human codon 129) with sporadic and acquired transmissible spongiform encephalopathies.[19]

Materials and Methods

Eyes and brain were collected postmortem from 27 Rocky Mountain elk diagnosed with CWD by immunohistochemistry assay of antemortem biopsy samples of rectal mucosal-associated lymphoid tissue[31,34] or postmortem assay of brain and lymphoid tissues.[33] Negative-control elk included 2 ranch-raised elk from a herd free of a history of CWD and 1 free-ranging elk with no detectable PrP^{CWD} in brain or lymphoid tissues.

The open reading frame of the *PRNP* gene was sequenced in all the elk as described.[22] The previously described coding change at codon 132 was detected; elk were identified as homozygous for the wild-type allele encoding methionine (132MM), homozygous for the alternative allele encoding leucine (132LL), or heterozygous for the alleles (132ML).

Fourteen elk (47%) were free-ranging adult cows (eleven 132MM, three 132ML) originating from north-central Colorado and 11 elk (37%) originating from captive herds in northern Colorado. The captive group included 8 bulls (seven 132MM, one 132ML) and 3 cows (two 132MM, one 132ML). In addition, two 132LL elk experimentally fed a pooled brain homogenate from two 132ML CWD-positive elk were examined.[8,23] Both were euthanized at 60 months postinfection.

Following euthanasia, eyes were collected within 1 to 5 minutes from each elk. The globe of each eye was opened via an incision through the sclera just posterior to the limbus, thus increasing exposure of the retina to fixative. Eyes were placed in Davidson's fixative for 3 days.[13] Brains were collected within 4 hours of death from each elk, hemisectioned, placed in 10% neutral buffered formalin, and fixed for 1 week. The formalin was changed at least twice for each brain. Sections from each brain were cut at 5 μm and immunolabeled as previously described with anti–prion protein monoclonal antibody F99/97.6.1[33] (mAb99; Anti-Prion (99), Ventana Medical Systems, Inc, Tucson, AZ) and monoclonal antibody P4 (mAbP4).[36] The latter binds an epitope at residues 93–99, near the amino terminus of the processed prion protein; the former binds an epitope at residues 220–225, near the carboxyl terminus of the processed prion protein.[21] Hematoxylin and eosin–stained sections were examined from all sections for spongiform degeneration.[29]

To confirm the CWD status, one section of obex was examined from each elk (ie, brain stem at the level where the fourth ventricle converges into the central canal of the spinal cord); this section contained the dorsal motor nucleus of the vagus nerve (DMNV), medial retropharyngeal lymph node, and palatine tonsil. In addition, the stage of the disease was estimated by scoring the degree of detectable positive chromogen immunoreactivity in the nuclei and white matter tracts, combined with the degree of spongiform degeneration in this section. The evaluated nuclei included the DMNV, area postrema, solitary nucleus, cuneate nucleus, nucleus of the spinal tract of the trigeminal nerve, nucleus ambiguus, reticular formation area/nucleus, olivary nuclei, medial raphe, and hypoglossal nucleus.[39] The obex was scored as a progressive increase in intensity of PrP^{CWD} immunoreactivity and distribution, essentially as described,[30] with additional classification criteria based on the intensity of spongiform degeneration and PrP^{CWD} in axonal tracts. An obex score of 0 was assigned to elk with no detectable PrP^{CWD} in the brain stem. Scoring of samples with detectable PrP^{CWD} in the obex ranged from 1, characterized by minimal immunoreactivity of the ventral aspects of the DMNV (Fig. 1), to 10, characterized by all the nuclei and axonal tracts containing a moderate to heavy PrP^{CWD} immunoreactivity (Fig. 2). All nuclei had mild to severe spongiform degeneration. Intermediate stages were defined by an increase in immunoreactivity intensity and spongiform degeneration. A detailed description of this scoring system is in preparation.

Eyes from each animal and brain sections of the proposed visual pathways were trimmed and embedded in paraffin blocks. The visual pathways within the brain of elk have not been described; therefore, the visual pathways described in humans were applied. The pathway is assumed to include, in order, retina, optic nerve, optic chiasm, optic tract, lateral geniculate nucleus, optic radiations, and visual cortex of the occipital lobe—all of which were sampled. Impulses leaving the lateral geniculate nucleus also travel to the superior colliculus (sampled) and midbrain tectum; these are primarily reflex. The oculomotor nucleus (sampled), even though not directly a part of the visual pathway, was examined because this nucleus controls multiple functions of the eye, including pupillary size.[1,2,26] Immunoreactivity intensity was scored as 0 (no labeling detected), 0.5 (trace amounts of chromogen in some areas of the region), 1 (light labeling within the region), 2 (moderate labeling in the region), and 3 (heavy chromogen deposits throughout the region). Elk were grouped by obex score, and the median value was calculated for each group at each anatomic site in the visual pathway.

Results

Clinical Status and PRNP Genotype of the Test Population

Of the 27 CWD-positive elk, 20 were nonclinical and 7 showed typical clinical signs of CWD at the time of euthanasia. Clinical signs included behavior changes, mild to moderate aggression when cornered, weight loss, excessive salivation, grinding of the teeth, and emaciation. *PRNP* genotypes in the 25 naturally infected elk included twenty 132MM elk (80%) and five 132ML elk (20%), a ratio similar to that observed in the natural population.[22,24] Elk homozygous for the 132L allele are relatively rare in the population; therefore, 2 experimentally

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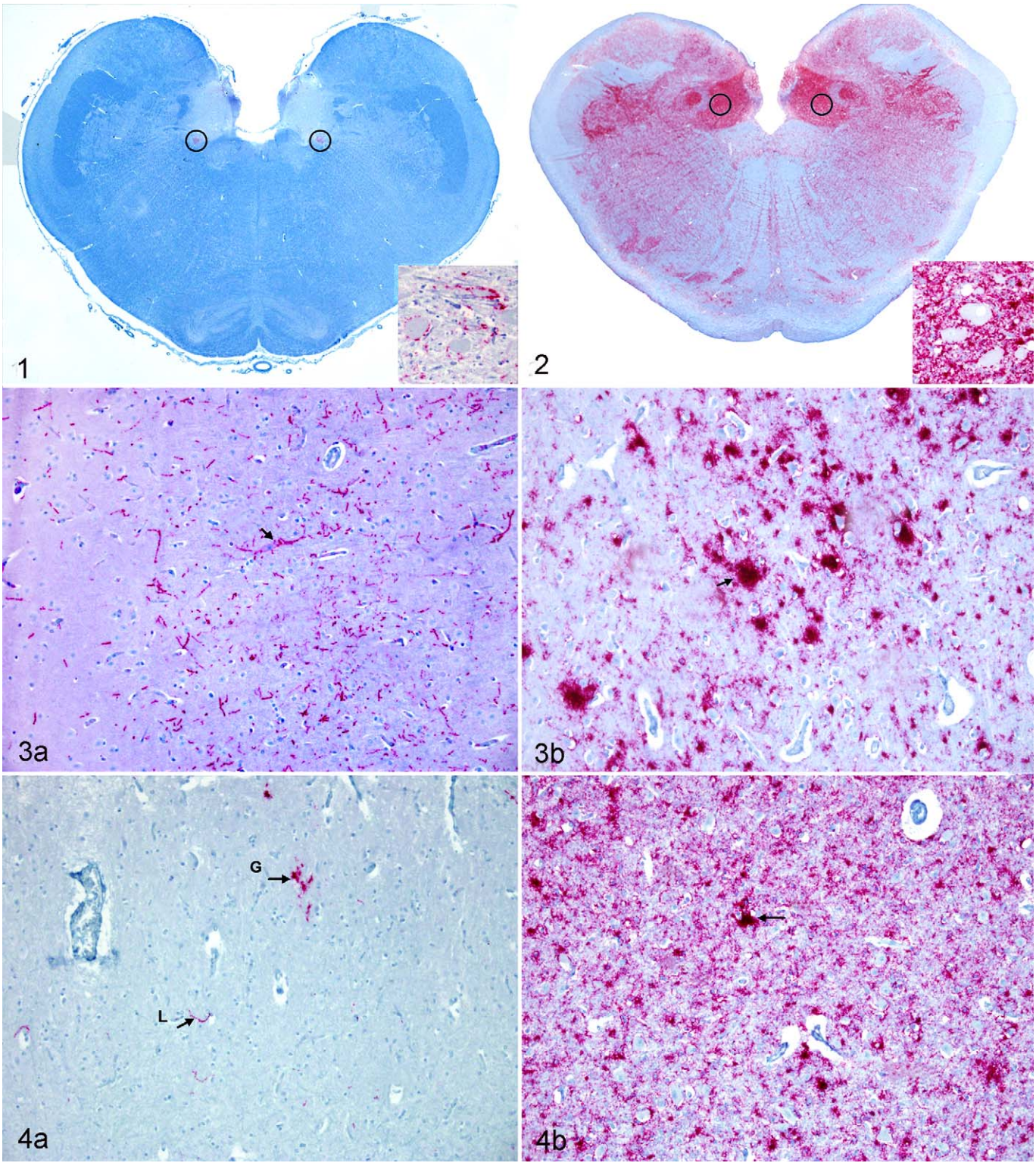

Figure 1. Subgross photograph of a cross section of the brain stem at the level of the obex of an elk with an obex score of 2. Note the scant red granular staining in the ventral aspects of the dorsal motor nucleus of the vagus nerve (circled). *Inset*: Circled area. Note the minimal degree of red granular PrP^{CWD} staining on the periphery of neurons and within the neuropil of the dorsal motor nucleus of the vagus nerve. Immunohistochemical staining: Anti Prion (99) monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. PrP^{CWD}, prion protein associated with chronic wasting disease. **Figure 2.** Subgross photograph of a cross section of the obex of an elk with an obex score of 10. Note the extensive red granular staining in all the nuclear areas, with less staining in axonal tracts. *Inset:* Circled area within the vagus nucleus. Note the extensive red granular PrP^{CWD} staining within the neuropil and on the periphery of neurons throughout

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infected animals described in previous studies[8,23] were added to this study.

Obex Scores of Preclinical and Clinically Affected Elk

The obex was scored in all 30 elk based on the distribution and intensity of immunoreactivity combined with the degree of spongiform degeneration. Neither PrP^{CWD} detected by immunoreactivity nor spongiform degeneration was observed in the brain stem from the 3 negative control elk. Of the twenty 132MM CWD-positive elk, obex scores were as follows: one 1, two 2s, one 3, one 4, one 6, three 7s, three 8s, three 9s, and five 10s (Table 1). Of the five 132ML-positive elk, the obex scores were as follows, one of each: 4, 5, 6, 8, 10. One 132LL elk had an obex score of 8 and the other, a score of 9. Elk with obex scores of < 9 were clinically normal except for 2 elk, 1 with a score of 8 and the other, a score of 9; both these elk had pneumonia at the time of necropsy.

PrP^{CWD} in the Brain and Visual Pathway of Elk at Different Stages of CWD

Immunoreactivity of the visual pathway was scored at 9 neuroanatomic locations in samples from elk with obex scores ranging from 1 to 10. Table 2 shows the median scores for each location in relation to the obex score. The earliest sites of PrP^{CWD} immunoreactivity were the visual cortex (1 of 2) and superior colliculus (2 of 2) in the brain of elk with an obex score of 2. Heaviest PrP^{CWD} immunoreactivity was observed in the superior colliculus, oculomotor nucleus, visual cortex, lateral geniculate nucleus, and retina in elk with an obex score of 9 or 10. At each location, visual pathway scores were higher in elk with higher obex scores. PrP^{CWD} immunoreactivity was detected in the retina of elk with obex scores of 7 or higher. The character and location of the PrP^{CWD} immunoreactivity varied with obex score and genotype, as follows.

Obex score = 0. Neither histological lesions nor PrP^{CWD} immunoreactivity was detected in obex, brain, lymphoid tissues, or sections of the visual pathway including the eye of the 3 control elk.

Obex score = 1. One 132MM elk had an obex score of 1. This elk had no detectable PrP^{CWD} immunoreactivity in the visual pathways of the brain or retina. Spongiform degeneration was not found in any of the nuclei of the visual pathway. Histological lesions were not detected in any of the layers of the retina.

Obex score = 2. Two 132MM elk had an obex score of 2. PrP^{CWD} immunoreactivity was detected in the visual cortex of 1 elk and in the superior colliculus in both elk. The pattern of PrP^{CWD} immunoreactivity was considered to be trace and primarily linear. Evidence of spongiform degeneration was not found in any of the nuclei of the visual pathway. Histological lesions or PrP^{CWD} immunoreactivity was not detected in any of the layers of the retina.

Obex score = 3. One 132MM elk had an obex score of 3. PrP^{CWD} immunoreactivity was detected in the visual cortex in this elk (Fig. 3A). The pattern of PrP^{CWD} immunoreactivity was considered to be mild and primarily linear. No evidence of spongiform degeneration was found in any of the nuclei of the visual pathway. Histological lesions or PrP^{CWD} immunoreactivity was not detected in any of the layers of the retina in this elk.

Obex score = 4. Two elk (one 132MM and one 132ML) had an obex score of 4. PrP^{CWD} immunoreactivity was detected in the lateral geniculate nucleus, the oculomotor nucleus, superior colliculus, and visual cortex in the 132MM elk but only in the visual cortex in the 132ML elk. The pattern of PrP^{CWD} immunoreactivity was primarily linear, but coarse granules of chromogen were found in the visual cortex and lateral geniculate nucleus in the 132MM elk and in the superior colliculus in both elk. Spongiform degeneration was confined to the DMNV in both elk and not found in nuclei of the visual pathway. Histological lesions or PrP^{CWD} immunoreactivity was not detected in any of the layers of the retina in these 2 elk.

Obex score = 5. One elk (132ML) had an obex score of 5. PrP^{CWD} immunoreactivity was detected in the lateral geniculate nucleus, superior colliculus, visual cortex, and oculomotor nucleus in this elk. Both linear and granular patterns were found in this stage, but the linear pattern was more prominent. Spongiform degeneration was confined to the obex and not found in nuclei of the visual pathway. Histological lesions or PrP^{CWD} immunoreactivity was not detected in any of the layers of the retina in this elk.

Figure 2. (continued) the dorsal motor nucleus of the vagus nerve. Immunohistochemical staining: Anti Prion (99) monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. PrP^{CWD}, prion protein associated with chronic wasting disease. **Figure 3.** Photomicrograph of the visual cortex from two 132MM CWD-positive elk. A, an elk with an obex score of 3. B, an elk with an obex score of 10. Note the linear staining (arrow) of PrP^{CWD} (A) compared to the course granular staining (arrow) with plaque formation (B). Immunohistochemical staining: Anti Prion (99) monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. 132MM: codon 132, elk identified as homozygous for the wild-type allele encoding methionine. CWD, chronic wasting disease; PrP^{CWD}, prion protein associated with chronic wasting disease. **Figure 4.** Photomicrograph of the superior colliculus from two 132MM positive CWD elk. A, an elk with an obex score of 6. Note the minimal amount of linear (L) and granular (G) staining of PrP^{CWD}. B, an elk with an obex score of 10. Note the lack of linear staining with the abundance of granular staining and plaque formation (arrow). Immunohistochemical staining: Anti Prion (99) monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. 32MM: codon 132, elk identified as homozygous for the wild-type allele encoding methionine. CWD, chronic wasting disease; $PrP^{CWD,}$ prion protein associated with chronic wasting disease.

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Table 1. Rocky Mountain Elk in Which Eyes Were Collected For Examination

Elk No.	*PRNP* Genotype[a]	Source[b]	Clinical Signs	Obex Score[c]	PrP^{CWD} in Retina[d]
T78-6110	MM	F	No	1	N
T78-6121	MM	F	No	2	N
T78-6111	MM	F	No	2	N
T78-6473	MM	R	No	3	N
T78-6054	ML	F	No	4	N
T78-6071	MM	F	No	4	N
T78-6130	ML	F	No	5	N
T78-6113	ML	F	No	6	N
T78-6136	MM	F	No	6	N
T78-6070	MM	F	No	7	N
T78-6073	MM	F	No	7	P
T78-6098	MM	F	No	7	P
T78-6055	MM	F	No	8	N
T67-6540	MM	R	No	8	P
T67-4413	LL	E	No	8	P
T89-0104	ML	R	Yes	8	P
T67-6790	MM	R	No	8	P
T78-6487	MM	R	No	9	P
T67-4412	LL	E	Yes	9	P
T78-1987	MM	F	No	9	P
T67-6541	MM	R	No	9	P
T78-6474	MM	R	Yes	10	P
T56-7353	MM	R	Yes	10	P
T56-1821	MM	R	No	10	P
T45-7445	ML	R	Yes	10	P
T78-0368	MM	F	Yes	10	P
T89-3530	MM	R	Yes	10	P

n, 27.
[a] Deduced amino acid at residue 132 of the *PRNP* gene. M, methionine; L, leucine.
[b] R, ranch-raised elk; F, free-ranging elk; E, experimental oral exposure.
[c] Obex score based on absence (0) or relative intensity of immunolabeling and spongiform degeneration (1–10) in a single section of medulla at the level of the dorsal motor nucleus of the vagus.
[d] PrP^{CWD}, prion protein associated with chronic wasting disease. Immunolabeling of the retina: P, elk with; N, elk without.

Table 2. Median PrP^{CWD} Immunoreactivity Intensity Scores of Rocky Mountain Elk With Disease Progression as Estimated From Obex Scores (Number of Elk Affected)

Obex Score	No. Elk	Retina	Optic Nerve	Optic Chiasm	Optic Tract	Lateral Geniculate Nucleus	Optic Radiations	Visual Cortex	Oculomotor Nucleus	Superior Colliculus
1	1	0	0	0	0	0	0	0	0	0
2	2	0	0	0	0	0	0	0.5 (1)	0	0.8 (2)
3	1	0	0	0	0	0	0	1.0 (1)	0	0
4	2	0	0	0	0	0.5 (1)	0	1.0 (2)	0.5 (1)	0.5 (1)
5	1	0	0	0	0	1.0 (1)	0	1.0 (1)	2.0 (1)	1.0 (1)
6	2	0	0	0	0	0.5 (1)	0	2.0 (2)	1.3 (2)	2.0 (2)
7	3	0.7 (2)	0	0	0.2 (1)	0.7 (2)	0.7 (2)	1.0 (3)	1.7 (3)	1.7 (3)
8	5	1.4 (4)	0.9 (4)	0.7 (4)	0.7 (4)	1.9 (5)	1.6 (5)	2.0 (5)	2.0 (5)	2.2 (5)
9	4	2.8 (4)	1.3 (3)	0.6 (3)	0.9 (4)	2.0 (4)	1.8 (4)	1.5 (4)	2.3 (4)	2.3 (4)
10	6	2.5 (6)	1.5 (6)	2.7 (3)[a]	1.7 (6)	3.0 (6)	2.0 (6)	3.0 (6)	3.0 (6)	3.0 (6)

PrP^{CWD}, prion protein associated with chronic wasting disease. Immunoreactivity in the retina was not observed until an obex score of > 7 was obtained. Immunoreactivity intensity was scored as 0 (no labeling detected), 0.5 (trace amounts of chromogen in some areas of the region), 1 (light labeling within the region), 2 (moderate labeling in the region), and 3 (heavy chromogen deposits throughout the region). The number in parentheses is the number of elk in each category affected.
[a] The optic chiasm was collected in only 3 of these 6 elk.

Obex score = 6. Two elk (one 132MM and one 132ML) had an obex score of 6. PrP^{CWD} immunoreactivity was detected in the superior colliculus (Fig. 4A), visual cortex, and oculomotor nucleus in the 132ML elk. PrP^{CWD} immunoreactivity was detected in the lateral geniculate nucleus, superior colliculus, visual cortex, and oculomotor nucleus in the 132MM elk. Both

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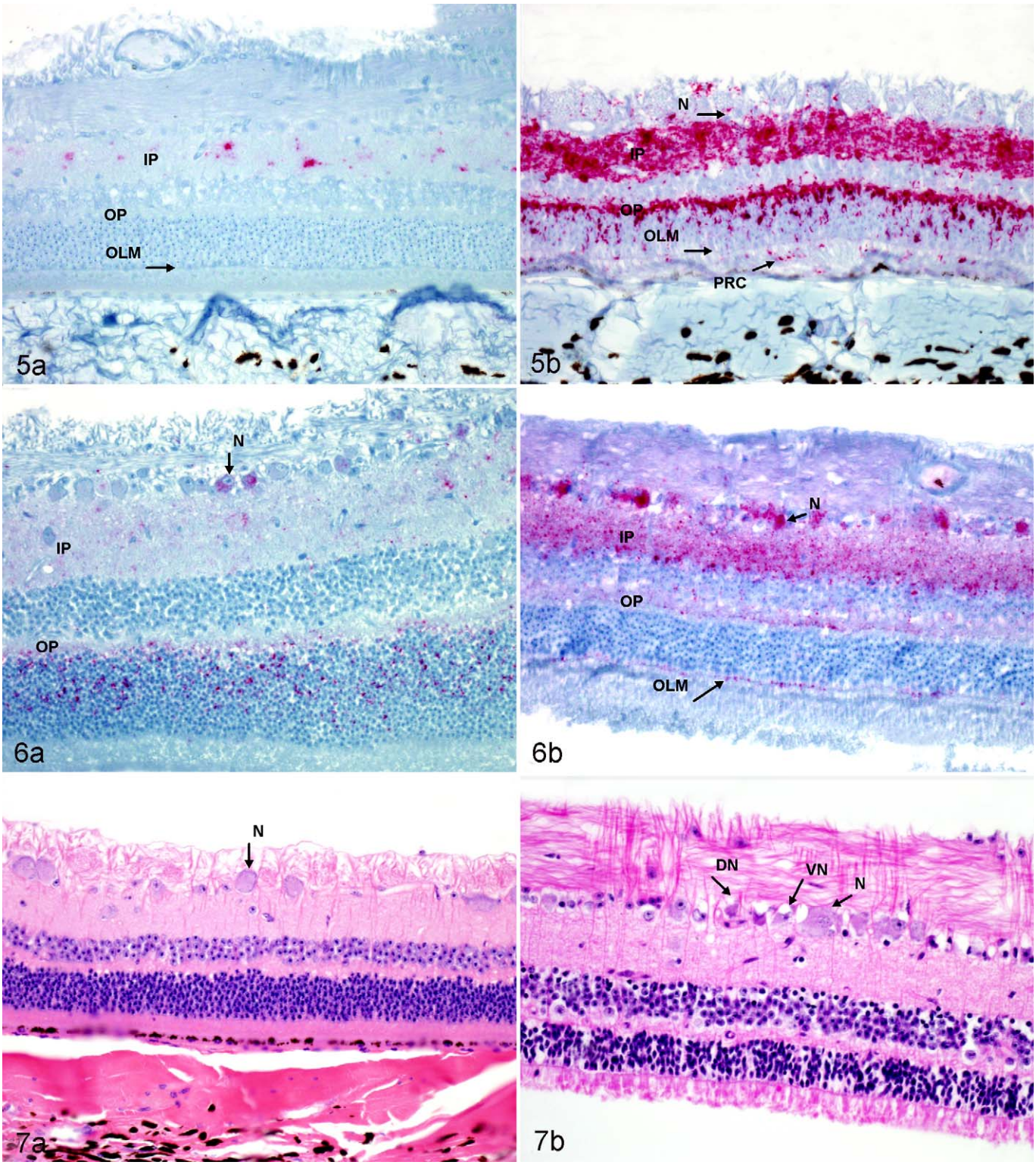

Figure 5. Photomicrograph of the retina in 2 CWD-positive 132MM elk. A, an elk with an obex score of 7. Note the minimal bright red granular chromogen deposition in the inner plexiform (IP) layer and the absence of staining in the outer plexiform (OP) and outer limiting membrane (OLM) layers. B, an elk with an obex score of 9. Note the heavy staining in the IP and OP layers, with less staining in other layers; the absences of intracytoplasmic staining of the neurons (N) of the ganglion cell layer and the lack of accumulation of small aggregates of chromogen on the inner surface of the OLM in both elk; and the granular staining in the layer of photoreceptor cells (PRC). Immunohistochemical staining: Anti Prion (99), monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. 132MM: codon 132, elk identified as homozygous for the wild-type allele encoding methionine. CWD, chronic wasting disease. **Figure 6.** Photomicrograph of the retina of a CWD-positive 132ML Rocky Mountain elk with an obex score of 10 (A) and a 132LL elk with an

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linear and granular patterns were found in these elk, but the linear pattern was more prominent. Spongiform degeneration was confined to the obex and not found in nuclei of the visual pathway. PrP^{CWD} immunoreactivity or histological lesions were not detected in any layer of retina in these 2 elk.

