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Southeast Poultry Research Laboratory

The Agricultural Research Service's Southeast Poultry Research Laboratory (SEPRL) brings together scientific expertise in an integrated research approach that includes virology, molecular biology, pathology, and immunology. This expertise is key to developing prevention, management, and control and eradication strategies to protect the $25-billion-a-year U.S poultry industry, which contributes $2.5 billion in U.S exports annually. SEPRL is a major international resource to control highly pathogenic Avian Influenza and virulent Newcastle Ddisease, which pose a major threat to the U.S poultry industry as well as a severe risk to public health.

Research at the laboratory has been expanded in the last five years to address priority domestic viral diseases that also impact the U.S poultry industries. SEPRL, which was established in 1960, has two research units: the Exotic and Emerging Avian Viral Diseases Research Unit and the Endemic Poultry Viral Diseases Research Unit.

This research provides government regulatory agencies and the poultry industries with improved intervention strategies against poultry viral diseases. SEPRL will require new laboratory and animal housing facilities if it is to continue providing rapid responses to new and emerging disease threats and effectively respond to the research needs of the United States.

Mission

Provide scientific solutions to national and international exotic, emerging, and endemic poultry viral diseases through a comprehensive research program emphasizing basic and applied research in diagnostics, prevention, and control strategies, prediction of disease outbreaks, molecular epidemiology, and understanding disease pathogenesis.

Program Objectives

SEPRL's objectives are to produce new research knowledge and technology to--

- prevent, reduce, or eliminate losses from impaired performance and increased deaths and condemnations;
- develop more sensitive, specific, and faster diagnostic tests;
- Improve vaccines and vaccine delivery methods;
- improve our understanding of the ecology and epidemiology of viruses at the wild bird-domestic poultry interface; and
- improve our understanding of the genetic and pathobiological basis of virulence.

Priority Diseases

High-pathogenicity Avian Influenza virus – Select Agent
Virulent Newcastle Disease virus – Select Agent
Low-pathogenicity Avian Influenza virus – Nonselect Agent
Low-virulence Newcastle Disease virus – Nonselect agent
Marek's Disease herpesvirus - Nonselect Agent
Enteric viruses of poultry - Nonselect Agent

Stakeholder groups

National Turkey Federation
National Chicken Council
United Egg Producers
State poultry associations
State Departments of Agriculture, Animal Bureaus, and Animal Commissions
Animal and Plant Inspection Service
Food Safety and Inspection Service
U.S. Food and Drug Administration
Centers for Disease Control and Prevention
University diagnostic laboratories
U.S. veterinary colleges

Research Facilities

- 32 acres, 29 buildings (25 completed in 1963, 1 BSL-3AG completed 1976, 2 SPF flock houses completed in 1983, replacement sewage decontamination facility 2001)
- Laboratory spaces and decontamination procedures
- BSL-2/3E, ABSL-2/3E, and animal holding spaces
- BSL-3Ag loose animal rooms
- Administrative, storage, support, and utility spaces
- Breeding (SPF) and production spaces

Personnel

Scientists: 11
Technicians: 19
Administration and support staff: 13
Total: 43

Accomplishments

- Developed and used rapid, real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) diagnostic tests that were used in live bird markets in Virginia in 2001-2002.
- Collaborated with corporate partners in research that resulted in the licensing and commercialization of two avian influenza vaccines: fowlpox recombinant-avian influenza H5 gene insert and inactivated H5 avian influenza.
- Collaborated with corporate partners in research that resulted in two licensed ELISA test kits for avian influenza diagnostics.
- Used molecular epidemiology to track sources and links in the Mexican H5 outbreak (1994-95), H7N2 outbreak in LBM of northeastern USA (1994-2002), Hong Kong H5N1 outbreaks (1997-2001), and Chilean H7N3 outbreak (2002).
- Provided scientific data to USDA to support negotiations on trade in poultry and poultry products including validating pasteurization as a way to inactivate LPAI virus in egg products and determining that there was a low risk of LPAI virus being transmitted in broiler meat.
- Demonstrated the susceptibility of turkeys to 2010 pandemic H1N1 and modified RT-PCR diagnostic tests for biosurveillance in poultry.
- Used molecular epidemiology to track sources of the virulent Newcastle Disease (NDV) in the Mexican outbreak (2000) and the California and Texas outbreaks (2002-2003).
- Demonstrated that recent NDV isolates, isolates recovered during disease outbreaks since the 1970s, and new clades of virus emerging in Asia are phylogenetically distinct from current vaccine viruses and standard challenge strains.
- Completed a genetic characterization of the first U.S. avian pneumovirus isolate, which resulted in it being classified as type C, different from European types A and B.
- Demonstrated that waterfowl outside of Minnesota can carry and potentially disseminate avian metapneumoviruses.
- Isolated the first astrovirus and demonstrated its involvement in poultry enteritis mortality syndrome (PEMS).
- Developed and transferred to diagnostic laboratories a multiplex PCR test for simultaneous demonstration of astrovirus, reovirus, and coronavirus.
- Demonstrated that astrovirus is resistant to inactivation by common disinfectants and that Virkon S is the only effective disinfectant.
- Demonstrated that inoculation of turkeys with West Nile Virus (WNV) did not produce disease and that WNV replicates poorly in turkeys, so there is minimal potential to infect mosquitoes and contribute to WNV ecology.
- Demonstrated that WNV can cause severe neurological disease and death in young domestic geese.

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Avian influenza: our current understanding

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Abstract

Avian influenza (AI) virus is one of the most important diseases of the poultry industry around the world. The virus has a broad host range in birds and mammals, although the natural reservoir is wild birds where it typically causes an asymptomatic to mild infection. The virus in poultry can cause a range of clinical diseases and is defined either as low pathogenic AI (LPAI) or highly pathogenic AI (HPAI) depending on the type of disease it causes in chickens. Viruses that replicate primarily on mucosal surfaces and cause mild disease with low mortality are termed LPAI. Viruses that replicate on mucosal surfaces and systemically and cause severe disease with a mortality rate of 75% or greater in experimentally infected chickens are referred to as HPAI. A virus that is highly pathogenic in chickens may infect but result in a completely different disease and replication pattern in other host species. Outbreaks of HPAI have been relatively uncommon around the world in the last 50 years and have had limited spread within a country or region with one major exception, Asian lineage H5N1 that was first identified in 1996. This lineage of virus has spread to over 60 countries and has become endemic in poultry in at least four countries. AI virus also represents a public health threat, with some infected humans having severe disease and with a high case fatality rate. AI remains a difficult disease to control because of the highly infectious nature of the virus and the interface of domestic and wild animals. A better understanding of the disease and its transmission is important for control.

Keywords: avian influenza, wild birds, LPAI, HPAI, H5N1

Origins of avian influenza

Wild birds, primarily wild ducks, gulls, and shorebirds are the natural host and reservoir for all type A influenza viruses (Slemons *et al.*, 1974; Kawaoka *et al.*, 1988; Stallknecht, 1998). The virus, however, has an extremely wide host range and can on rare occasions spread from wild birds to either domestic poultry or mammalian species. The viruses typically are not well adapted to these new hosts and replicate and transmit poorly, resulting in dead end hosts. Therefore most introductions of virus into new hosts are often not recognized and rarely cause clinical disease (Suarez, 2000). However, in rare cases, avian influenza (AI) viruses can become adapted to the new host. Often our commercial animal rearing practices, which concentrate large numbers of susceptible animals in confined spaces, aid in the transmission of virus. Poor biosecurity can also facilitate transmission of viruses between different poultry farms. The longer a virus is allowed to persist in a poultry population, the more likely it is that the virus can become adapted to the new host. This adaptation process results in an increased ability of the virus to replicate within and transmit more efficiently in the new host species. The result may be a more severe clinical disease with the potential for the virus to mutate to the highly pathogenic (HP) phenotype with some subtypes of AI viruses (H5 or H7). The more adapted a virus is to the new host species, the more difficult it will be to eradicate the virus from that species.

Host susceptibility

AI has been isolated from over 100 species of birds, which represent at least 12 different Orders. However, birds of the Orders Anseriformes and Charadriiformes, which include ducks, gulls, and shorebirds, are considered to be the primary natural reservoir of the virus because the virus

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is reliably isolated from certain species from these Orders of birds (Stallknecht and Brown, 2008). However, the Anseriformes and Charadriiformes Orders of birds represent a diverse group of bird species, and it is clear that particular bird species are infected at a high prevalence every year and are important for the maintenance of the virus in the environment. Mallards, Northern Pintail, Green-winged teal, and Northern Shoveller ducks are four Anseriformes species from which AI virus can be commonly isolated, particularly in the autumn as the birds are marshalling for or traveling to wintering grounds on their southern migration (Stallknecht and Brown, 2008). Other Anseriformes species are susceptible to infection based on experimental studies; these include Canada geese or wood ducks, but based on the rates of isolation in the wild, these species do not appear to be important in the natural maintenance of the virus (Deibel *et al.*, 1985; Slemons *et al.*, 1991; Brown *et al.*, 2006, 2008). In the Charadriiformes Order of birds, the only species from which AI virus is isolated with high frequency is the Ruddy Turnstones in the spring in Delaware Bay. The high isolation rate from Ruddy Turnstones has been proposed to be the result of the unique concentration of migratory birds in Delaware Bay because of the abundance of high-quality feeding grounds at that time of year (Hanson *et al.*, 2008).

We currently have a poor understanding of why some species appear to be important in the ecology of AI, while closely related species are not. Factors that appear to be common between infected species is that they concentrate in high numbers for at least part of the year, particularly at marshaling grounds, which likely facilitates transmission of the virus. Similar observations have been made with poultry and for mammalian species in which influenza has become endemic, in that the affected species are often crowded together, thereby facilitating transmission (Suarez, 2008). Other factors are likely to be involved. For example, the incidence of infection appears highest at the higher latitudes. This may be related to the hypothesis that AI viruses can overwinter in water, reinfecting returning migratory waterfowl in the spring (Zhang *et al.*, 2006). Mounting evidence supports this idea because AI viruses appear to be viable for long periods of time at low temperatures in water (Brown *et al.*, 2009a). Another proposed factor may be the biology of the birds themselves, as the feeding style and differences in the density of lamellae (filter plates) of ducks appear to be positively correlated with AI infection (Hill *et al.*, 2010). Our understanding of the ecology of AI viruses in the natural host, however, remains rudimentary, and the complete host range important for propagation of the virus may never be clearly defined.

The movement of AI viruses from the natural wild bird host to aberrant hosts including gallinaceous poultry species, such as chickens, turkeys, quail, and pheasants, appears to happen relatively frequently, but is likely not often recognized (Suarez, 2000). With the increased concern for H5N1 highly pathogenic AI (HPAI), the surveillance of poultry for AI virus infections has greatly increased and documents that a number of commercial poultry flocks are infected with a variety of different AI viruses every year. Most of these cases are recognized based on seroconversion of the flock without clear evidence of clinical disease, and most of these infections are transient with the virus not spreading to other flocks (Alexander, 2007; Senne, 2007). This lack of persistence is believed to be because most of these viruses are not well adapted to the new host and cannot efficiently maintain the transmission chain. However, some viruses do become established in the new host population, in part because of the natural variation of AI viruses that exist in the wild bird population that allows some viruses to more easily replicate and transmit (Swayne and Slemons, 2008). On rare occasions, an introduced virus can become established, and by an adaptation process, increase its ability to replicate and transmit in poultry populations. Although the exposure of low pathogenic AI (LPAI) viruses from wild birds to poultry species is likely to be similar, the number of outbreaks of HPAI in the last 20 years is higher than the number of HPAI outbreaks reported in the previous 40 years. One hypothesis for the increase in the number of unique HPAI outbreaks in recent years is that there has been an increase in the number of high-density poultry rearing operations, and this increased density of birds within a flock increases opportunities for virus transmission, providing greater opportunity for the virus to adapt and mutate to the HP form of the virus (Swayne, 2008a).

One method of evaluating virus adaptation for a particular species is to experimentally determine the minimal infectious dose 50 (MID_{50}) or the dose for a particular strain of virus that will infect half the birds of a particular species (Tumpey *et al.*, 2004; Spackman *et al.*, 2007b). A highly host-adapted virus is expected to have a low MID_{50} and a poorly adapted virus will have a high MID_{50}. However, the MID_{50} can be influenced by a number of factors including the age of the bird, the route of inoculation, the breed of animal, and concurrent infection (Swayne and Slemons, 2008; Costa *et al.*, 2010; Pantin-Jackwood *et al.*, 2010; Ramirez-Nieto *et al.*, 2010). Calculating the MID_{50} can be difficult because it requires the inoculation of a large number of animals, which must be separated based on challenge dose. For this reason relatively few viruses have been characterized with respect to infectious dose, and almost all of these have been in chickens. In the studies performed in chickens with virus given by the mucosal route, viruses in three general groups have been tested: (i) LPAI from wild birds, (ii) LPAI of poultry origin, and (iii) HPAI (Table 1) (Chen *et al.*, 2003; Subbarao *et al.*, 2003; Tumpey *et al.*, 2004; Swayne and Beck, 2005; Brown *et al.*, 2007; Okamatsu *et al.*, 2007; Spackman *et al.*, 2007b; Tsukamoto *et al.*, 2007). For the wild-bird origin LPAI viruses, the chicken

Table 1. Chicken infectious doses for viruses in various pathogenicity categories

Isolate	Subtype	Chicken infectious dose (log 10)	Reference
Wild bird low pathogenic isolate			
Mallard/Ohio/338/1986	H4N8	4.4	Swayne and Slemons (2008)
Mallard/Ohio/184/1986	H5N1	6.7	Swayne and Slemons (2008)
Cinnamon Teal/Bolivia/4537/2001	H7N3	6.2	Spackman *et al.* (2006)
Mallard/Maryland/791/2002	H5N2	>5.3	Spackman *et al.* (2007a)
Ruddy Turnstone/New Jersey/1148676/2004	H5N7	>7.5	Spackman *et al.* (2007b)
DK/Pennsylvania/454069-9/2006	H5N1	7.5	Spackman *et al.* (2007b)
Mute Swan/Michigan/451072-2/2006	H5N1	6.3	Spackman *et al.* (2007b)
Poultry low pathogenic isolate			
CK/Alabama/1975	H4N8	3.0	Swayne and Slemons (2008)
Emu/Texas/39924/1993	H5N2	4.2	Swayne *et al.* (1996)
Rhea/North Carolina/39482/1993	H7N1	6.9	Swayne *et al.* (1996)
CK/Hong Kong/G9/1997	H9N2	<2.9	Chen *et al.* (2003)
TK/Virginia/15821/2002	H7N2	2.8	Tumpey *et al.* (2004)
Parrot/California/D0406032/2004	H5N2	4.1	Swayne and Slemons (2008)
HP isolate			
CK/Rostock(Germany)/1934	H7N1	1.2	Swayne and Slemons (2008)
CK/Scotland/1959	H5N1	3.0	Swayne and Slemons (2008)
Tern/South Africa/1961	H5N3	3.4	Swayne and Slemons (2008)
TK/Ontario/7732/1966	H5N9	3.4	Swayne and Slemons (2008)
CK/Victoria/1975	H7N7	2.9	Swayne and Slemons (2008)
TK/Ireland/1983	H5N8	4.7	Swayne and Slemons (2008)
CK/Pennsylvania/1370/1983	H5N2	3.0	Swayne and Beck (2005)
Turkey/England/50-92/1991	H5N1	3.9	Swayne and Slemons (2008)
CK/Queretaro/14588-19/1995	H5N2	3.0	Swayne and Slemons (2008)
Hong Kong/486/1997	H5N1	2.4	Swayne and Slemons (2008)
Hong Kong/491/1997	H5N1	2.3	Subbarao *et al.* (2003)
TK/Italy/4580/1999	H7N1	2.0	Swayne and Slemons (2008)
CK/Korea/ES/2003	H5N1	2.5, 3.1	Swayne and Beck (2005)
CK/Yamaguchi/7/2004	H5N1	2.0	Nakamura *et al.* (2008)
Whooper Swan/Mongolia/7/2005	H5N1	2.8	Brown *et al.* (2007)

MID_{50} was greater than 10^6 EID_{50} with one exception (A/Mallard/Ohio/338/1986 at a MID_{50} of $10^{4.4}$). For LPAI viruses of poultry origin, most of the MID_{50}s were in the range of $10^{2.8}$–$10^{4.2}$. This difference in the infectious dose clearly highlights that wild-bird origin viruses are generally not well adapted to chickens. Most of the wild-bird origin viruses required 100–1000 times more virus to infect a chicken than did the poultry-adapted viruses. Some of the poultry origin viruses have a well-described history of infection in chickens or other gallinaceous birds, such as A/Turkey/Virginia/15821/2002 (H7N2), with a MID_{50} of $10^{2.8}$. The roots of this H7N2 virus can be documented in poultry back to 1994 in the live bird markets (LBMs) in the Northeast United States, and it is considered a poultry-adapted virus. This virus also highlights the differences within poultry species, because this strain infected turkeys at an infectious dose 50 of $10^{0.8}$, which is almost 100 times lower than that required to infect chickens. HPAI viruses have a MID_{50} that ranges from $10^{1.2}$ to $10^{4.7}$, with an average of around $10^{3.0}$, which demonstrates that these viruses are also well adapted to poultry (Subbarao *et al.*, 2003; Swayne and Beck, 2005; Brown *et al.*, 2007; Swayne and Slemons, 2008).