Obex score = 7. Three elk (132MM) had an obex score of 7. PrP^{CWD} immunoreactivity was detected in the optic tracts of 1 elk; in the retina, lateral geniculate nucleus, and optic radiations of 2; and in the visual cortex, oculomotor nucleus, and superior colliculus of all 3. The patterns of PrP^{CWD} immunoreactivity were predominately granular, but areas of linear staining were still found. An increased number of plaques of PrP^{CWD} immunoreactivity were found in all visual pathways in the brain. In most areas, these plaques appeared to be surrounding and filling the cytoplasm of astrocytes. Mild spongiform degeneration was found within ocular motor nucleus and superior colliculus. PrP^{CWD} immunoreactivity was detected in the inner plexiform layer of the retina in one (Fig. 5A) and in the inner and outer plexiform layer in the other. The pattern of PrP^{CWD} immunoreactivity in the retina of the 132MM elk was characterized by light red granular chromogen staining that was first detected in the inner plexiform layer.

Obex score = 8. Five elk (three 132MM, one 132ML, and one 132LL) had an obex score of 8. PrP^{CWD} immunoreactivity was detected in the retina of two 132MM elk and the 132ML and 132LL elk. PrP^{CWD} immunoreactivity was detected in the optic nerve, optic chiasm, and optic tracts in two 132MM elk. PrP^{CWD} immunoreactivity was detected in the lateral geniculate nucleus, optic radiations, visual cortex, oculomotor nucleus, and superior colliculus in all 5 elk. The pattern of immunoreactivity in these elk was of the coarse granular pattern with an increased number of plaques. Linear staining was minimal. Mild to moderate spongiform degeneration was found in the nuclei of the visual pathways within the brain. PrP^{CWD} immunoreactivity was detected in multiple layers of the retina in all 5 elk. All layers had some degree of PrP^{CWD} immunoreactivity, except the outer limiting membrane and the pigmented epithelium. Layers of the retina with the most intense PrP^{CWD} immunoreactivity were the inner and outer plexiform layers. Intracytoplasmic PrP^{CWD} immunoreactivity within neurons of the ganglion cell layer was present in the 132ML elk but was a prominent feature in the 132LL elk (Fig. 6A, 6B). Intracytoplasmic PrP^{CWD} immunoreactivity within neurons of the ganglion cell layer was not present in the 132MM elk. Small aggregates of PrP^{CWD} immunoreactivity were present on the inner surface of the outer limiting membrane in 132LL elk but not in 132MM or 132ML elk.

Obex score = 9–10. Four (three 132MM and one 132LL) elk had an obex score of 9, and 6 had an obex score of 10 (five 132MM and one 132ML). PrP^{CWD} immunoreactivity was found in all the neuroanatomical visual pathways in the brain (Figs. 3B, 4B). The patterns of PrP^{CWD} immunoreactivity were similar to those described in elk with an obex score of 8. Linear staining was rare. Mild to severe spongiform degeneration was found in the nuclei of the visual pathways in all these elk. PrP^{CWD} immunoreactivity was found in all layers of the retina except the outer limiting membrane and the pigmented epithelial layers in the 132MM elk. Small aggregates of PrP^{CWD} immunoreactivity were present on the inner surface of the outer limiting membrane in 132LL elk but not in the 132MM elk (Fig. 5B). A few ganglia of the retina showed evidence of central chromatolysis, vacuolation, and necrosis (Fig. 7A, 7B) but with slightly more frequency in the elk with a score of 10. The overall degree of spongiform degeneration of the retina was minimal in all genotypes.

Epitope Mapping With Amino Terminal Antibody P4 and Carboxyl Terminal Antibody 99

The intensity and location of PrP^{CWD} immunoreactivity observed with mAbP4 in the brain and retinal sections were similar to those of the brain and retinal sections in the 132MM and 132ML elk stained with mAb99. Immunoreactivity with mAbP4 was identical in character and pattern to that observed with mAb99 except in one location in the 132LL elk. The accumulation of red chromogen granules on the inner surface of the outer limiting membrane as seen in the 132LL elk immunostained with mAb99 was not observed with mAbP4.

Lack of PrP^{CWD} Immunoreactivity in Other Structures in the Eye

PrP^{CWD} immunoreactivity or histological lesions were not found in the lens, ciliary body, iris, cornea, or sclera in any of the 3 genotypes of the 27 CWD-positive elk.

Figure 6. (continued). obex score of 9 (B). Both elk were showing clinical signs when euthanized. Note the reduced amount of red chromogen deposition of PrP^{CWD} in the inner plexiform (IP) and outer plexiform (OP) layers of the retina as compared to that of the 132MM elk. Note also the heavy intracytoplasmic staining of the neurons (N) of the ganglion cell layer and the accumulation of small aggregates of chromogen on the inner surface of the outer limiting membrane (OLM) in the 132LL elk as compared to the 132 ML elk. Immunohistochemical staining: Anti Prion (99), monoclonal antibody 99, streptavidin–alkaline–phosphatase method. Hematoxylin/bluing counterstain. 132MM: codon 132, elk identified as homozygous for the wild-type allele encoding methionine; 132LL: codon 132, elk identified as homozygous for the alternative allele encoding leucine; 132ML: codon 132, elk identified as heterozygous for the wild-type allele encoding methionine and for the alternative allele encoding leucine. CWD, chronic wasting disease; PrP^{CWD}, prion protein associated with chronic wasting disease. **Figure 7.** Photomicrograph of the retina from a CWD-positive 132ML Rocky Mountain elk with an obex score of 10. A, early lesions characterized by central chromolysis of neurons (N) of the ganglia cell layer. B, retina of the same elk. Note unaffected (N), vacuolated (V), and dead neurons (DN) within the ganglia cell layer. HE. 132ML: codon 132, elk identified as heterozygous for the wild-type allele encoding methionine and for the alternative allele encoding leucine. CWD, chronic wasting disease.

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Discussion

The degree of PrP^{CWD} immunoreactivity and spongiform encephalopathy is thought to correlate with the duration of disease. Researchers have suggested that for mule deer and white-tailed deer, the more detectable PrP^{CWD} and severity of spongiform degeneration in neural tissues, the longer the animal has been incubating the disease[6,16,35]; however, this relationship has not yet been delineated in elk. Neither the pathogenesis nor the dissemination of the agent through the host is well defined in Rocky Mountain elk. In this study, we examined the presumed progression of PrP^{CWD} dissemination along the visual pathway. Detection of PrP^{CWD} immunoreactivity in the retina has been reported in mule deer[35] and white-tailed deer[16] with CWD, ovine scrapie,[4,12] and bovine spongiform encephalopathy,[7] but the pathway and kinetics of prion protein accumulation are not reported. Data from this study demonstrated that detectable PrP^{CWD} immunoreactivity first accumulated in the visual pathways of the brain, before the retina. Elk with an obex score of < 6 had no evidence of PrP^{CWD} immunoreactivity in the optic nerve or retina but did have PrP^{CWD} immunoreactivity in neural regions of the visual pathway in the brain. The first nuclear area where PrP^{CWD} immunoreactivity was detected was in the superior colliculus, followed by the visual cortex (obex score of 2). Next PrP^{CWD} immunoreactivity was detected in the lateral geniculate nucleus and oculomotor nucleus (obex scores of 3 to 6). PrP^{CWD} immunoreactivity was first detected in the retina in elk with an obex score of 7. At this time, PrP^{CWD} immunoreactivity was beginning to accumulate to detectable levels in the axonal tracts of the visual pathway (the optic radiations, optic tracts, optic chiasm, and optic nerve). Elk with an obex score of 7 usually had PrP^{CWD} immunoreactivity in the optic nerve and retina but at much lower intensity as compared to that of the more advanced elk with an obex score of 9 or 10.

Elk with an obex score of < 5 were thought to be in relatively early stages of disease; elk with an obex score of 6 to 7 were thought to be in the midstage of disease; and elk with an obex grade of > 8 were in a more advanced stage. Clinical signs were primarily observed in elk with an obex score of 10; however, 2 elk (one with a score of 8 and the other, a score of 9) did show mild signs, but both had pneumonia at the time of necropsy. In addition to differences in distribution, the immunoreactivity characteristics of PrP^{CWD} were different as the disease progressed. The pattern of PrP^{CWD} immunoreactivity in the brain in early cases was mostly linear, apparently composed of small granular particles of PrP^{CWD} immunoreactivity attached to the periphery of axons or dendrites. The pattern of PrP^{CWD} immunoreactivity in later stages of disease was more granular, including plaque formation.

Retina had detectable PrP^{CWD} immunoreactivity in elk with an obex score of > 7. Prion was first detected in the inner plexiform layer of the retina, then the inner and outer plexiform layers. The prion then spread throughout the remaining retinal layers, sparing the pigmented epithelial layer. In domestic sheep with scrapie, the inner plexiform layer appears to be affected first; then, as the disease progresses, the outer plexiform layer accumulates PrP^{Sc} (abnormal prion protein associated with spongiform encephalopathy).[7] Further evidence suggests that as PrP^{Sc} accumulates, there are morphologic changes in retinal ganglion cells, rod bipolar cells, and Müller glia that are associated with functional abnormalities.[28] The spread of PrP^{CWD} in the retina of elk may be similar to that of sheep.

The amino acid sequence of the *PRNP* gene at codon 132 plays a role during the incubation period of elk developing CWD. The 132MM genotype is the most common in free-ranging and captive elk, and it is associated with an estimated incubation period of approximately 2.5 years. The 132ML genotype is not uncommon in captive and free-ranging elk.[24] CWD was identified in this genotype at a frequency in proportion to its abundance in the entire sampled population in a recent study.[24] The experimental incubation period in elk of this genotype was approximately 3.5 years. Finally, 132LL elk are rare in free-ranging and captive elk (< 4%) and have an experimental incubation period of approximately 5 to 6 years or longer.[8,22] The elk in this study (except for the two 132LL elk) had been incubating the disease for an unknown period, and the number of times these elk were naturally exposed was not known.

There were 2 patterns of PrP^{CWD} immunoreactivity in the retinal layers among the 132MM, 132ML, and 132LL elk. Intracytoplasmic accumulation of PrP^{CWD} immunoreactivity within neurons of the ganglion cell layer of the retina was not found in the 132MM elk, but a few neurons with intracytoplasmic PrP^{CWD} immunoreactivity were detected in the 132ML elk. Intracytoplasmic PrP^{CWD} immunoreactivity granules within neurons within the ganglion cell layer were a prominent feature in the 132LL elk. This pattern was identical with the mAb99 and mAbP4. It also appeared that even though the overall severity of retinal degeneration was minimal, it was nearly nonexistent in the 132MM elk and only sporadically observed in the 132ML and 132LL elk. In mouse models, retinal degeneration was noted in mice expressing PrP^{c} (the normal cellular isoform of the prion protein) in astrocytic, microglial, and neuronal cells in the retina but not in mice expressing PrP^{c} solely in neurons, thereby suggesting that microglial/astrocytic prion protein expression may be required for scrapie-induced retinal damage.[17,18] In regard to the elk in this study with naturally occurring CWD, data suggest that the lack of intraneuronal PrP^{CWD} immunoreactivity in the 132MM elk indicates minimal neuronal PrP^{C} expression. However, if leucine is incorporated into the *PRNP* gene, then neuronal PrP^{C} expression is increased and, therefore, PrP^{CWD}. Likewise, the 132LL genotype may be associated with higher neuronal PrP^{C} expression.

The second difference was the detection of small aggregates of PrP^{CWD} located on the inner surface of the outer limiting membrane in 132LL elk but not in 132MM or 132ML elk. This pattern was observed with mAb99 but not with mAbP4. The outer limiting membrane (or external limiting membrane) is not actually a membrane but a series of junctional complexes between the photoreceptor cells and the Müller cells. The outer limiting membrane is formed by the distal ends of the Müller cells that attach to the photoreceptor cells by zonula

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adherens–type junctions. The filamentous material associated with these intercellular zonula adherens junctions with light microscopy appears to be a membrane.[5,15] This PrP^{CWD} immunoreactivity at the inner surface of the outer limiting membrane suggests that this junction can impede movement of PrP^{CWD} to some degree in the 132LL elk but not in the 132MM and 132ML elk. This may reflect a functional difference between the Müller cells of 132MM and 132ML elk and those of the 132LL elk.

Further epitope mapping with antibodies specific for the amino and carboxyl termini of the processed prion protein will be needed to demonstrate a difference in intracellular processing of abnormal PrP^{CWD} in tissues of the visual pathway in 132LL elk. We have reported a novel proteinase K cleavage site in PrP^{CWD} extracted from brain homogenate from 132LL elk.[23] In this study, we demonstrate the novel processing in vivo in ganglia of the retina. The reason for these differences was not determined, but it may relate to the mechanisms by which the leucine plays a role in the metabolism or expression of PrP^{CWD} or PrP^{C} within ganglia and Müller cells.

Data from this study suggest that the retina of elk may be affected in the latter stages of CWD, as similar to the findings of white-tailed deer in which PrP^{CWD} was detected in only 4 of 63 CWD-positive white-tailed deer; all 4 white-tailed deer were in advanced disease based on obex scoring.[16]

Prions in the nervous system have been found to localize in the presynaptic junctions in the brain, thereby suggesting that prions may have a function at the synaptic junctions in humans suffering with sporadic and variant Creutzfeldt–Jakob disease.[11,20] This may explain why the inner and outer plexiform layers of the retina were affected first and had most of the detectable PrP^{CWD} immunoreactivity. In domestic sheep, PrP^{Sc} has been reported to be heavily concentrated in the synaptic layers of the retina (inner and outer plexiform layers).[7,28]

In the terminal stages of CWD, there is heavy immunoreactivity in neuronal regions of the brain and retina but low immunoreactivity in such regions the optic nerve, optic chiasm, optic tract, and optic radiations. This finding may be due to the lack of synaptic junctions in these predominately large axonal tracts; therefore, these regions would not produce as much PrP^{C} and, as such, not support as much PrP^{CWD} production or accumulation.

Literature describing clinical signs of CWD has not mentioned visual impairment.[32,37,38] Elk in terminal stages of CWD often appear to see movement, but if one walks slowly or does not move quickly near an affected elk, the elk does not seem to pay much attention (personal observations, T.R.S.). This behavior has been attributed to lesions in the central nervous system rather than in the eye. The literature does suggest that abnormal prion alters localization of synaptic proteins.[25] Contributing to this behavior, by causing visual impairment in elk in the latter stages of CWD, is the accumulation of PrP^{CWD} within the retina—especially in the inner and outer plexiform layers—with neuronal degeneration of the neurons of the ganglia cell layer, as well as PrP^{CWD} accumulation and spongiform degeneration of the nuclei of the visual pathways within the brain.

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Declaration of Conflicting Interests

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Ornithobacterium rhinotracheale North American Field Isolates Express a Hemolysin-Like Protein

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SUMMARY. *Ornithobacterium rhinotracheale* is a gram-negative bacterium responsible for the sporadic outbreaks of airsacculitis in poultry, accounting for millions of dollars in losses to the poultry industry annually. Although the organism was originally classified as non–β-hemolytic, recent North American field isolates of *O. rhinotracheale* obtained from pneumonic lungs and air sacs indicated hemolytic activity on blood agar plates upon extended incubation for 48 hr at room temperature in air after initial incubation at 37 C for 48 hr under 7.5% CO_2. This report characterizes the β-hemolytic activity of *O. rhinotracheale* isolates by using *in vitro* kinetic hemolysis assays with sheep red blood cells, western blotting with leukotoxin-specific monoclonal antibodies, and isobaric tagging and relative and absolute quantitative (iTRAQ) analysis of *O. rhinotracheale* outer membrane protein digest preparations. The kinetic analyses of the hemolytic activity with red blood cells indicated that the protein is a pore former. iTRAQ analysis with membrane preparations revealed four peptides with homology to *Mannheimia haemolytica* leukotoxin and two peptides with homology to *Actinobacillus actinoacetemcomitans* leukotoxin. This is the first report that North American field isolates of *O. rhinotracheale* may express a hemolysin-like activity.

RESUMEN. Los aislamientos de campo de *Ornithobacterium rhinotracheale* de América del Norte expresan una proteina tipo hemolisina.

Ornithobacterium rhinotracheale es una bacteria gram-negativa responsable de brotes esporádicos de aerosaculitis en aves comerciales, lo que representa millones de dólares en pérdidas a la industria avícola por año. Aunque el organismo originalmente fue clasificado como no β-hemolítico, aislamientos recientes de *O. rhinotracheale* de América del Norte, obtenidos de pulmones con neumonía y de sacos aéreos, mostraron actividad hemolítica en placas de agar sangre después de una incubación prolongada durante 48 horas a temperatura ambiente en el aire después de una incubación inicial a 37 C durante 48 horas en un ambiente con 7.5% de CO_2. En este reporte se caracterizó la actividad β-hemolítica de los aislamientos de *O. rhinotracheale* mediante ensayos de la cinética de la hemólisis *in vitro* con eritrocitos de oveja, inmunoelectrotransferencia con anticuerpos monoclonales específicos contra leucotoxina y por los análisis cuantitativos relativos y absolutos de las preparaciones de la digestión de la proteína externa de membrana de *O. rhinotracheale* (con las siglas en inglés iTRAQ). El análisis cinético de la actividad hemolítica con los eritrocitos indicaron que la proteína forma poros. El análisis iTRAQ con preparaciones de membrana reveló cuatro péptidos que mostraban homología con la leucotoxina de *Mannheimia haemolytica* y dos péptidos con homología con la leucotoxina de *Actinobacillus actinoacetemcomitans*. Este es el primer reporte que describe que los aislamientos de campo de *O. rhinotracheale* de América del Norte pueden expresar una actividad similar a la de una hemolisina.

Key words: *Ornithobacterium rhinotracheale*, hemolysin, hemolysis

Abbreviations: BHI = brain-heart infusion; HEPES = 4-(2-hydroxyethyl) piperizane-1-ethanesulfonic acid; iTRAQ = isobaric tagging and relative and absolute quantitative; lktA = leukotoxin A; PBS = phosphate-buffered saline; RBC = red blood cell; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ornithobacterium rhinotracheale is a gram-negative emerging pathogen of adult poultry (12) and occurs worldwide (1). In the United States, despite the availability of autogenous vaccines, economic losses to the poultry industry are estimated in hundreds of millions of dollars annually (12). Clinical signs include sinusitis, airsacculitis, arthritis, and increased mortality (10,17,36,38,43) and lowered egg production in turkey layers (28) and laying chickens (44). Poultry can carry *O. rhinotracheale* and seroconvert in the absence of overt clinical signs (3,25). Outbreaks of *O. rhinotracheale* are seasonal and usually occur as a secondary infection (34,51), and often have minimal effect on the outcome of the primary infection (27,36). Outbreaks of *O. rhinotracheale* as the primary agent have also been reported (52).

In the absence of information about the genome sequence it is not possible to query the sequence and identify potential virulence factors for *O. rhinotracheale*, but a few reports describe potential virulence factors. For example, a potential virulence-associated plasmid-encoded protein of unknown function was described (26). Most pathogens are known to express redundant mechanisms of virulence, including iron acquisition mechanisms (14,15,16,24,45), colonization strategies (20,21,33,35,37), and adaptations that alter immune cell function through the toll-like receptor–mediated signaling pathway (7). Some pathogens secrete cytolytic toxins that affect host cell function, such as leukotoxin A (lktA) of *Mannheimia haemolytic*a (32), or the hemolysin HlyA of *Escherichia coli* (18,23). No cytolytic activities have been reported for *O. rhinotracheale*. Indeed, the ATCC 51463 strain of *O. rhinotracheale* was described as non–β-hemolytic (49). We observed, however, that some field strains of *O. rhinotracheale* exhibited typical but weak β-hemolytic

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Table 1. Description of *O. rhinotracheale* isolates used in this study.

Isolate no.	Serotype	Tissue source	Designation/origin
P5883	C	Unknown	Strain 31, University of Minnesota
P5884	A	Unknown	Strain 83, University of Minnesota
P5886	E	Unknown	Strain 87, University of Minnesota
P5887	A	Lung (pneumonia)	Strain 88, University of Minnesota
ATTC strain 51463	A	Respiratory tract	ATCC strain 51463, U.K.
Isolates capable of growth under iron-restricted conditions[A]			
P5902		Lung	3311-05/AR
P5917		Unknown[B]	05-573/CA
P5921		Unknown[B]	Ko500325/CA
P5932		Lung	H06-030791/NC
P5938		Lung	06-Jo/NC
P5945		Lung	D05-045749/MN
P5952		Liver	D05-062700/MN
P5967		Unknown	D06-10652/MN
P5969		Unknown	D06-12314/MN
Isolates incapable of growth under iron-restricted conditions[A]			
P5907		Trachea	167-06/MO
P5908		Lung	1012-06/CO
P5909		Trachea	1513-06/AR
P5913		Not known[B]	K02-181/CA
P5926		Not known[B]	97-7091/GA
P5936		Lungs	R06-025944/NC
P5940		Trachea	D05-043244/MN
P5963		Unknown[B]	D06-30480/MN
P5970		Unknown[B]	D0611105/MN

[A]Cultures grew in the presence and absence of 100 μM 2,2′-dipyridyl (see reference 41).
[B]Data not available.

reactions on sheep blood agar plates (46) similar to what has been observed for *M. haemolytica* (8). Depending on the strain used, development of β-hemolytic activity of *O. rhinotracheale* was a delayed reaction and was often observed after 48 hr of incubation under 7.5% CO_2 followed by 48 hr of aerobic incubation at room temperature.

The objective of this work was to compare and characterize biochemically and kinetically the hemolysin-like activity of *O. rhinotracheale* field isolates.

MATERIALS AND METHODS

Cultures and growth conditions. *Ornithobacterium rhinotracheale* used in this study were turkey isolates and are summarized in Table 1. Stock cultures were stored at −80 C in brain-heart infusion (BHI) broth (Becton-Dickinson, Sparks, MD) containing 20% glycerol. Isolates from our culture collection were previously selected for their ability or inability to grow in the presence of 100 μM 2,2′-dipyridyl (45) to stimulate iron acquisition mechanisms.

For assays of hemolytic activity on blood agar plates, a loop-full of stock culture was streaked on a trypticase soy agar plate containing 5% defibrinated sheep blood (Becton-Dickinson) and incubated (48 hr at 37 C, 7.5% CO_2, 15% relative humidity). Evidence for both α-hemolysis (greening of the blood agar under the confluent growth, but no clearing of the blood agar) and β-hemolysis (partial to complete clearing of the blood agar under confluent growth and individual colonies) was recorded daily.

For *in vitro* hemolytic assays, 10 colonies were removed and transferred to a 10 ml culture tube (Falcon; Becton-Dickinson, Franklin Lakes, NJ) containing 5 ml of BHI (Becton-Dickinson). Tubes were incubated aerobically with shaking (100 rpm) at 37 C for 24 hr. One milliliter of culture was removed for absorbance measurements at 600 nm using a Beckman DU-800 spectrophotometer (Beckman-Coulter, Fullerton, CA). Four milliliters of culture from each of two tubes were transferred to 100 ml BHI each in 125-ml Erlenmeyer flasks. Cultures were incubated with shaking (100 rpm) for 24 hr at 37 C and harvested by centrifugation at 10,000 × *g* at 5 C.

Protein extraction. Cell-free protein extracts were prepared as follows. The washed cells were resuspended in 20 mM 4-(2-hydroxyethyl) piperizane-1-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.15 M sodium chloride at a concentration of 30 A_{600} units/ml and was sonicated for 5 min on ice using a microprobe at 50% duty cycle and 50% power (Model 250 cell disruptor; Branson, New Brunswick, NJ) and centrifuged at 15,000 × *g*. The cell-free sonicates were stored at −80 C until used.

Blood collection and hemolysis assays. Sheep and turkey red blood cells (RBCs) were obtained aseptically (by the veterinary staff of the National Animal Disease Center) from blood defibrinated by collection into sterile Erlenmeyer flasks containing 3-to-4-mm glass beads and two 2.5-cm (1-inch) lengths of coiled copper wire while gently swirling the flasks. Blood was poured aseptically through sterile gauze into sterile Erlenmeyer flasks and stored at 5 C and used within 3 days.

In vitro hemolysis assays were performed with both sheep and turkey RBCs. Briefly, sheep and turkey blood was collected aseptically in ethylenediaminetetraacetic acid–coated blood collection tubes (Becton-Dickinson) and centrifuged in 50-ml sterile conical tubes in a swinging bucket centrifuge at 400 × *g* for 10 min at 20 C. Erythrocytes were washed three times with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer and 0.15 M sodium chloride, pH 7.4) until the supernatant was colorless. A suspension of 1% RBCs (determined from the hematocrit volume) was made in 50 mM PBS containing 0.1% bovine serum albumin.

The hemolysis assay was performed as described previously (19) with some modifications. Briefly, proteins in 0.5-ml volumes containing 0.5, 1.5, and 2.25 mg protein/ml in 50 mM PBS were added to microfuge tubes containing 0.5 ml of RBC suspension. The tubes were gently inverted and incubated in a 37 C water bath. RBC lysis control (100%) was prepared by adding 0.5 ml of a 0.4% saponin (Sigma Chemical Co., St. Louis, MO) solution in water to 0.5 ml of RBC suspension. At specified time intervals, 0.2-ml aliquots were removed and centrifuged at

5000 × *g* for 1 min at room temperature. Supernatants were removed and deposited in Falcon 3595 (Costar, Cambridge, MA) microtiter plates. The absorbance was read at 540 nm using a model 250 microplate reader (SpectraMax 250; Molecular Dynamics, Sunnyvale, CA). Results were plotted using Excel (Microsoft, Redmond, WA).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Protein samples were denatured and chemically reduced using sample buffer from Invitrogen (Carlsbad, CA) containing 1 mM dithiothreitol and loaded onto 10-or-15-well 4%–20% gradient gels (Invitrogen). Samples were run according to the manufacturer's instructions, stained with Coomassie blue R250, and destained as described (30). Western blotting was performed using nitrocellulose membranes as described (48). For leukotoxin visualization, membranes were probed with a 1:10,000 dilution of anti-leukotoxin IgG monoclonal antibodies 2C9-1E8 (neutralizing) and 6A7-2E7 (nonneutralizing; R. E. Briggs *et al.*, unpubl. data) and prepared from *Mannheimia haemolytica* strain D153 serotype 1 using preparative SDS electrophoresis (Biorad Preparative Cell 491; Biorad, Hercules, CA). The neutralizing antibody was similar to MM601 (22). Unrelated IgG2b monoclonal antibody at 1:10,000 (obtained from P. Kapke, Iowa State University, and prepared to an unrelated recombinant bacterial protein) served as a control. Blots were incubated with the appropriate conjugates, alkaline phosphatase-conjugated anti-mouse IgG at 1:1000, or alkaline phosphatase-conjugated anti-turkey IgG at 1:1000. Color was developed for 10 min at room temperature using one 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet per 10 ml (Sigma).

Isobaric tagging and relative and absolute quantitative (iTRAQ) analysis of *O. rhinotracheale* outer membrane proteins for leukotoxin peptides. Outer membrane proteins for iTRAQ analysis were prepared as follows. Four strains of *O. rhinotracheale* (P5883, P5884, P5886, ATTC 51463) were grown in 100 ml of BHI broth for 48 hr at 37 C with agitation (150 rpm), and centrifuged at 7500 × *g*. The cell pellet was washed with 0.1 M PBS, pH 7.2, and resuspended in a small volume (0.5 ml) of 2.5% NaCl in 20 mM HEPES, pH 7.4, and heated for 1 hr at 56 C to extract the outer membrane proteins (18). The extracted protein was dialyzed against 20 mM HEPES, pH 7.4. Protein concentration was determined using a bicinchoninic acid assay (Pierce Chemicals, Rockford, IL). Protein preparations were precipitated with ice-cold acetone (13), and 100 μg of each protein preparation was treated with one of four isobaric amino-modification reagents labeled with biotin (iTRAQ labels; Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The protein preparations were digested with trypsin and mixed, and the peptides were separated by affinity chromatography on an avidin column as per manufacturer's directions. Samples were analyzed by liquid chromatography/mass spectrometry/mass spectrometry using the Q-Star XL-TOF quadrupole tandem mass spectrometer (Applied Biosystems) located at the Plant Science Institute, Iowa State University. Results were analyzed by using the Multi-Q software program (http://ms.iis.sinica.edu.tw/Multi-Q-Web/) (54).

RESULTS

Hemolytic activity on blood agar plates. Hemolytic activity was observed for most of the field strains after 48 hr at room temperature aerobically following an initial 48-hr incubation under 7.5% CO_2. Initial observations were recorded for isolate P5883 (serotype C, a chicken isolate), isolates P5884 and P5887 (both serotype A isolates from turkey), isolate P5886 (serotype B from turkey), and the reference strain ATTC 51463 (serotype A, a turkey isolate designated as the type strain for *O. rhinotracheale*) (47). For these strains and the subsequently tested additional field strains, α-hemolysis (greening of the blood agar) was often observed after 48 hr of incubation and upon removal of the plates from the CO_2 environment. Upon further incubation at room temperature, partial or complete hemolysis was observed, depending on the isolate tested.

Fig. 1. Hemolytic reaction of *O. rhinotracheale* field isolate P5932 on sheep blood agar. The isolate was incubated for 48 hr at 37 C, 7.5% CO_2, 15% relative humidity, followed by incubation aerobically at ambient temperature for 48 hr. Complete clearing under the confluent growth is evident as well as clearing under and surrounding the isolated colonies.

One field strain, isolate P5932, (Table 1; Fig. 1) (45) showed the earliest and most complete hemolysis of the isolates examined, followed by isolates P5883, P5884, and P5887, whereas the type strain ATCC 51463 exhibited a weak or no β-hemolytic reaction even after extended incubation aerobically at room temperature. In addition, isolate P5886 was nonhemolytic (no clearing). Some field isolates tested, whether they were able to grow in the presence of 2,2′-dipyridyl or not, showed some degree of α-hemolysis and β-hemolysis on sheep blood agar. Similar observations were recorded for hemolysis reactions on defibrinated turkey blood trypticase soy agar plates. Because sheep blood agar plates gave more consistent results (sheep RBCs remain intact and do not release nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate into the medium (2)) all experiments were subsequently performed using sheep blood agar plates.

Mass spectrometry identification of hemolysin orthologs. Because of the initial unexpected observations of hemolysis on blood agar, comparative proteomics using iTRAQ were employed to specifically compare labeled peptide fragments derived from the same protein (or proteins) expressed by different strains of *O. rhinotracheale*. Outer membrane extracts were prepared from four of the five characterized (45) isolates: P5883, P5884, P5886, and reference strain ATCC 51463 (Table 1). The results indicate that isolate P5884-labeled outer membrane proteins contained four peptides that were identical to the lktA of *M. haemolytica* and two peptides that were identical to *Actinobacillus actinomycetemcomitans* hemolysin (Table 2). An iTRAQ mass spectrum for peptide ^{600}KETKIIAK^{609} is shown in Fig. 2. The labeled extracts from isolates P5883, P5886, and ATCC 51463 showed lower relative intensities of the iTRAQ tag released from the respective hemolysin-like peptides peptide ^{600}KETKIIAK^{609}.

***In vitro* hemolytic and kinetic assays.** In order to confirm the observation of β-hemolytic activity observed on sheep blood agar plates and the iTRAQ results, *in vitro* hemolysis assays with both sheep and turkey RBCs were performed. The results shown in Fig. 2

Table 2. Hemolysin peptide homologues identified from iTRAQ analysis of *O. rhinotracheale* P5884 outer membrane proteins.

Peptide	Homology/accession no.	Score
^{221}KGLSGFDK^{229}	*M. haemolytica*/LKA16_PASHA	8.2
^{600}KTKETKIIAK^{609}	*M. haemolytica*/LKA16_PASHA	11.5
^{824}DLTFEKVNHHLVITNTKQEK^{844}	*M. haemolytica*/ LKA16_PASHA	8.73
^{896}IAQSELTK^{904}	*M. haemolytica*/ LKA16_PASHA	25.4
^{122}KHLSNSVGSTGNLTKAIDK^{139}	*A. actinomycetemcomitans*/Q43892	7.09
^{506}KKGEELAKHSDKFTK^{520}	*A. actinomycetemcomitans*/Q43892	15.9

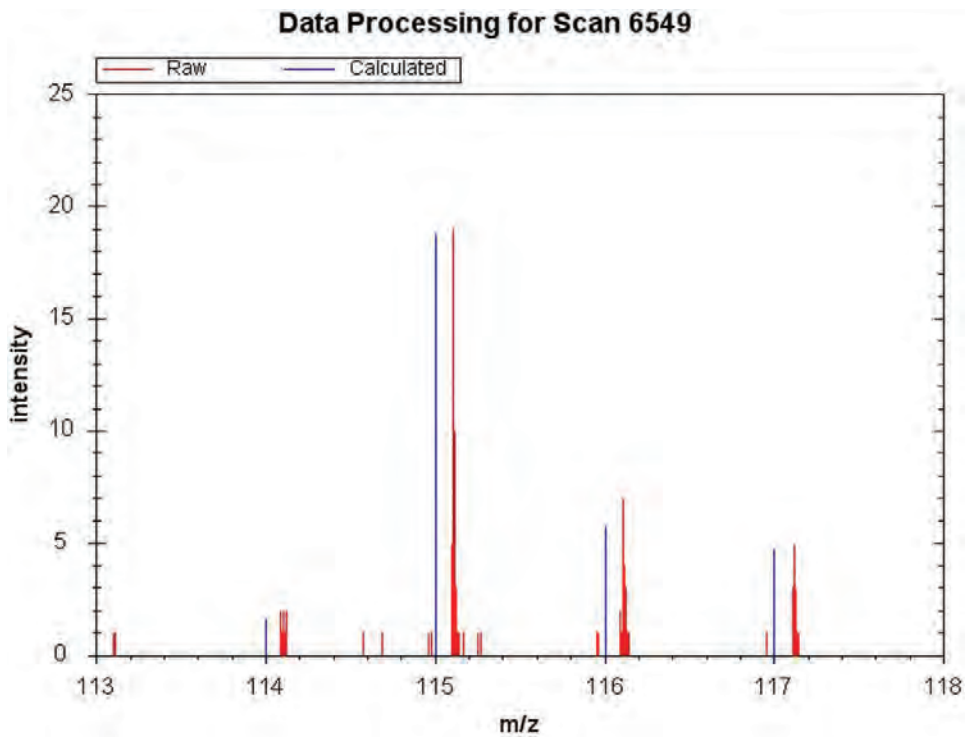

Fig. 2. iTRAQ results for the hemolysin-like activity of *O. rhinotracheale* strains. Relative intensities are shown for strains P5883 (tag 114), P5884 (tag 115), P5886 (tag 116), and ATCC 51463 (tag 117). Details are described under Materials and Methods. The red lines of the mass spectrum indicate the raw data; the blue lines show the normalized data.

depict a time-dependent assay using *O. rhinotracheale* cell-free sonicates obtained from isolates P5883, P5884, ATTC 51463, and P5932 and from sheep red blood cells. Results with turkey erythrocytes were comparable and are not shown. The highest hemolysin-like activity was observed from protein extracts prepared from field isolate P5932. At zero time, percentage of hemolysis of the buffer control, isolate P5883, and the reference strain ATCC 51463 were 11.7% compared to the positive control of saponin (set at 100% hemolysis); isolates P5884 and P5932 exhibited 29% and 71% hemolysis, respectively, compared to the positive control (Fig. 3). We observed that hemolysis takes place within the first minute of incubation at 37 C. Therefore, for subsequent assays, the first sample was removed at 30 sec, and at 2-to-3-min intervals thereafter. The highest hemolytic activity was observed for protein extracts from isolate P5932. Therefore protein extracts from this isolate were used in subsequent assays for concentration-dependent and kinetic analyses. Fig. 4 depicts the concentration-dependent hemolytic assay with the cell-free sonicates from strain P5932. Initial rates of hemolysis measured at the 30-sec time point were directly proportional to the protein concentration of the cell-free sonicates.

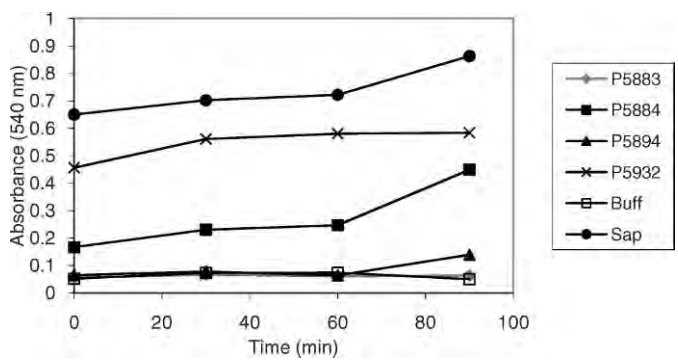

Fig. 3. Time-dependent hemolytic activity of *O. rhinotracheale* protein extracts. Cell-free sonicates were assayed at a final protein concentration of 1 mg/ml using the *in vitro* hemolytic assay with 0.5% sheep RBCs. Absorbance of the assay supernatants were measured after centrifugation at 540 nm. P5883, P5884, P5894 (ATCC 51463), and P5932 are the cell-free sonicates; Buff indicates the buffer control sample and Sap indicates the positive control sample of RBCs lysed with saponin.

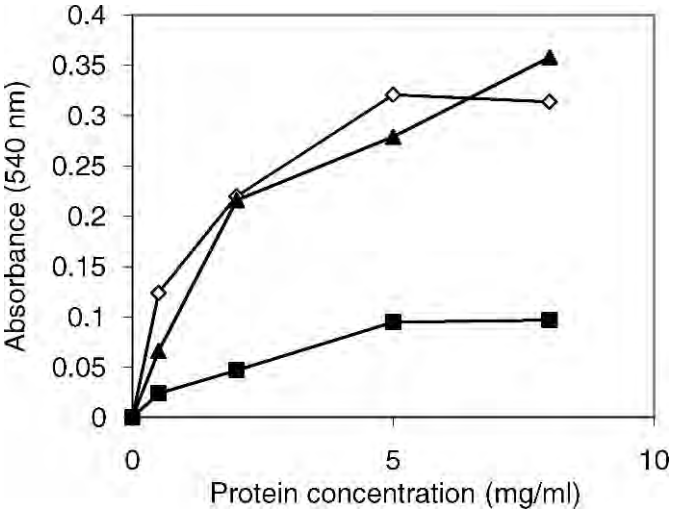

Fig. 4. Concentration-dependent hemolytic activity of *O. rhinotracheale* strain P5932 hemolysis-like activity. Plot of absorbance at 540 nm *vs.* protein concentration of the cell-free sonicates of strain P5932 are shown at protein concentrations of 0.5 (■), 1.5 (▲), and 2.25 (◇) mg/ml in the assay.

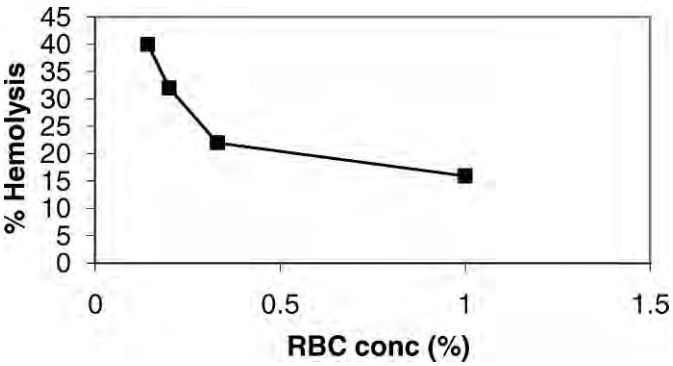

Fig. 5. Plot of percentage of hemolysis *vs.* RBC concentration. Percentage of hemolysis decreases with increasing RBC concentration suggesting that the hemolytic activity is a pore-forming protein. Protein concentration was at 2.5 mg/ml in the assay.

Kinetic analyses of the substrate-dependent data, the RBC concentration in the assay, (Fig. 5) suggests that the hemolytic activity was due to a pore-forming ability of the protein and was not due to enzyme action on the RBC membrane (39), as the percentage of hemolysis decreased with increasing RBC concentration. If the hemolytic activity were an enzyme, percentage of hemolysis would have increased with increasing RBC concentration.

1-Dimensional SDS-PAGE and western blotting of a hemolysin-like protein. Because iTRAQ comparative experiments for global analysis of the *O. rhinotracheale* proteome identified proteins with sequence identity toward four peptide sequences of lktA of *M. haemolytica*, western blotting was performed using highly specific IgG monoclonal antibodies prepared to lktA (Fig. 6). Numerous proteins appeared on the Coomassie-stained gel (Fig. 6A), but only three proteins were visualized on the western blot using the neutralizing monoclonal antibody, 2C9, and the nonneutralizing antibody, 6A7 (Figs. 6B and 6C, respectively). The weakly staining bands corresponded to proteins at 181 and 92 kDa and the strongly staining band corresponded to a protein (or proteins) at 56 kDa.

To rule out potential reactivity of the monoclonal IgG with a putative IgG binding protein, an unrelated IgG2b monoclonal antibody prepared to an unrelated recombinant protein expressed in *E. coli* (Paul Kapke, Iowa State University) was used in a separate western blot experiment (results not shown). This monoclonal also reacted with a protein corresponding to a band at 56 kDa, but at a lower intensity than the monoclonal antibodies to lktA. The unrelated IgG2b monoclonal antibody did not react with the 181- or 92-kDa proteins on the western blot. The difference in intensities could be due to variation in titer of the specific and nonspecific monoclonal antibodies or due to the fact that less putative IgG-binding protein was present in the band. It is possible that staining of the 56-kDa protein (or proteins) resulted because of binding to an as yet unidentified IgG-binding protein in addition to an unidentified hemolysin-like protein of low molecular weight.

DISCUSSION

In 1994, an *O. rhinotracheale* isolate from clinical samples from turkeys was characterized by Vandamme and colleagues (49). This isolate of *O. rhinotracheale* was designated as the reference strain for

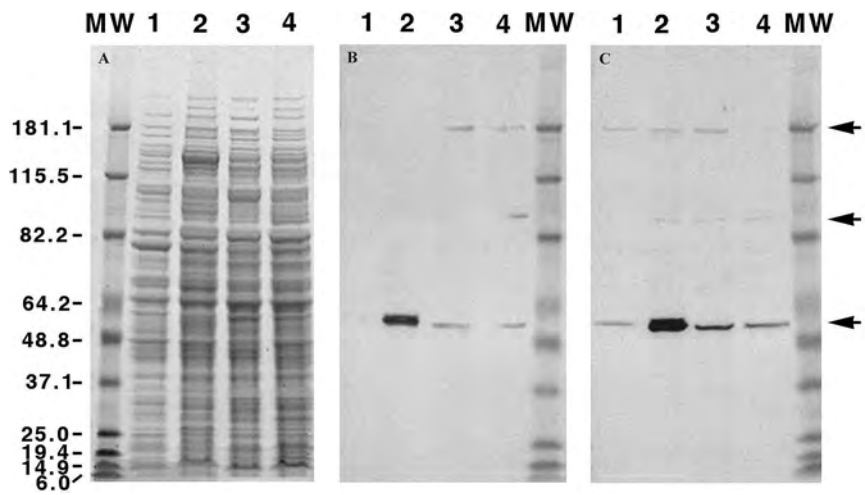

Fig. 6. SDS-PAGE and western blot of cell-free sonicates from *O. rhinotracheale* strains. (A) SDS-PAGE of cell-free lysates from P5883 (lane 1), P5884 (lane 2), ATCC 51463 (lane 3), P5932 (lane 4). (B) Western blot developed with anti-lktA monoclonal antibody 2C9-IE8. (C) Western blot with anti-lktA monoclonal antibody 6A7-2E7. For B and C, lanes are the same as indicated in A. MW, molecular weight markers in kilodaltons. Arrows indicate the bands at 181, 92, and 56 kDa.

Table 3. β-Hemolytic activity on sheep blood agar plates of the *O. rhinotracheale* strains used in this study.

Strain no.	Reaction after 48 hr, 37 C, CO_2[A]	Reaction after 48 hr, room temp, air[B]
Original strains used		
P5883	1 mm, clearing initiated	Complete clearing
P5884	1 mm, clearing initiated	Complete clearing
P5886	1 mm, no clearing	No clearing
P5887	1 mm, clearing initiated	Complete clearing
ATCC 51463	1 mm, no clearing	No clearing
Isolates capable of growth under iron-restricted conditions		
P5902	1 mm, clearing initiated	Complete clearing
P5917	1 mm, clearing initiated	Complete clearing
P5921	1 mm, clearing initiated	Complete clearing
P5932	1 mm, clearing initiated	Complete clearing
P5938	1 mm, no clearing	No clearing
P5945	1 mm, no clearing	No clearing
P5952	2 mm, no clearing	No clearing
P5967	1 mm, clearing initiated	Complete clearing
Isolates incapable of growth under iron-restricted conditions		
P5907	1 mm, clearing initiated	Complete clearing
P5908	1 mm, clearing initiated	Complete clearing
P5909	0.5 mm, clearing initiated	Complete clearing
P5913	1 mm, clearing initiated	Complete clearing
P5926	1 mm, no clearing	No clearing
P5936	1 mm, no clearing	No clearing
P5940	1 mm, clearing initiated	Complete clearing
P5963	2 mm, clearing initiated	Complete clearing
P5970	1 mm, clearing initiated	Complete clearing

[A]Reaction on blood agar after 48 hr of incubation under CO_2; sizes of colonies are indicated; clearing of blood agar observed under confluent growth, but not yet under the individual colonies.

[B]Reaction on blood agar after 48 hr of incubation at room temperature in air; clearing of blood agar observed under most individual colonies.

O. rhinotracheale (ATCC 51463) and was described as a non–β-hemolytic species using sheep blood agar. The isolates used for the current study were North American clinical isolates obtained from the culture collection of K. V. Nagaraja (University of Minnesota, St. Paul, MN; Table 1). Interestingly, the majority of the North American field isolates of *O. rhinotracheale* showed β-hemolytic reactions on sheep blood agar when the incubation period was extended beyond 48 hr under CO_2 with further incubation at ambient temperature for an additional 48 hr (Table 3; Fig. 1). This observation is not unusual for certain microbes and incubation under aerobic conditions and has been done to identify oxygen-stable hemolysins (53). One explanation for the observed difference between the data reported here and those reported by Vandamme *et al.* (49) may be the acquisition of the hemolysin/cytolysin gene by horizontal transfer. Alternatively, it is possible that the gene encoding the hemolysin/cytolysin is not expressed in the type strain described by Vandamme *et al.* (49). Genetic analysis of these strains, although not within the scope of this study, may resolve this issue. Furthermore, it is unknown if the hemolytic-like activity is related to virulence of *O. rhinotracheale*. The hemolytic reactions on sheep blood agar reported here were also observed on turkey blood agar plates. Although of interest, no other cytolytic activities or cell substrates were examined in this study. Furthermore, this study did not directly associate the hemolytic activity with any one of the three proteins identified by western blotting with the lktA-specific monoclonal antibody. It would be of interest to compare the relative virulence of the hemolytic and nonhemolytic phenotypes in the turkey model described by Sprenger *et al.* (43).

In order to identify the hemolytic activity of *O. rhinotracheale* strains, secreted proteins, outer membrane proteins, and whole-cell lysates were examined by iTRAQ mass spectrometry methodology. iTRAQ methodology is the technique of choice for comparative global analysis of proteins expressed by various strains. In this particular study, the data of the iTRAQ experiments were searched for potential hemolysins to corroborate the hemolytic reactions observed on blood agar. Indeed, analysis of iTRAQ experiments identified peptides from *O. rhinotracheale* protein extracts that were identical to peptides of lktA and peptides of *Actinobacillus actinoacetemcomitans* as shown in Fig. 2 and Table 2.

Kinetically, three different types of hemolytic (cytolytic) mechanisms have been described based on the analysis of concentration-dependent *in vitro* assays (4,9). These types are 1) porins, 2) enzymes, and 3) surfactants. Based on kinetic analysis of the hemolytic activity of isolate P5932, we determined that the hemolytic activity was a pore-forming molecule rather than an enzyme molecule (47). However, because the kinetic analysis cannot distinguish between a pore-former and a molecule with surfactant properties, complete DNA and protein sequence information is needed to make this determination. Attempts to clone a hemolysin-like gene with degenerative primers based on the peptides identified by mass spectrometry and southern blotting experiments using probes based on *M. haemolytica* leukotoxin were not successful (F. Tatum, B. Briggs, and L. B. Tabatabai, unpubl. data). Genomic sequencing will eventually provide the results of the nature of the hemolysin/cytolysin molecule.

Western blots prepared from 1-D gels of *O. rhinotracheale* whole-cell lysates and treated with the monoclonal antibodies 2C9-1E8 and 6A7-2E7 to *M. haemolytica* leukotoxin showed three bands at 181, 92, and 56 kDa. A search of the literature for hemolytic molecules indicated hemolysin-like molecules with molecular masses of approximately 180,000, 102,000, and 56,000. These molecules include cytolysins (4,29,31,32,47), Zn-metalloprotease (11), and certain amino-peptidases (5) of both gram-negative and gram-positive microbes. Interestingly, a western blot treated with an

unrelated IgG2b monoclonal antibody indicated that some reactivity occurred with a band at 56 kDa, and may be due to an as yet unidentified IgG-binding protein, which is expressed by many gram-negative pathogens.

In summary, this is the first report that North American field isolates of *O. rhinotracheale* express a hemolysin-like protein present in the whole cell lysate. This hemolytic activity may contribute to the overall virulence of *O. rhinotracheale* (6,40,41,42,50).

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Original Article

Experimental inoculation of pigs with pandemic H1N1 2009 virus and HI cross-reactivity with contemporary swine influenza virus antisera

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Background A novel A/H1N1 was identified in the human population in North America in April 2009. The gene constellation of the virus was a combination from swine influenza A viruses (SIV) of North American and Eurasian lineages that had never before been identified in swine or other species.