The introduction, transmission, and adaptation of a wild bird AI virus to a poultry-adapted AI virus can be a complex chain of events, and the steps required are rarely clear. The term 'poultry' typically is defined as domesticated birds that are kept for their food, feathers, or sport and include several orders of birds. The actual list of poultry species is large, but ducks, chickens, turkeys, quail, pheasant, and pigeons are commonly kept species. Many backyard or village farmers have multiple species in close association, including ducks and gallinaceous birds, and LBMs often sell multiple species under crowded conditions. It is presumed, although not well tested experimentally, that domestic ducks are more susceptible to wild duck AI viruses than are gallinaceous birds (Spackman *et al.*, 2007b). Domestic ducks also often have closer association with wild birds because duck rearing practices often allow access to open lakes where wild and domestic birds can mingle. This provides the potential for wild ducks to infect domestic ducks, which then have opportunities to spread the virus to gallinaceous poultry (Swayne, 2008b). The role of LBMs in harboring and potentiating the adaptation of AI viruses to gallinaceous poultry is also well known (Senne *et al.*, 1992; Suarez *et al.*, 1999; Bulaga *et al.*, 2003; Liu *et al.*, 2003; Spackman

et al., 2003; Choi *et al.*, 2005; Nguyen *et al.*, 2005; Yee *et al.*, 2009).

In addition to the interaction between ducks and gallinaceous poultry, certain gallinaceous species have been identified as being more susceptible to infection with AI viruses and therefore may play a special role in the adaptation or spread of the virus to chickens. Japanese Quail, a commonly reared poultry species, has been proposed as an important bridge species, based on an increased susceptibility to some AI viruses compared to chickens and to the concentration of both α 2,3 and α 2,6 sialic acid receptors in the respiratory tract (Perez *et al.*, 2003a, b; Wan and Perez, 2006; Sorrell and Perez, 2007). The concern about quail as a bridge species even prompted regulatory officials in Hong Kong in 2002 to ban the sale of live quail in LBMs just as they had banned the sale of ducks and geese in 1998 (Lau *et al.*, 2007). Another species proposed as an important bridge species is the turkey (Tumpey *et al.*, 2004; Pillai *et al.*, 2010). In the United States, based on over 30 years of surveillance activity, turkeys are more commonly infected with avian and swine origin influenza viruses than chickens, supporting observations of an increased susceptibility of turkeys in the field (Halvorson *et al.*, 1985; Senne, 2007). Changes in turkey rearing practices in the United States to a more confinement rearing approach, which reduces exposure to wild birds, has resulted in a greatly reduced number of outbreaks of AIV infection (Halvorson, 2008). The interactions among various species are critical in the spread and maintenance of AI in poultry, but because the relations between species vary considerable in different production systems and regions of the world, it remains difficult to model. Approaches to reduce contact, like banning certain species in LBMs, appear to have been successful in reducing the number of influenza outbreaks, but this approach has not been widely adopted (Lau *et al.*, 2007). Increased biosecurity to reduce contact with wild birds, also shown to be effective, is also not a widely adopted practice in part because of the cost involved. Prevention, although in the long run perhaps the most cost effective approach, has been difficult to apply consistently around the world.

An alternative way to compare viruses is to measure virus shedding, typically oropharyngeal and cloacal shedding, after experimental inoculation. The presumption is that a well-adapted virus will replicate better and shed more virus compared to a poorly adapted virus. For example, a recent mute swan H5N1 LPAI virus from Michigan when challenged by the choanal mucosal route replicated to higher levels in domestic ducks as compared to chickens or turkeys (Spackman *et al.*, 2007b). Viral shedding is also dependent on a number of host factors including the age of the bird, the challenge dose, the route of challenge, and the breed of bird.

Another common method of evaluating host adaptation is to determine transmission of the virus within a species. These types of experiments are usually performed as either direct exposure experiments, where uninfected birds are placed directly in contact with infected birds or indirect transmission experiments where the birds remain separated but share common air space or are otherwise in close proximity (Shortridge *et al.*, 1998). The direct transmission model has also been used to model virus transmission to show the efficacy of vaccination in controlling disease outbreaks using the SEIR epidemic model (van der Goot *et al.*, 2005). This model evaluates susceptible, latently infected, infectious, recovered (SEIR) animals to provide a statistical basis to determine the reproductive ratio (R) to estimate if the transmission chain is robust enough to result in an epizootic outbreak. If the transmission model shows an $R>1$, meaning one infected bird infects more than one susceptible bird, then an epidemic spread can occur in that species. If $R<1$, then an infected bird infects less than one animal, and the transmission chain will fizzle out (van der Goot *et al.*, 2003). These types of experiments can provide a direct assessment of the transmissibility of a virus, but they can be difficult to repeat or replicate because of the many variables that contribute to the transmission of a virus.

Genetic markers of species adaptation

The adaptation process of influenza to a new host currently is unpredictable, with few genetic markers to predict host adaptation. As previously described, wild bird viruses generally replicate poorly in chickens, but several studies have shown that by repeated passaging of viruses in chickens, the viruses can replicate to higher levels and be more readily transmitted from chicken to chicken (Sorrell and Perez, 2007; Ramirez-Nieto *et al.*, 2010). However, in these studies, genetic changes include amino acid differences that directly contribute to adaptation and those that are neutral, and it has been difficult to separate what changes are important. In addition, considerable variation in the external environment or the host can occur to allow a virus to be better adapted to the new host. In other words, a virus can adapt to the new host species in many ways that are not predictable. When comparing AI viruses in wild bird species, at the nucleotide level, considerable variation occurs so that viruses from the Americas can usually be distinguished from viruses from Eurasia. The data document that these two populations of virus appear to be evolving separately. However, at the amino acid level, all the influenza genes that have been examined closely seem to be highly conserved between the two populations (Suarez, 2000). The only clear exception is within gulls, whose AI viruses appear to have some distinctive amino acid changes that identify them as a unique lineage (Swayne *et al.*, 2008). However, gulls are susceptible to both the gull-specific lineage viruses and the general AI virus lineage. Once AI viruses have jumped species and become established in new hosts, the viruses do start to accumulate amino acid

changes unique to that lineage, but comparison of different viral lineages from the same species do not show clear patterns or specific mutations that are required for virus adaptation, again demonstrating that adaptation can follow many paths (Suarez, 2008).

The clearest changes that are suggestive of adaptation to gallinaceous birds are in the hemagglutinin and neuraminidase proteins. One change that is thought to occur that has a major effect on virulence is the addition of amino acids, typically the basic amino acids lysine and arginine, at the cleavage site of the hemagglutinin gene of viruses of the H5 and H7 subtype. The mutation or insertion of basic amino acids at the cleavage site allows for different proteases to cleave the hemagglutinin protein into the HA1 and HA2 subunits, which is required for the virus to be infectious (Perdue, 2008). Typically, trypsin-like proteases cleave the hemagglutinin protein extracellularly in the enteric and respiratory tracts, which accounts for the restriction of LPAI viruses to replicate primarily on mucosal surfaces. The change in the cleavage site by substitution to additional basic amino acids or by insertion of additional amino acids allows for the hemagglutinin protein to be cleaved by a wider range of proteases, including ubiquitous proteases. The ubiquitous proteases can be found in the endoplasmic reticulum and the hemagglutinin protein can be cleaved during the translation process, allowing the virus to be infectious before it is released from the cell. This increases the types of cells that the virus can productively infect, allowing both mucosal and systemic replication, which results in an increase in virulence that typically results in the HP phenotype (Suarez, 2008).

HPAI is defined by the ability to infect and kill chickens using a standardized dose given intravenously (World Organization for Animal Health, 2006). This definition has generally been a practical and useful definition, but several examples have been described where the genotype and the phenotype of the virus in chickens have not correlated, including the recent outbreak of H5N2 in Texas in 2004. In this case, the HA cleavage site had four basic amino acids compatible with earlier HP viruses, but the virus did not cause mortality in chickens infected by the standard intravenous pathogenicity test (Lee *et al.*, 2005). The phenotypes of HPAI viruses are even less predictable in other species, although experimental data show some similarity in the pathotype in gallinaceous birds (Perkins and Swayne, 2001). However, most HPAI viruses from chickens are non-pathogenic in domestic ducks. The one major exception is the Asian lineage H5N1 HPAI viruses that were first isolated in China in 1996. The initial viruses that were examined could infect but were non-pathogenic in ducks, but over time these viruses became more and more pathogenic in ducks (Pantin-Jackwood and Swayne, 2007; Pfeiffer *et al.*, 2009). Similar observations have been made with the Asian H5N1 lineage in wild duck species, with many species being infected but with unpredictable virulence. Again the genetic differences accounting for these phenotypic differences are not clear. Finally, some species, like pigeons, appear to be highly refractory to experimental infection at typical challenge doses of 10^5–10^6 EID_{50} of virus (Perkins and Swayne, 2002; Fang *et al.*, 2006; Liu *et al.*, 2007; Werner *et al.*, 2007; Brown *et al.*, 2009a, b).

Our current understanding is that HPAI viruses are created by the passage of H5 or H7 LPAI viruses in gallinaceous birds such that an unknown selective pressure pushes for the creation of the virulent virus. The virus once created in poultry can then spread to other species including, in some cases, wild birds, although usually only transiently. HPAI viruses are not thought to normally exist in wild birds. This theory is supported by the isolation of thousands of LPAI viruses from wild birds, with only two known exceptions. The first known exception of HPAI virus in wild birds not associated with poultry was in terns in South Africa in 1961. A HPAI virus was isolated during this outbreak associated with mortality in the terns, but the virus did not appear to persist in terns nor was a similar virus found in poultry (Becker, 1966). The second known exception is the Asian lineage H5N1 HPAI virus that was endemic in poultry for almost 9 years before it appeared to jump into and persist in wild birds. The Asian lineage H5N1 HPAI virus was first isolated in domestic geese (Xu *et al.*, 1999), and from 1996 to 2005 it was associated almost exclusively with poultry species (Sims and Brown, 2008). However, in 2005 viruses from this lineage caused a large disease outbreak in wild birds in China (Liu *et al.*, 2005). This specific variant virus then appeared to move large distances, fueling the spread of the virus into Europe and Africa (Sims and Brown, 2008). Although large epidemic outbreaks in wild birds have rarely been seen since 2006, the virus appears to be maintained in the wild bird population, providing opportunities for spread back to domestic poultry (Lvov *et al.*, 2010; Sharshov *et al.*, 2010). To conclude, the changes at the HA cleavage site appear to be primarily a gallinaceous bird adaptation, but the actual sequence for this change is quite variable. In addition the cleavage site changes are not major species barriers that prevent the viruses from spreading to other species of birds or to mammals.

The second recognized adaptation to gallinaceous birds is the presence of stalk deletions in the neuraminidase gene (Matrosovich *et al.*, 1999). The stalk region of the NA protein is typically around 30 amino acids in length, predicted to be hydrophilic, and is between the transmembrane region and the globular head that contains the enzymatic site of the protein. The stalk is extremely variable in sequence between subtypes, and the principal function of the stalk is to extend the enzymatically active site of the protein away from the cell or viral surface. The neuraminidase protein removes sialic acid from both viral and host proteins and is important in the release of the virus from the infected cell, in part by preventing the virus from sticking to other influenza viruses as it exits

the host cell (Liu *et al.*, 1995). In wild birds, the stalk length within a subtype is highly conserved, but when AI viruses replicate in poultry, stalk deletions often occur. The number of amino acids that are deleted is extremely variable from just a few to over 20. Most commonly the deletion length is 12–20 amino acids. Although the emergence of stalk deletion mutants appears to be the result of positive selection pressure, paradoxically the stalk deletion actually greatly decreases the activity of the protein. The enzymatically active site remains undisturbed, but the shorter stalk reduces the flexibility or reach of the enzyme's receptor site, effectively reducing the enzymatic activity. Experimentally, stalk deletions appear to cause the virus to aggregate on the cell surface, effectively reducing the amount of virus being released from the cell (Colman, 1989; Suzuki, 2005). In one experimental study the introduction of a stalk deletion in a wild duck isolate increased the virulence of the virus for chickens, but the mechanism is still unknown (Munier *et al.*, 2010).

Stalk deletions are often closely aligned to increased numbers of glycosylation sites in the hemagglutinin gene. Increased N-linked glycosylation near the receptor-binding site appears to reduce the affinity of the receptor for sialic acid, essentially making the virus less sticky. The changes in the HA and NA proteins appear to be compensatory changes, where one seems to negate the change of the other. The less sticky virus does not require an efficient NA gene, and a poorly efficient NA gene can increase viral spread with a hemagglutinin protein with lower affinity to sialic acid (Matrosovich *et al.*, 1999; Mitnaul *et al.*, 2000). However, it is unclear what change occurs first or what advantage either change may have for the virus in gallinaceous birds.

For the internal proteins, no clear pattern has emerged for determining whether a virus is adapted to wild birds or poultry. One of the most commonly studied changes is whether there is glutamic acid or lysine at position 627 of the PB2 protein. The presence of lysine appears to strongly correlate with viruses that are adapted to mammals. The marker appears to primarily differentiate adaptation to birds and mammals, but it does not differentiate between birds species (Subbarao *et al.*, 1993). The primary function of the protein appears to be adaptation to temperature, as the lysine at this position allows the virus to replicate at lower temperatures, which correlates with the lower temperature of the respiratory tract in mammals compared to birds (Massin *et al.*, 2001; Hatta *et al.*, 2007). However, in the H5N1 Asian lineage viruses, a subset of viruses with lysine at this position have been isolated from many different species of birds with no apparent effect on the virulence observed in birds (Chen *et al.*, 2006a). Other markers in a number of different internal genes have been identified that separate avian from mammalian species adaptation, but little information is available that separates adaptation of viruses for various avian species.

Control of AI virus outbreaks in poultry

The movement of AI viruses from the wild bird reservoir to domestic bird species is not uncommon, but rarely results in viruses becoming endemic in poultry. The direct exposure of poultry to wild birds is the most likely source of introduction, with some of the best documented cases of exposure being in commercial turkeys in Minnesota where multiple outbreaks of AI were observed yearly in the 1980s and early 1990s (Halvorson and Kelleher, 1985). AI viruses of many different HA and NA subtypes were isolated from turkeys in different outbreaks, and typically at times when wild ducks were migrating to or from their summer breeding grounds. During the migratory wild duck season, turkeys were raised outside and the wild birds could fly over or actually land in the turkey pens. During the 1990s the management system was changed so that the turkeys were reared in confinement for their entire lives, and the incidence of AI virus was greatly decreased (Swayne and Suarez, 2005). Other known risks of exposure to AI viruses have been through contaminated drinking water. Outbreaks in the United States, Canada, Chile, and Australia have all been linked to using unpurified surface water where wild ducks have access to the drinking water supply for poultry (Hinshaw *et al.*, 1979; Sivanandan *et al.*, 1991; Suarez *et al.*, 2004; Pasick *et al.*, 2010). Another unusual source of infection of poultry with an HPAI virus is through contaminated meat and offal. Because HPAI is systemic, the virus is found in most if not all the internal organs including skeletal and heart muscle. These tissues experimentally have been shown to be infectious from oral feeding, and a recent case in Germany of feeding uncooked meat and offal scraps from infected domestic ducks to chickens initiated an outbreak of H5N1 in that country (Tumpey *et al.*, 2002; Swayne and Beck, 2005; Harder *et al.*, 2009). Limiting exposure of poultry to wild birds through confinement rearing and other biosecurity measures likely provides the best opportunity to reduce the risk of AI virus introduction from wild birds.

Once a virus has become adapted to and becomes endemic in a poultry population, control becomes difficult. Transmission of infectious virus between farms or different regions can occur in many ways, but the movement of infected poultry is the most common. A standard outbreak control tool is to quarantine infected flocks and control animal movement within an outbreak zone. The outbreak zone is usually a prescribed distance (e.g. 5 or 10 km) from an outbreak site or is defined by specific geographic borders. Outbreaks of HPAI result in restrictions on trade in poultry or poultry products that is usually immediate and can affect the entire country, resulting in serious economic losses for the producer (Swayne and Suarez, 2005). Outbreaks of LPAI virus in poultry may also result in trade restrictions.

In the US and for most countries with a poultry export market, the main control tool for HPAI and for

Table 2. Notable events in the H5N1 Asian lineage epizootic

Guangdong, China	1996	First report of HPAI Asian H5N1 lineage (HA gene)	Xu *et al.* (1999)
Hong Kong, SAR	1997	H5N1 outbreak in live bird markets in spring with the first human case	Subbarao *et al.* (1998), Suarez *et al.* (1998)
Hong Kong, SAR	1999	H5N1 detected from geese imported from Guangdong	Cauthen *et al.* (2000)
Hanoi, Vietnam	2001	H5N1 isolated from live bird markets	Nguyen *et al.* (2005)
Quarantine station, South Korea	2001	H5N1 isolated from frozen duck meat from China	Tumpey *et al.* (2002)
Hong Kong, SAR	2002	H5N1 on commercial farms. Vaccine used as part of control	Ellis *et al.* (2006)
Hong Kong, SAR	2002	H5N1 causes mortality in wild bird species	Sturm-Ramirez *et al.* (2004)
Indonesia, Thailand, Japan, Vietnam, South Korea, China, Cambodia, Laos	2003–2004	H5N1 spreads rapidly in many nations in Southeast Asia	CDC (2004)
Vietnam	2004	H5N1 detected in humans emphasizing zoonotic risk	Tran *et al.* (2004)
China	2005	H5N1 causes lethal outbreak in wild birds at Qinghai Lake	Chen *et al.* (2005), Liu *et al.* (2005)
Mongolia, Russia, Kazakhstan	2005	H5N1 spread through migratory birds to summer breeding grounds and into poultry	L'Vov *et al.*, (2006), Gilbert *et al.* (2006)
Europe, Middle East, Africa	2006	H5N1 spreads through migratory birds to wintering grounds	Alexander (2007)
Vietnam, Indonesia, Egypt, China	2007	H5N1 officially reported as endemic in three countries and unofficially in China	Domenech *et al.* (2009)

LPAI of the H5 and H7 subtypes is the identification and euthanasia of infected birds. This 'stamping out' approach, although costly, can result in rapid control of an outbreak when the infrastructure for diagnosis, indemnity, euthanasia, and disposal of infected birds is readily available, as occurred in the H5N2 outbreak in Texas in 2004 (Pelzel *et al.*, 2006). The stamping out approach has not been as effective in countries with poor veterinary infrastructure or where the virus is widespread in the country when it was first diagnosed.

One tool for the control of AI that is getting more attention is the vaccination of susceptible poultry. Proper vaccination for AI virus can prevent clinical disease, reduce virus shedding if vaccinated birds become infected with the virus, and can increase the amount of virus required to infect vaccinated birds (Lee and Suarez, 2005). Vaccination needs to be part of an integrated approach that includes intensive surveillance, quarantine, animal movement controls, increased biosecurity, and education. Additionally, the availability of well-matched and efficacious vaccines to provide optimal protection is also important (Lee and Suarez, 2005).

For most outbreaks of HPAI in poultry, quarantine, animal movement controls, increased biosecurity, stamping, and in rare cases the addition of vaccination have been sufficient to eradicate the virus. However, the Asian lineage H5N1 HPAI virus has not been controlled, and has become the most widespread and devastating HPAI outbreak reported. Why this lineage of virus has resulted in such a different result will be discussed further.

Asian lineage H5N1 HPAI

The Asian lineage H5N1 HPAI virus was first isolated in domestic geese in 1996 from Guangdong China (Xu *et al.*, 1999). The virus appeared to primarily circulate in southern China for almost 7 years before it began an unprecedented spread across Asia and eventually into Europe and Africa, where it has subsequently been reported from over 60 countries and has become endemic in poultry in at least four countries. This lineage of virus has a number of unusual features that has made it difficult to control. Key events in the history of this virus lineage are listed in Table 2. These events help us to understand why the H5N1 appears to be a unique event in the history of infectious disease in veterinary medicine (Sims and Brown, 2008). Some of these factors will be discussed in more detail in the remainder of this paper.

Viral variation within the Asian H5N1 HPAI lineage

The Asian H5N1 HPAI lineage has extensive viral variation in every gene, and the only constant in this viral lineage is the hemagglutinin gene. The first reported isolation of this lineage of virus was in domestic geese in Guangdong, China in 2006. This outbreak had clinical disease with some mortality in the affected goose flocks, but there was no report of involvement of chickens in this original outbreak. This virus had a hemagglutinin gene of Eurasian origin, had an insertion of multiple basic

amino acids at the cleavage site consistent with a HPAI virus, and experimentally caused mortality in chickens. The virus had a neuraminidase gene with a full length stalk, which is suggestive of a virus not well adapted to gallinaceous poultry (Xu *et al.*, 1999). Even the origin of this first reported case is unusual because most HPAI outbreaks have been associated with outbreaks in turkeys or chickens, although secondary spread to domestic waterfowl has been reported previously (Capua and Mutinelli, 2001). The origin of HPAI is generally believed to follow the model of transmission of LPAI from wild birds to gallinaceous poultry, possibly through intermediate species, and then mutation in gallinaceous poultry to the HP form of the virus. This case leaves two distinct possibilities. First, with no known association of the Goose/Guangdong HPAI virus in gallinaceous poultry, consideration must be given that LPAI viruses can mutate to HPAI in other species. Alternatively, the goose outbreak in 1996 was not the first or only H5N1 outbreak in the region, but it was the only one reported. The second possibility has at least some support because the Chinese government did not report to the World Organization of Animal Health (OIE) that an HPAI outbreak occurred in the country at this time and a published report from 1997 stated that no HPAI had ever been diagnosed in China (Sun, 1997). The incomplete or non-reporting of AI outbreaks is not unique to China, because many countries have restricted information about the disease because of the trade implications, but the early history of H5N1 will likely never be well understood because of the lack of transparency in the region.

The first isolate in 1996 appears to be the source of the hemagglutinin gene for all the subsequent outbreaks of this lineage of virus, but all the seven other influenza gene segments appear to come from different viral origins depending on the isolate. This variation of the internal genes occurs by reassortment of gene segments between different AI viruses that circulate in the region. One of the best studied cases is the Hong Kong H5N1 outbreak in 1997. This outbreak was centered at the LBMs in the city with the first reported outbreaks occurring in early 1997. The virus remained in the LBMs through the fall of the year, and it was not until 18 human cases occurred that intense international interest resulted in extreme control measures. The decision was made to depopulate all the poultry in Hong Kong in an attempt to eradicate the virus; this action was successful in eradicating this variant of the virus (Sims and Brown, 2008). When the viruses from Hong Kong were sequenced, the H5 gene was similar to the Goose/Guandong/96 virus, but all the other genes were different (Xu *et al.*, 1999). Because of the surveillance occurring in the LBMs, several LPAI viruses were isolated, including H6N1 and H9N2 viruses. The internal genes from some of these viruses appeared to be closely related to the Hong Kong H5N1 virus, and it is speculated that a reassortment event occurred between the Goose/Guangdong/96-like virus and one or both of these LPAI viruses in the LBMs of Hong Kong (Guan *et al.*, 1999; Hoffmann *et al.*, 2000). The Hong Kong 1997 H5N1 viruses appear to have a unique constellation of gene segments, and Hong Kong/97-like viruses have not been seen since the eradication program in Hong Kong at the end of 1997.

Changes were made in the Hong Kong LBM system to reduce the risk of the LBMs harboring AI, but because almost all the poultry coming into Hong Kong originated in southern China, the prevention program was not completely successful. The next reported occurrence of H5N1 was again in Hong Kong in 1999. Indeed, much of the early history of H5N1 seems to be centered in Hong Kong because of the commendable transparency of veterinary officials in Hong Kong for disease reporting in the city, and Hong Kong became a unique window into what was occurring in southern China. As part of the testing and surveillance of birds coming into Hong Kong from China, a consignment of geese were tested for antibodies to H5 influenza and were positive. However, by the time serology results were available, the geese had already been slaughtered, but environmental samples from the cages of the geese were tested and H5N1 was isolated. These viruses were sequenced and characterized biologically. Sequence similarity showed the environmental (Goose)/Hong Kong/99 viruses to have an H5 gene closely associated with the earlier Hong Kong and Guangdong viruses. However, the other viral genes were most closely associated with the Goose/Guangdong/96 virus, including the N1 gene with a full-length stalk. Biologically, the Hong Kong/99 viruses were highly pathogenic for chickens with 100% mortality, but only moderately pathogenic in geese with 50% mortality (Cauthen *et al.*, 2000; Webster *et al.*, 2002). Additional reports of H5N1 viruses being isolated in Hong Kong from 2000 to 2002, either in the LBMs or as part of the surveillance efforts of flocks from China, documented the continued circulation of H5N1 in mainland China. In addition, evidence of viruses with different combinations of internal genes from reassortment was observed (Guan *et al.*, 2002a, b). Reports of H5N1 circulating in apparently healthy ducks in China from 1999 to 2002 were eventually published in the scientific literature (Chen *et al.*, 2004).

The next unusual isolation of H5N1 was from a shipment of duck meat at a quarantine station in South Korea. Because of concerns about HP H5N1, poultry products from China were being tested for AI, and in May 2001, a virus was isolated from duck meat coming from Shanghai, China. The virus had H5 and N1 genes that were clearly in the Goose/Guangdong/96 lineage, but the N1 gene had a 20-amino-acid deletion in the stalk, suggestive of gallinaceous poultry adaptation. Some of the internal gene segments were most closely related to the Goose/Guangdong/96 lineage, but several genes were unrelated and the virus was clearly a reassortant virus. The virus was highly pathogenic in chickens. In Pekin ducks, the virus could infect the ducks, but they

showed no clinical signs. However, immunohistochemistry and virus isolation tests demonstrated that the virus replicated inside skeletal muscle, which corroborates the finding of the virus in the frozen duck meat (Tumpey *et al.*, 2002). This case demonstrated for the first time that apparently clinically healthy birds could be a source of infectious virus that could have public health implications. In addition, as was later shown in Germany, infected poultry products can be a direct source of infection for poultry (Harder *et al.*, 2009).

Evidence of H5N1 infection continued to mount in China, associated with circulation of the virus primarily in domestic ducks and geese, but some outbreaks in chickens were also observed (Guan *et al.*, 2002a, b; Sims *et al.*, 2003; Chen *et al.*, 2004). However, an outbreak of H5N1 in late 2002 in Hong Kong in a zoological collection as well as in a small number of wild birds demonstrated a different pattern. This outbreak was associated with mortality in a variety of avian species and was one of the first well-documented cases of wild birds being infected and having mortality with the H5N1 virus (Guan *et al.*, 2004). The viruses from this period also had considerable variability in the combinations of internal genes found in the different viruses, but a dominant constellation of genes referred to as the Z genotype began to emerge (Li *et al.*, 2004). The non-structural gene with an unusual genotype, a 5-amino-acid deletion in the NS1 protein, was first identified in 2000, but quickly became the most commonly seen segment associated with the H5N1 lineage. This deletion in the NS1 protein was reported to enhance the virulence of the virus in chickens (Long *et al.*, 2008). This plethora of gene combinations has made it extremely difficult to correlate the differences in phenotype to the genotype.

The stage was set in late 2003 for the unprecedented spread of H5N1 across Asia. The first country to officially report H5N1 HPAI to OIE was South Korea in December 2003, and a number of countries followed with reports in the first few months of 2004. However, retrospective analysis shows that the virus had been spreading in some Asian countries much earlier and, in some cases, was diagnosed but purposely not reported for trade reasons. One of the most egregious offenders was Thailand. Thailand was a major exporter of fresh poultry meat to the European Union, and they continued to deny that they had H5N1 in their country despite evidence to the contrary until the first human case was reported from Thailand in 2004. This denial of H5N1 in the poultry sector had serious ramifications as it undermined the country's credibility with trading partners and seriously damaged their long-term poultry exports (McLeod, 2008).

Antigenic drift and the emergence of clades

The hemagglutinin protein of AI from the beginning had some genetic and antigenic drift. However, these differences became more prominent when the virus began spreading across Asia in 2004 and it became apparent that multiple sublineages of the hemagglutinin gene were appearing. So, although all the viruses had a similar origin, at least for the hemagglutinin gene, the viruses were becoming more genetically and phenotypically different. Under the current classification, ten different viral lineages, referred to as clades, have been defined. The clades are labeled from 0 to 9, with the clade 0 group having the Goose/Guangdong/96 virus as the origin (World Health Organization Global Influenza Program Surveillance Network, 2005). The WHO/OIE/FAO H5N1 Evolution working group (http://h5n1.flugenome.org/method.php) defines a clade as having pairwise sequence distance of >1.5% in the hemagglutinin gene. A clade should contain a minimum of representative isolates that cluster together with <1.5% pairwise sequence difference within the group. A clade can also be further divided into subclades if they also meet these criteria. Ideally, antigenic properties, as measured in cross hemagglutination inhibition (HI) titers, should be considered in the identification of clades. There is good evidence that genetic clades do correlate with antigenic differences, but not all clades have been adequately antigenically characterized. This is due, in large part, to the fact that there is not widespread access to AI viruses from many parts of the world, including China that appears to be the origin of most of the defined clades (Chen *et al.*, 2006b; Wallace and Fitch, 2008; Wang *et al.*, 2008).

The spread of H5N1 in 2004 included two genetically distinct groups of viruses, clades 1 and 2. The clade 1 viruses spread to Vietnam, Cambodia, Thailand, Laos, Malaysia, and Hong Kong. The clade 2 viruses spread to South Korea and Indonesia (Wang *et al.*, 2008). After this initial introduction of virus, a variety of outcomes was possible. One outcome was that the virus could be eradicated relatively quickly, as occurred in South Korea. Other possibilities were that the virus could be eradicated after lengthy circulation of the virus (Thailand), or it could persist and become endemic (Vietnam and Indonesia). With all these scenarios, the potential for introduction of new viruses was also possible. Once a virus was introduced into a country, if the virus was allowed to persist, then the geographic separation of the viruses allowed them to independently evolve compared to viruses in other countries. For example, a single introduction of virus of the Clade 2 type appears to have occurred in Indonesia (Wang *et al.*, 2008). This virus, although from a single source or closely related group of viruses, quickly spread to a number of Indonesia islands. As control measures were increased, the virus within Indonesia became separated geographically, both domestically and internationally, and the virus started to evolve independently from the Clade 2 viruses found in China and other countries (Wallace and Fitch, 2008). The viruses that originally started the outbreak in Indonesia were

identified as Clade 2.1 viruses to acknowledge the differences in sequence of viruses found in other countries. However, the Clade 2.1 viruses continue to evolve in several independent lineages within Indonesia, and further division into subclades was necessary so that clades 2.1.1, 2.1.2, and 2.1.3 were observed (data not shown). Again there are genetic and antigenic differences between these sublineages that can affect available control measures, specifically vaccination.

Other countries like Vietnam have a different story. In Vietnam, clade 1, 2 and 7 viruses have been identified (Dung Nguyen *et al.*, 2008; Wang *et al.*, 2008; Nguyen *et al.*, 2009). The clade 1 viruses appear to have been introduced first and were found in both the northern and southern parts of the country. However, additional introduction of virus, both clades 2.3.2 and 2.3.4, from China occurred in the north in late 2005 (Pfeiffer *et al.*, 2009). Although vaccination and other control measures are being applied in Vietnam, there is rampant illegal movement of poultry from China, which continues to allow the introduction of new viruses, with clade 7 being the latest (Nguyen *et al.*, 2009). Currently, multiple clades continue to circulate in Vietnam, but a pattern has emerged of clade 1 viruses being endemic in the south in the Mekong River delta and clade 2 viruses being endemic in the north around the Red River delta, but with the introduction of some clade 7 viruses on the Chinese border (Pfeiffer *et al.*, 2009). This correlation with the major river systems likely reflects how important these rivers are for moving poultry within the country.

Currently, H5N1 is known to be endemic in Vietnam, Egypt, Indonesia, and China (Domenech *et al.*, 2009). Other countries including Bangladesh are suspected of having endemic HP H5N1. Unfortunately, accurate and transparent reporting of AI outbreaks continues to be the exception rather than the rule, and true distribution of the virus in poultry is not known. Over 60 countries have had HP H5N1 and most have been able to control and eliminate the infection. The countries that have been successful in eliminating the virus have typically had a better veterinary infrastructure that allows earlier diagnosis and more rapid controls. However, reintroduction is common for countries that border these endemic countries. Often this is because of illegal movement of poultry between countries. Countries like China, Indonesia, Egypt, and Vietnam, partly for economic reasons, used vaccination as the primary control tool, and unfortunately this approach did not eradicate the virus from the country.