Objectives The objectives were to (i) evaluate the clinical response of swine following experimental inoculation with pandemic H1N1 2009; (ii) assess serologic cross-reactivity between H1N1 2009 and contemporary SIV antisera; and (iii) develop a molecular assay to differentiate North American-lineage SIV from H1N1 2009.

Methods Experiment 1: Weaned pigs were experimentally infected with A/California/04/2009 (H1N1). Experiment 2: The cross-reactivity of a panel of US SIV H1N1 or H1N2 antisera with three isolates of pandemic A/H1N1 was evaluated. Experiment 3: A polymerase chain reaction (PCR)-based diagnostic test was developed and validated on samples from experimentally infected pigs.

Results and Conclusions In experiment 1, all inoculated pigs demonstrated clinical signs and lesions similar to those induced by endemic SIV. Viable virus and antigen were only detected in the respiratory tract. In experiment 2, serologic cross-reactivity was limited against H1N1 2009 isolates, notably among virus antisera from the same HA phylogenetic cluster. The limited cross-reactivity suggests North American pigs may not be fully protected against H1N1 2009 from previous exposure or vaccination and novel tests are needed to rapidly diagnose the introduction of H1N1 2009. In experiment 3, an RT–PCR test that discriminates between H1N1 2009 and endemic North American SIV was developed and validated on clinical samples.

Keywords H1N1 2009, hemagglutination inhibition assay, influenza A virus, pathogenesis, swine.

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Introduction

In March–April 2009, a novel pandemic H1N1 emerged in the human population in North America.[1] The gene constellation of the emerging virus was demonstrated to be a combination of genes from swine influenza A viruses (SIV) of North American and Eurasian lineages that had never before been identified in swine or other species. The emergent H1N1 quickly spread in the human population and the outbreak reached pandemic level 6 as declared by the World Health Organization on 11 June 2009. Although the eight gene segments of the novel virus share lineage with available sequences of corresponding genes from SIV from North America and Eurasia, no closely related ancestral SIV with this gene combination has been identified in North America or elsewhere in the world.[2,3] Other than sporadic transmission to humans,[4,5] SIV of the H1N1 subtype historically have been distinct from avian and other mammalian H1N1 influenza viruses in characteristics of host specificity, serologic cross-reactivity, and/or nucleotide sequence. Since 1997–1998 in North America, multiple subtypes of endemic SIV (H3N2, H1N1, and H1N2) co-circulate in most major swine producing regions of the USA and Canada (reviewed in Ref. 6). The North American SIV have a triple reassortant internal gene (TRIG) constellation consisting of six genes excluding the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA), with the six TRIG genes being derived from swine, avian, and human influenza viruses.

Additionally, introduction of H1N1 and H1N2 viruses with the HA and NA genes originating from contemporary human seasonal influenza A viruses (hu-like H1) that are genetically and antigenically distinct from the classical swine H1 lineage were reported in pigs in Canada.[7] The viruses identified in Canadian pigs were human virus lineage in entirety or double (human-swine) reassortants. Since 2005, hu-like H1N1 and H1N2 viruses have emerged in swine herds across the USA as human-swine reassortants possessing the TRIG.[8] Four phylogenetic clusters (α, β, γ, and δ) of H1 SIV are now endemic in US swine.[8,9] However, to date, Eurasian lineage SIVs have not been reported in the USA, thus the potential impact of transmission of the pandemic H1N1 virus to the US pig population is unknown.

In experiment 1, weaned pigs were experimentally inoculated with A/CA/04/2009 H1N1 to evaluate clinical signs and lesions during acute infection with the human pandemic virus in the swine host. As North American lineage H1 SIV are endemic in the USA, it is also important to understand the role that existing herd immunity against endemic swine H1 may play in protecting the swine population from the human pandemic H1N1 2009. The serologic cross-reactivity of a panel of swine antisera generated against H1N1 or H1N2 SIV was investigated against three novel human A/H1N1 isolates in experiment 2 to predict the susceptibility of pigs with pre-existing immunity against North American lineage H1 swine viruses against the pandemic H1N1 2009. To rapidly differentiate the novel H1N1 from endemic North American H1 SIV, in experiment 3 a restriction fragment length polymorphism (RFLP) developed for the matrix (M) gene was evaluated for use as a method of discriminating H1 genotypes in swine specimens.

Materials and methods

Virus isolates

A/California/04/2009 (CA/09) (cell passage 1), A/New York/18/2009 (NY/09) (egg passage 3), and A/Mexico/4108/2009 (MX/09) (egg passage 2) received from the Centers for Disease Control and Prevention (CDC) were propagated in Madin–Darby Canine Kidney (MDCK) cells for use in the studies described below.

In vivo study

In experiment 1, four 5-week-old cross-bred pigs from a herd free of SIV and porcine reproductive and respiratory syndrome virus (PRRSV) were housed in ABSL3 isolation and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. Pigs were inoculated intra-tracheally with 2 ml of 1×10^5 50% tissue culture infectious dose ($TCID_{50}$) of CA/09 (H1N1)v as previously described.[9] Four additional age- and source-matched pigs were maintained as negative controls. All pigs were screened for influenza A nucleoprotein antibody by ELISA (MultiS ELISA; IDEXX, Westbrook, ME, USA) before the start of the study to ensure absence of prior immunity. Pigs were observed twice daily for signs of clinical disease and fever. Nasal swabs (Fisherbrand Dacron swabs; Fisher Scientific, Pittsburg, PA, USA) were taken and placed into 2 ml minimal essential medium (MEM) on 0, 1, 2, 3, 4, and 5 days post-infection (dpi) to evaluate nasal virus shedding and stored at −80°C until study completion. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleep-away; Fort Dodge Animal Health, Fort Dodge, IA, USA) on 5 dpi to evaluate lung lesions and viral load in the lung and other selected tissues. Fresh samples were taken from lung, tonsil, inguinal lymph node, liver, spleen, kidney, semitendinosus skeletal muscle (ham), and colon contents (feces) using individual sterile instruments for each tissue. Fresh necropsy samples were stored at −80°C until processed for downstream assays. Additional samples of the same tissues were fixed in 10% buffered formalin and processed by routine methods for histopathologic and immunohistopathologic examination. Immunohistochemical methods for the detection of influenza antigen in tissues using monoclonal antibody against type A nucleoprotein were employed as previously described.[10] Bronchoalveolar lavage fluid (BALF) samples from 5 dpi were screened for aerobic bacterial growth on blood agar and Casmin (Nicotinamide adenine dinucleotide (NAD) enriched) plates. Diagnostic polymerase chain reaction (PCR) for PCV2 [11] and *Mycoplasma hyopneumoniae*,[12] or an in-house reverse transcriptase PCR (RT–PCR) for PRRSV were conducted on nucleic acid extracts from BALF.

Virus isolation from tissues

Frozen necropsy samples were utilized to assess tissue viral load. Approximately 500 mg of tissue or colon contents (feces) were placed in a 1·5-ml pestle tube and homogenized in 300 μl of PBS with antibiotics. Subsequently, 200 μl of the tissue homogenate, serum, or nasal swab sample was then placed on confluent MDCK cells in 24-well plates to incubate for 1 h. After 1 h of incubation, the sample was removed and 400-μl MEM w/TPCK trypsin was added. The plate was checked at 24 and 48 h for cytopathic effects. After 48 h, 200 μl of cell culture supernatant from each well of the 24-well plate was subsequently passed onto a confluent 48-well plate after a freeze and thaw cycle. After 48 h, evidence of cytopathic effects was evaluated and presence of virus antigen confirmed by immuno-cytochemical staining. Two replicates of tissue virus isolation were conducted. Virus titers in BALF and nasal swabs were determined on MDCK cells in 96-well plates.

RNA extraction from tissues and fluids

The MagMax Microarray (Ambion, Austin, TX, USA) protocol for RNA extraction from tissues was followed.

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Approximately 10 mg of each tissue was placed in a 1·5-ml pestle tube and homogenized in 300 μl of PBS w/antibiotics. Tissue homogenate (100 μl) from above was added to bromo–chloro–propane (10 μl), incubated for 5 min at room temperature, centrifuged at 12 000 × ***g*** for 10 min at 4°C and 100 μl of the aqueous phase was transferred to the processing plate for MagMax RNA extraction as per manufacturer's instructions. The MagMax Viral RNA Isolation (Ambion) kit protocol was used as per manufacturer's instructions for serum, nasal swab, or BALF by adding 50 μl of serum, nasal swab, or BALF sample to the Mag Max plate for RNA extraction.

Real time RT–PCR

A TaqMan assay targeting the matrix gene was performed as previously described [13] with modification as per the USDA–APHIS National Veterinary Services Laboratory protocol to increase sensitivity for the pandemic virus matrix gene by adding a pandemic H1N1 2009 matched reverse primer.

Serologic assays

In experiment 2, 38 polyclonal antisera from pigs immunized with 19 H1 SIV isolated during 1999–2008 were tested against the pandemic viruses in a standard hemagglutination inhibition (HI) assay.[14] Each of the four phylogenetic clusters (α, β, γ, and δ) [8,9] of endemic North American H1 SIV were represented in the panel of sera as previously described [15] and with additional SIV isolates using the same methodology (A.L. Vincent, unpublished). Virus isolates used to generate antisera are listed in Table 3. Immunized pigs received intramuscular injections of 1×10^6 $TCID_{50}$ per ml or approximately 64–128 HA units of UV-inactivated influenza virus combined with a commercial adjuvant (Emulsigen D; MVP Laboratories, Inc., Ralston, NE, USA), followed by one or two booster doses 2–3 weeks apart until sufficient homologous HI titers were reached. For use in the HI assay, sera were heat inactivated at 56°C for 30 min, then treated to remove non-specific HA inhibitors and natural serum agglutinins with receptor destroying enzyme followed by treatment with a 20% suspension of kaolin (Sigma Aldrich, St Louis, MO, USA) and adsorption with 0·5% turkey red blood cells (RBCs). The HI assays were then performed with the CA/09, NY/09, and MX/09 viruses as antigens and turkey RBC using standard techniques.[14] Additionally, 14 antisera from pigs immunized with five commercial vaccines or vaccines in the process of eventual licensure from the USDA–APHIS Center for Veterinary Biologics were tested against the pandemic H1N1 2009 viruses. Commercial vaccine antisera against either individual virus components or the combination of vaccines were supplied by the manufacturers for use in the study. Vaccine A (FluSure® XP; Pfizer Animal Health, New York, NY, USA) is a fully licensed trivalent commercial product containing cluster IV H3N2, γ-cluster H1N1, and δ-cluster H1N1 SIV as vaccine seed viruses. Vaccine B (MaxiVac Excell® 5.0; Intervet/Schering-Plough, Boxmeer, the Netherlands) is a pentavalent product under review for full licensure containing clusters I and IV H3N2 and β-, γ-, and δ-cluster H1 SIV. Vaccine C (Pneumostar® SIV; Novartis Animal Health, Basel, Switzerland) is a fully licensed bivalent commercial product containing H3N2 and α-cluster H1N1, and a trivalent product that is under review for full licensure with the addition of a γ-cluster H1 SIV to the H3N2 and α-cluster H1N1 in the bivalent product. Vaccine D (Newport Labs, Worthington, MN, USA) is a bivalent autogenous vaccine containing β- and γ-cluster H1 SIV. Vaccine E (Boehringer-Ingelheim-Vetmedica, St Joseph, MO, USA) is a subunit vaccine for H1 SIV in the research and development stage. The reported HI titer is the reciprocal of the highest dilution of serum where inhibition of viral agglutination of RBC was observed with a particular serum and virus pair.

RFLP analysis

To identify the Eurasian lineage matrix gene found in pandemic H1N1 2009 by RFLP analysis, isolated RNA was amplified by RT–PCR using forward primer 5′-CATCCCGTCAGGCCCCCTCA and reverse primer 5′-CATGGCCTCCGCTGCCTGTT in experiment 3. Specifically, 4 μl RNA was subjected to amplification by the One-Step RT–PCR Kit (Qiagen, Valencia, CA, USA) using a final concentration of each primer at 0.6 μm with 5 units of RNase inhibitor (Invitrogen, Carlsbad, CA, USA) included in a total volume of 25 μl. Cycling conditions were as follows: 1 cycle of 50°C for 30 min followed by 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 1 min; 1 cycle of 72°C for 10 min followed by a 4°C hold. After amplification, 17 μl was digested for 2 h with restriction endonuclease *AlwNI* under manufacturer's suggested conditions (New England BioLabs, Ipswich, MA, USA). Digested samples were analyzed using a 1·2% agarose gel. Products migrated as an uncleaved 568-bp fragment for endemic North American SIV matrix genes or as cleaved (40, 185, and 343 bp) fragments for the Eurasian SIV-lineage matrix gene in the pandemic H1N1 2009.

Results

A/CA/04/2009 induced clinical disease in pigs

All pigs were free of influenza A virus and influenza A virus antibodies prior to the start of the experiment. All pigs were negative for extraneous viral and *M. hyopneumoniae* in the BALF at 5 dpi and were negative for significant aerobic bacterial growth from BALF. Negative control pigs remained negative for influenza A virus. All pigs infected with CA/09 developed clinical disease and elevated rectal

temperatures beginning as early as 24 h post-infection and extending to 4–5 dpi. Mean rectal temperatures peaked on 2 dpi at 40·4°C with a maximum individual temperature of 41·4°C. Clinical signs were typical of acute respiratory illness with SIV, including lethargy, inappetence, and increased respiration rate and respiratory effort. Nasal virus shedding was detected as early as 1 dpi by RT–PCR and 2 dpi by virus isolation on MDCK cells (Table 1). Virus isolation identified no biologically viable virus outside the respiratory tract whereas viral nucleic acid was identified at low levels in serum and lymph node (Table 2).

All pigs had cranioventral lung consolidation with the percentage of lung involved ranging from 10% to 22%. Microscopic lesions in lungs were typical of influenza virus infection in pigs and were characterized by multifocal to widespread necrotizing bronchitis and bronchiolitis, light peribronchiolar lymphocytic cuffing, and mild multifocal interstitial pneumonia. Airway damage involved all levels of the intrapulmonary respiratory tract and varied from acute epithelial necrosis and sloughing or bronchioles lined by attenuated epithelium and filled with necrotic cellular debris to subacute lesions characterized by disorganized proliferative epithelium lining recovering bronchi and bronchioles. Occasional alveoli contained necrotic cellular debris, but most were clear of debris or inflammatory infiltrates. Tracheal epithelium was focally attenuated in two pigs. No microscopic lesions were observed in other tissues. Lung tissues from all pigs were positive for immunohistochemical staining of influenza A viral antigen. Virus antigen was detected in attached and sloughing necrotic epithelial cells in large to small bronchioles and occasionally in cells or necrotic debris in alveolar lumens (Figure 1B). Only an occasional tracheal epithelial cell contained virus antigen. No viral antigen was detected in liver, kidney, spleen, lymph nodes, tonsil, or skeletal muscle in any of the pigs (data not shown).

Serologic cross-reactivity

The cross-reactivity of the panel of swine H1 antisera against the three pandemic H1N1 2009 isolates is summarized in Table 3. A reciprocal HI titer for individual pig antiserum is reported for each virus isolate. Cross-reactivity ranged from minimal to moderate among the three viral antigens based on reduction between the homologous and heterologous reciprocal HI titers. The greatest cross-reactivity was demonstrated between A/Mexico/4108/2009 and antisera generated against SIV from the H1γ phylogenetic cluster. Cross-reactivity with antisera from vaccinated pigs was limited against all three 2009 pandemic viral antigens.

Discrimination of pandemic H1N1 2009 by RFLP analysis of the matrix gene

An alignment of endemic swine influenza matrix gene nucleotide sequences with CA/09 (FJ966085, 972 bp) revealed two unique sites (nt 72 and 415) for restriction endonuclease *AlwNI*. To assess whether this site could be used to differentiate the novel H1N1 isolate from the pres-

Table 1. Nasal shedding and viral load in the lung by RT–PCR and virus titration*

	Nasal swab					Bronchoalveolar lavage fluids
Pig	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	5 dpi
446	+/0	+/0·5	+/3·5	+/4·5	+/4·5	+/3·7
447	−/0	−/0	+/0·5	+/1·5	+/2·3	+/4·3
448	+/0	−/0	+/1·7	+/2·5	+/3·5	+/4·3
449	−/0	+/2·5	+/4·5	+/4·5	+/3·5	+/4·7

*RT–PCR result/$\log_{10}$ $TCID_{50}$ titer/mL.

Table 2. Detection of virus by RT–PCR and virus isolation in blood and tissues at 5 dpi*

Pig	Lung	Tonsil	Lymph node	Serum	Liver	Kidney	Spleen	Muscle	Feces
446	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
447	+/+	+/+	+/−	+/−	−/−	−/−	−/−	−/−	−/−
448	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−
449	+/−	+/−	−/−	+/−	−/−	−/−	−/−	−/−	−/−

*RT–PCR result/virus isolation result.

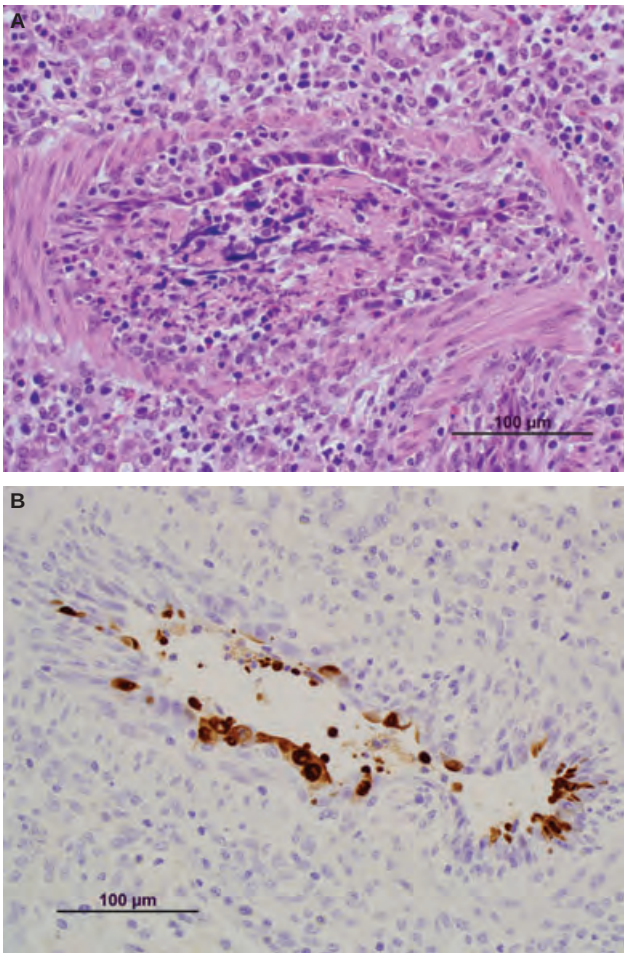

Figure 1 (A) Large bronchiole with severe acute necrosis and sloughing of epithelial cells. Remaining epithelial layer of attenuated cells only partially lines the lumen that is filled with necrotic debris. Light mixed leukocytic infiltrate is evident in the lamina propria and surrounding tissue. (B) Necrotizing bronchiolitis with influenza virus-infected epithelial cells sloughing into the lumen identified by immunohistochemistry.

ent circulating SIV based on the matrix gene, a panel of 24 SIV isolated from cases of respiratory disease from US swine and three pandemic isolates were analyzed using the RFLP test. As shown in Figure 2, the M genes of none of the 24 viruses isolated from US swine were susceptible to digestion with *AlwNI* (lanes 1–24), whereas the M genes of all three human isolates were cleaved into 40, 185, and 343 bp fragments (lanes 25, 30, 31). RNA extracted directly from BALF from animals infected with CA/09 were also examined by this method (lanes 26–29) and produced identical results as the human isolates.

Discussion

Although the pandemic H1N1 2009 viruses are genetically related to SIV of North American and Eurasian lineages, the constellation of the eight gene segments in the pandemic H1N1 2009 is not known to circulate widely in pigs. Additionally, the HA from the pandemic H1N1 viruses circulating in the human population are phylogenetically distinct from the nearest swine virus sequences. As the virulence of the pandemic H1N1 2009 in pigs was not known, we experimentally infected weaned pigs with one isolate of pandemic H1N1 2009, CA/09. We demonstrated that weaned pigs are susceptible to infection and clinical disease induced by CA/09. Importantly, the infection was characteristic of acute influenza illness in swine. Clinical signs, pathologic changes, and virus replication were restricted to the respiratory tract. The results reported here are consistent with recent reports of experimental infections with other strains of pandemic H1N1 virus, where pigs developed pyrexia, anorexia, and dyspnea within several days following challenge.[16,17] Likewise, there have been reports of swine becoming infected in the field with the pandemic H1N1 virus in which the pigs displayed mild respiratory disease (http://www.oie.int/eng/en_index.htm), similar to outbreaks with endemic SIV.

In the study reported here, tissues outside the respiratory tract were found to be negative by virus isolation at 5 dpi. Only respiratory tract samples were positive by both real-time RT–PCR and virus isolation. The inguinal lymph node from one pig and serum from two pigs were positive for viral RNA, but lymph node and serum samples from all pigs were negative by virus isolation. This demonstrates the sensitivity of the RT–PCR assay in that viral nucleic acid in lymph tissue and blood can be detected by RT–PCR when infectious virus cannot. In contrast, all day 5 post-infection BALF, nasal swabs, and lung tissue homogenates from two out of four pigs were positive by virus isolation and RT–PCR. Lung samples from two pigs were negative in both replicates of virus isolation but positive by RT–PCR. The negative virus isolation results from lung tissues were likely due to sampling and/or the presence of a severe inflammatory response in the lungs of these two pigs leading to virus inactivation. A future study is planned to evaluate influenza levels in tissues by VI and real-time RT–PCR at additional time points.

Limited serologic cross-reactivity with the pandemic H1N1 2009 isolates was demonstrated in HI tests with sera from pigs infected or vaccinated with contemporary H1 SIV, although variation in cross-reactivity between viral antigens was apparent. The reduction in reactivity was demonstrated by the eightfold or greater decrease in titer when compared to homologous HI titer. An eightfold or greater loss in reactivity is considered a significant reduction that may be predictive of decreased protection from challenge. NY/09 was used as antigen against the swine anti-SIV panel with results similar to those obtained with CA/09. However, MX/09 demonstrated broader

Table 3. Serologic cross-reactivity between swine anti-influenza sera and pandemic H1N1 2009

Antiserum	Homologous	CA/09	NY/09	MX/09
H1α phylogenetic cluster				
A/sw/MN/37866/99	1280	20	40	160
	2560	80	40	320
A/sw/MN/02053/08	320	<10	<10	20
	320	<10	<10	<10
A/sw/MN/02093/08	320	<10	<10	<10
	320	<10	<10	<10
H1β phylogenetic cluster				
A/sw/NC/36883/02	640	40	40	160
	640	20	20	80
A/sw/IA/00239/04	1280	20	10	80
	1280	20	20	40
A/sw/KY/02086/08	640	<10	<10	<10
	80	<10	<10	<10
A/sw/NE/02013/08	640	<10	<10	<10
	160	<10	<10	<10
A/sw/NC/03084/08	640	<10	<10	<10
	320	<10	<10	<10
H1γ phylogenetic cluster				
A/sw/MN/1192/01	320	<10	40	160
	80	<10	<10	20
A/sw/MN/00194/03	1280	<10	40	160
	320	<10	40	320
A/sw/KS/00246/04	1280	<10	40	160
	1280	<10	40	160
A/sw/OH/511445/07	640	20	20	160
	2560	80	40	640
A/sw/MO/02060/08	1280	40	20	160
	640	80	40	320
A/sw/OH/02026/08	640	40	<10	80
	160	<10	<10	10
A/sw/NC/02023/08	320	<10	<10	40
	320	<10	20	80
A/sw/IA/02096/08	160	<10	<10	<10
	80	<10	<10	<10
H1δ phylogenetic cluster				
A/sw/TX/01976/08	320	<10	<10	<10
	160	<10	<10	<10
A/sw/IA/02039/08	320	<10	<10	<10
	160	<10	<10	<10
A/sw/MN/02011/08	2560	<10	<10	<10
	640	<10	<10	<10
Commercial vaccine strains				
Vaccine A	1280	10	<10	20
	1280	<10	<10	<10
	640	<10	<10	<10
	640	<10	<10	<10
Vaccine B	320	<10	10	40
	640	<10	<10	<10
	640	40	10	40
Vaccine C	–	<10	<10	<10
	–	10	<10	10
Vaccine D	1280	20	10	40
	1280	40	20	80
	1280	20	10	40
Vaccine E	–	<10	<10	<10
	–	<10	<10	<10

CA/09, A/California/04/2009; NY/09, A/New York/18/2009; MX/09, A/Mexico/4108/2009.

cross-reactivity with the NADC H1 serum reference panel. This was especially apparent for sera from pigs immunized with viruses from the H1γ HA phylogenetic cluster. The cross-reactivity with antisera from viruses of this cluster is important as this is the HA clade in which the HA from the 2009 pandemic H1N1 is most closely related.[1] Thus, it is possible to speculate that pre-existing immunity to certain currently circulating H1 SIV strains may provide some level of protection in pigs against the pandemic virus. However, the differences between the pandemic H1N1 2009 isolates suggest a possibility of biologic variation in host and/or virus properties responsible for the variation in serologic cross-reactivity. The CA/09 HA gene has 99·1% identity at the amino acid level with the NY/09 and MX/09 viruses, with four to five amino acid changes over the entire HA molecule depending on the reference sequences compared. However, none of these changes occurs at the receptor binding site or at putative antigenic sites, making the mechanism behind the differences in antigenicity unclear. The effect this variation would have on preventing transmission of the pandemic H1N1 2009 from the human population to pig populations with prior immunity to SIV remains unknown.

Serologic cross-reactivity with antisera from pigs vaccinated with five US commercial vaccines was additionally assessed by HI with the three pandemic H1N1 isolates. Cross-reactivity was consistently low between the vaccine antisera and all pandemic H1N1 2009 novel viruses tested, although titers were slightly higher with the isolate from Mexico. This suggests that currently available vaccines may provide only limited protection against infection with the novel human H1N1. Future studies will investigate a subset of the vaccines from this *in vitro* study in a vaccination efficacy challenge model to validate the serum cross-reactivity.

To discriminate between the endemic North American SIV isolates and the pandemic H1N1 2009, an RT–PCR–RFLP assay was developed and evaluated on a panel of virus isolates as well as clinical samples from the pigs experimentally infected with CA/09. None of the matrix gene amplicons from the panel of endemic SIV were cut by *AlwNI*. The matrix genes from the three examined pan-

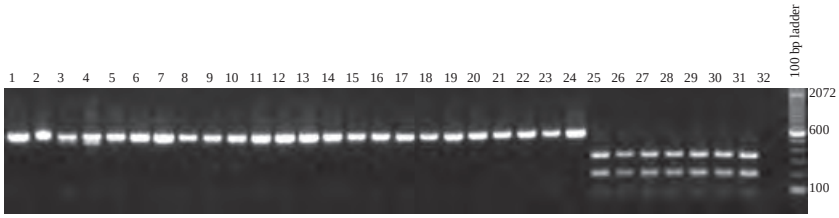

Figure 2. Restriction fragment length polymorphism analysis of different swine influenza isolates. 1: A/Sw/MN/002083/2007 (HuH1N1), 2: A/Sw/MN/00194/2003 (H1N2), 3: A/Sw/IA/00239/2004 (H1N1), 4: A/Sw/MN/37866/1999 (H1N1), 5: A/Sw/NC/36883/2002 (rH1N1), 6: 2007 H1N1 field isolate (H1N1), 7: A/Sw/OH/51445/1/2007 (H1N1), 8: A/Sw/NC/02084/2008 (H1N1), 9: A/Sw/MN/02093/2008 (H1N1), 10: A/Sw/IA/02096/2008 (H1N1), 11: 2005 H1N1 field isolate (H1N1), 12: 2005 H1N1 field isolate (H1N1), 13: A/Sw/IL/3743/2007 (H1N2), 14: A/Sw/IL/00685/2005 (H1N1), 15: A/Sw/TX/01976/2008 (H1N2), 16: A/Sw/MN/02011/2008 (H1N1), 17: A/Sw/NE/02013/2008 (H1N1), 18: A/Sw/NC/02023/2008 (H1N1), 19: A/Sw/IA/02039/2008 (H1N2), 20: A/Sw/OH/02026/2008 (H1N1), 21: A/Sw/MN/02053/2008 (H1N1), 22: A/Sw/MO/02060/2008 (H1N1), 23: A/Sw/NC/00573/2005 (H1N1), 24: A/Sw/MN/1192/2001 (H1N2), 25: A/CA/04/2009 (H1N1)v, 26: A/CA/04/09 (H1N1)v BALF 446, 27: A/CA/04/09 (H1N1)v BALF 447, 28: A/CA/04/09 (H1N1)v BALF 448, 29: A/CA/04/09 (H1N1)v BALF 449, 30: A/NY/18/09 (H1N1)v, 31: A/Mexico/168/09 (H1N1)v, 32: RT–PCR Negative Control.

demic H1N1 2009 isolates as well as the BALF samples from pigs infected with CA/09 were cleaved by *AlwNI*, demonstrating the usefulness of the diagnostic test to discriminate North American SIV lineage matrix genes from Eurasian SIV lineage matrix genes present in the pandemic H1N1 2009. In addition to monitoring for the emergence of the pandemic H1N1 2009 in the swine population *in toto*, tests that identify specific gene segments such as the one described here will be useful for monitoring changes in the gene constellation of viruses should reassortment between the pandemic virus and endemic SIV occur.

The pig has been suggested to be a 'mixing vessel' for emergence of influenza viruses with pandemic potential due to the presence of both avian and mammalian receptors expressed by respiratory tract epithelial cells.[18] However, both receptors linkage types have been additionally detected in the respiratory tract of humans[19] and in the trachea and intestine of quail.[20] These findings suggest the potential hosts for reassortment events between avian and mammalian influenza A viruses are not as limited as once thought. The pandemic H1N1 2009 demonstrates the potential for viruses with genes from swine lineages to emerge and spread in the human population. Although the novel reassortant H1N1 has not been identified circulating endemically in swine, reassortants between the North American and Eurasian lineage swine viruses have been identified from pigs in China.[2] It is apparent that pigs may be infected at least transiently with wholly avian and/or human viruses, allowing reassortment with swine viruses to acquire avian and/or human virus gene segments.[7,21–26] The 2009 pandemic H1N1 underscores the potential risk to human and animal populations of other influenza virus subtypes and genotypes that may evolve with the SIV TRIG backbone. Increased surveillance and monitoring for the pandemic A/H1N1 as well as other SIV in both the swine and human populations are critical to understand the dynamic ecology of influenza A viruses in susceptible host populations.

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Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs

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ABSTRACT

The gene constellation of the 2009 pandemic A/H1N1 virus is a unique combination from swine influenza A viruses (SIV) of North American and Eurasian lineages, but prior to April 2009 had never before been identified in swine or other species. Although its hemagglutinin gene is related to North American H1 SIV, it is unknown if vaccines currently used in U.S. swine would cross-protect against infection with the pandemic A/H1N1. The objective of this study was to evaluate the efficacy of inactivated vaccines prepared with North American swine influenza viruses as well as an experimental homologous A/H1N1 vaccine to prevent infection and disease from 2009 pandemic A/H1N1. All vaccines tested provided partial protection ranging from reduction of pneumonia lesions to significant reduction in virus replication in the lung and nose. The multivalent vaccines demonstrated partial protection; however, none was able to prevent all nasal shedding or clinical disease. An experimental homologous 2009 A/H1N1 monovalent vaccine provided optimal protection with no virus detected from nose or lung at any time point in addition to amelioration of clinical disease. Based on cross-protection demonstrated with the vaccines evaluated in this study, the U.S. swine herd likely has significant immunity to the 2009 A/H1N1 from prior vaccination or natural exposure. However, consideration should be given for development of monovalent homologous vaccines to best protect the swine population thus limiting shedding and the potential transmission of 2009 A/H1N1 from pigs to people.

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1. Introduction

Since 1997–98 multiple subtypes of endemic SIV, H3N2, H1N1, and H1N2 with a triple reassortant internal gene (TRIG) constellation derived from swine, avian and human influenza viruses, co-circulate in most major swine producing regions of the U.S. and Canada (reviewed in Ref. [1]). Additionally, introduction of H1N1 and H1N2 viruses with the HA and NA genes originating from contemporary human seasonal influenza A viruses (hu-like H1) that are genetically and antigenically distinct from the classical swine H1 lineage were reported in pigs in Canada [2]. The viruses identified in Canadian pigs were human lineage in entirety or double (human–swine) reassortants. Since 2005, hu-like H1N1 and H1N2 viruses have emerged in swine herds across the U.S. as human–swine reassortants possessing the TRIG (H1 δ-cluster) [3]. As a result of these interspecies reassortment events and antigenic drift, 4 phylogenetic clusters (α, β, γ, and δ) of H1 SIV are now endemic in U.S. swine [3,4]. Eurasian lineage SIVs have not been reported to circulate in U.S. swine.

In March–April 2009, a novel pandemic A/H1N1 emerged in the human population in North America [5]. The gene constellation of the emerging virus was demonstrated to be a combination of genes from swine influenza A viruses (SIV) of North American and Eurasian lineages that had never before been identified in swine or other species. The emergent A/H1N1 quickly spread in the human population and the outbreak reached pandemic level 6 as declared by the World Health Organization on June 11, 2009. Although the 8 gene segments of the novel virus have lineages with available sequences of corresponding genes from SIV from North America or Eurasia, no closely related ancestral SIV with this gene combination has been identified in North America or elsewhere in the world [6,7]. Other than sporadic transmission to humans [8,9], swine influenza A viruses of the H1N1 subtype historically have been distinct from avian and other mammalian H1N1 influenza viruses in

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characteristics of host specificity, serologic cross-reactivity, and/or nucleotide sequence. Although the HA gene from the 2009 pandemic A/H1N1 has ancestry with the γ-cluster H1 SIV from North America, it is unknown whether prior immunity to endemic SIV or SIV vaccines would cross-protect against the 2009 pandemic A/H1N1.

Vaccines against swine influenza virus (SIV) are commonly used in the U.S. swine industry. Most fully licensed vaccines are multivalent in nature with H1 and H3 subtypes included. In recent years, use of autogenous vaccines [10] has become increasingly widespread in an attempt to address the dynamic nature of SIV in the U.S. In 2008, over half of the doses of SIV vaccine released for sale were autogenous (Dr. Rick Hill, USDA-APHIS–Center for Veterinary Biologics, personal communication). These vaccines require basic purity and safety testing, but not the rigorous purity, safety, potency and efficacy testing required of fully licensed SIV vaccines; therefore they can have only limited distribution in that they must only be used in the herd of origin. Most autogenous vaccines are also multivalent with various combinations of H1 and H3 subtypes.

In this study, weaned pigs were vaccinated with 4 different inactivated vaccines produced from endemic North American SIV. Following 2 doses of vaccine, pigs were challenged with a 2009 A/H1N1 virus to evaluate the effectiveness of the vaccines to reduce virus shedding, pneumonia, and clinical disease. Significant protection from virus replication and shedding was demonstrated for 3 of the vaccines. In particular, the homologous monovalent vaccine induced optimal protection by complete prevention of viral replication in the nasal epithelium and lungs as well as significant reduction in pneumonia and febrile response to challenge with 2009 A/H1N1. Strong serologic cross-reactivity with a γ-cluster H1 SIV was demonstrated with 2009 A/H1N1 vaccine antisera, suggesting robust titers against γ-cluster H1 SIV may be correlated with partial cross-protection against pandemic A/H1N1.

2. Materials and methods

2.1. In vivo vaccine study

Sixty three-week old cross-bred pigs were obtained from a herd free of SIV and porcine reproductive and respiratory syndrome virus (PRRSV) and treated with ceftiofur crystalline free acid according to label (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the study. Pigs were housed in biosafety level 2 (BSL2) containment during the vaccine phase of the study. On the day of challenge, pigs were transferred to ABSL3 containment for the remainder of the experiment. Pigs were cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. A/California/04/2009 H1N1 (CA/09) (cell passage 1) was received from the Centers for Disease Control and Prevention (CDC) and propagated in Madin-Darby Canine Kidney (MDCK) cells for use in the studies described below. Three inactivated vaccines (Vaccines A, B, and D) were selected based on serologic evidence of cross-reactivity in a previous study using vaccine antisera supplied by commercial vaccine manufacturers [11]. Commercial vaccines were supplied by the manufacturers for use in the study and administered as per label or manufacturer recommendation. Vaccine A (FluSure® XP, Pfizer Animal Health, New York, NY) is a fully licensed trivalent commercial product containing cluster IV H3N2, γ-cluster H1N1, and δ-cluster H1N1 SIV as vaccine seed viruses. Vaccine B (MaxiVac Excell® 5.0, Intervet/Schering-Plough, Boxmeer, The Netherlands) is a pentavalent product under review for full licensure containing clusters I and IV H3N2 and β-, γ-, and δ-cluster H1 SIV as vaccine seed viruses. Vaccine D (Newport Labs, Worthington, MN) is a bivalent autogenous vaccine containing β- and γ-cluster H1 SIV as vaccine seed viruses. A monovalent experimental vaccine (Vaccine E) was prepared from CA/09 at 8 HA units per 50 μL and $1 \times 10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$) per mL with inactivation by ultraviolet irradiation and addition of a commercial adjuvant (Emulsigen D, MVP Labs) at a v:v ratio of 4:1 virus to adjuvant. Pigs ($N = 10$ per group) were vaccinated with 2 mL of each vaccine by the intramuscular route at approximately 4 weeks of age, boosted at 7 weeks of age, and challenged at 10 weeks of age.

For viral challenge, pigs were inoculated intratracheally with 2 mL of 1×10^5 $TCID_{50}$ of CA/09 as previously described [4]. Pigs were observed daily for signs of clinical disease and fever. Nasal swabs (Fisherbrand Dacron swabs, Fisher Scientific, Pittsburg, PA) were taken on 0, 3, and 5 days post-infection (dpi) to evaluate nasal virus shedding by dipping the swab in minimal essential medium (MEM) and inserting the swab approximately 2.5 cm into each nares. Swabs were then placed into 2 mL MEM and stored at −80° C until study completion. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) on 5 dpi to evaluate lung lesions and viral load in the lungs.

2.2. Pathologic examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with plum colored and well-demarcated consolidated lesions typical of influenza virus infection in pigs. The percentage of the surface affected with pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume as previously described [12]. Each lung was then lavaged with 50 mL MEM to obtain bronchoalveolar lavage fluid (BALF). Tissue samples from the trachea and right cardiac lung lobe and other affected lobes were taken and fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Lung sections were given a score from 0 to 3 to reflect the severity of bronchial epithelial injury using previously described methods [13].

2.3. Diagnostic microbiology

All pigs were screened for influenza A nucleoprotein antibody by ELISA (MultiS ELISA, IDEXX, Westbrook, Maine) prior to the start of the study to ensure absence of prior immunity. BALF samples from 5 dpi were screened for aerobic bacterial growth on blood agar and Casmin (NAD enriched) plates. Diagnostic PCR for PCV2 [14] and *Mycoplasma hyopneumoniae* [15] or an in-house RT-PCR for PRRSV was conducted on nucleic acid extracts from BALF.

2.4. Viral replication and shedding

Nasal swab samples were subsequently thawed and vortexed for 15 s, centrifuged for 10 min at 640 × *g* and the supernatant was passed through 0.45 μm filters to reduce bacterial contaminants. Ten-fold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibiotics were made with each BALF sample and nasal swab filtrate sample. Each dilution was plated in triplicate in 100 μL volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48–72 h post-infection. At 72 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry with an anti-influenza A nucleoprotein monoclonal antibody as previously described [16]. A $TCID_{50}$ titer was calculated for each sample using the method of Reed and Muench [17].

2.5. Serologic assays

For use in the hemagglutination inhibition (HI) assay, sera were heat inactivated at 56 °C for 30 min, then treated to remove non-specific hemagglutinin inhibitors and natural serum agglutinins by treatment with a 20% suspension of kaolin (Sigma Aldrich, St. Louis, MO) and adsorption with 0.5% turkey red blood cells (RBC). The HI assays were then performed with CA/09 and A/Mexico/4108/2009 (MX/09) pandemic A/H1N1, A/Swine/MN/37866/1999 H1N1 (MN/99; α-cluster SIV), A/Swine/NE/2013/2008 H1N1 (NE/08; β-cluster SIV), and A/Swine/OH/51145/2007 H1N1 (OH/07; γ-cluster SIV) viruses as antigens and turkey RBC using standard techniques [18]. Log_2 transformations were analyzed and geometric mean reciprocal titers reported.

ELISA assays to detect total IgG and IgA antibodies specific to 2009 pandemic H1N1 present in serum and BALF were performed as previously described [19] with modifications. Concentrated A/California/07/2009 New York Medical Center reassortant (X-179A) virus was resuspended in Tris–EDTA basic buffer, pH 7.8, and diluted to an HA concentration of 100 HA units/50 μL. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 μL of antigen solution and incubated at room temperature overnight. Serum was diluted 1000-fold followed by 2-fold serial dilutions in PBS. BALF was diluted by 2-fold serial dilutions in MEM. The assays were performed on each sample in duplicate. The mean of duplicate wells was calculated and antibody titers were designated as the highest dilution with an OD greater than 2 standard deviations above the mean of the non-vaccinated, non-challenged negative controls. Log_2 transformations were analyzed and geometric mean reciprocal titers are reported.

2.6. Statistical analysis

Macroscopic pneumonia scores, microscopic pneumonia scores, log_{10} transformed BALF and nasal swab virus titers, and log_2 transformations of HI reciprocal titers or ELISA reciprocal titers were analyzed using analysis of variance (ANOVA) with a P-value ≤ 0.05 considered significant (GraphPad Prism, GraphPad Software, La Jolla, CA). Response variables shown to have a significant effect by treatment group were subjected to pair-wise comparisons using the Tukey–Kramer test. Rectal temperature data were analyzed using a mixed linear model for repeated measures (SAS, SAS Institute, Cary, NC). Linear combinations of the least squares means estimates for rectal temperatures were used in a priori contrasts after testing for a significant treatment group effect ($P < 0.05$). Comparisons were made between each treatment group at each time point using a 5% level of significance ($P < 0.05$) to assess statistical differences.

3. Results

3.1. Inactivated vaccines reduced CA/09 virus levels and clinical disease

All pigs were free of influenza A virus and influenza A virus antibodies prior to the start of the experiment. All pigs were negative for extraneous viral and *M. hyopneumoniae* in the BALF at 5 dpi. All pigs except one were negative for significant aerobic bacterial growth at necropsy. A single pig had significant colony counts of a *Streptococcus* species in the BALF. Two doses of the homologous CA/09 vaccine prevented nasal shedding in all pigs challenged with CA/09 A/H1N1 at both time points after challenge (Table 1). In addition, infectious virus was not detected from the lungs of any pigs vaccinated with the homologous vaccine. There were statistically significant reductions in group mean virus titer levels in the A and D vaccine groups in nasal swabs at 3 and 5 dpi and in BALF at 5 dpi. However, both groups exhibited detection of virus in the nose or lung.

Table 1
Virus titers in nasal swabs and BALF.[a].

Group	Nasal swabs		BALF
	3 dpi	5 dpi	5 dpi
Vaccine A	0.1 ± 0.1/1	0.4 ± 0.3/3*	1.3 ± 0.6/4*
Vaccine B	0.8 ± 0.3/5	3.0 ± 0.3/10	2.9 ± 0.7/9
Vaccine D	0.0 ± 0.0/0	0.3 ± 0.3/2*	0.3 ± 0.2/2*
Vaccine E	0.0 ± 0.0/0	0.0 ± 0.0/0*	0.0 ± 0.0/0*
NV/Challenge	0.5 ± 0.3/4	2.7 ± 0.2/10	4.3 ± 0.2/10
NV/NC	0.0 ± 0.0/0	0.0 ± 0.0/0*	0.0 ± 0.0/0*

Vaccine A, fully licensed trivalent vaccine; Vaccine B, fully licensed pentavalent vaccine; Vaccine D, bivalent autogenous vaccine; Vaccine E, monovalent experimental CA/09 vaccine; NV/Challenge, non-vaccinated, challenged positive control group; NV/NC, non-vaccinated, non-challenged negative control group.

[a] Log_{10} geometric mean titer ± standard error of the mean/number positive out of 10 per group. BALF, bronchoalveolar lavage fluid; dpi, days post-infection.

* Significantly different from NV/Challenge control group at $P < 0.05$.

Macroscopic pneumonia in the non-vaccinated challenge control group was typical of influenza induced lesions in a 10-week old pig (Table 2). All vaccine groups demonstrated statistically significant reduction in percentage of macroscopic pneumonia over the positive controls. Additionally, the E and D vaccines were statistically indistinguishable from the non-challenged negative controls. Microscopic changes associated with influenza infection in the lungs were reflective of the macroscopic pneumonia (Table 2).

Pigs challenged intratracheally with the CA/09 H1N1 pandemic virus had a significant ($P < 0.0001$) febrile response (1.0 °C increase) to infection compared with healthy, non-challenged controls (Fig. 1). The febrile response peaked 24 h following challenge. As expected, the homologous CA/09 vaccine (Vaccine E) provided significant ($P < 0.0001$) protection against a febrile response following challenge with the same virus. Moreover, the homologous CA/09 vaccine (Vaccine E) was significantly ($P < 0.0001$) better than any of the 3 commercial vaccines (licensed vaccine A and B or autogenous vaccine D) at reducing a febrile response to the virus challenge. All pigs vaccinated with commercial vaccine exhibited significantly ($P < 0.0015$) elevated rectal temperatures versus non-challenged control pigs 24 h following challenge. Although there was no significant difference in protection against the febrile response between any of the 3 vaccines tested (licensed or autogenous), the autogenous vaccine had a significantly reduced febrile response compared with the non-vaccinated, challenged controls ($P = 0.006$) (Fig. 1).

Table 2
Macroscopic pneumonia and microscopic pneumonia lesion severity at 5 days post-infection.[a].

Group	Macroscopic	Microscopic
Vaccine A	3.6 ± 0.6*	0.7 ± 0.4*
Vaccine B	3.5 ± 0.8*	1.4 ± 0.3
Vaccine D	1.8 ± 0.8*	0.4 ± 0.3*
Vaccine E	0.2 ± 0.1*	0.0 ± 0.0*
NV/Challenge	8.2 ± 1.3	2.4 ± 0.2
NV/NC	0.1 ± 0.1*	0.0 ± 0.0*

Vaccine A, fully licensed trivalent vaccine; Vaccine B, fully licensed pentavalent vaccine; Vaccine D, bivalent autogenous vaccine; Vaccine E, monovalent experimental CA/09 vaccine.

[a] NV/Challenge, non-vaccinated, challenged positive control group; NV/NC, non-vaccinated, non-challenged negative control group.

* Significantly different from NV/Challenge control group at $P < 0.05$.

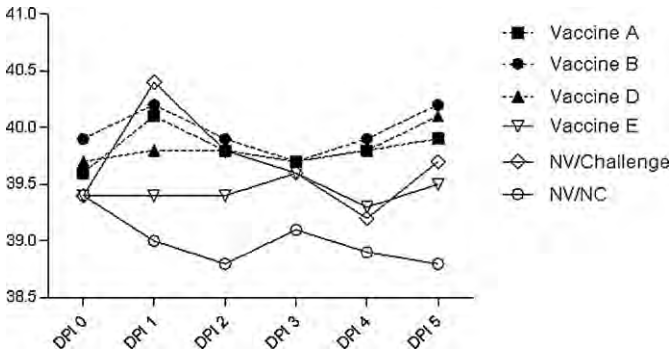

Fig. 1. Rectal temperatures were taken daily from 0 to 5 days post-infection (dpi). The monovalent homologous CA/09 vaccine (Vaccine E) significantly reduced fever compared to the non-vaccinated challenge positive control group. Vaccine A, fully licensed trivalent vaccine; Vaccine B, fully licensed pentavalent vaccine; Vaccine D, bivalent autogenous vaccine; Vaccine E, monovalent experimental CA/09 vaccine; NV/Challenge, non-vaccinated, challenged positive control group; and NV/NC, non-vaccinated, non-challenged negative control group.

3.2. Humoral immune response to vaccines

Homologous vaccine administration induced robust serum HI antibody titers against CA/09, but mean titers in the other vaccine groups were below the detectable threshold or positive cut-off (Table 3). Similar titers were induced against a second 2009 A/H1N1 isolate, A/Mexico/4108/2009 (data not shown). In contrast to the CA/09 HI, the A and D vaccine groups, as well as the homologous vaccine group, all had significant titers to the γ-cluster OH/07 and α-cluster MN/99 swine influenza viruses. Surprisingly, the homologous monovalent CA/09 vaccine group had significant cross-reacting antibody titers to the β-cluster SIV NE/08 as well. The D bivalent vaccine containing both γ- and β-cluster SIV also demonstrated significant HI titers to the β-cluster NE/08 virus as expected.

By ELISA assay, only the D and E groups had levels of IgG significantly higher than negative controls against the 2009 A/H1N1 virus in the serum or BALF (Table 3). The D and E vaccinated pigs were significantly higher in total IgG, but not significantly different from each other. IgA levels were not significantly different than negative controls for any of the inactivated vaccine groups in serum or BALF (data not shown).