Wild bird involvement in the epidemiology of H5N1

As previously described, HPAI viruses as a general rule do not persist in wild bird populations. However, the H5N1 lineage of virus no longer follows this rule. During the period 1996–2001, there was little evidence of wild bird involvement with H5N1. The first example showing that wild birds might be important in the spread of the virus was the previously mentioned outbreak in a zoological collection in late 2002 in Hong Kong (Guan *et al.*, 2004). With the emergence of H5N1 in a large number of countries in 2004, some speculation of wild bird involvement was raised as a possible explanation for the rapid spread of the virus, but with little scientific support. Expert opinion felt the spread of the virus was still primarily through movement of infected poultry or poultry products (Sims and Brown, 2008). However, in April 2005, a paradigm shift occurred when a large outbreak of H5N1 with high mortality occurred in variety of wild ducks and gulls in Qinghai Lake, China (Liu *et al.*, 2005; Chen *et al.*, 2005, 2006a). Subsequently, strong evidence of wild bird infection and movement of the virus over large geographic areas resulted in the spread of HPAI virus into Europe and Africa (Chen *et al.*, 2006b; L'Vov *et al.*, 2006). In particular, during the severe winter of 2006, a large number of wild birds were shown to be infected in Europe (Sims and Brown, 2008). The current evidence suggests that the virus is still present in wild birds, but at a lower level than was seen in 2006 (Lvov *et al.*, 2010; Sharshov *et al.*, 2010).

With the important role of wild birds in the epidemiology of H5N1 HPAI viruses, the control and eradication of the virus was greatly complicated. Most previous outbreaks of HPAI have been eradicated, in part because the virus was maintained only in poultry. Previous experience with LPAI viruses shows that you can reduce exposure to wild birds, but you cannot completely prevent exposure to wild birds or to the viruses they excrete. Additional preventive measures were instituted in Europe to try to reduce the risk of infection, including increased biosecurity and involved confinement rearing of poultry. However, confinement rearing for backyard and small holder flocks is difficult and expensive to maintain (Pittman and Laddomada, 2008). An additional difficulty is the increased susceptibility of poultry to the H5N1 HPAI lineage of viruses as measured by the low MID_{50} in chickens (Table 1) (Subbarao *et al.*, 2003; Swayne and Beck, 2005; Swayne and Slemons, 2008). Therefore, poultry flocks exposed to only small amounts of virus may be infected. The true prevalence and persistence of H5N1 HPAI in wild birds is still not known. Although the surveillance of wild birds has increased on a massive scale, there is a lack of understanding of the ecology of HPAI, including what species are infected and how long they can shed (Feare, 2010). Unfortunately, because the virus continues to change and the limited research done with one viral strain may not apply to other viral strains, a complete understanding of the virus in wild birds is not likely to be attained. With the apparent reduction of H5N1 HPAI in wild birds, at least based on reported mass mortality events in wild birds, it is unclear whether the virus will be maintained in wild birds.

Conclusions

AI viruses remain one of the most important challenges in veterinary medicine, and long-term control appears unlikely because of the wild bird reservoir of LPAI virus. Over the last 20 years, the incidence of new HPAI outbreaks appears to be increasing, suggesting that current poultry-rearing practices may increase the opportunities for outbreaks. Lastly, the emergence and spread of H5N1 HPAI in Asia, Europe, and Africa has greatly increased the stakes for both veterinary and public health communities. AI continues to be a global health issue, and control efforts need to be coordinated before the virus can be eliminated. As an example, the veterinary officials in Hong Kong have instituted the most rigorous controls and have maintained a high level of surveillance for over 13 years, but they continue to face H5N1 and have to respond to outbreaks almost every year. AI also continues to affect a disproportionate number of smallholder and backyard poultry farmers, which results in economic and food security concerns for the poorest people.

The last 13 years have resulted in a huge influx of resources into the veterinary community, both for control and research, and although progress has been made, we still remain far from our goal. An increased understanding of our adversary and innovative approaches for control and eradication is still needed. The most obvious control tools are vaccines, and the next generation of viral-vectored vaccines may have distinct advantages over our current vaccines. However, AI, like other influenza viruses, suffers from antigenic drift, and vaccines will need to be targeted for the best results. Other technologies may include the use of transgenic animals that are resistant to influenza infection. These technologies are on the near horizon, but are currently out of reach. International cooperation, virus sharing, and transparency in disease reporting are all necessary first steps to achieve the goal of eradication of H5N1 HPAI and the prevention of outbreaks with either LP or HPAI viruses.

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ORIGINAL ARTICLE

Pathobiological characterization of low-pathogenicity H5 avian influenza viruses of diverse origins in chickens, ducks and turkeys

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Abstract We undertook one of the most comprehensive studies on the replication and intraspecies transmission characteristics of low-pathogenicity avian influenza viruses in ducks, chickens and turkeys. Our results indicated that most of these isolates could replicate and be transmitted in poultry without inducing clinical disease. However, differences in transmission to contact control birds were noted, emphasizing the importance of having contact control cage mates in biological characterization experiments. Ducks supported the replication of viruses of wild aquatic bird origin in their respiratory and digestive tracts equally well. The viruses from wild aquatic birds were not effectively transmitted among chickens. In contrast, the wild-bird isolates and viruses of domestic bird origin from live-bird markets and commercial poultry operations replicated and were transmitted more efficiently in turkeys than in chickens or ducks. We also found a lower minimal infectious dose requirement for infection of turkeys compared to chickens and ducks. Our data support an important role of turkeys as being more susceptible hosts for avian influenza viruses than domestic ducks and chickens. These results highlight the role of turkeys as intermediate or bridging hosts in the transmission of influenza viruses from wild birds to land-based domestic poultry or among different land-based bird species.

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Introduction

Wild aquatic and shore birds act as the global reservoirs of influenza viruses in nature, in which all 16 hemagglutinin and 9 neuraminidase subtypes have been found [27]. Among wild birds, the most frequent isolations have been made from members of the order Anseriformes, with the highest numbers from the family Anatidae. In wild-bird reservoirs, infections are usually asymptomatic and follow the fecal–oral route of transmission [32, 35]. Most of the wild-bird influenza viruses in their natural hosts have developed highly evolved relationships that depend on a multitude of interactions among viral components, the intracellular machinery of the host, the cell surface and its receptors, and host innate and adaptive immune responses [18]. Phylogenetic evidence suggests that all influenza A viruses in birds and mammals are derived from viruses circulating in aquatic bird species [36]. For example, reports on H7N3 viruses from turkeys in northern Italy in 2002 showed that these viruses were closely related to H7N3 viruses that circulated in wild ducks in 2001 [3]. These H7N3 viruses also seemed to have infected humans who were in close contact with the infected turkeys [21]. However, the replication of most avian influenza (AI) viruses in mammalian species is inefficient, and it has been postulated that an intermediate

host such as swine may be needed to overcome the species barrier [1, 11, 22]. Nevertheless, recent human infections caused by H5N1 and H9N2 viruses indicated that AI viruses could be transmitted directly to humans from their precursors in terrestrial poultry [2, 8, 24]. These findings have led to the hypothesis that domestic poultry, like swine, can act as potential intermediate hosts for the transmission of influenza viruses from wild aquatic birds to humans. Many land-based poultry, such as chickens, turkeys and quail, have been found to support the replication and transmission of a variety of influenza viruses of different subtypes [14, 19]. The detection of α2,3 (avian type) and α2,6 (mammalian type) sialic-acid-linked receptors in the tracheal epithelium of domestic poultry [7] further indicate that they can be infected with avian and mammalian viruses and serve as adaptation hosts for changing the binding preference from avian to mammalian receptors. Reports indicate that the transmission of influenza viruses from their wild-bird reservoirs to domestic poultry might be a frequent event [4, 10, 36]. In most cases, low-pathogenicity avian influenza (LPAI) infections in domestic poultry result in mild respiratory disease, reduction in egg production and, occasionally, increased mortality [32]. In some cases, following transmission from wild-bird reservoirs, influenza viruses undergo rapid evolution [15]. Although changes in nucleotide bases or amino acids can occur in all gene segments, it is most apparent in the hemagglutinin (HA) and neuraminidase (NA) genes, where the changes can result in antigenic drift [1, 36]. In some H5 and H7 viruses, additional insertions of basic amino acids at the cleavage site of HA can contribute to the development of highly pathogenic strains [32]. In this study, we were specifically interested in the biological properties of low-pathogenic H5 AI viruses from diverse sources, which may represent different levels of poultry adaptation, in different domestic bird species. Adaptation of LPAI viruses to domestic poultry is considered to be a necessary first step in the evolution of highly pathogenic AI viruses. Studies on infection, replication and transmission properties of wild aquatic bird and domestic bird viruses in different host systems are a good initial step towards understanding viral and host ecologies. Such studies are important for identifying the susceptibility of different avian species to influenza viruses and their potential role as sentinels. The seasonal pattern of distribution of AI viruses in wild waterfowl is often detected by means of sentinel domestic birds that are highly susceptible to AI viruses [37].

In order to get a detailed understanding of the biological properties of low-pathogenic AI viruses, we studied the replication, pathogenicity and transmission characteristics of 20 low-pathogenic H5 subtype viruses of different origins in three different domestic bird species.

Materials and methods

Virus

The viruses used in this study (listed in Tables 1, 2, 3) were obtained from the repository of Southeast Poultry Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Athens, Georgia. The viruses were passaged once or twice in 10-day-old embryonated chicken eggs (ECE) to make working stocks of the virus. The eight wild bird isolates that were used in the study included four isolates from mallard ducks, one from mute swan and three from ruddy turnstones. Along with the wild-bird isolates, we included low-pathogenicity domestic bird viruses from live-bird markets (LBMs) as well as chicken and turkey isolates from commercial poultry operations. All of the isolates tested belong to the North American lineage AI viruses, and within this lineage, some isolates belong to the LBM sublineage. The parrot isolate belongs to the Mexican sublineage [12, 20, 26].

Comparative pathogenicity, replication and intraspecies transmission of viruses in chickens, ducks and turkeys

Four-week-old SPF chickens (Charles River Laboratories, Inc. Wilmington, MA, USA), two-week-old commercial Pekin ducks (Ridgeway Hatcheries, Inc. LaRue, OH, USA) and three-week-old breeder turkeys (flocks maintained at Ohio Agricultural Research and Development Center) were used in the present study. Eleven to twelve birds of each species were inoculated with 0.2 ml of 10^6 50% egg infectious dose (EID_{50}) of the virus through the intrachoanal route. Three or four birds were introduced as contact controls at 1 day postinfection (DPI). Tracheal and cloacal swabs were collected from all the birds at 2, 4 and 7 DPI (1, 3, 6 DPI for contact controls) to determine virus shedding. Individual swabs were placed in 2.0 ml of sterile phosphate-buffered saline (PBS) containing gentamycin (1 mg per 100 ml). RNA was extracted from the tracheal and cloacal swabs using an RNeasy Mini Kit or Viral RNA Extraction Kit (Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions. The virus was quantified by real-time RT-PCR (RRT-PCR) directed to the matrix (M) gene as reported previously [13]. For quantitation, swab samples were run together with known amounts of control viral RNA. We used RNA extracted from 10^1 to 10^5 EID_{50}/0.2 ml dilutions of the homologous virus as standard controls. Standard curves were generated with those control viral RNAs, and the amount of RNA in the swab samples was converted to EID_{50}/ml by interpolation. A high correlation ($r^2 > 0.99$) between cycle number and dilution factors was observed with standard controls. Viral titers are

Table 1 Replication and transmission of H5 subtype LPAI viruses of different origins in chickens

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Wild bird isolates										
Mallard/WI/42/75	0.9 ± 0.5	(2/11)[h]	1.2 ± 0.6	(6/8)	5.3 ± 1.2	0.8	(1/4)	2.1 ± 1.2	(3/4)	4.8 ± 1.3
H5N3	1.1 ± 0.9	(2/11)	1.5 ± 1.6	(2/8)		0.0	(0/0)	1.2 ± 0.9	(2/4)	
Mallard/MN/182742/98	2.3 ± 0.7	(12/12)	1.7 ± 0.9	(8/9)	5.1 ± 1.1	1.5 ± 0.1	(3/3)	1.2 ± 0.0	(2/3)	0.0
H5N2	1.3 ± 0.2	(8/9)	1.0 ± 0.1	(4/9)		1.0 ± 0.2	(1/3)	1.5 ± 0.4	(2/3)	
Mallard/MN/479/00	3.8 ± 1.3	(8/12)	2.7 ± 0.4	(8/9)	4.7 ± 1.9	2.4	(1/3)	2.6 ± 0.2	(2/3)	2.0
H5N3	2.3 ± 0.2	(3/12)	0.0	(0/0)		0.0	(0/0)	0.0	(0/0)	(1/3)
A/Mallard/MN/3 5581/00	1.7 ± 0.9	(11/12)	1.7 ± 1.3	(5/9)	4.0 ± 1.5	0.9 ± 0.1	(2/3)	0.0	(0/0)	0.0
H5N5	1.2 ± 0.2	(11/12)	1.0 ± 0.3	(2/9)	(6/7)	1.4 ± 0.1	(3/3)	0.9	(1/3)	
Muteswan/MI/451072-2/06	4.2 ± 0.4	(4/11)	3.1 ± 0.4	(5/8)	7.0 ± 1.5	3.3	(1/4)	0.0	(0/0)	5.5 ± 3.7
H5N1	0.0	(0/0)	5.0 ± 1.2	(2/8)		0.0	(0/0)	0.0	(0/0)	
RuddyTurnstone/NJ2242/00	1.4 ± 0.3	(12/12)	1.1 ± 0.4	(3/9)	4.0 ± 1.5	1.3 ± 0.1	(3/3)	0.0	(0/0)	0.0
H5N3	1.1 ± 0.2	(11/12)	0	(0/9)		2.2 ± 0.1	(3/3)	0.0	(0/0)	
RuddyTurnstone/DE/2046/01	2.6 ± 0.4	(3/12)	3.8 ± 0.2	(9/9)	3.7 ± 1.2	0.0	(0/0)	4.0 ± 0.1	(3/3)	0.0
H5N7	2.4 ± 0.4	(3/12)	2.3 ± 0.2	(2/9)		0.0	(0/0)	2.5 ± 0.2	(2/3)	
RuddyTurnstone/DE/85/03	1.7 ± 0.5	(9/12)	2.6 ± 0.2	(9/9)	4.4 ± 1.3	0.0	(0/0)	2.4 ± 0.1	(3/3)	0.0
H5N9	1.2 ± 0.3	(6/12)	1.2 ± 0.4	(3/9)		1	(1/3)	1.1	(1/3)	
Domestic bird isolates										
Live bird market										
Duck/NJ/117228-7/01	1.4 ± 0.6	(11/12)	0.8 ± 0.2	(2/9)	4.1 ± 1.7	1.0 ± 0.3	(2/3)	0.0	(0/0)	0.0
H5N2	0.9 ± 0.1	(10/12)	0.0	(0/0)		0.9 ± 0.2	(2/3)	0.0	(0/0)	
Duck/ME/151895-7A/02	1.7 ± 0.5	(11/12)	3.5 ± 1.8	(4/9)	7.0 ± 1.7	1.2 ± 0.1	(3/3)	0	(0/0)	6.5 ± 3.5
H5N2	1.7 ± 1.3	(9/12)	2.9 ± 2.6	(2/9)		1.2	(1/3)	0.7	(1/3)	(2/3)
Duck/NY/185 502/02	2.6 ± 0.5	(12/12)	2.4 ± 1.1	(5/9)	3.2 ± 1.6	1.8 ± 0.4	(2/3)	3.8 ± 0.6	(2/3)	5.5 ± 2.1
H5N2	2.7 ± 1.8	(5/12)	2.5 ± 1.4	(6/9)	(5/9)	0.0	(0/0)	0.0	(0/0)	(2/3)
Chicken/PA/13 609/93	3.5 ± 0.6	(12/12)	4.1 ± 0.5	(9/9)	7.0 ± 1.3	2.8 ± 0.1	(3/3)	3.6 ± 0.9	(3/3)	4.3 ± 2.9
H5N2	2.2 ± 0.4	(2/12)	2.7 ± 0.3	(9/9)		2.3	(1/3)	2.7 ± 0.1	(3/3)	
Pheasant/NJ/1355/98	2.6 ± 0.5	(12/12)	2.3 ± 1.0	(7/9)	6.0 ± 2.8	1.0 ± 0.1	(3/3)	0.0	(0/0)	2.0
H5N2	2.4 ± 1.4	(11/12)	2.2 ± 1.3	(7/9)		1.6 ± 0.1	(3/3)	2.6 ± 1.6	(2/3)	(1/3)
Pheasant/MD/4457/98	1.4 ± 0.7	(4/11)	1.3 ± 0.6	(2/8)	6.3 ± 0.7	0.0	(0/0)	1	(1/8)	3.0 ± 1.0
H5N2	1.0 ± 0.9	(1/11)	1.0 ± 0.9	(1/8)		0.0	(0/0)	0.0	(0/0)	
Avian/NY/31588/00	1.6 ± 0.2	(12/12)	1.7 ± 0.3	(9/9)	3.5 ± 1.3	1.5 ± 0.2	(3/3)	1.4 ± 0.3	(3/3)	2.0
H5N2	1.6 ± 0.1	(12/12)	1.6 ± 0.2	(9/9)		1.4 ± 0.1	(3/3)	1.6 ± 0.1	(3/3)	(1/3)
Emu/NY/12716/94	2.4 ± 0.2	(12/12)	2.5 ± 0.2	(9/9)	4.1 ± 1.7	2.2 ± 0.2	(3/3)	2.4 ± 0.0	(3/3)	0
H5N2	2.2 ± 0.3	(8/12)	1.7	(1/9)		2.1	(1/3)	2.7 ± 0.2	(3/3)	
Farms										
Chicken/TX/167280-4/02	4.7 ± 0.6	(11/11)	3.6 ± 0.8	(8/8)	6.6 ± 1.4	1	(1/4)	1.4	(1/4)	3.3 ± 0.7
H5N3	0.0	(0/0)	1.0 ± 0.6	(1/8)		0.0	(0/0)	0.0	(0/0)	(3/4)
Turkey/MN/10734-2/95	3.3 ± 0.4	(12/12)	3.3 ± 0.3	(9/9)	5.1 ± 0.6	2.5 ± 0.0	(3/3)	2.4 ± 0.1	(3/3)	5.0
H5N2	0.0	(0/0)	1.0 ± 0.2	(4/9)		0.0	(0/0)	1.0 ± 0.2	(2/3)	(1/3)
Turkey/CA/8651-C/04	1.0 ± 0.2	(12/12)	1.2 ± 0.6	(9/9)	3.8 ± 3.7	0.8	(3/3)	0.9 ± 0.1	(3/3)	0.0
H5N2	1.0 ± 0.3	(8/12)	0.0	(0/0)		0.0	(0/0)	0.0	(0/0)	