4. Discussion

Although the pandemic 2009 A/H1N1 viruses are genetically related to SIV of North American and Eurasian lineages, the constellation of the 8 gene segments in the pandemic A/H1N1 was unique and not known to circulate in pigs prior to the pandemic. Reassortants between the North American and Eurasian lineage swine viruses have been identified from pigs in China [6], but a direct ancestor of the 2009 human pandemic virus has yet to be reported. Additionally, the 2009 pandemic A/H1N1 viruses circulating in the human population have hemagglutinin genes that are phylogenetically distinct from the nearest swine virus sequences, the North American-lineage γ-cluster H1. We have demonstrated that weaned pigs are susceptible to infection and clinical disease induced by CA/09 [11], and others have demonstrated infection of pigs with other isolates of 2009 A/H1N1 [20,21]. The experimental infections in pigs were characteristic of acute influenza illness in swine. Clinical signs, pathologic changes, and virus replication were restricted to the respiratory tract. Likewise, reports of naturally occurring transmission events to pigs suggest that clinical disease is relatively mild and indistinguishable from SIV.

Limited serologic cross-reactivity with the 2009 pandemic A/H1N1 isolates was demonstrated in HI assays with sera from pigs infected or vaccinated with contemporary H1 SIV [11]. Although variation in cross-reactivity between viral antigens was apparent, consistent cross-reactivity was demonstrated between 2009 A/H1N1 and sera from pigs immunized with SIV from the H1γ HA phylogenetic cluster. The cross-reactivity with antisera from these viruses is important since this is the HA cluster to which the HA from the 2009 pandemic H1N1 is most closely related [5]. Thus, pre-existing immunity to certain currently circulating H1 SIV strains may provide some level of protection in pigs against the pandemic virus. Serologic cross-reactivity with antisera from pigs vaccinated with 5 U.S. commercial vaccines was additionally assessed by HI with 3 pandemic H1N1 isolates [11]. Cross-reactivity was consistently low between the vaccine antisera and all 2009 A/H1N1 novel viruses tested, but 3 vaccines (Vaccines A, B, and D) demonstrated some cross-reactivity, suggesting that currently available vaccines may provide some level of protection against infection with the novel human A/H1N1. Here, we investigated a subset of the vaccines from the previous *in vitro* study in a vaccine efficacy experiment to validate the serum cross-reactivity.

In this study, the homologous monovalent inactivated vaccine provided optimal protection against 2009 A/H1N1 challenge in all parameters evaluated. No virus was isolated from the lung or nose at any time points evaluated. Rectal temperatures were significantly reduced over the non-vaccinated challenged controls during the acute 5-day post-infection period, including protection from the peak febrile response at 24 h post-infection. The homologous vaccine also conferred complete protection from macroscopic and microscopic pneumonia. The *in vivo* protection corresponded with

Table 3
Geometric mean reciprocal titers of 0 dpi serum against H1 influenza viruses in the hemagglutination inhibition assay or 0 days post-infection for serum and 5 days post-infection for BALF in a total IgG ELISA.[a].

Group	Hemagglutination inhibition				IgG ELISA	
	MN/99 (α)	NE/08 (β)	OH/07 (γ)	CA/09 pH1N1	CA/09 X-179A	
					Serum	BALF
Vaccine A	92*	17	53*	≤10	≤1000	≤4
Vaccine B	21	11	20	≤10	≤1000	≤4
Vaccine D	92*	53*	113*	12	7131*	8*
Vaccine E	485*	86*	557*	243*	15287*	11.3*
NV/Challenge	≤10	≤10	≤10	≤10	≤1000	≤4
NV/NC	≤10	≤10	≤10	≤10	≤1000	≤4

Vaccine A, fully licensed trivalent vaccine; Vaccine B, fully licensed pentavalent vaccine; Vaccine D, bivalent autogenous vaccine; Vaccine E, monovalent experimental CA/09 vaccine.

[a] NV/Challenge, non-vaccinated, challenged positive control group; NV/NC, non-vaccinated, non-challenged negative control group; BALF, bronchoalveolar lavage fluid. A representative virus from each H1 cluster of North American SIV (α, β, and γ) was included in HI assays.

* Significantly different than NV/NC negative controls.

robust HI antibody titers specific to 2009 A/H1N1, only detected in the Vaccine E group. The CA/09 homologous vaccine also induced cross-reacting HI antibodies to the γ-cluster SIV OH/07, but at a higher magnitude than expected. Surprisingly, the CA/09 vaccine antisera also demonstrated significant titers of cross-reacting antibodies to the α-cluster MN/99 and β-cluster NE/08. The Vaccine A commercial trivalent vaccine and the autogenous bivalent Vaccine D induced HI titers to the α-cluster H1 SIV, but significantly lower than the homologous monovalent vaccine. The autogenous bivalent vaccine induced equivalent levels of β-cluster HI antibodies as the CA/09 vaccine, but this was to be expected since the bivalent vaccine included both a β-cluster and a γ-cluster SIV. The lack of response to the β-cluster SIV by Vaccine A antisera and to each of the SIV tested by Vaccine B antisera may indicate a lack of cross-reactivity induced by the vaccine strain to the selected HI test strains or a suboptimal immune response induced by the vaccine. Vaccine B was in the process of being licensed at the time of this experiment, but not yet commercially available, and the vaccine available for testing was at a minimum protective dose (MPD). Commercial SIV manufacturers normally formulate commercial serials of their vaccines at higher HA levels than the MPD and was since done for this vaccine to induce higher HI titers.

The H1 SIV-specific antibodies induced by the commercial and autogenous vaccines are likely to have contributed to the partial protection demonstrated against CA/09 pandemic A/H1N1. Only the homologous vaccine and the autogenous SIV vaccine had significant levels of 2009 A/H1N1-specific IgG in the serum and lower respiratory mucosa measured by ELISA. As the Vaccine D vaccinated pigs had minimal HI activity against the 2009 A/H1N1 isolates tested but had significant protection against CA/09 challenge, it is likely that these non-HI antibodies aided in protection, as was previously shown between subtypes in a mouse model [22]. Of note, all of the vaccines tested provided protection from pneumonia. Importantly, none of the vaccines demonstrated aggravated pneumonia, a phenomenon periodically seen with selected mismatched inactivated vaccine and challenge virus in experimental studies [19,23]. Nonetheless, based on the results demonstrated here, consideration should be given for development of monovalent homologous vaccines to best protect the swine population and to limit shedding of virus, thus reducing the potential transmission of 2009 A/H1N1 among pigs or from pigs to people. As vaccine formulation, including type and amount of adjuvant, is proprietary, the role of adjuvants in inducing the variable responses to the vaccines tested here cannot be ruled out.

Vaccinating pigs against influenza virus is a common practice in the U.S. swine industry. Inactivated influenza vaccines became commercially available in 1994. In 1995, influenza vaccine usage was not reported in the National Animal Health Monitoring System survey of U.S. swine operations [24]. However, by 2000, over 40% of large producers reported that they vaccinated breeding females and approximately 20% vaccinated weaned pigs [25]. In the survey conducted in 2006, the number of producers vaccinating large herds of breeding females increased to 70%, whereas vaccinating weaned pigs remained relatively unchanged [26]. Importantly, of those farms that vaccinated breeding females in 2006, approximately 20% reported using autogenous SIV vaccines rather than commercial vaccines. Autogenous vaccine usage against influenza virus has continued to increase due to the diversity of viruses circulating in the North American pig population and the inability of the animal biologics industry to change the vaccine composition as rapidly as the viruses are changing. In fact, over half of the doses of SIV vaccine released in 2008 were autogenous (Rick Hill, USDA–APHIS–Center for Veterinary Biologics, personal communication). The inclusion of the autogenous vaccine in this study was to understand the current immune status of the U.S. swine population due to prior vaccine usage and not to promote use of γ-cluster autogenous vaccines to protect against 2009 A/H1N1 in U.S. hogs.

Autogenous vaccines have the advantage that they can be produced relatively quickly for use in the herd of origin in response to a specific or unique veterinary need. The disadvantages are that they are only minimally tested for purity and safety, untested for potency and efficacy, and thus cannot be distributed outside the herd of origin. Very limited peer-reviewed scientific information is publicly available on the impact of autogenous vaccine usage in controlling SIV. Fully licensed commercial SIV vaccines must meet the USDA–Center for Veterinary Biologics' requirements for purity, safety, potency, and efficacy, requiring significant investments of time and resources to develop a new product. One of the highest priorities for the recently launched USDA SIV surveillance plan is to enhance monitoring of the evolution of viruses for relevant commercial vaccine strain selection and diagnostic reagent development.

The pig has been suggested to be a "mixing vessel" capable of generating reassorted influenza viruses with pandemic potential due to the presence of both avian and mammalian receptors expressed by respiratory tract epithelial cells [27]. The 2009 pandemic A/H1N1 underscores the potential risk to the human population of other influenza virus subtypes and genotypes with the SIV TRIG backbone and demonstrates the potential for viruses with genes from swine lineages to emerge and cause a pandemic in the human population. Based on the results demonstrated here, consideration should be given for development of monovalent homologous vaccines to be used in all age groups of naive pigs to best protect the swine population and to limit the potential transmission of 2009 A/H1N1 among pigs or from pigs to people. Increased surveillance and monitoring for the pandemic A/H1N1 as well as other SIV in the swine population worldwide are critical to understand the dynamic ecology of influenza A viruses in this susceptible host population. Likewise, development of a vaccine strain selection system through the USDA surveillance plan is critical for controlling SIV and reducing the risk of such reassortment events with the current 2009 A/H1N1 or other emerging viruses in the future.

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ACVIM Consensus Statement
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Control of Bovine Viral Diarrhea Virus in Ruminants

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Key words: Bovine viral diarrhea virus, BVDV, Pestivirus, Persistent infection, Biosecurity; Epizootic; Immune System; Ruminant.

Bovine viral diarrhea virus (BVDV) is a diverse group of viruses responsible for causing disease in ruminants worldwide. Since the first description of BVDV as a cause of disease, it has undergone surges and lulls in importance. Epizootics of disease caused by BVDV are described. Although naming of the virus and illness implies gastrointestinal disease in cattle, BVDV is a pathogen that affects multiple organ systems in many animal species. Infection, disease, or both have been described in cattle, sheep, goats, pigs, bison, alpacas, llamas, and white-tailed deer, among others. In 2007, the Office of International Epizootics added bovine viral diarrhea to its list of reportable diseases, but the listing is as a reportable disease of cattle rather than as a reportable disease of multiple species. Although initial descriptions of disease caused by BVDV were of digestive disease, respiratory disease and reproductive losses because of BVDV are the most important economically. BVDV uses multiple strategies to ensure survival and successful propagation in mammalian hosts, and this includes suppression of the host's immune system, transmission by various direct and indirect routes, and, perhaps most importantly, induction of persistently infected (PI) hosts that shed and transmit BVDV much more efficiently than non-PI animals. Successful control and eventual eradication of BVDV requires a multidimensional approach, involving vaccination, biosecurity, and identification of BVDV reservoirs. The following consensus statement reflects current knowledge and opinion regarding the virus, prevalence and host range, clinical manifestations, and most importantly, the control and potential for ultimate eradication of this important viral pathogen of ruminants.

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Virus Description

BVDV is an enveloped, single-stranded RNA virus, and is the prototypic member of the genus *Pestivirus* within the family Flaviviridae. Currently recognized species within the *Pestivirus* genus include BVDV1, BVDV2, border disease virus, and classical swine fever virus (hog cholera virus).[1] Strains of BVDV can exist as different biotypes, which are either cytopathic (CP) or noncytopathic (NCP). The classification of biotype is independent of genotype, as there exist CP and NCP BVDV1 strains and CP and NCP BVDV2 strains. Only NCP strains of BVDV induce persistent infection.[2] CP BVDV strains are relatively rare, with NCP isolates accounting for approximately 90% of BVDV isolates at a diagnostic laboratory.[3] The NCP biotype is the source for CP strains, which arise by mutations and recombinations in the NCP strain. A 3rd biotype of BVDV, the lymphocytopathic biotype, consists of a subpopulation of NCP strains that are capable of causing CP effect in lymphocytes cultured in vitro. NCP strains that are lymphocytopathic have been associated with severe clinical disease.[4]

As BVDV is an RNA virus, genetic mutations occur readily, leading to substantial genetic, antigenic, and pathogenic variation. Because of frequent mutation in viral RNA replication, BVDV exists as a quasi-species, which are different but closely related mutant viral genomes subjected to continuous competition and selection, thus resulting in genetic and antigenic variation. Nucleotide sequence differences are the most reliable criteria for differentiation of BVDV species. The differences between BVDV species are not restricted to any 1 genomic region and are found throughout the genome[5]; however, some BVDV genomic regions are more amenable to comparison or have greater biological importance between BVDV1 and BVDV2. The 5′

untranslated region (5′-UTR) is the most commonly used region for detection and characterization of BVDV because of highly conserved areas that are favorable to PCR amplification, but the first nonstructural protein region is unique to pestiviruses, and comparison of this region among BVDV strains is being used for characterization of putative pestivirus species.[6] Subgenotypes of BVDV are described within BVDV1 and BVDV2 species, 12 among BVDV1 viruses (BVDV1a through BVDV1l)[7] and 2 among BVDV2 viruses (BVDV2a and BVDV2b).[8] Phylogenetic survey of the 5′-UTR genomic sequences of BVDV1 and BVDV2 strains reveals a similar level of sequence variation within each species,[9] and this finding suggests that these 2 species have been evolving for a similar time span. Within the U.S. cattle population, there are 3 major subtypes, BVDV1a, BVDV1b, and BVDV2a, with the BVDV1b subtype predominating from diagnostic laboratory submissions and PI prevalence studies, accounting for 78% of persistent infections in cattle in one North American study.[10]

Prevalence and Host Range

Cattle are the natural host for BVDV, and BVDV is distributed in cattle populations throughout the world as indicated by serologic surveys. The prevalence of seropositive cattle varies among countries, and is influenced by vaccine use and management practices. Surveys in North America have indicated individual-animal seropositive rates between 40 and 90%.[11,12] Herd-level prevalence, ie, the percentage of herds with unvaccinated cattle that are seropositive to BVDV, varies from 28 to 53% depending on geographic region.[13–15] In contrast, the prevalence of PI cattle is much lower and is generally believed to be <1% of all cattle.[16] PI cattle can cluster within groups of cattle, elevating the prevalence within populations. There are no random surveys that estimate the prevalence of PI cattle in North America. Despite reduced survivability, the prevalence of PI calves arriving at feedlots in the United States is between 0.1 and 0.4%,[17–19] which is similar to the 0.17% reported for U.S. beef cow-calf operations.[16]

BVDV does not possess strict host specificity. Classically, pestivirus isolates have been assigned according to the species from which they were isolated, with most BVDV, classical swine fever virus, and border disease virus isolates being recovered from cattle, pigs, and sheep, respectively. Evidence of BVDV infection as demonstrated by the identification of serum antibodies exists in over 50 species within 7 of the 10 families of the mammalian order Artiodactyla.[20–22] Species that are susceptible to BVDV infection include cattle, pigs, sheep, goats, bison, captive and wild cervids, and Old World and New World camelids, with recent accounts of BVDV infections in alpacas and wild cervids in North America receiving much attention. Clustering of pestivirus strains among 3 host groups (domestic ruminants, camelids, deer) has been proposed; however, the implications for transmission between these clusters are unknown.[23] Identification of heterologous PI hosts might have important implications for the epidemiology of BVDV, most importantly as these nonbovid PI animals can serve as reservoirs for BVDV.

BVDV infections have been identified in Old and New World camelids. In New World camelids, seroprevalence rates <20% have been reported in both North and South America.[24–26] In North America, highest antibody titers to BVDV were detected on farms on which PI crias were present.[26] The herd-level prevalence is 25% where crias were tested in 63 alpaca herds in the United States.[27]

Historically, seroepidemiologic and experimental infection studies suggested that New World camelids could be infected with BVDV but have few or no clinical signs of disease.[25] Reports of BVDV isolation and identification of PI alpacas have concerned the alpaca industry, and the virus is now considered an emerging pathogen of New World camelids.[28] The first description of a PI alpaca was made in Canada where a BVDV1b strain was isolated from a PI cria after natural exposure of its dam to a chronically ill cria.[29] Several cases of PI alpacas have since been reported in North America and Great Britain.[28,30–32] PI alpacas can survive for several months, but low birth weights, failure to thrive, and chronic respiratory and gastrointestinal infections occur in PI alpacas. Diagnosis of BVDV infection in PI alpacas has been made through traditional virological techniques, by RT-PCR, and through immunohistochemistry (IHC); however, these tests have not been formally validated for camelids. Similar to PI cattle, BVDV antigen is identified in many tissues of PI alpacas.[28–30] All isolates examined in North America and the United Kingdom belonged to BVDV 1b genogroup when subgenotyping was performed.[28–30] All 46 BVDV isolates from alpacas in North America were NCP BVDV1b strains[31]; furthermore, the nucleotide identity in 45 of 46 isolates was ≥99% using the highly conserved 5′-UTR genomic region. This finding suggests an association of the BVDV1b genotype with infections in North American alpacas.[31] Possible explanations for this predominance of BVDV1b strains in alpacas include introduction and intraspecific spread and maintenance of BVDV1b into North American alpaca populations or that unique BVDV1b subgenotypes are able to establish transplacental infections in alpacas.[31] When simultaneous intranasal inoculation of pregnant alpacas with 3 different BVDV strains (BVDV1b of cattle origin, BVDV 1b of alpaca origin, or BVDV2 of cattle origin) was performed, PI crias were born with only BVDV1b strains of cattle or alpaca origin, but not BVDV2,[33] providing further support for a unique role of BVDV1b in alpacas. Both species of BVDV were isolated from Chilean alpacas and llamas, contrasting findings from North America and Great Britain.[34] Viremia, nasal shedding, and seroconversion were observed when alpacas were inoculated with BVDV1b or BVDV2 strains.[35] Irrespective of BVDV genotypes, biosecurity and surveillance principles are important for BVDV control in alpacas, as movement of alpacas, including dams with crias, between farms for breeding purposes is associated with reproductive disease and birth of PI offspring.[27,29,30]

Some wildlife are serologically positive to BVDV, and the virus has been isolated from individual animals. The

livestock-wildlife interface is of great concern for a number of infectious diseases, including classical swine fever virus, but less is known about the role of wildlife in the epidemiology of BVDV. Wildlife can become infected with BVDV, but other factors, including shedding of the virus, intrapopulation maintenance, and amount of interspecies contact might influence the establishment of BVDV wildlife reservoirs. Similar to cattle, PI wildlife are likely a central factor in the establishment of wildlife reservoirs, and PI animals have been identified in free-ranging and captive species. PI animals were detected among free-ranging eland (*Taurotragus oryx*) in Zimbabwe and white-tailed deer (*Odocoileus virginianus*) in the United States.[36–38] Apparent prevalence rates of persistent infections in U.S. cervid populations are 0.2% in Alabama, 0.03% in Colorado, and 0.3% in Indiana.[36–38] Whether the source for BVDV infection in these populations is contact with cattle, or the result of an endemic cycle is unknown, but evidence for both hypotheses exists, and both explanations are not mutually exclusive. Although 1 study did not identify a correlation between cattle stocking densities and BVDV seroprevalence rates in wildlife,[39] seroprevalence rates in white-tailed deer are higher on ranches where cattle were present.[40] Also, the management of cattle could have an important impact on interspecific transmission of BVDV, as there is likely less wildlife contact with housed dairy cattle compared with beef cattle in pastures.[41] Endemic presence of BVDV is indicated by seroprevalence rates exceeding 60% of caribou (*Rangifer tarandus*) that had no contact to cattle, and 60% of a mule deer population in Wyoming.[22,42] In a group of captive white-tailed deer, BVDV was maintained by exposure of pregnant does to a PI fawn, resulting in birth of PI offspring.[43] Vertical transmission of BVDV by transplacental infection resulted in continued birth of PI animals in a maternal lineage of lesser Malayan mouse deer in a zoological collection, emphasizing the potential for maintenance of BVDV in wildlife.[44]

White-tailed deer are the most abundant free-ranging ruminants in North America. Contact between white-tailed deer and cattle can occur in a typical North American pastoral setting, and this species has potential to be a reservoir for BVDV. Infections of white-tailed deer with BVDV occur by experimental and natural exposure.[45,46] Similar to cattle, the most dramatic effects of BVDV infections in white-tailed deer are fetal resorption, fetal mummification, stillbirth, and abortion.[46,47] Nasal or rectal shedding occurs in acutely infected and PI white-tailed deer, and results in transmission to other white-tailed deer.[43,48] In contrast to transmission of BVDV among white-tailed deer, spill-back infections, or infection of cattle as a result of exposure to white-tailed deer has not yet been demonstrated, but because of its importance, warrants further evaluation.

The discovery of novel pestiviruses in wildlife species might lead to new classifications within the genus Pestivirus.[49] An isolate from a giraffe is different from pestiviruses of domestic species, based on comparison of complete genomic sequences and palindromic nucleotide substitutions in the 5′-UTR.[50] A pestivirus isolated from an immature blind pronghorn is highly divergent from other pestiviruses.[51]

Although BVDV is not considered a human pathogen, its highly mutable nature, ability to replicate in human cell lines,[52] similarity to human hepatitis C virus,[53] and isolation from 2 clinically healthy people,[54,55] a Crohn's disease patient,[56] and feces of children under 2 years old who had gastroenteritis[54,55,57] create some concern regarding zoonotic potential.

Clinical Disease Syndromes and Pathogenesis

A wide range of clinical manifestations from subclinical to fatal disease occur in association with BVDV infection. The clinical presentation and the outcome of BVDV infection depend on numerous factors, with host influences being very important, and these include immune status, the species of host, pregnancy status and gestational age of the fetus, and the presence of concurrent infections with other pathogens. Viral factors influence clinical presentation and these include biotypic variation, genotypic variation, and antigenic diversity, but it is important to note that BVDV1 and BVDV2 strains can be involved in the entire spectrum of clinical disease.

Acute (Transient or Primary) Infections

The terms "acute," "transient," and "primary" have been interchangeably used to describe BVDV infection in postnatal cattle, with the ability to respond immunologically to BVDV. The source of most acute infections is cattle PI with BVDV, although acutely infected cattle can be a source of virus to other susceptible cattle.[58] The most effective route of transmission appears to be nose-to-nose contact. The majority of BVDV infections in immunocompetent and seronegative cattle are subclinical; however, truly benign BVDV infections probably do not exist, as cattle undergoing an "inapparent" infection could exhibit mild fever, leukopenia, anorexia, and decrease in milk production if observed closely. Moreover, if the infected animal is pregnant, deleterious effects can occur in the fetus. Acute BVDV infections result in signs that include diarrhea, depression, oculonasal discharge, anorexia, decreased milk production, oral ulcerations, and pyrexia, with laboratory findings including leukopenia characterized by lymphopenia and neutropenia. Peracute BVDV infections originally described in Canada and the United States result in severe clinical disease manifestations and higher than expected case fatality rates.[59] Genomic analysis of BVDV isolates from infected cattle from these outbreaks indicated the BVDV2 genotype, and this ultimately raised a renewed interest in acute BVDV infections.[9,59]

Another clinical disease manifestation in cattle acutely infected with BVDV is the hemorrhagic syndrome, which is characterized by thrombocytopenia.[60,61] The first descriptions of hemorrhagic syndrome included both calves and adult cattle naturally infected with BVDV, with severe depressions in platelet count.[60] Clinical manifestations of the hemorrhagic syndrome are primarily related to

thrombocytopenia and include bloody diarrhea, epistaxis, petechial hemorrhages, ecchymotic hemorrhages, and bleeding from injection sites or insect bites. Thrombocytopenic BVDV infections have been experimentally reproduced, almost exclusively with NCP BVDV2 strains.[61,62] Platelet dysfunction has also been described with experimental BVDV infection,[63] thus quantitative and qualitative platelet defects contribute to the hemorrhagic diathesis observed in infected cattle. BVDV infection of bone marrow megakaryocytes is important in the etiology of BVDV-induced thrombocytopenia.[62,64]

BVDV is lymphotrophic, and acutely infected cattle are immunosuppressed as a result of reduction in circulating immune cells and diminished function of immune cells. The consequence of immunosuppression is an increased susceptibility to other infectious disease agents, and the bovine respiratory disease complex is an example where BVDV plays an important role in polymicrobial disease. Cells of both the innate and adaptive immune responses are affected by BVDV. Leukopenia occurs in most acutely infected cattle, but the severity of leukopenia can be influenced by BVDV strain. Decreases in total leukocyte count and in leukocyte subpopulations appear to be less dramatic in calves experimentally infected with BVDV1 strains than with some BVDV2 strains; however, this can be simply because of the selection of BVDV strains chosen for study.[62,65,66] In general, highly virulent strains of BVDV induce greater declines in the white blood cell count than less virulent strains. Lymphopenia (T-lymphocytes and B-lymphocytes) and neutropenia are the major hematologic abnormalities.[66,67] Removal of BVDV-infected leukocytes by the immune system, destruction of immune cells by BVDV, and increased trafficking of immune cells into tissue sites of viral replication are all responsible for leukopenia. During acute infection, lymphoid depletion in the thymus, spleen, lymph nodes, and gut-associated lymphoid tissues (Peyer's patches), and the severity might also be strain dependent.[62] Diminished function in immune system cells has also been described during acute BVDV infection, and affected cells include lymphocytes, neutrophils, and monocytes and macrophages.[68–70]

Reproductive Tract Infections

The importance of BVDV on the male reproductive tract has not received the attention equivalent to the effects on female reproduction. Bulls infected with BVDV are capable of shedding virus in semen.[71–73] The virus can survive cryopreservation and processing of semen for artificial insemination.[74] Although acutely infected bulls shed lower concentrations of BVDV in semen than PI bulls, infection of artificially inseminated heifers can result from insemination with semen collected from acutely infected bulls before seroconversion.[72] Acute BVDV infections generally result in a transient viremia with subsequent clearance of the virus by the host immune system; however, prolonged infection of testicular tissue has been described under both natural and experimental conditions.[71,73] Prolonged testicular infection with BVDV was first identified in the testes of a seropositive, nonviremic bull at an artificial insemination center.[73] This bull continuously shed infectious BVDV in semen throughout his life despite the absence of a viremia and the presence of consistently high concentrations of circulating serum antibodies that neutralized the specific viral strain that was persistently shed in the semen.[75] Localized, prolonged testicular infections with BVDV have also been experimentally reproduced after acute infection of peripubertal bulls with BVDV. Viral RNA has been detected in semen for 2.75 years after BVDV exposure, and infectious virus grown from testicular tissue has been detected up to 12.5 months after BVDV exposure.[71] Protection from a systemic immune response because of a blood-testes barrier is believed to be the mechanism for the localized, prolonged testicular infection. Uncertainty currently exists regarding whether bulls with a prolonged testicular infection can become viremic and infectious to other animals.