Table 1 continued

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Others										
Parrot/CA/406032/04	5.4 ± 0.5	(11/11)	5.3 ± 0.8	(8/8)	6.9 ± 0.8	4.3 ± 0.2	(3/4)	5.7 ± 0.4	(4/4)	6.5 ± 1.3
H5N2	0	(0/0)	0.0	(0/0)		0.0	(0/0)	0.0	(0/0)	

[a] Chickens infected with 10^6 50% egg infectious dose (EID_{50})/0.2 ml of virus through intra-choanal route

[b] Contact control chickens introduced into the cage 1 day post infection

[c] Average viral titers expressed as EID_{50}/ml of swab fluid ± standard deviation

[d] Log_2 hemagglutination inhibition (HI) titer of the sera ± standard deviation

[e] Number of days post infection

[f] Viral titers determined from the tracheal swab fluid

[g] Viral titers determined from cloacal swab fluid

[h] No. of positive samples/total number of samples tested

Table 2 Replication and transmission of H5 subtype LPAI viruses of different origins in ducks

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Wild bird isolates										
Mallard/WI/42/75	1.8 ± 1.2	(6/12)	2.4 ± 2.0	(2/9)	4.9 ± 1.1	0.0	(0/0)	1.4	(1/3)	3.7 ± 1.2
H5N3	1.5 ± 0.4	(12/12)	2.1 ± 0.8	(8/8)		1.3 ± 0.1	(3/3)	1.6 ± 0.1	(3/3)	
Mallard/MN/182742/98	1.1 ± 0.3	(3/12)	2	(1/9)	4.2 ± 1.1	0.9	(0/0)	0.0	(0/0)	2.3 ± 1.5
H5N2	1.6 ± 1.0	(6/12)	2.4 ± 0.6	(3/9)		1.8 ± 0.1	(2/3)	1.0 ± 0.3	(2/3)	
Mallard/MN/479/00	3.7 ± 1.5	(12/12)	4.1 ± 2.1	(5/9)	4.5 ± 0.8	1.9 ± 0.3	(1/3)	4.4 ± 1.4	(2/3)	3.7 ± 0.6
H5N3	2.1 ± 0.2	(6/12)	4.0 ± 1.5	(7/9)		2.2 ± 0.6	(1/3)	3.6 ± 2.7	(2/3)	
Mallard/MN/35581/00	1.8 ± 1.0	(10/12)	2.6 ± 2.0	(4/9)	4.4 ± 0.9	0.0	(0/0)	0.0	(0/0)	4.7 ± 0.6
H5N5	0.0	(0/12)	2.3 ± 0.9	(4/9)		0.0	(0/0)	4.3 ± 0.1	(2/3)	
Mutesw an/MI/451072-2/06	4.5 ± 1.6	(7/11)	2.7 ± 0.2	(3/8)	6.4 ± 0.9	0.0	(0/0)	2.6 ± 0.2	(2/4)	6.0 ± 2.2
H5N1	3.6 ± 1.3	(5/11)	5.2 ± 0.8	(8/8)		0.0	(0/0)	4.5 ± 1.4	(3/4)	
RuddyTurnstone/NJ/2242/00	2.4 ± 0.2	(2/12)	0.0	(0/0)	5.2 ± 0.9	1.5	(1/3)	0.0	(0/0)	3.0 ± 1.4
H5N3	1.0 ± 0.2	(10/12)	1.4 ± 0.6	(9/12)		0.9 ± 0.2	(3/3)	1.1 ± 0.1	(3/3)	
RuddyTurnstone/DE2046/01	2.7 ± 0.7	(6/12)	2.7 ± 0.5	(3/9)	6.0 ± 1.0	3.1 ± 0.4	(2/3)	2.5 ± 0.3	(2/3)	4.5 ± 0.7
H5N7	2.8 ± 0.4	(8/12)	0.0	(0/0)		0.0	(0/0)	0.0	(0/0)	
RuddyTurnstone/DE85/03	1.7 ± 0.6	(10/12)	1.8	(1/9)	6.9 ± 1.5	1.8 ± 0.4	(2/3)	0.0	(0/0)	4.7 ± 0.6
H5N9	1.2 ± 0.3	(10/12)	3.1 ± 0.2	(2/9)		0.0	(0/0)	0.0	(0/0)	
Domestic bird isolates										
Live bird market										
Duck/NJ/117228-7/01	0.0	(0/12)	0.0	(0/0)	3.0 ± 1.3	0.0	(0/0)	0.0	(0/0)	0.0
H5N2	1.0 ± 0.1	(9/12)	1.0 ± 0.2	(8/9)		0.9 ± 0.1	(3/3)	1.2 ± 0.1	(2/3)	
Duck/ME151895-7A/02	1.6 ± 0.5	(10/12)	1.1	(1/9)	4.6 ± 0.7	1.8 ± 0.8	(3/3)	0.0	(0/0)	4.7 ± 1.1
H5N2	1.1 ± 0.3	(5/12)	1.5 ± 0.9	(3/9)		0.0	(0/0)	1.6 ± 0.7	(2/3)	

Table 2 continued

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Duck/NY/185502/02	2.3 ± 0.5	(11/12)	0.0	(0/0)	2.9 ± 1.3	1.5 ± 0.3	(2/3)	0.0	(0/0)	0.0
H5N2	2.6 ± 0.7	(11/12)	2.9 ± 0.4	(9/9)	(7/8)	1.5 ± 0.1	(3/3)	2.7 ± 0.5	(3/3)	
Chicken/PA/13609/93	3.9 ± 1.4	(12/12)	4.2 ± 1.6	(7/9)	4.2 ± 1.0	2.2 ± 0.1	(2/3)	2.2 ± 0.1	(3/3)	1.5 ± 0.7
H5N2	2.5 ± 0.7	(12/12)	2.0 ± 0.1	(1/9)		2.2 ± 0.4	(3/3)	0.0	(0/0)	(2/3)
Pheasant/NJ/1355/98	2.4 ± 0.5	(1/12)	0.0	(0/0)	4.0 ± 1.2	0.0	(0/0)	0.0	(0/0)	0.0
H5N2	2.6 ± 0.4	(11/12)	2.5 ± 0.4	(9/9)		2.4 ± 0.1	(3/3)	3.1 ± 0.0	(3/3)	
Pheasant/MD/4457/98	2.7 ± 0.7	(4/12)	0.9	(1/9)	5.5 ± 0.9	0.0	(0/0)	0.0	(0/0)	5.0
H5N2	2.7 ± 0.7	(0/12)	3.3 ± 0.3	(8/9)		0.0	(0/0)	3.1 ± 0.2	(3/3)	(1/3)
Avian/NY/31588/00	0.0	(0/12)	0.0	(0/0)	7.0 ± 1.2	0.0	(0/0)	0.0	(0/0)	4.3 ± 1.2
H5N2	1	(1/12)	1.9 ± 0.4	(9/9)		0.0	(0/0)	1.7 ± 0.1	(3/3)	
Emu/NY/12716/94	0.0	(0/12)	2.0 ± 0.5	(1/9)	3.0 ± 1.3	0.0	(0/0)	0.0	(0/0)	0.0
H5N2	1.7 ± 0.2	(12/12)	0	0		1.7 ± 0.2	(3/3)	0.0	(0/0)	
Farms										
Chicken/TX/167280-4/02	2.2 ± 1.3	(5/11)	2.6 ± 1.2	(2/8)	6.1 ± 2.8	0.0	(0/0)	0.0	(0/0)	0.0
H5N3	0.0	(0/12)	0.0	(0/0)		0.0	(0/0)	0.0	(0/0)	
Turkey/MN/10734-2/95	1.5 ± 0.3	(8/12)	1.5 ± 0.2	(4/9)	4.1 ± 0.9	0.0	(0/0)	3.2	(1/3)	2.7 ± 0.6
H5N2	1.5 ± 0.2	(11/12)	1.9 ± 0.4	(9/9)		0.9 ± 0.1	(3/3)	2.9 ± 0.5	(3/3)	
Turkey/CA/8651-C/04	2.0 ± 0.8	(12/12)	1.7 ± 0.4	(4/9)	5.3 ± 0.7	0	(1/3)	0.8	(1/3)	1.7 ± 0.6
H5N2	1.0 ± 0.3	(9/9)	0.8 ± 0.4	(5/9)		0.9 ± 0.1	(2/3)	0	(2/3)	
Others										
Parrot/CA/406032/04	4.7 ± 1.3	(7/11)	2.9 ± 0.5	(5/8)	5.2 ± 0.8	0.0	(0/0)	3.6 ± 0.5	(4/4)	1.8 ± 0.5
H5N2	0.0	(0/12)	4.4 ± 2.0	(2/8)		0.0	(0/0)	0.0	(0/0)	

[a] Ducks infected with 10^6 50% egg infectious dose (EID_{50})/0.2 ml of virus through intra-choanal route

[b] Contact control ducks introduced into the cage 1 day post infection

[c] Average viral titers expressed as EID_{50}/ml of swab fluid ± standard deviation

[d] Log_2 hemagglutination inhibition (HI) titer of the sera ± standard deviation

[e] Number of days post infection

[f] Viral titers determined from the tracheal swab fluid

[g] Viral titers determined from cloacal swab fluid

[h] No. of positive samples/total number of samples tested

reported for 2 and 4 DPI for the infected birds and 1 and 3 DPI for the contact control birds.

At 3 DPI, three infected chickens, turkeys and ducks were euthanized, and tissues (trachea, lungs, kidney, bursa, cloaca, spleen, portions of small and large intestine, cecal tonsils) were collected for histopathology. Tissue samples were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin (H & E) and examined by light microscopy. A duplicate 4 µm section from selected samples was immunohistochemically stained for detection of influenza virus antigen in tissues as described previously [29].

All of the infected birds were bled for serum collection at 7 and 14 DPI (6 and 13 DPI for contact controls). The hemagglutination inhibition (HI) titers were determined using one percent turkey erythrocytes as described [33] and homologous antigen that was used for infection of each group of birds.

Determination of 50% infectious dose of A/mute swan/Michigan/451072-2/06, A/parrot/California/406032/04 and A/mallard/Minnesota/35581/00 viruses in chickens, ducks and turkeys

Four to five birds of each species were inoculated with virus dilutions ranging from 10^1 to 10^6 EID_{50}/0.2 ml.

Table 3 Replication and transmission of H5 subtype LPAI viruses of different origins in turkeys

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Wild bird isolates										
Mallard/WI/42/75	0.7 ± 0.4	(1/9)[h]	1.3 ± 0.7	(4/6)	7.0 ± 0.9	0.0	(0/0)	2.4 ± 1.7	(3/3)	6.7 ± 0.6
H5N2	2.4 ± 1.2	(2/9)	1.5 ± 0.6	(4/6)		0.0	(0/0)	0.0	(0/0)	
Mallard/MN/182742-133/98	2.9 ± 1.1	(6/9)	1.9 ± 1.0	(4/6)	5.2 ± 1.8	0.0	(0/0)	1.6	(1/3)	3.7 ± 2.1
H5N2	1.9 + 0.6	(7/9)	2.1 + 1.0	(4/6)		1.5 + 0.7	(2/3)	1.1	(1/3)	
Mallard/MN/479/00	2.8 ± 0.7	(8/9)	2.2 ± 0.9	(2/6)	4.7 ± 0.8	2.5 ± 1.1	(2/3)	2.0 ± 0.5	(1/3)	4.3 ± 2.1
H5N3	0.0	(0/0)	3.0 ± 0.3	(4/6)		1.6	(1/3)	1.8	(0/0)	
Mallard/MN/35581 (MNOO-105)/00	2.4 ± 0.8	(3/9)	2.2 ± 0.8	(3/6)	2.3 ± 0.8	0.0	(0/0)	0.0	(0/0)	0.0
H5N5	1.0 ± 0.1	(6/9)	1.3 ± 0.1	(3/6)		1.2 ± 0.1	(2/3)	1	(1/3)	
Mutesw an/MI/451072-2/06	2.7 ± 0.7	(9/11)	0.0	(0/0)	6.6 ± 1.3	2.4 ± 0.4	(3/4)	0.0	(0/0)	6.5 ± 1.7
H5N1	2.4 ± 0.8	(4/11)	3.1 ± 0.7	(3/8)		0.0	(0/0)	3.7	(1/4)	
RuddyTurnstone/NJ/2242/00	0.0	(0/0)	1.1	(1/6)	3.5 ± 1.1	0.0	(0/0)	0.0	(0/0)	2.7 ± 2.1
H5N3	1.2 ± 0.3	(6/9)	0.9	(1/6)		0.9	(1/3)	1.2 ± 0.3	(2/3)	
RuddyTurnstone/DE/2046/01	2.5 ± 0.3	(1/9)	2.4 ± 0.4	(1/6)	5.9 ± 1.8	0.0	(0/0)	0.0	(0/0)	4.7 ± 0.6
H5N8	2.7 ± 0.6	(2/9)	2.5 ± 0.5	(3/6)		2.5 ± 0.4	(2/3)	0.0	(0/0)	
RuddyTurnstone/DE/85/03	1.4 ± 0.7	(3/9)	1.7 ± 0.2	(4/6)	7.4 ± 2.1	2.9 ± 0.2	(2/3)	0.0	(0/0)	4.7 ± 0.6
H5N9	2.6 ± 0.6	(8/9)	2.6 ± 1.3	(6/6)		1.4 ± 0.5	(2/3)	2.7 ± 1.7	(3/3)	
Domestic bird isolates										
Live bird market										
Duck/NJ/117228-7/01	1.4 ± 0.4	(6/9)	1.5 ± 1.0	(4/6)	6.8 ± 0.8	0.0	(0/0)	0.0	(0/0)	4.0 ± 1.0
H5N2	1.6 ± 0.5	(4/9)	2.5 ± 0.5	(4/6)		0.0	(0/0)	0.0	(0/0)	
Duck/ME151895-7A/02	1.3 ± 0.4	(7/9)	2.8 ± 1.1	(3/6)	6.2 ± 1.0	1.7	(1/3)	0.0	(0/0)	4.5 ± 2.1
H5N2	1.8 ± 0.9	(9/9)	2.1 + 1.1	(5/6)		1.3 ± 0.3	(3/3)	2.0 ± 0.9	(3/3)	
Duck/NY/185502/02	1.5 + 0.4	(3/9)	3.8 ± 1.5	(4/6)	5.0 ± 1.0	0.0	(0/0)	2.2 ± 0.4	(2/3)	4.7 ± 0.6
H5N2	0.9 ± 0.2	(9/9)	1.7 ± 0.4	(3/6)		1.1	(1/3)	2.9 ± 1.9	(3/3)	
Chicken/PA/13609/93	3.2 ± 0.9	(9/9)	5.5 ± 0.6	(6/6)	6.7 ± 0.6	2.6 ± 0.5	(3/3)	4.7 ± .1	(3/3)	5.5 ± 2.1
H5N2	3.0 ± 0.3	(9/9)	4.5 ± 0.8	(6/6)		3.2 ± 0.8	(3/3)	3.8 ± 0.7	(3/3)	
Pheasant/NJ/1355/98	2.3 ± 0.6	(7/9)	2.5 ± 1.8	(3/6)	4.7 ± 1.9	1.7	(1/3)	0.0	(0/0)	5.3 ± 0.6
H5N2	2.7 ± 0.9	(8/9)	2.6 ± 0.9	(5/6)		3.0 ± 0.8	(1/3)	1.4 ± 0.6	(2/3)	
Pheasant/MD/4457/93	3.1 ± 0.6	(9/9)	3.5 ± 1.3	(5/6)	5.3 ± 0.5	2.6 ± 1.6	(2/3)	1.8 ± 0.3	(1/3)	5.0 ± 0.2
H5N2	2.8 ± 0.5	(8/9)	1.5	(1/6)		0.0	(0/0)	0.5 ± 0.1	(2/3)	
Avian/NY/31588-3/00	0.9 ± 0.4	(2/9)	2.7 ± 1.5	(6/6)	5.0 ± 0.6	2.0 ± 1.0	(1/3)	1.8 ± 0.6	(3/3)	3.7 ± 1.5
H5N2	1.7 ± 0.1	(9/9)	1.9 ± 0.8	(6/6)		2.4 ± 0.0	(2/3)	1.6 ± 0.2	(3/3)	
Emu/NY/12716/94	1.9 ± 0.6	(7/9)	2.3 ± 1.0	(3/6)	6.8 ± 0.8	1.8 ± 0.4	(2/3)	2.1 ± 1.3	(2/3)	4.0 ± 1.0
H5N2	2.7 ± 0.6	(4/9)	0.9 ± 0.2	(3/6)		3.5 ± 0.4	(3/3)	0.8	(1/3)	
Farms										
Chicken/tX/167280-4/02	3.3 ± 1.7	(8/11)	3.4 ± 2.7	(4/8)	9.1 ± 1.0	1.3 ± 2.3	(1/4)	3.1 ± 2.8	(2/4)	9.0 ± 0.8
H5N3	0.0	(0/0)	1.5 ± 1.4	(1/8)		0.0	(0/0)	2	(1/4)	
Turkey/MN/10734-2/95	2.5 ± 0.7	(9/9)	3.4 ± 1.1	(5/6)	6.8 ± 0.8	1.5 ± 0.5	(3/3)	3.1 ± 0.9	(3/3)	6.3 ± 0.6
H5N2	2.0 + 0.3	(8/9)	3.4 + 0.6	(5/6)		1.8 + 0.4	(3/3)	0.8	(1/3)	
Turkey/CA/D0208651-C/02	1.2 ± 0.5	(7/9)	1.5 ± 0.9	(5/6)	4.8 ± 0.8	0.8 ± 0.1	(2/3)	0.9 ± 0.2	(2/3)	2.7 ± 0.6
H5N2	1.8 ± 0.6	(8/9)	1.8 ± 0.8	(5/6)		2.2 ± 0.3	(3/3)	1.1	(1/3)	

Table 3 continued

Virus	Infected[a]					Contact control[b]				
	Virus detection[c]				HI titer[d]	Virus detection				HI titer
	2 DPI[e] (Tracheal[f] Cloacal[g])		4 DPI (Tracheal Cloacal)		14 DPI	2 DPI (Tracheal Cloacal)		4 DPI (Tracheal Cloacal)		14 DPI
Others										
Parrot/CA/406032/04	3.1 ± 1.2	(7/11)	4.8 ± 0.5	(7/8)	8.0 ± 1.4	3.5 ± 0.4	(3/4)	4.4 ± 0.8	(4/4)	7.3 ± 1.9
H5N2	2.4 ± 0.6	(3/11)	3.6 ± 1.0	(5/8)		2.1 ± 0.3	(3/4)	3.7	(1/4)	

[a] Turkeys infected with 10^6 50% egg infectious dose (EID_{50})/0.2 ml of virus through intra-choanal route

[b] Contact control turkeys introduced into the cage 1 day post infection

[c] Average viral titers expressed as EID_{50}/ml of swab fluid ± standard deviation

[d] Log_2 hemagglutination inhibition (HI) titer of the sera ± standard deviation

[e] Number of days post infection

[f] Viral titers determined from the tracheal swab fluid

[g] Viral titers determined from cloacal swab fluid

[h] No. of positive samples/total number of samples tested

Tracheal and cloacal swabs were collected from the infected birds at 2 and 4 DPI. The virus was detected and quantitated by RRT-PCR as described above. The birds were bled at 14 DPI for serum collection to confirm the infection by HI test.

Statistical analysis

Students' unpaired *t* test was used to compare the HI titers among groups of birds of the three species infected with mallard and ruddy turnstone isolates as well as to compare the HI titers among virus infected and contact control birds of each group.

Results

Clinical signs

Most of the isolates tested did not produce clinical signs in the infected or contact control birds of the three species. With the chicken/Pennsylvania/13609/93 (CK/PA/93) isolate, clinical signs such as gasping, respiratory distress and swollen heads were observed in infected chickens and turkeys. The virus caused mortality in one infected turkey at 4 DPI, 2 at 5 DPI and 1 at 6 DPI, and resulted in the death of two infected chickens at 4 DPI. Infection with chicken/Texas/167280-4/02 (CK/TX/02) virus resulted in respiratory distress in infected chickens and turkeys without causing death. The turkey/Minnesota/10734-2/95 (TK/MN/95) and turkey/California/D0208651-C/02 (TK/CA/02) isolates also caused transient mild respiratory clinical signs in infected turkeys at 3–5 DPI.

Replication, pathogenicity and intraspecies transmission of viruses of wild aquatic bird origin in chickens, ducks and turkeys

The wild-bird viruses used in the study showed low viral shed titers from the respiratory and digestive tracts of the infected and contact control ducks (Table 1). With the eight wild-bird isolates tested, viral shedding was observed with ≥50% of the infected and contact control ducks of each group. Except for a mute swan isolate and two mallard isolates, the average Ct values from tracheal and cloacal swab samples from the infected and contact control ducks as determined by RRT-PCR were above 30.0 (equivalent to EID_{50} titers of $<10^3$ EID_{50}/0.2 ml). All of the infected and contact control ducks seroconverted, indicating efficient intraspecies transmission of the viruses. The average HI titers from the treatment groups of ducks infected with ruddy turnstone viruses (5.6 log_2) were higher than the average HI titers from the groups infected with mallard viruses (4.3 log_2). Although we did not observe a preferential tropism of the wild-bird viruses for the digestive or respiratory tract of infected ducks, we observed a higher tendency for virus detection from the digestive tract of contact control ducks in the groups infected with mallard viruses. Of the eight wild bird isolates that we tested, the mute swan isolate transmitted most efficiently to contact control ducks based on average HI titers (6.0 log_2). Our results indicate that influenza

viruses of wild aquatic bird origin can infect and transmit among domestic ducks although the viral shedding was relatively low ($<10^3$ EID_{50}/0.2 ml) and differed according to the origin (e.g. mallard vs. ruddy turnstone) of isolates tested.

In comparison to ducks, in chickens, the intraspecies transmission of the wild-bird viruses was poor or not observed (Table 2). Positive viral shed titers ($<10^3$ EID_{50}/0.2 ml) were detected from the infected chickens, and they seroconverted. However, except for the contact control chickens of the mute swan/Michigan/451072-2/06 (MS/MI/06) and mallard/Wisconsin/42/75 (mallard/WI/75) groups, contact control chickens from other groups showed either no seroconversion or low HI titers (≈ 2.0 $\log_2$). Similar to the observation for infected ducks, no preferential tropism of these viruses was detected for the respiratory or digestive tracts of chickens.

In turkeys, on the other hand, efficient replication and intraspecies transmission of the wild-aquatic-bird viruses were observed (Table 3). The only exception was the mallard/Minnesota/35581/00 (mallard/MN/00) isolate, which did not transmit to contact control turkeys. Although viruses from wild aquatic birds did not show a distinct tropism for the digestive or respiratory tracts of turkeys, it was interesting to note that in the groups infected with ruddy turnstone virus, comparatively higher viral titers were observed, and more virus shedding was observed in the digestive tracts in comparison to the respiratory tract. As seen for ducks and chickens, the mute swan virus replicated and was transmitted more efficiently than other isolates in turkeys. Based on the viral shed titers, the number of birds showing positive viral shed titers from tracheal and cloacal swabs, and the average HI titers from the infected and contact control birds, turkeys were found to support the replication and intraspecies transmission of wild-bird viruses better than chickens and ducks.

Replication, pathogenicity and intraspecies transmission of viruses of non-wild aquatic bird origin in chickens, ducks and turkeys

As observed with the wild-bird isolates, we observed differing levels of viral replication and seroconversion with the isolates from non-wild aquatic birds in the three species (Tables 1, 2, 3). An important finding was that there were higher viral shed titers, a larger number of positive birds, and higher average HI titers in infected and contact control turkeys than in chickens and ducks, regardless of the origin of the viruses. In contact control ducks, a lower HI titer (<3.0 $\log_2$, except for duck/Maine/151895-7A/02 (DK/ME/02) and avian/NewYork/31588/00 (avian/NY/00) isolates) or no seroconversion was observed, indicating poor transmissibility of these viruses among domestic ducks. Two exceptions were noted with viruses of LBM origin, pheasant/New Jersey/1355/98 (pheasant/NJ/98) and avian/NY/00, which replicated and were transmitted efficiently in ducks. Two of the three duck viruses from LBM did not transmit to contact control ducks, but all three strains were efficiently transmitted to turkeys, and two of them to chickens, indicating their higher adaptation for terrestrial poultry. Two viruses of chicken origin, CK/PA/93 and CK/TX/02, which have been found to be highly poultry-adapted in previous studies [12], replicated to higher titers ($>10^3$ EID_{50}/0.2 ml) and induced higher HI antibody titers in infected and contact control chickens (7.0 and 6.6 $\log_2$, respectively) and turkeys (6.7 and 9.1 $\log_2$, respectively).

The pheasant isolates, pheasant/Maryland/4457/98 (Pheasant/MD/98) and pheasant/NJ/98, showed poor intraspecies transmission in ducks, similar to the other LBM isolates used in the study. Although their replication and seroconversion in infected chickens was higher than in turkeys, viral titer and serology indicated that they transmit better to contact control turkeys than to chickens. Unlike other poultry-adapted LBM isolates, these isolates showed tropism for the digestive tracts of chickens. The two turkey isolates, TK/MN/95 and TK/CA/02, showed replication and transmissibility in turkeys and ducks; however, the TK/CA/02 isolate did not transmit among chickens. The emu isolate replicated to similar titers in infected chickens, ducks and turkeys; however, while it was not transmissible between ducks and chickens, it could be transmitted among turkeys. The parrot isolate, parrot/CA/406032/04 (PS/CA/04), showed moderate ($\geq 10^3$ EID_{50}/0.2 ml) viral shed titers in infected and contact control chickens and turkeys and higher seroconversion as indicated by HI titers in infected and contact control chickens (6.9 and 6.5 $\log_2$, respectively) and turkeys (8.0 and 7.3 $\log_2$, respectively). In ducks, following infection with PS/CA/04, although the viral shed titers from infected and contact control birds were comparable to those in chickens and turkeys, the seroconversion, as indicated by average HI titers in contact control ducks, was low.

Taken together, our data indicate that, similar to the wild-bird viruses, turkeys support initial infection and transmission of domestic-bird viruses better than chickens or ducks.

Histopathology

Microscopic lesions in tissues of the infected birds of the three species were mostly confined to the respiratory tract (Supplementary Table 1). Tracheitis and bronchitis were the most common lesions observed, with viral antigen staining found in the tracheal and bronchial epithelial cells and infiltrating mononuclear cells. Lesions in the trachea and bronchus were mild to severe and consisted of congestion and edema, sloughing of epithelial cells, and an increased

presence of mucin in the tracheal lumen. Also common were deciliation, hyperplasia and metaplasia of the epithelium, epithelial degeneration, and lymphoplasmacytic infiltration. In the lungs, non-specific mild congestion, edema, and mild focal to multifocal lymphohistiocytic interstitial pneumonia were observed in some cases. In turkeys infected with CK/PA/93, these lesions were severe. No microscopic lesions were observed in the intestine of most of the birds. A mild increase in lymphocytes in the submucosa and mild focal to multifocal desquamation of the intestinal epithelium were rarely present. Among other lesions, mild atrophy of lymphoid follicles of the bursa, increased numbers of lymphoid follicles in cecal tonsils and spleen, and mild lymphocytic infiltration in kidneys were observed in some birds from all three bird species. Infrequently, minimal antigen staining was also found in the lung, intestine, spleen and bursa. In general, we did not observe marked differences in microscopic lesions and viral staining in tissues between birds infected with the wild-bird viruses and the domestic-bird viruses.

An unexpected finding was the detection of severe tracheitis in ducks infected with wild- and domestic-bird viruses. Although we did not see a strict correlation between the tracheal lesions and virus detection from the trachea of infected ducks, in infections with avian/NY/00, TK/CA/04 and CK/PA/93 viruses, the tracheal lesions matched with the viral shed titers from infected ducks. Of the different viruses of domestic bird origin tested in ducks, avian/NY/00 was the only domestic-bird virus that showed higher average HI titers in infected and contact control ducks than in chickens or turkeys. With the avian/NY/00 isolate, mild antigen staining was also observed in the intestinal tract. In general, no marked lesions were observed in the intestines of the ducks infected with wild- or domestic-bird influenza viruses. In some cases, an increased number of lymphoid follicles in cecal tonsils, and infrequently, desquamation of the intestinal epithelium and increased lymphocytes in intestinal submucosa were observed. Immunohistochemistry revealed minimal or no antigen staining in the trachea, intestines and other organs from the infected ducks in most cases.

In turkeys, two of the wild-bird viruses, mallard/MN/00 and ruddy turnstone/Delaware/2046/01 (RT/DE/01) caused moderate tracheitis. Of the domestic bird isolates, the CK/PA/93 and CK/Texas/02 viruses caused severe trachietis and lymphocytic infiltration in infected turkeys. In the infected birds from the two groups, severe bronchitis, pneumonia, congestion of lungs and staining for influenza viral antigen were demonstrated in the tracheal and bronchial epithelium and macrophages. Infections with these two viruses resulted in clinical signs, including respiratory distress, swollen head and death of infected turkeys. The PS/CA/04 virus caused moderate tracheitis and congestion of the lungs, with moderate staining for influenza viral antigen. Similar to ducks, lesions in other organs and antigen staining were minimal with other wild- and domestic-bird viruses.

Chickens seemed to be more resistant to disease caused by LPAI viruses, as they showed almost no lesions in the different organs examined. Of the wild-bird viruses tested, only RT/DE/01 isolate caused moderate tracheitis. The lesions observed in the trachea were minimal or non-apparent for the other isolates tested. Of the domestic-bird isolates, only emu/New York/12716/94 and avian/NY/00 viruses caused mild to moderate tracheitis and mild antigen staining in the tracheal epithelium and macrophages. Although CK/TX/02, CK/PA/93 and PS/CA/04 viruses showed viral shed titers in chickens that were comparable to those in turkeys and produced respiratory signs in infected chickens, no antigen staining was detectable in the tracheal sections from the infected birds. It is possible that the small amount of tissue collected and used for histopathological evaluation might not have represented the lesion areas, as infection of chickens with CK/TX/02 and CK/PA/93 viruses has been reported to result in histopathological changes in the trachea, lung, spleen, cecal tonsils and kidneys [16].

Minimum infectious dose titers of A/mute swan/Michigan/451072-2/06, A/parrot/California/406032/04 and A/mallard/Minnesota/35581/00 viruses for chickens, ducks and turkeys

The 50% minimum infectious dose titers (MID_{50}) of three selected isolates confirmed our hypothesis that turkeys support initial infections with wild-aquatic- and domestic-bird viruses better than chickens or ducks (Table 4). The

Table 4 Minimum infectious dose titers of selected low-pathogenicity avian influenza viruses for chickens, ducks and turkeys

Virus	Subtype	Species		
		Chickens	Ducks	Turkeys
A/mute swan/MI/06	H5N1	7.0[a, b]	4.2	4.0
A/parrot/CA/04	H5N2	3.3	3.7	2.1
A/mallard/MN/OO	H5N5	3.9	2.3	3.2

[a] The minimum infectious dose titer expressed as $\log_{10}$ 50% egg infectious dose (EID_{50})/ml of the swab supernatant determined by real time RT-PCR

[b] The minimum infectious dose titer of A/mute swan/MI/06 for chickens has been published previously [26]

MID_{50} value for the viruses MS/MI/06 and PS/CA/04 were lower in turkeys than in chickens and ducks. Even for the mallard/MN/00 isolate, the infectious dose was lower for turkeys than chickens. It was interesting that mute swan virus, which is an aquatic-bird isolate that showed efficient replication and transmission in ducks, also had lower minimum infectious dose for turkeys than for ducks. The three viruses tested required a comparatively higher infectious dose to infect chickens. Only the parrot isolate showed a comparatively lower infectious dose for chickens than for ducks. The lower 50% infectious dose, better replication and efficient intraspecies transmission in turkeys indicate a higher susceptibility of turkeys for low-pathogenic influenza viruses in comparison to chickens and ducks.