Infection of pregnant cattle with BVDV can result in transplacental transmission and infection of the developing fetus. The economic damage caused by BVDV in susceptible breeding herds is mainly associated with the outcomes of intrauterine infections, which are dependent upon 3 main factors: (1) gestational age of the fetus at the time of infection; (2) organ system involved in the infection; and (3) biotype, virulence, and target cell range of the virus. Besides persistent infection, other outcomes of reproductive tract infections include abortion, embryonic or fetal resorption manifesting as repeat breeding, congenitally malformed offspring, mummification, and congenital infections manifesting as normal calves or calves of poor vigor. Although embryonic/fetal death and abortion are most common during the first trimester, mid- and late-term abortions and stillbirths can be caused by BVDV.[76] Congenital malformations are produced by BVDV infection between days 100 and 150 of gestation, and include cerebellar hypoplasia, hypomyelinogenesis, hydranencephaly, alopecia, cataracts, optic neuritis, brachygnathism, hydrocephalus, microencephaly, thymic aplasia, hypotrichosis, pulmonary hypoplasia, and growth retardation.[76]

The ability of BVDV to cause early embryonic death has been somewhat controversial. Infection of cattle before insemination reduces conception rates.[77] This could be due in part to ovarian infection and dysfunction as a result of BVDV viremia. Oophoritis and the presence of viral antigen in ovarian tissue occur in cattle acutely infected with BVDV.[78,79] Conception and pregnancy rates are lower if the animals are viremic at the time of insemination. Cattle viremic with NCP BVDV at the time of insemination had a 44% conception rate as compared with 79% for the control animals.[77] Further field studies have supported this theory that BVDV is involved in early embryonic death and repeat breeding syndrome.

Persistent Infection

Persistent infection is considered by many the most important aspect of BVDV infection as this is the key mode by which the virus maintains and perpetuates itself in the cattle population. Additionally, developments in diagnostic assays have focused on identification of PI

cattle, and a central component of BVDV control is the identification and elimination of this major reservoir of the virus. PI calves are the result of in utero BVDV infection during the period of fetal development from gestation day 45 to gestation day 125, which is the gestational period bracketed by the end of the embryonic stage and the development of fetal immunocompetence. Biological variation is clearly apparent regarding the gestational age at which developing bovine fetuses become immunocompetent, and it is important to note that day 125 is not an absolute date for immune system competence.[80] Biotypic variation is important, and whereas infection with either biotype is capable of causing fetal death, only NCP strains are associated with persistent infection.[2] To our knowledge, all genotypes and subgenotypes appear to be capable of causing PIs. Persistent BVDV infection appears to arise from specific B- and T-lymphocyte immunotolerance. Immunotolerance is specific to the infecting NCP strain of BVDV, and postnatal PI animals can respond immunologically to heterologous strains of BVDV.[81] For this reason, PI animals can be seropositive to BVDV, and seropositive status cannot be utilized diagnostically to rule out persistent infection. Virus is found in many tissues in PI animals and shed from multiple sites, including nasal and ocular discharges, urine, semen, colostrum/milk, and feces, thus making PI animals efficient transmitters. Vertical transmission rate is 100% as all PI cows will give birth to PI offspring. Most PI calves are born weak, stunted, and die shortly after birth or fall behind their cohorts as they mature, but some PI calves are born without observable abnormalities and are impossible to distinguish phenotypically from cohorts. PI animals can have an impaired immune response, making them more susceptible to opportunistic pathogens, and this could contribute to early death. Regardless of the clinical outcome, the true importance of PIs is the fact that they shed large amounts of virus thus serving as the major source of virus spread both within and between farms.

Mucosal disease is the most dramatic form of BVDV-associated clinical disease because of the severity and characteristics of lesions. Mucosal disease occurs when PI cattle become superinfected with a CP BVDV.[82] Because PI cattle comprise $<1\%$ of the cattle population, mucosal disease is characterized by a low case attack rate but high case fatality rate. The origin of the CP BVDV can be external, such as modified-live virus vaccines containing CP BVDV, or internal as the result of mutations of the NCP BVDV (the PI biotype) resulting in CP BVDV.[83] Cohorts of PIs that originate from the same strain of BVDV often succumb to mucosal disease in a narrow window of time. This occurs when 1 PI develops a mutation of the NCP BVDV resulting in a CP BVDV, which is then subsequently spread to PI cohorts. Multiple clinical forms of mucosal disease exist and can be divided into acute fatal mucosal disease, chronic mucosal disease, chronic mucosal disease with recovery, and delayed onset mucosal disease.[84] The clinical variations of mucosal disease are attributable to the antigenic relationship between the PI NCP strain and the superinfecting CP strain.[84]

Diagnosis

Many diagnostic tests are available for BVDV detection, and the choice of test depends on the clinical problem, the local availability of tests, and financial considerations. The majority of diagnostic tests developed are used to identify PI animals. Accurate diagnosis of BVDV infection relies upon laboratory testing, and once an accurate, positive diagnosis is made, further losses are prevented by implementation of rational management decisions and control procedures.

Isolation of BVDV in cell cultures using validated methodology is the gold standard for diagnosis of BVDV infection,[85] but because of the greater expense and time taken to report a result for this method, antigen detection or nucleic acid detection has largely replaced virus isolation for diagnosis of BVDV infection. The virus can be cultured and isolated from a variety of samples including serum, whole blood, semen, nasal swabs, and various tissues. Buffy coat cells from whole blood are the preferred sample for antemortem diagnosis, whereas lymphoid organ-related tissues are preferred samples from necropsies.[86] A microtiter virus isolation (immunoperoxidase monolayer assay) has been developed and utilized as a herd screening virus isolation assay, primarily for the detection of PI animals.[87] This test is not recommended for detecting acute infections, nor is it advisable to use this assay for testing calves <3 months of age, as passively derived colostral antibodies interfere with the test.

Antigen detection methods such as IHC and antigen capture ELISA (ACE) are used for BVDV detection largely because they provide rapid and inexpensive detection when compared with virus isolation. Additionally, results from antigen detection-based tests are highly reproducible between laboratories. The IHC and ACE tests performed on skin samples have become widely used and applied for the detection of PI cattle.[88] Skin biopsies are easy to obtain, and testing can be performed on young PI animals that would test negative by virus isolation, microplate virus isolation, and ACE testing on serum because of inhibition of the tests by acquired colostral antibodies.[89] The IHC and ACE tests are ideally suited for the detection of PI animals. Although these tests do not detect acutely infected cattle,[90–92] a single report indicates positive results might be observed for acutely infected cattle.[93] Whereas IHC is performed using monoclonal antibodies that detect an epitope which is not destroyed by formalin fixation and the U.S.-licensed antigen-capture ELISA kit also uses a monoclonal antibody detecting the same antigenic epitope, clinicians should note that a strain of BVDV has been detected in the United States that is not detected by conventional IHC and ACE tests.[94]

Molecular techniques for diagnosis of BVDV infection have gained widespread use as a routine diagnostic method.[88] Development of commercial kits, with rapid and simple viral RNA extraction techniques, has made molecular techniques ideal for detection of viral genomic nucleic acids. The RT-PCR assay is specific and can detect from 10^1- to 10^4-fold lower concentrations of virus than virus isolation, thus making RT-PCR more sensitive

than virus isolation.[95] The high sensitivity of RT-PCR has allowed it to be adapted for pooled testing of tissues, whole blood, serum, or milk samples, making this an economical way to detect BVDV infection in herd screening strategies. Pooling of samples and testing by RT-PCR is controversial, and if RT-PCR pooling protocols are not validated and continually assessed, their value in BVDV control programs might be counterproductive. Clinicians should also note that detection of viral RNA does not always equate to detection of infectious virus.[96]

Serologic testing can also be used to demonstrate BVDV infection but there is difficulty in differentiating antibodies produced in response to a natural infection, after vaccination, or as a result of transfer of maternal antibodies from dam to offspring. Serologic testing can be used to assess vaccine efficacy and vaccine protocol compliance, and by testing of sentinel animals to determine if BVDV exposure has occurred in the herd.[97] The serum virus neutralization test is the most commonly used serologic assay to determine BVDV specific antibody titers. This test can be used for the detection of antibodies against BVDV1 or BVDV2 strains depending upon the reference viral strain used in the test. However, there are no universally accepted reference strains for the VN test, which makes interpretation of results obtained from different laboratories difficult.

Prevention and Control

Eliminating pathogen reservoirs and limiting transmission from infected individuals to susceptible animals are the major principles for infectious disease control. PI cattle are the major reservoir of BVDV, although transiently infected animals can, to a lesser extent, also serve as a reservoir. Therefore, prevention or elimination of PIs is central to BVDV control. Development and implementation of herd health programs that limit exposure of pregnant cattle to BVDV are important for successful control. When developing a BVDV prevention and control program, 3 aspects should be considered: (1) identification and elimination of PI animals, (2) enhancing immunity through vaccination, and (3) implementing biosecurity measures to prevent BVDV exposure of susceptible cattle. Each of these three principles has been applied to BVDV control and greater success can be expected when used simultaneously in BVDV control programs.[98,99] Several European countries have successful eradication programs,[100–102] and this has encouraged veterinary and cattlemen's organizations in the United States to adopt control strategies.[103]

Identification and Elimination of PI Cattle

The major source for BVDV transmission is cattle PI with BVDV. Removal of PI animals should occur before their entry into breeding herds. This can be more easily achieved in beef cow-calf operations that follow a controlled breeding season. In this situation, all calves, replacement heifers, bulls, and nonpregnant cows without calves should be tested for PI status before entry of the bull.[104] Because PI cows always produce PI calves, a negative test result of a calf indicates a negative PI status for the dam.[105] Dams of test-positive calves need to be tested for PI status. Most PI calves result from acute infection of their dam, so dams that test negative could reenter the breeding herd. If pregnant cattle are present at the time of testing in herds with a controlled breeding season, they should be segregated and their calves be tested before return to the breeding herd. In herds without a controlled breeding season, young calves should be tested and removed as soon as possible to avoid transmission to the breeding herd. Screening young calves for PI status is best accomplished by PCR, ACE, or IHC on skin samples. The use of skin samples for testing young calves is advantageous in that sample collection is simple, samples can be taken from calves that have maternal antibodies, and a single positive test usually indicates PI status. Because the occasional acutely infected animal might be PCR, IHC, or ACE positive,[93] valuable cattle should be retested after 30 days using virus isolation or RT-PCR assays on blood samples.

Screening all individuals of a herd is very costly and other strategies can be more cost-effective. These strategies include evaluation of production records, BVDV evaluation of aborted fetuses, use of sentinel animals, pooling strategies by RT-PCR testing, and BVDV testing on sick or dead cattle.[104] Monitoring breeding records, calf morbidity and mortality rates, and weaning proportions are considered the minimal level of surveillance and are the least expensive, but this level of surveillance lacks sensitivity in detecting a PI animal.[16,104] As an example of the difficulty in utilizing clinical suspicion as a reason to perform herd testing, BVDV was isolated from cattle in 53% of herds where there was no suspicion of the infection,[106] and BVDV PI animals were not identified in 81% of herds where veterinarians suspected BVDV was present.[16] On the other hand, identifying BVDV in sick or dead animals, or in aborted fetuses provides the justification for further whole-herd testing for BVDV PI animals.

Because of high sensitivity, RT-PCR assays using pooled samples have been developed to screen herds for PI animals.[107] Pooled samples of serum, whole blood, bulk tank milk, and skin have been utilized in RT-PCR assays.[95,107–110] Pooled sample testing by RT-PCR is rapid and cost-effective for screening populations of cattle for PI animals. However, failed attempts to replicate this work in multiple labs indicate the sensitivity of the assay to changes in sample handling or operator variability. Subsequent testing of individuals within the positive pools can be performed by IHC, ACE, virus isolation, or RT-PCR methods.

Vaccination

Many vaccines or vaccine combinations are available for BVDV, and the majority of these USDA licensed vaccines contain BVDV in combination with other bovine respiratory and reproductive pathogens. In the past, most BVDV vaccines contained only BVDV1 strains, but because of antigenic diversity, modified-live and inactivated vaccines containing both BVDV1 and BVDV2 strains are now widely available. There are advantages

and disadvantages to use of BVDV modified-live viral vaccines and inactivated vaccines.[111] One disadvantage of inactivated BVDV vaccines is that two doses are required for the initial immunization, and a major problem with programs using inactivated vaccines is the widespread lack of compliance among producers by failing to booster the primary series.[112]

Vaccines are an important component to BVDV prevention, and their effectiveness has been to limit transmission and clinical disease rather than completely prevent infections with BVDV, as has been demonstrated in experimental and field studies using either inactivated or modified-live BVDV vaccines.[59,113] Protection from clinical disease is important for stocker/backgrounder and feedlot operations, and cattle that arrive at a feedlot with antibody titers to BVDV tend to have protective immunity against bovine respiratory disease complex.[114–116] Preconditioning cattle by preweaning and vaccinating against BVDV and other respiratory pathogens before commingling and shipping reduces the incidence of bovine respiratory disease in feedlot cattle.[115]

Vaccination against BVDV should protect against viremia and prevent dissemination of virus throughout the host, including preventing infection of the reproductive tract and fetus. The focus for vaccine efficacy has shifted from protection against clinical disease to protection against fetal infection. Protection against fetal infections after BVDV vaccination varies, being influenced by use of inactivated or modified-live vaccine, the timing of challenge, and the degree of homology between vaccine and challenge strains. Fetal protection is superior when animals are challenged with strains from the same genotype. Although protection is not 100%, the level of protection is superior to that observed when proper vaccination is not utilized as evidenced by higher rates of PI animals in unvaccinated cattle.

Biosecurity

After the elimination of PI animals, strict biosecurity is essential to prevent reintroduction of the virus. All purchased cattle should be isolated and tested for PI status before entry into the herd. Isolation of new additions for 3 weeks before entry into the resident herd should prevent transmission of BVDV from acutely infected animals. Most lapses in herd biosecurity involve purchasing PI cattle or purchasing pregnant cattle with unknown BVDV status of the fetus. Purchased pregnant cattle should be isolated and their offspring tested to ensure that they are free of BVDV. Semen should only be used from bulls that have been tested for BVDV infection. For purebred herds marketing valuable embryos and livestock, testing of embryo transplantation recipients for PI status is essential. Exposure of cattle to other ruminants at exhibitions should be limited, and animals should be quarantined for 3 weeks before reentry into the breeding herd. Most biosecurity principles instituted for BVDV control will benefit disease control of other pathogens. Further biosecurity principles include elimination of fence-line contact with neighboring livestock and sanitation of equipment and people entering the farm.

Outline of a BVDV Control Program

Since the discovery of BVDV, control programs have been developed and successfully implemented at the herd level. These control measures need to be multidimensional and cannot rely on 1 aspect, such as vaccination. Therefore, BVDV control requires a comprehensive programmed approach that begins with first understanding the virus, its associated clinical presentations and how it might affect the livestock industry. Producers with this understanding are better able to analyze risks and make more informed decisions. Second, it involves setting goals related to BVDV control that can be different for every operation. By understanding individual operation goals and risk tolerance, a control program can be effectively designed. Goals range from eliminating BVDV from a herd with an existing problem to keeping the virus from entering a BVDV free herd. Achieving these 2 goals will require very different diagnostic testing, vaccination, and biosecurity plans. Therefore, goals should be determined using information about the herd BVDV status, current management practices, and the likelihood of future introduction of the virus (based on animal movement and biosecurity practices). If the herd BVDV status is unknown, a strategy of serologic or virologic testing to determine the presence of the virus can optimize the control program. Measurable outcomes need to be established to evaluate progress toward goals. Objective criteria such as performance measures, reproduction data, number of health problems, or number of BVDV positive animals can be used to gauge the changes the control program has made. Accurate records provide information on the long-term viability of the control program.

Development of effective control plans requires that producers understand risks and the cost to reduce them through a risk analysis. Risks are defined by a probability of occurrence and a magnitude of loss associated with that occurrence. The magnitude of loss is termed the impact. Either part of the risk equation can be decreased through management. There is a probability that BVDV will be introduced to the herd and there is the impact of disease if it is introduced. There are also costs associated with strategies implemented to decrease the probability or impact.

Once goals are set and risk is understood, effective control strategies can be implemented. Initially, producers should be encouraged to determine if BVDV is circulating in their herd. Methods to answer this question vary in cost and reliability. Most importantly, PI animals need to be identified and eliminated. If BVDV is detected in the herd, then biocontainment protocols to minimize the negative impact of infection or eliminate circulating virus on the farm should be implemented. If BVDV is not present in the herd, appropriate biosecurity protocols to keep the herd free of BVDV should be in place.

BVDV Eradication

Many European countries have initiated BVDV control or eradication programs, with several Scandinavian

countries achieving near elimination of BVDV in their cattle population.[100–102,117] The European perspective has provided evidence that BVDV can be successfully controlled and potentially eradicated; however, designing an eradication program for 1 part of the world might not apply to other geographic regions. Control or eradication programs should be carefully constructed with information on virus characteristics and producer management practices incorporated into the program design. A key component for success of a program is level of producer compliance and program funding. Predominant type of cattle production unit, density of animal populations, amount of animal movement, and potential for contact with wildlife reservoirs are other nonviral factors that can influence implementation and success of a BVDV control program. Variation among circulating BVDV strains and vaccine usage in the region could also impact success of control. All factors need to be considered carefully if time and investment are put toward BVDV eradication in countries or regions outside of Europe. In North America, veterinary and producer organizations have formulated and/or adopted position statements on BVDV for control and eventual eradication of the virus in North America.[103] Multiple states have initiated voluntary BVDV control programs, and at present it is too early to determine their effect.

Summary and Future Directions

Considerable advancements have been made regarding our understanding of BVDV, its associated diseases, and the methods for control; yet BVDV infections remain a source for economic losses in the cattle industries worldwide. Genetic and antigenic diversity of BVDV strains, potential for nonbovine reservoir hosts, and limitations in vaccine efficacy and diagnostic accuracy are immediate and future areas of concern as control and eradication efforts are begun. Equally important to virus attributes are producer willingness and compliance with control and eradication programs, thus education efforts are imperative. Advances achieved through research have led to improved and expanded testing strategies aimed at the detection and removal of PI animals, and this has contributed to the increasing number of regional control programs. Removal of PI animals is the cornerstone for BVDV control and eradication, but enhancing herd immunity and implementing reasonable and sound biosecurity practices are important for ultimate success. Veterinarians are in a unique position to significantly impact the goal of controlling and eventually eradicating BVDV. Their broad knowledge and training provide them with the best tools to help the cattle industry make significant strides toward meeting these goals.

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Immune Responses in Cattle Inoculated with *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium kansasii*▽

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Cattle were inoculated with *Mycobacterium bovis*, *Mycobacterium tuberculosis*, or *Mycobacterium kansasii* to compare the antigen-specific immune responses to various patterns of mycobacterial disease. Disease expression ranged from colonization with associated pathology (*M. bovis* infection) and colonization without pathology (*M. tuberculosis* infection) to no colonization or pathology (*M. kansasii* infection). Delayed-type hypersensitivity and gamma interferon responses were elicited by each mycobacterial inoculation; however, the responses by the *M. bovis*- and *M. tuberculosis*-inoculated animals exceeded those of the *M. kansasii*-inoculated animals. Specific antibody responses were detected in all *M. tuberculosis*- and *M. bovis*-inoculated cattle 3 weeks after inoculation. From 6 to 16 weeks after *M. tuberculosis* inoculation, the antibody responses waned, whereas the responses persisted with *M. bovis* infection. With *M. kansasii* inoculation, initial early antibody responses waned by 10 weeks after inoculation and then increased 2 weeks after the injection of purified protein derivative for the skin test at 18 weeks after challenge. These findings indicate that antibody responses are associated with the antigen burden rather than the pathology, cellular immune responses to tuberculin correlate with infection but not necessarily with the pathology or bacterial burden, and exposure to mycobacterial antigens may elicit an antibody response in a presensitized animal.

Tuberculosis (TB) in humans and animals may result from exposure to bacilli within the *Mycobacterium tuberculosis* complex (i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. pinnipedi*, *M. microti*, *M. caprae*, or *M. canetti* [8]). Despite their ~99.95% sequence identity (12), *M. bovis* and *M. tuberculosis* exhibit distinct differences in virulence and host adaptation. Compared to *M. tuberculosis*, experimental *M. bovis* infection of mice or rabbits results in a more severe pathology and shorter mean survival times (9, 17, 18). *Mycobacterium tuberculosis* is primarily a human pathogen that demonstrates a high level of attenuation in cattle (as reviewed by Francis in 1947 [10]), whereas *M. bovis* has a wider host range and affects many domesticated and free-ranging mammals as well as humans. Prior to the initiation of control and eradication campaigns in the early to mid-1900s, *M. bovis* infection accounted for up to 30% of human tuberculosis cases, with *M. bovis* being transmitted to humans primarily by the consumption of unpasteurized dairy products and contact with infected livestock. Control efforts, including slaughter surveillance and test/cull campaigns, have dramatically reduced the prevalence of *M. bovis* infection in domestic cattle herds, thereby reducing the spread of *M. bovis* to humans. However, in developing countries, *M. bovis* infection of humans persists as a serious and relatively common zoonosis (16).

Although *Mycobacterium kansasii* is not a member of the *M. tuberculosis* complex, it may cause disease in otherwise healthy humans, albeit infrequently, that is clinically indistinguishable from *M. tuberculosis* infection (1, 3). As with humans, *M. kansasii* infection of cattle is uncommon; however, it is occasionally associated with granulomatous lesions within lymph nodes and the respiratory tract of cattle (B. Harris, unpublished observations). Of particular relevance for the diagnosis of tuberculosis, *M. kansasii* infection/sensitization may elicit responses to antigens generally considered to be tuberculosis specific, such as ESAT-6, CFP-10, and MPB83 (2, 30, 35).

With experimental *M. bovis* infection of cattle, the levels of MPB83-specific antibody correlate with disease severity, bacterial burden, and specific cell-mediated immune responses (15, 33). With this particular scenario, disease severity (i.e., pathology) and bacterial burden are intimately linked; thus, it is difficult to define a potential correlation of a particular immune response to either readout independently. Prior studies have demonstrated that virulent and attenuated strains of *M. bovis* induce similar delayed-type hypersensitivity responses in cattle; however, only the virulent *M. bovis* strain induces a persistent gamma interferon (IFN-γ), interleukin-2 (IL-2), and antibody response (34). The objective of the present study was to compare mycobacterium-specific immune responses to the patterns of mycobacterial disease expression in which the mycobacterial burden is uncoupled from pathological changes. Disease expression patterns included persistent colonization with an associated pathology (i.e., *M. bovis* infection), colonization without an associated pathology (i.e., *M. tuberculosis*

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infection), and no colonization or pathology (i.e., *M. kansasii* infection). Antigen-specific immune responses were evaluated for their correlation to manifestations of disease expression.

MATERIALS AND METHODS

Calves, challenge inoculum, and necropsy. Friesian or Friesian cross castrated male calves of ~6 months of age were obtained from bovine TB-free herds and were housed within either biosafety level 3 facilities at the Veterinary Laboratory Agencies (VLA), Addlestone, United Kingdom (*M. bovis*- and *M. tuberculosis*-inoculated animals) or biosafety level 2 facilities at the National Animal Disease Center (NADC), Ames, IA (*M. kansasii*-inoculated animals). All cattle experiments were cleared by local ethical review, and animal procedures were performed in accordance with either British Home Office requirements [i.e., A(SP)A, 1086; VLA studies] or according to institutional guidelines and approved animal care and use protocols (NADC studies). The treatment groups included *M. tuberculosis*-inoculated calves ($n = 5$), *M. bovis*-inoculated calves ($n = 5$), and *M. kansasii*-inoculated calves ($n = 4$). The inoculation of *M. tuberculosis* (2.8×10^6 CFU) and *M. bovis* (1.0×10^6 CFU) was by direct instillation of the challenge inoculum into the tracheas of sedated calves, as described previously (5, 27). The inoculation of *M. kansasii* (4×10^8 CFU) was by direct instillation of the inoculum into the tonsillar crypts of sedated calves, as described previously (19). In general, these two routes of inoculation of tuberculous mycobacteria to cattle (i.e., intratracheal and intratonsillar) result in similar disease severities and infections (5, 20, 27). Whole blood was collected at 0, 2, 4, 6, 8, 10, 12, and 16 weeks after challenge for IFN-γ assays and placed in tubes with heparin; and serum was collected at 0, 3, 6, 7, 9, 10, 12, 13, 16, and 18 weeks after challenge for antibody detection assays (the slight variations in collection time points were dependent upon the treatment groups and assay). The 100-fold larger dose of *M. kansasii* was used on the basis of the findings of prior studies (19) and the relatively low virulence of *M. kansasii* compared to that of *M. bovis* and *M. tuberculosis*.

M. bovis field isolate AF2122/97 was grown to mid-log phase in Middlebrook 7H9 medium (Becton, Dickinson and Company [BD], Oxford, United Kingdom) supplemented with 10% (vol/vol) Middlebrook acid-albumin-dextrose-catalase enrichment (BD), 4.16 g/liter sodium pyruvate (Sigma-Aldrich Co., Poole, United Kingdom), and 0.05% (vol/vol) Tween 80 (Sigma-Aldrich); and stocks were stored frozen at −80°C. Stocks of *M. tuberculosis* $H_{37}R_v$ were prepared in the same way, except that the 7H9 medium was supplemented with 0.2% (vol/vol) glycerol (Sigma-Aldrich) rather than sodium pyruvate. *M. kansasii* field isolate 03-6931 (subtype 1, based on the 16S-23S rRNA sequence [1, 30]) was grown to mid-log phase in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Becton Dickinson, Franklin Lakes, NJ) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO), and stocks were stored frozen at −80°C. Enumeration of the bacterial stocks (CFU/ml) was by serial dilution on modified Middlebrook 7H11 agar (11). In addition, the virulence of the *M. tuberculosis* and *M. bovis* strains was confirmed in guinea pigs prior to the start of the cattle challenge experiment (data not shown). The *M. kansasii* strain used in the present study was isolated from a tuberculous lesion (i.e., a pyogranuloma) detected within an adult cow upon routine slaughter surveillance. The *M. kansasii* isolate had 100% identity with the subtype 1 genotype, which is most commonly associated with disease in immunocompetent humans (30).

Postmortem examination. Approximately 16 to 18 weeks after inoculation, all cattle were euthanized by the intravenous injection of sodium pentobarbital and examined. Specimens of various tissues were collected (tonsil, lung, lung-associated lymph nodes, and head-associated lymph nodes) for bacteriologic culture (described below) and microscopic examination, including Ziehl-Neelsen staining for acid-fast bacilli as well as hematoxylin-eosin staining for morphological assessment of the tissues, as described previously (4, 31). All major visceral organs were visually inspected for lesions, and tissue specimens were collected for further microscopic and bacteriologic assessment, when it was warranted. The lungs were examined externally and were sliced into 0.5- to 1-cm-thick sections for the detection of visible lesions. Lymph node tissues were sliced into thin sections (1 to 2 mm thick) and were examined for the presence of visible lesions. The severity of the gross pathological changes was scored by using a semiquantitative scoring system, as described previously (29). In summary, each of seven lung lobes was scored from 0 to 5, depending on the number of lesions and the extent of the pathology observed, with 0 being no pathology and 5 being extensive gross coalescing lesions. The lymph nodes of the upper and lower respiratory tracts were similarly scored but by use of a score of from 0 to 3. The scores of the individual lung lobes were added to calculate the lung score, and the scores of the individual lymph nodes were added to calculate the lymph node score. Both lymph node and pathology scores were combined to determine the total pathology score for each animal.

Bacterial enumeration. Tissue sections collected from lymph node and lung samples postmortem were individually homogenized in 5 ml of sterile phosphate-buffered saline (PBS) with a rotating-blade macerator system. Enumeration of the CFU in each sample was done by inoculating modified 7H11 agar plates with 50 μl of tissue homogenate and counting the colonies after incubation at 37°C for 4 to 6 weeks (11). A semiquantitative bacterial burden score of 0 to 4 was determined for each tissue sample, where 0 indicates no colonies, 1 indicates 1 to 10 CFU, 2 indicates 11 to 100 CFU, 3 indicates 101 to 1,000 CFU, and 4 indicates >1,000 CFU per sample. The score for all tissues was combined to provide a total bacteriology burden score per animal. All culture-positive samples from the *M. tuberculosis*-infected animals and a representative sample from four of the *M. bovis*-infected cattle were submitted to the VLA Molecular Strain Typing Laboratory to confirm the strain identity by spoligotyping. Strains were spoligotyped by the method of Kamerbeek et al. (14) with minor modifications (6).

Tuberculin skin test procedures. For the studies at VLA (i.e., for *M. bovis*- and *M. tuberculosis*-inoculated animals), skin tests were performed as specified in European Economic Community Directive 80/219EEC and amending directive 64/422/EEC, annex B. For the studies at NADC (i.e., for the *M. kansasii*-inoculated animals), skin tests were performed as specified in the circular on uniform methods and rules for the eradication of bovine tuberculosis of the Animal and Plant Health Inspection Service (APHIS), USDA (APHIS circular 91-45-011 [24]). At approximately 16 weeks after inoculation, the skin thickness was measured with calipers immediately prior to the administration of *M. bovis* purified protein derivative (PPD), and the skin thickness was again measured 72 h after injection. The skin tests were performed at the midcervical region and the caudal fold for the studies at VLA and NADC, respectively. Data are presented as the change in the skin thickness (mm) from the preinjection measurements (mean ± standard error of the mean [SEM]).

IFN-γ assay. Duplicate 250-μl heparinized whole-blood aliquots were distributed in 96-well plates with *M. bovis* PPD (10 μg/ml; VLA or Prionics Ag, Schlieren, Switzerland) or no antigen and were incubated at 37°C in a 5% CO_2 atmosphere for 20 h. The IFN-γ concentrations in stimulated plasma were determined by using a commercial enzyme-linked immunosorbent assay (ELISA)-based kit (Bovigam; Prionics Ag). The absorbances of the standards (recombinant bovine IFN-γ; Endogen, Rockford, IL) and the test samples were read at 450 nm by using an ELISA plate reader (Molecular Devices, Menlo Park, CA). Duplicate samples for individual treatments were analyzed, and the data are presented as the optical densities at 450 nm of the response to *M. bovis* PPD minus the response to no antigen (mean ± SEM).

Lateral-flow assay. The VetTB Stat-Pak lateral-flow assay (Chembio Diagnostic Systems, Inc., Medford, NY) was performed as previously described for use with samples from cattle (33). Briefly, the lateral-flow device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with test antigen (i.e., MPB83, ESAT-6, and CFP-10) and laminated with several pads made of glass fiber and cellulose. Thirty microliters of serum and 3 drops of sample diluent are sequentially added to the sample pad. As the diluted test sample migrates to the conjugate pad, the latex particles conjugated to antigen bind to antibody (IgM, IgG, and IgA), if antibody is present, thus creating a colored immune complex. This complex flows laterally via capillary forces across the nitrocellulose membrane impregnated with specific antigen and binds to the immobilized antigen, producing a visible blue band. In the absence of specific antibody, no band is visible.

MAPIA. The multiantigen print immunoassay (MAPIA) was performed as previously described for use with samples from cattle (30, 33). Briefly, *M. tuberculosis* complex antigens were immobilized on nitrocellulose membrane strips, blocked for 1 h with 1% nonfat skim milk in PBS with 0.05% Tween 20, and then incubated for 1 h with serum samples diluted 1:40 in blocking solution. After the strips were washed, they were incubated overnight with peroxidase-conjugated protein G (Sigma) diluted 1:1,000, washed, and developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Responses to individual *M. tuberculosis* complex antigens were identified by MAPIA.

ELISA for serum IgG to MPB83 and *M. bovis* culture filtrate. The amount of antigen-specific IgG in serum from clotted blood was measured as described previously (28). Briefly, Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with either recombinant MPB83 (0.1 μg/ml; Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany) or an *M. bovis* AN5 culture filtrate previously prepared at VLA (1 μg/ml). Serum samples were diluted 1:100 prior to addition to precoated and preblocked assay plates. Wells not coated with

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TABLE 1. Disease expression upon mycobacterial inoculation

Group	Gross pathology[a]	Culture score[b]
M. bovis (n = 5)	All positive	27.2 ± 7.3
M. tuberculosis (n = 5)	All negative	13.9 ± 5.5
M. kansasii (n = 4)	All negative	0 ± 0

[a] Gross lesions were confirmed to be tuberculous upon histologic evaluation and culture of lung tissue as well as lung- and head-associated lymph node tissues.

[b] Cumulative score (mean ± SEM) based on a ranking of the total numbers of CFU per plate of lung tissue and lung-associated lymph node tissue homogenates at the highest dilution. Mycobacteria were not detected in any tissues collected from *M. kansasii*-inoculated animals. Mean scores reflect the results from each animal within the treatment group, including culture-negative animals. The results differ ($P < 0.05$) according to mycobacterial treatment: *M. bovis*-inoculated animals > *M. tuberculosis*-inoculated animals > *M. kansasii*-inoculated animals.

antigen were used to ensure the absence of nonspecific responses, and the prechallenge responses were used for comparison to the specific responses after challenge. Total IgG was detected by using a horseradish peroxidase-conjugated sheep anti-bovine IgG (AbD Serotec, Oxford, United Kingdom), with the data being expressed as the optical densities at 450 nm (mean ± SEM).

ELISA for serum Ig to *M. bovis* LAM. An ELISA for serum Ig to *M. bovis* lipoarabinomannan (LAM) was performed as described previously (30, 33). Briefly, Immulon II 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with LAM-enriched mycobacterial antigen (8 μg, prepared as described previously [32]). Serum samples were diluted 1:100 prior to addition to precoated and preblocked assay plates. Total Ig was detected by using horseradish peroxidase-conjugated goat anti-bovine IgG heavy and light chains (Kirkegaard & Perry Laboratories Inc.). Data are presented as sample/positive (S/P) values (mean ± SEM). S/P values of test samples were calculated from absorbency values by using the following formula: sample − negative control/positive control − negative control. Positive and negative control sera with known reactivities were obtained from previous studies.

Statistics. Data were analyzed by analysis of variance, followed by the Bonferroni multiple comparisons test or Student's t test, using a commercially available statistics program (Prism, version 4.0; GraphPAD Software, La Jolla, CA).

RESULTS AND DISCUSSION

Disease progression. Granulomatous lesions containing acid-fast bacilli were detected in the lungs as well as the lung- and head-associated lymph nodes from five of five, five of five, and three of five *M. bovis*-infected animals, respectively (Table 1). Lesions with acid-fast bacilli were not detected in any of the *M. tuberculosis*- or *M. kansasii*-inoculated animals (Table 1). *Mycobacterium bovis* was isolated by culture from five of five animals, whereas *M. tuberculosis* was isolated from three of five animals inoculated with the respective mycobacteria. For the *M. bovis*-infected cattle, *M. bovis* was isolated by culture from lung-associated lymph nodes and lungs from five of five animals and from medial retropharyngeal lymph nodes from three of five cattle. For the *M. tuberculosis*-infected cattle, *M. tuberculosis* was isolated by culture from lung-associated lymph nodes from three of five animals and from the medial retropharyngeal lymph nodes from one of five cattle. Analysis of the spoligotype patterns confirmed that the mycobacterial strains isolated from tissues matched the respective challenge strains (i.e., $H_{37}R_v$ for *M. tuberculosis* and AF2122/97 for *M. bovis*; data not shown). *Mycobacterium* spp. were not detected in *M. kansasii*-inoculated animals. The mycobacterial burden differed ($P < 0.05$) between challenge species (Table 1), as follows: *M. bovis* > *M. tuberculosis* > *M. kansasii*.

Cell-mediated responses. Injection of *M. bovis* PPD for the skin test (at ~16 weeks postinoculation) resulted in palpable reactions in all calves, regardless of the mycobacterial treatment; however, differences were detected, with the responses (means ± SEMs) of the *M. tuberculosis*-inoculated cattle (22.6 ± 5.1) and *M. bovis*-inoculated cattle (12.8 ± 1.9) exceeding ($P < 0.05$) the responses of the *M. kansasii*-inoculated cattle (4.5 ± 0.3). Similarly, IFN-γ responses to *M. bovis* PPD were elicited in all calves regardless of the mycobacterial treatment. Two weeks after inoculation, the mean IFN-γ responses did not differ ($P > 0.05$) between treatment groups. From 4 weeks to the end of the study, the mean IFN-γ responses to *M. bovis* PPD by *M. tuberculosis*- and *M. bovis*-inoculated cattle exceeded ($P < 0.05$) the mean response of the *M. kansasii*-inoculated cattle (Fig. 1). Throughout the study, the IFN-γ responses to *M. bovis* PPD did not differ ($P > 0.05$) between the *M. tuberculosis*- and the *M. bovis*-inoculated cattle. The patterns of the IFN-γ responses to the ESAT-6 and CFP10 antigens were similar to the pattern of responses to *M. bovis* PPD (data not shown) (30). Thus, the delayed-type hypersensitivity and IFN-γ responses did not correlate with the disease progression differences detected between *M. tuberculosis*- and *M. bovis*-inoculated cattle. Likewise, transient IFN-γ responses may be elicited to mycobacterial species (e.g., *M. kansasii*) that are efficiently cleared by cattle. It is interesting to note that the IFN-γ response kinetics toward challenge with all three pathogens developed very similarly up to 2 weeks postinfection, after which time the responses in the *M. kansasii*-infected calves were curtailed and remained at a significantly lower level throughout the rest of the observation period. One caveat concerning this observation is that *M. bovis* antigens were used for the assay; thus, the responses may be indicative of early cross-reactive responses. Two weeks postinfection is most likely the time point when a developing cellular immune response becomes effective, and this response appears to be capable of eliminating *M. kansasii*, while it is unable to fully control *M. bovis* infection and, to a lesser extent, *M. tuberculosis* infection. This response kinetic is very similar to that observed after mouse aerosol infection, in which *M. tuberculosis* is exported to the regional lymph nodes for the development of the initial primary response (7, 21, 36). This delayed

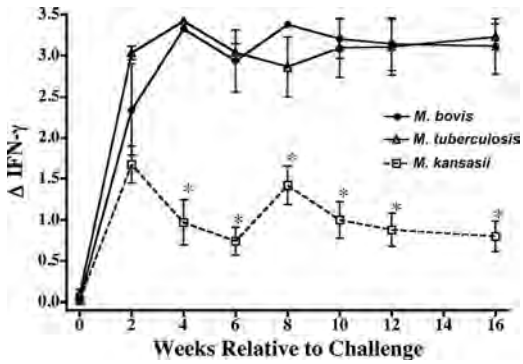

FIG. 1. IFN-γ responses upon experimental inoculation of calves with *M. bovis* (n = 5), *M. tuberculosis* (n = 5), or *M. kansasii* (n = 4). Data are presented as optical densities (means ± SEMs) at 450 nm of the response to *M. bovis* PPD minus the response to no antigen (ΔIFN-γ). *, the response was significantly different ($P < 0.5$) from the responses by the *M. bovis*- and M. *tuberculosis*-inoculated animals at the same time point.

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TABLE 2. Kinetics of serum antibody responses to MPB83, ESAT-6, and CFP-10 in cattle during experimental mycobacterial infection detected by VetTB Stat-Pak lateral-flow assay

Group	No. of animals positive/no. of animals tested at the following wk after inoculation:					
	0	3	6	9	12	18[a]
M. bovis[b]	0/4	4/4	4/4	3/4	3/4	NA[c]
M. tuberculosis	0/5	5/5	4/5	2/5	0/5	NA
M. kansasii	0/4	0/4	0/4	2/4	1/4	4/4

[a] For the *M. kansasii*-inoculated cattle, the responses at 18 weeks were 2 weeks after the injection of PPD for the skin test.
[b] One of the *M. bovis*-infected cattle was euthanized at week 6, and therefore, kinetics data were available for only four of the five original study animals.
[c] NA, animals in the *M. tuberculosis*- and *M. bovis*-inoculated groups were euthanized 16 weeks after inoculation; thus, samples were not available for analysis at that time point.

immune kinetic in mice is associated with the slow and delayed migration of parenchymal dendritic cells to pulmonary lymph nodes (26). It would be of interest to conduct similar studies with *M. kansasii* to see if the migration kinetics are identical to those for its more virulent cousins, *M. tuberculosis* and *M. bovis*.

Antibody responses. Specific antibody responses were detected in all *M. tuberculosis*- and *M. bovis*-inoculated cattle 3 weeks after inoculation (Table 2). At 12 and 16 weeks after inoculation, the IgG responses to MPB83 and *M. bovis* culture filtrate by *M. bovis*-inoculated cattle exceeded ($P < 0.05$) the respective responses by the *M. tuberculosis*-inoculated cattle (Fig. 2). Antibody levels to MPB83 persisted from 3 to 16 weeks after challenge with the *M. bovis* group, whereas the responses to MPB83 by the *M. tuberculosis*-inoculated cattle waned from 6 to 16 weeks after challenge (Fig. 2). Using a nonquantitative assay (i.e., MAPIA), three of four *M. bovis*-inoculated cattle responded to MPB83 by 3 weeks after challenge and four of four cattle responded at all other subsequent time points, whereas three of five, four of five, four of five, and three of five *M. tuberculosis*-inoculated cattle responded to MPB83 at 3, 6, 9, and 12 weeks after challenge, respectively. *Mycobacterium kansasii* inoculation elicited LAM-specific IgG: the preinoculation response (given as the mean ± SEM S/P ratio) was 0.12 ± 0.13, whereas the response was 1.01 ± 0.28 at 7 weeks after inoculation ($P < 0.05$). The initial early responses waned by 10 weeks after challenge to 0.50 ± 0.20 and then increased ($P < 0.05$) 2 weeks after injection of PPD for the skin test at 18 weeks after challenge to 1.47 ± 0.26. Prior to the skin test, only two of four *M. kansasii*-inoculated calves had antibody specific to the MPB83, ESAT-6, and CFP10 antigen cocktail (i.e., as determined by the VetTB Stat-Pak lateral-flow assay), whereas after the skin test, all four animals had responses to this antigen cocktail (Table 2).

The strong and persistent generation of MPB83-specific IgG following infection with *M. tuberculosis* is an interesting observation, given that *in vitro M. tuberculosis* expresses very small amounts of MPB83 compared with the amount expressed by *M. bovis* (35). Potent immune responses to MPB83 have previously been observed in *M. tuberculosis*-infected mice (13), and the response is also upregulated in *M. tuberculosis*-infected macrophages (i.e., *in vitro*) (22, 23). Together with the present findings of MPB83-specific responses by *M. tuberculosis*-inoculated cattle, these data support the notion that MPB83 expression is upregulated by *M. tuberculosis in vivo*.

Immune response-disease progression associations. As indicated in the early 1900s (25), the administration of culture-derived *M. tuberculosis* (in this case, $H_{37}R_v$) to healthy cattle elicits an immune response without the expression of disease (i.e., pathology), thus providing the rationale for early 20th century studies evaluating live *M. tuberculosis* as a vaccine for bovine tuberculosis. In the present study, *M. tuberculosis* was detected in three of five inoculated animals upon necropsy, which was at ~16 weeks after inoculation. MPB83-specific antibody was detectable early after challenge in all five *M. tuberculosis*-inoculated animals, yet these responses waned to preinoculation levels by the end of the study (Fig. 2A). Thus, with experimental *M. tuberculosis* $H_{37}R_v$ infection of cattle, antibody responses are associated with mycobacterial burden rather than pathology. A wane in the early MPB83-specific antibody response likely followed the clearance of *M. tuberculosis* from the host. In contrast, persistent *M. bovis* infection resulted in lesions and sustainable specific antibody responses (Fig. 2). Together, these findings indicate that antibody responses upon mycobacterial infection are indicative of the antigen load rather than the mycobacterium-induced pathology.

Inoculation of *M. kansasii* elicited LAM-specific responses in four of four cattle and MPB83-specific responses in two of four cattle, as detected by MAPIA. Injection of PPD for the skin test boosted both the LAM- and the MPB83-specific re-

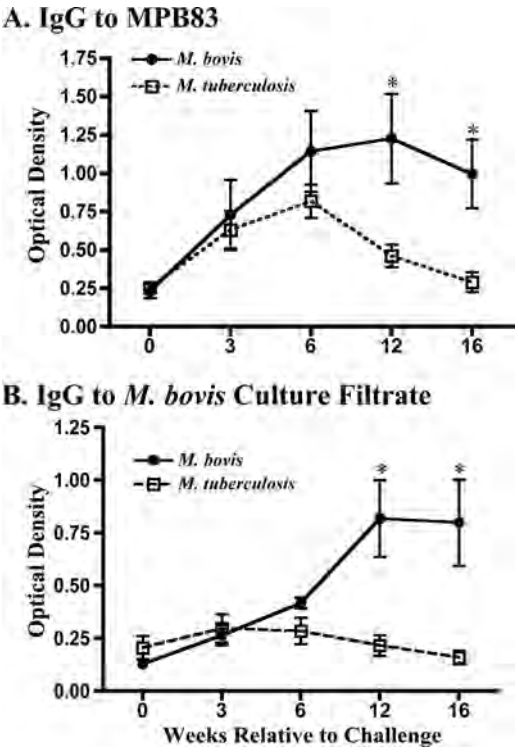

FIG. 2. Antibody responses upon experimental inoculation of calves with *M. bovis* ($n = 5$) or *M. tuberculosis* ($n = 5$). Data are presented as the optical densities (means ± SEMs) at 450 nm of the response to MPB83 (A) or *M. bovis* culture filtrate (B). *, the response was significantly different ($P < 0.5$) from the response by *M. tuberculosis*-inoculated animals at the same time point.

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sponses in all four animals, as previously detected with *M. bovis* infection of cattle (33). Mycobacteria were not isolated upon necropsy, and lesions were not detected. These findings demonstrate the potential for a correlation of the specific antibody responses to a boosting effect resulting from exposure to cross-reactive antigens, even when the mycobacteria have likely been cleared weeks prior to subsequent exposure.

Conclusions. In this study, cellular immune responses correlated with mycobacterial infection but not necessarily with the pathology or bacterial burden. The findings of the present study highlight several scenarios for the interpretation of antibody responses by cattle to mycobacterial exposure. (i) The first scenario is the classic model, in which infection (e.g., *M. bovis* infection of cattle) is not controlled by the host, resulting in significant pathology and mycobacterial burden. With this scenario, the association of the antibody responses to pathology (15) is likely coincident with the antigen burden. (ii) The second scenario is an antigen load model in which the antibody responses are positively correlated to the antigen burden. With *M. tuberculosis* $H_{37}R_v$ infection of cattle, as the mycobacterium is cleared, the antibody responses wane. With *M. bovis* infection of cattle, as the infection progresses and the mycobacteria persist, the antibody responses also persist or increase. (iii) The final scenario is the sensitization model, in which exposure to a mycobacterium (e.g., *M. kansasii* inoculation) induces an antibody response which wanes over time, yet the response may be boosted significantly by reexposure to mycobacterial antigens (e.g., PPD) or other live mycobacteria.

Good and reliable models of human latent tuberculosis are not readily available for support of both applied tuberculosis research (e.g., vaccinology and diagnostic studies) and basic tuberculosis research (e.g., immunopathogenesis and bacterial pathogenesis). An animal model in which different infection outcomes can be modeled with organisms with overlapping antigen repertoires, such as the three organisms studied here, would be of advantage. *Mycobacterium bovis* infection of cattle could be considered a model of clinical tuberculosis, while *M. tuberculosis* $H_{37}R_v$ infection could be akin to latent, subclinical tuberculosis. Infection with *M. kansasii*, on the other hand, might be a model for a human tuberculous infection that has successfully been cleared from its human host. Therefore, we propose that these three infection systems be developed into useful models mimicking different stages of human *M. tuberculosis* infection.

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