Discussion

Although several reports are available on the biological properties of LPAI viruses of different subtypes in domestic poultry [16, 28, 32], previous studies in general have been restricted to the comparison of a few strains in single bird species. In order to gain a detailed understanding of the biological properties of LPAI viruses in domestic birds, we compared the replication and intraspecies transmission characteristics of 20 H5 subtype LPAI viruses of wild and domestic bird origin in chickens, ducks and turkeys. To our knowledge, this is one of the most comprehensive characterization studies that included viral shed titers from infected and contact control birds of three species at two different time points, their serology, and histopathology and immunohistochemistry for antigen detection in different tissues of the infected birds. In addition, the minimum infectious dose titers of three selected viruses were determined in the three species of birds.

As observed in previous studies [5], the isolates of wild bird origin that we used in our study did not produce clinical signs in the infected or contact control birds, and the viral titers from tracheal and cloacal swabs were lower than $10^{3.0}$ EID_{50}/0.2 ml. Positive viral shed titers from the tracheal and cloacal swabs and the lack of pathological lesions and antigen detection from other organs indicated the presence of localized infections of respiratory and digestive tracts of the infected birds. The trypsin-like activity of epithelia and luminal contents of respiratory and digestive organs presumably make them primary sites for replication of LPAI viruses [29]. It is also possible that the intrachoanal route of inoculation might have favored the localized infection observed in this study.

Dabbling ducks of the genus *Anas*, which includes mallards, are the most extensively studied species as influenza viral hosts and have been found to be more frequently infected with influenza viruses than other species [9, 27]. Domestic ducks are genetically close to their wild bird counterparts, and our results indicate that they could support influenza viruses from wild aquatic and shorebirds without any prior adaptation. Although the wild-bird isolates did not preferentially replicate in the digestive tracts of infected ducks, among the contact control ducks, a higher tendency was noted for viral detection from cloacal swabs, and more birds were positive by RRT-PCR from the cloacal swabs than from the tracheal swabs. These discrepancies in results between the infected and contact control groups underscore the importance of having contact controls in biological characterization studies. In experimentally infected ducks, the high viral titers used ($10^{6.0}$ EID_{50}) and the choanal route of inoculation might have favored a localized infection of the respiratory and digestive system, as choana are known to connect the respiratory and digestive tracts. Although these results could vary with route of inoculation and viral strains, we believe that in surveillance studies for AI viruses in ducks, tracheal swab samples should also be included whenever possible. A recent surveillance study for AI viruses in mallard ducks in Sweden lends credence to this hypothesis, as 3% of infections in their study were found positive only by oropharyngeal swabs, and these infections went undetected when only cloacal swabs were used [6].

Among the wild-bird isolates that we studied in ducks, our data indicated higher average HI titers in groups of ducks infected with the ruddy turnstone isolates than with mallard isolates ($P < 0.001$). We do not know the intricate details of the differing ecologies of influenza viruses in the two different host systems, the ruddy turnstones and mallards. Phylogenetic analysis of H5 gene segments from recent wild-bird isolates grouped two of the three ruddy turnstone isolates in one group and another isolate grouped together with mallard and mute swan viruses [26]. Although these genetic similarities or dissimilarities do not correlate with antigenic relatedness, similar studies combining antigenic, genetic and biological characterization could throw light on the selection of candidate vaccine strains for poultry.

Our studies revealed that wild-bird viruses were not effectively transmitted between chickens. The HI titers in contact control chickens were low or zero in comparison to the treatment groups ($P < 0.001$); however, we could detect low and inconsistent viral replication in the respiratory and digestive tracts of the contact control birds. The low viral titers detected by RRT-PCR led us to speculate whether actual infection occurred in the contact control chickens or if the titers observed were nonspecific. The low HI titers in the contact control chickens might also indicate that infection did not result in efficient enough replication

to reach the threshold viral titer required to initiate a humoral immune response. In the infected birds, on the other hand, the high viral titers in the inoculum (10^6 EID_{50}) might have accounted for infection and seroconversion. In turkeys, the viral shed titers and HI titers were comparable among infected and contact control cage mates, indicating efficient intraspecies transmission of the wild bird viruses. The poor intraspecies transmission in chickens in comparison to turkeys indicated that chickens might not be good initial hosts in terms of viral amplification and transmission. It might take multiple passages for successful viral adaptation and transmission of wild-bird influenza viruses in chickens. Along with data on biological characterization, available data on molecular determinants of interspecies transmission of influenza viruses also indicate a higher susceptibility of turkeys to wild-aquatic-bird viruses. While data from H5 subtype viruses are lacking, no species-specific molecular changes in duck H7 HA were found to be required for infection of turkeys by influenza virus [25]. In contrast, host-species-related mutations in duck H9 HA were found to be required for infection of chickens [19]. The results from biological and genetic characterization of the viruses imply the importance of turkeys as good initial hosts for wild-bird viruses that have no previous adaptation for terrestrial domestic poultry. Our findings also reiterate the importance of having control cage mates in biological characterization experiments. The use of inoculated birds alone would have revealed similar titers of viral shedding in the three bird species, and the striking differences in the biological properties of the viruses would have been missed.

Along with the wild-bird viruses, we studied the biological properties of low-pathogenic influenza viruses showing differing degrees of adaptation to domestic poultry. Replication characteristics of a few of the isolates, including CK/PA/93, CK/TX/02, DK/ME/02 and TK/CA/02, had been studied in chickens previously [12]. As reported, these isolates showed differing degrees of adaptation to chickens in our studies. With ducks, the transmission of most of these isolates was poor, suggesting a higher adaptation for terrestrial domestic poultry. An interesting observation was the comparatively higher viral titers and seroconversion in the infected and contact control turkeys with the domestic-bird isolates, irrespective of the species from which they were isolated and their degree of adaptation to domestic poultry. Although detailed experimental studies on the replication and transmission characteristics of influenza viruses of domestic bird origin in turkeys are lacking, the available evidence indicates that turkeys are susceptible to LPAI viruses isolated from commercial poultry operations and LBMs [30, 31, 34]. Among commercial poultry operations, more low-pathogenicity influenza outbreaks have been reported in turkeys than in chickens [32]. In addition, viruses isolated from different bird species from LBMs that included ducks, chickens, and pheasants replicated and were transmitted to higher titers in turkeys, indicating again that turkeys are better hosts even for chicken- and duck-adapted LPAI viruses. LBMs provide a favorable environment for adaptation of influenza viruses to a variety of hosts, including chickens, turkeys, pheasants, quails and ducks, and have been implicated as the source of influenza outbreaks in commercial poultry operations as well as in humans [17]. LBMs in New York and New Jersey have been implicated as significant sources of influenza viruses for commercial chicken and turkey operations in the US [17, 23]. The high susceptibility of turkeys to domestic-bird isolates again underscores their suitability as hosts for influenza viruses from different origins, including domestic poultry.

The high susceptibility of turkeys to influenza viruses might be accounted for by the low minimum infectious dose titers required for infection. Our studies using two wild-bird viruses and a highly poultry-adapted strain showed a lower 50% infectious dose titer for turkeys than for chickens. The low ID_{50} dose titers imply that turkeys can be easily infected following a low-dose exposure, and the low dose is sufficient for efficient viral replication in these hosts. Although the mallard/MN/00 virus replicated to low titers in turkeys and were not transmitted efficiently to contact control turkeys, ID_{50} studies revealed the virus to have a comparatively lower viral dose requirement for infection ($3.5 \times 10^{2.0}$ EID_{50}). Similarly, the mute swan/MI/06 virus replicated and was transmitted well between chickens, although it was found to require a higher dose ($2 \times 10^{6.0}$ EID_{50}) for infection. These findings further suggest that the minimum dose of influenza virus required for infection might differ from the dose required for transmission. However, it should be noted that infectious dose titers vary among viral strains and depend on the genetic constitution of the birds used in the studies. Conditions within the incubator, such as the temperature, humidity, airflow and the bird density, are also found to have significant effects on the viral load required for infection and transmission [31]. However, an important observation from our studies was the low infectious dose requirement for turkeys in comparison to chickens or ducks under similar experimental conditions, thereby supporting our findings that turkeys are more susceptible to influenza viruses from different sources.

Histopathology revealed mild tracheitis to be one of the consistent findings in infected birds of the three species. This was expected, as LPAI viruses mainly cause localized infections of the respiratory and digestive tracts. The absence of pathological lesions and antigen staining in the intestines could be due to the very low levels of viral replication in the digestive tract. Our study did not reveal

any pathology or positive antigen staining in kidney sections following infection. Several influenza viruses of wild waterfowl origin have been found to be nephrotropic following intravenous exposure [25]. It is possible that the choanal route of infection that we employed in our study resulted in this finding, as it could have favored localized infections of the respiratory and digestive tracts. An interesting finding was the severe tracheitis in ducks infected with several wild-and domestic-bird isolates. However, antigen staining in the tracheal epithelial cells was minimal or infrequent, which could be due to the sloughing away of the infected dead cells. It is also possible that the tracheitis observed was the result of physical injury while swabbing, or it could be associated with respiratory illness unrelated to influenza infections. The ducks used in our studies were not specific-pathogen-free birds, and their eating habits resulted in accumulation of fecal matter and water below their cages, which could have resulted in bacterial infections of the upper respiratory tract and consequent tracheitis. In turkeys, the comparatively higher viral replication titers seen with most isolates were not reflected in the histopathology results except for the CK/PA/93 and CK/TX/02 isolates. In chickens, no lesions were observed with most of the isolates, and they appeared tolerant even to some of the highly poultry-adapted strains, which replicated relatively well in them. To summarize, results from histopathology and immunohistochemistry from the infected poultry tissues indicated that the higher replication titers do not necessarily match with pathology in terms of host susceptibility or adaptation.

In conclusion, our extensive data on pathobiological characterization of low-pathogenic influenza viruses of different origins in domestic birds elucidate the important role of turkeys as being more susceptible hosts for low-pathogenicity avian viruses from wild and domestic birds than are domestic ducks and chickens. The potential role of turkeys assumes a greater risk in areas where turkeys, waterfowl and other birds share common pasturelands. The higher susceptibility of turkeys to influenza viruses also indicates that they could be better sentinel hosts than chickens in influenza surveillance studies and/or better model hosts in studies on adaptation of influenza viruses of wild bird origin for domestic poultry. Our studies also show that wild-bird isolates of Anseriformes and Charadriiformes can replicate and be transmitted within domestic bird species without the need for any prior adaptation. Surveillance and characterization of wild bird isolates could provide early warning signals for the introduction and emergence of potential highly pathogenic viruses. Such characterization studies may accurately assess the threat that these viruses may pose for poultry and other animals.

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ORIGINAL ARTICLE

Pathogenicity of two Egyptian H5N1 highly pathogenic avian influenza viruses in domestic ducks

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Abstract Domestic ducks have been implicated in the dissemination and evolution of H5N1 highly pathogenic avian influenza (HPAI) viruses. In this study, two H5N1 HPAI viruses belonging to clade 2.2.1 isolated in Egypt in 2007 and 2008 were analyzed for their pathogenicity in domestic Pekin ducks. Both viruses produced clinical signs and mortality, but the 2008 virus was more virulent, inducing early onset of neurological signs and killing all ducks with a mean death time (MDT) of 4.1 days. The 2007 virus killed 3/8 ducks with a MDT of 7 days. Full-genome sequencing and phylogenetic analysis were used to examine differences in the virus genes that might explain the differences observed in pathogenicity. The genomes differed in 49 amino acids, with most of the differences found in the hemagglutinin protein. This increase in pathogenicity in ducks observed with certain H5N1 HPAI viruses has implications for the control of the disease, since vaccinated ducks infected with highly virulent strains shed viruses for longer periods of time, perpetuating the virus in the environment and increasing the possibility of transmission to susceptible birds.

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Introduction

Wild ducks are natural reservoirs of type A influenza viruses, and influenza infection in these birds is ubiquitous and mostly asymptomatic [72]. Domestic ducks that are in contact with wild waterfowl and also other poultry species can act as key intermediaries in the transmission of avian influenza among birds [41]. Avian influenza virus infections are constantly monitored worldwide, not only because of their negative effects on poultry, but also because of the potential spread to humans. The Asian lineage H5N1 highly pathogenic avian influenza (HPAI) viruses are widespread in poultry in Asia and have also spread to countries in the Middle East, Europe and Africa, causing great losses to the poultry industry. Domestic ducks have been implicated in the dissemination and evolution of H5N1 HPAI viruses, and their inclusion in disease control programs is therefore important [13, 28, 41, 71].

Before 2002, the Asian lineage H5N1 HPAI viruses did not cause severe disease or death in domestic ducks. However, since 2002, a number of H5N1 HPAI viruses have been shown to cause clinical signs and mortality in ducks. The mortality was documented by observations from the field and by experimental studies using standard challenge doses and routes of inoculation. However, the pathogenicity of different H5N1 HPAI viruses can vary,

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and the age and species of the ducks tested can influence the outcome of the infection as well (reviewed in Ref. [53]) [7, 23, 56, 74]. Most wild ducks infected with H5N1 HPAI viruses [9, 37] show no or mild clinical signs, and therefore, asymptomatic infected wild ducks could have contributed to the spread of H5N1 HPAI viruses westward from Qinghai Lake in 2005 to Europe, Africa, India, and the Middle East [11, 39]. On the other hand, the domestic duck population has a higher likelihood of perpetuating H5N1 HPAI viruses [39]. Free-range, as well as backyard domestic ducks, have been associated with the spread of H5N1 HPAI viruses in Southeast Asia [20]. Consequently, reducing the risk of H5N1 HPAI virus infection in ducks is important for controlling the continuing circulation and spread of H5N1 HPAI [38]. Vaccination has proven effective in protecting ducks against H5N1 HPAI and is being used in several countries to control the disease [65]. However, virus circulation may still occur in clinically healthy vaccinated populations, which may result in an endemic situation and in the emergence of antigenic variants [10].

Although widespread in Eurasia, H5N1 HPAI was not present in Africa until 2006, when it was detected in Nigerian poultry [33]. The H5N1 HPAI viruses spread quickly, in a matter of months, to Niger, Egypt, Cameroon and other African countries, but the virus has only been known to be endemic in Egypt. Egypt, which reported its first H5N1 HPAI outbreak in February 2006, has regular reporting of outbreaks in poultry in almost all of the 29 governorates [18], and it also has the third-highest number of confirmed human infections [78]. The current government policy is to allow commercial companies to vaccinate their flocks with registered vaccines of their choice. The government was providing vaccination of household/village birds free of charge. However, since July 2009, vaccination in backyard/household settings has been provisionally suspended until a new vaccination strategy is adopted [18]. Outbreaks in duck farms, backyard ducks, wild ducks, live-market ducks, and rooftop ducks have been reported, in many cases with high mortality [50]. The pathogenic potential of circulating Egyptian strains in ducks has not yet been investigated, but given the history of increased pathogenicity of H5N1 HPAI viruses for ducks in some countries in Asia, including Vietnam and Lao PDR [38, 56], it is of interest to study the pathogenicity of these viruses in countries like Egypt due to the parallels in geography and lifestyle that propagated the spread of H5N1 HPAI and interspecies transmissions in other countries.

In our laboratory, we routinely conduct studies in which we examine the pathogenicity of H5N1 HPAI viruses in different avian species following standard protocols [52, 56]. This has permitted the comparison of the pathogenicity of many different H5N1 HPAI viruses in domestic ducks using the Pekin duck as a model. In this study, two H5N1 HPAI viruses isolated in Egypt in 2007 and 2008 were analyzed to determine their pathogenicity in Pekin ducks. Full-genome sequencing and phylogenetic analysis complemented the pathogenesis data in order to better understand the molecular changes that occur in H5N1 HPAI viruses as they adapt and become more pathogenic to ducks.

Materials and methods

Viruses

The following H5N1 HPAI viruses were used in this study: A/chicken/Egypt/9402-CLEVB213/2007, isolated at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Egypt, and obtained from the Centers for Disease Control and Prevention, Atlanta, through Naval Medical Research Unit No. 3 (NAMRU3), Egypt; and A/chicken/Egypt/08124S-NLQP/2008, obtained from the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Animal Health Research Institute, Giza, Egypt. The viruses are abbreviated Egypt/07 and Egypt/08, respectively. Viruses were propagated in embryonated chicken eggs (ECE) as described previously [52], and virus 50% egg infectious doses (EID_{50}) were determined using the method of Reed and Muench [59]. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD). All experiments using H5N1 HPAI viruses, including work with animals, were performed in biosecurity level-3 enhanced facilities at Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service, United States Department of Agriculture (USDA), and all personnel were required to wear a powered air protection respirator with high efficiency particulate air (HEPA)-filtered air supply (3M™, St. Paul, MN).

Pathogenicity studies in 2-week-old ducks

Two-week-old Pekin-type domestic ducks (*Anas platyrhynchos*) were inoculated intranasally (IN) to determine the pathogenicity of the two Egyptian H5N1 HPAI viruses. The ducks were obtained at 1 day of age from a commercial farm and maintained at SEPRL facilities. At 2 weeks of age, ducks were housed in self-contained isolation units that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. Serum samples were collected from a representative number of ducks prior to beginning the experiment to

ensure that the birds were serologically negative for AIV, as determined with the agar gel precipitation test [5]. Feed and water were provided with ad libitum access. General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* [15]. The experimental design has been described previously [52]. Briefly, ducks were separated into a control group and two virus-inoculated groups. The control group contained 10 ducks, and these were inoculated IN with 0.1 ml of the sham inoculum. The two virus-inoculated groups, each also containing 10 ducks, were inoculated IN with inoculum containing 10^5 EID_{50} of the viruses in 0.1 ml. Two birds from each group were euthanized at 2 days post-inoculation (dpi), and the following tissues were collected in 10% neutral buffered formalin solution to determine microscopic lesions and the extent of virus replication in tissues: trachea, lung, heart, brain, adrenal gland, proventriculus, duodenum, jejunum, ceca, pancreas, liver, kidney, spleen, bursa, thymus, Harderian gland, tongue, and feathered skin with follicles and skeletal muscle from the left thigh. Portions of the brain, lung, skeletal muscle, heart and spleen were also collected in BHI medium with antibiotics for virus isolation. The remaining birds were observed for signs of illness over a 9-day period during which clinical signs were recorded. Body temperatures were taken at 3 dpi using a rectal thermometer. Oropharyngeal and cloacal swabs were collected from three ducks per group at 3 dpi. Ducks that showed severe neurological signs, stopped eating or drinking, or remained recumbent were euthanized and counted as dead the next day. Sample birds, moribund birds, and all birds remaining at the end of the 9-day period were euthanized by the intravenous (IV) administration of sodium pentobarbital (100 mg/kg body weight). Mean death times (MDTs) were calculated by determining the sum of the day of death and dividing by the total number of dead ducks.

Virus titrations

Portions of the brain, lung, muscle, heart and spleen as well as oral and cloacal swabs collected in BHI medium were stored at −70°C until use. Titers of infectious virus were determined by weighing, homogenizing tissues, and diluting in BHI to a 10% [wt/vol] concentration. Tissue homogenates or swab supernatants (100 µl) were inoculated into the allantoic sac of ECEs and incubated for 4 days at 37°C. Subsequently, allantoic fluid from eggs was harvested and 50% egg infectious dose (EID_{50}) titers were determined by testing hemagglutination activity. Titration endpoints were calculated by the method of Reed and Muench [59]. The threshold of detection for virus titers in tissues was $10^{1.9}$ EID_{50}/g of tissue. Virus titers in oral and cloacal swabs were determined as above using ten-fold dilutions and are reported as log10 EID_{50}/ml. The threshold of detection was $10^{0.97}$ EID_{50}/ml.

Statistical analysis

Data were analyzed using Prism v.5.01 software (GraphPad Software Inc.), and values are expressed as the mean ± SEM. The survival rate data was analyzed using the Kaplan–Meier log-rank test. One-way ANOVA with Tukey post-test was used to analyze body temperatures and virus titers. For statistical purposes, all oropharyngeal and cloacal swabs and tissue samples from which virus were not isolated were given a numeric value of $10^{0.97}$ EID_{50}/ml and $10^{1.9}$ EID_{50}/g, respectively. These values represent the lowest detectable level of virus in these samples based on the methods used. Statistical significance was set at $p < 0.05$.

Histopathology and immunohistochemistry (IHC)

Samples were prepared as described previously [52]. Briefly, collected tissues were fixed by submersion in 10% neutral buffered formalin and embedded in paraffin. Sections were made at 5 µm and were stained with hematoxylin and eosin (HE). A duplicate 4-µm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2,000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein (developed at Southeast Poultry Research Laboratory, USDA) was applied and allowed to incubate for 2 h at 37°C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using a biotin-streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex). Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain. All tissues were systematically screened for microscopic lesions. Lesions were scored as follows: − = no lesions; + = mild; ++ = moderate; +++ = severe lesions. The intensity of viral antigen staining in each section was scored as follows: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread staining.

Sequencing and phylogenetic analysis

Viral RNA was extracted from allantoic fluid using TRIzol LS (Invitrogen, Inc., Carlsbad, CA) and RNeasy Mini Kits (Qiagen, Valencia, CA). Virus genes were amplified using a One-Step Reverse Transcription PCR

Kit from Qiagen with gene-specific primers (available upon request). PCR products were purified after agarose gel electrophoresis using QiaQuick gel extraction kits (Qiagen). Sequences were obtained using an ABI Big Dye Terminator v.1.1 sequencing kit and run on a 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA). Gene and protein sequences were analyzed using DNASTAR analysis software (Madison, WI). The accession number for the partial nucleotide coding sequence of the HA gene of A/ck/Egypt/9402-CLEVB213/2007 is EU623467. The accession number for A/chicken/Egypt/08124S-NLQP/2008 HA is GQ184246, and for NA, it is GQ184285. The A/chicken/Egypt/9402-CLEVB213/2007 accession numbers are CY057193-CY057199 and A/chicken/Egypt/08124S-NLQP/2008 CY057200-CY057205.

Phylogenetic analysis of the nucleotide coding regions of the 9 viral gene products was done using virus sequences from fully-sequenced isolates or nearly complete sequences from African countries and sequences of other viruses representative of the H5N1 clades (Figs. 3 and 4; Figure S1-9) [1, 11, 51, 56, 76]. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [73] was used to align sequences using the Clustal W algorithm. Phylogenetic trees were constructed using the neighbor-joining method, with 1,000 bootstrap replicates, and evolutionary distances were computed using the Kimura 2-parameter method [19, 40, 60] and are given in units of base substitutions per site. The trees were rooted to the A/goose/Guangdong/1/1996 virus sequence. The two Egyptian virus isolates referred to in this study are indicated in bold.

Individual gene sequences were analyzed and compared to the sequences from viruses that induced different pathogenicity in ducks in comparable pathogenicity studies done at SEPRL [52, 56].

Results

Pathogenicity of the H5N1 Egyptian viruses in ducks

The outcome of intranasal infection of 2-week-old Pekin ducks is shown in Table 1. Infection with the A/chicken/Egypt/9402-CLEVB213/2007 (Egypt/07) resulted in 7 of 8 ducks becoming sick and 3 ducks dying at 7 days post-inoculation (dpi). The ducks also showed a significant increase in body temperatures at 3 dpi when compared with the controls. Mild neurological signs, consisting of lack of coordination and tilted head, were seen in 4 of the 8 ducks, with an average onset of 4.5 days. Three of these ducks died, and the fourth duck survived, showing only mild torticollis. The remaining four ducks presented no ($n = 1$) or mild to moderate depression ($n = 3$), recovering by the end of the 9-day period. The A/chicken/Egypt/08124S-NLQP/2008 (Egypt/08) virus was more pathogenic than Egypt/07 virus in ducks, with 100% of the ducks becoming sick and dying with a MDT of 4.1 days, compared to 7 days for Egypt/07 (Table 1). All ducks presented severe depression and anorexia as early as 1 dpi. Five ducks displayed mild to severe neurological signs beginning at 2 dpi, which included whole-body tremors, uncontrollable shaking, marked loss of balance, tilted head, seizures, loss of vision, and paralysis. Three ducks died presenting only severe depression. Body temperatures at 3 dpi were similar to those of the Egypt/07 group and were significantly different from the control group. Survival of the Egypt/07- and Egypt/08-infected groups were significantly different from the controls over the 9-day period. No clinical signs were observed in the sham-inoculated control ducks.

Replication of Egyptian H5N1 HPAI viruses in ducks

Virus replication was examined at 2 dpi in brain, heart, lung, spleen, and muscle tissue from ducks following the intranasal infection with the H5N1 HPAI viruses. As shown in Fig. 1a, infection of ducks with each of the H5N1 viruses studied resulted in detectable virus in all organs examined, indicating systemic viral replication. Egypt/08-virus-infected ducks had significantly more virus present in the lung, spleen, muscle and brain tissues at 2 dpi than Egypt/07-infected ducks, with muscle showing the overall lowest titers in Egypt/07 ducks, and the highest virus titers ($10^{9.8}$ EID_{50}/g) were found in the lungs of the Egypt/08-infected ducks. Virus titers obtained with Egypt/08 infection were 10^2 to 10^5 times higher in all tissues examined than the titers obtained with Egypt/07 infection. Infectious virus was also recovered from oropharyngeal and cloacal

Table 1 Morbidity, mortality, and mean death times (MDT) of 2-week-old domestic ducks inoculated intranasally with A/ck/Egypt/9402-CLEVB213/2007 and A/ck/Egypt/08124S-NLQP/2008

Virus	Ducks with neurological signs/total ducks	Average onset of neurological signs	Dead ducks/total ducks	Mean death time	Body temperatures at 3 dpi (°F)[a]
Controls	0/0	–	0/8	–	106.7 ± 0.42
A/ck/Egypt/9402-CLEVB213/2007	4/8	4.5 days	3/8	7	109.6 ± 0.32
A/ck/Egypt/08124S-NLQP/2008	5/8	2.6 days	8/8	4.1 ± 1.3	109.0 ± 0.51

[a] Mean ± SEM

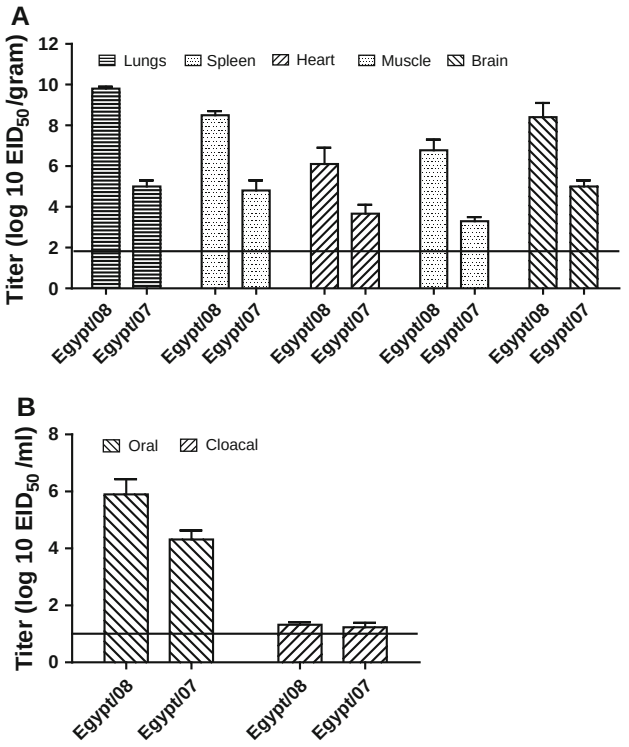

Fig. 1 Virus titers in tissues and swabs from domestic ducks infected with A/ck/Egypt/9402-CLEVB213/2007 and A/ck/Egypt/08124S-NLQP/2008. **a** Tissues collected at 2 dpi were homogenized to a 10% (wt/vol.) final concentration. Ten-fold dilutions of the 10% homogenates (100 µl) were inoculated into 10-day-old embryonated chicken eggs (ECE), and virus titers were calculated as log 10 EID_{50}/g of tissue. Values are the means ± SEM ($n = 2$). The threshold of detection was $10^{1.9}$ EID_{50}/g. **b** Oral and cloacal swabs were collected at 3 dpi, tenfold dilutions (100 µl) were inoculated into 10-day-old ECEs, and virus titers were calculated as log 10 EID_{50}/ml. Values are the means ± SEM ($n = 3$). The limit of detection was $10^{0.97}$ EID^{50}/ml

swabs collected at 3 dpi from infected ducks (Fig. 1b). The oropharyngeal virus titers were significantly higher in ducks inoculated with the Egypt/08 virus than Egypt/07, whereas cloacal virus titers were similar for ducks inoculated with either virus.

Gross and microscopic lesions and viral antigen distribution in tissues

Two ducks per group were necropsied and examined at 2 dpi. No gross lesions were observed in the sham-inoculated ducks or in the ducks infected with the Egypt/07 virus. Ducks infected with the Egypt/08 virus presented with dehydration, empty intestines, splenomegaly, thymic atrophy, dilated and flaccid hearts with increased pericardial fluid, and congested, malacic brains.

The presence of microscopic lesions and virus antigen in tissues was studied in order to determine potential tissue tropism differences among the two viruses. A summary of viral antigen staining and histological lesions in tissues is shown in Table 2. No microscopic lesions were present in tissues from the sham-inoculated ducks. In the ducks inoculated with Egypt/07, mild lesions were found in the trachea, lung, brain, spleen, Harderian gland, adrenal gland, pancreas, liver, thymus, and proventriculus. Microscopic lesions were more severe and widespread in tissues from ducks inoculated with Egypt/08. In the brain, randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema were observed. Severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium occurred in the sampled ducks. Multifocal to confluent areas of vacuolar degeneration to necrosis of the adrenal corticotrophin and chromaffin cells and epithelia of the Harderian glands, degeneration to necrosis of individual myofibers in skeletal muscle, and mild to moderate necrotizing tracheitis were also observed. Moderate to severe proventriculitis with diffuse lymphoid infiltration and moderate to severe lymphoid depletion in the bursa and thymus were present. The intestinal epithelium was mildly affected, with mild inflammatory changes in the lamina propria.

Viral antigen staining was present in multiple tissues of ducks infected with either virus, indicating systemic infection. Viral antigen was observed in the pancreatic acinar epithelium, neurons and glial cells of the brain, trachea epithelium, alveolar epithelium, fragmented cardiac and skeletal myofibers, adrenal corticotrophic cells, Harderian gland epithelia, and tongue epithelia (Fig. 2). In lymphoid organs, viral antigen was identified in resident and infiltrating phagocytes but not in apoptotic lymphocytes. Viral antigen was also identified in the glandular epithelium of the proventriculus, in hepatocytes and Kupffer cells in the liver, smooth muscle of the ventriculus, autonomic ganglia of the enteric tract, and feather epidermal cells. Consistent with the outcomes of infection, Egypt/08 infection resulted in more widespread viral antigen staining in tissues compared with the Egypt/07 group. Virus titers in the lung, spleen, heart, muscle, and brain tissues (Fig. 1a) correlated with the antigen staining and the similar pattern of increased virus load in Egypt/08-infected ducks versus Egypt/07-infected ducks.

Sequence and phylogenetic analysis

Sequence similarity of the viral genes

Nucleotide similarities between the viral genes of the two Egyptian viruses studied ranged from 97.8% (NP) to 99.5% (M1 and NS2) (Table 3). Amino acid similarities ranged from 97% (HA) to 100% (M1). The number of amino acid changes was highest in the hemagglutinin (HA) proteins, with 16 amino acid changes. A complete listing of the

Table 2 Distribution of microscopic lesions and viral antigen staining in tissues resulting from intranasal inoculation of 2-week-old domestic ducks with A/ck/Egypt/9402-CLEVB213/2007 and A/ck/Egypt/08124S-NLQP/2008

Tissue[a]	A/Ck/Egypt/9402-CLEVB213/2007		A/Ck/Egypt/08124S-NLQP/2008	
	Lesions[b]	IHC[c]	Lesions	IHC
Trachea	++	++	+	++
Lung	+	+	+++	+++
Heart	−	+	++	+++
Brain	+	++	++	+++
Adrenal gland	−	−	++	+++
Enteric tract	−	−	+	+
Pancreas	++	++	+++	+++
Liver	+	−	+++	++
Kidney	−	−	+	+
Spleen	+	+	++	++
Bursa	+	−	+++	+
Thymus	+	+	+++	+++
Skeletal muscle	−	+	+	+++
Proventriculus	+	−	+	+
Feather follicles	−	+	++	+++
Tongue	−	−	+	++
Harderian gland	+	++	+	++

[a] Results are the average score from tissues collected from 2 ducks at 2 days postinfection

[b] HE microscopic lesions: − no lesions; + mild; ++ moderate; +++ severe

[c] IHC immunohistochemical staining: − no antigen staining; + infrequent; ++ common; +++ widespread

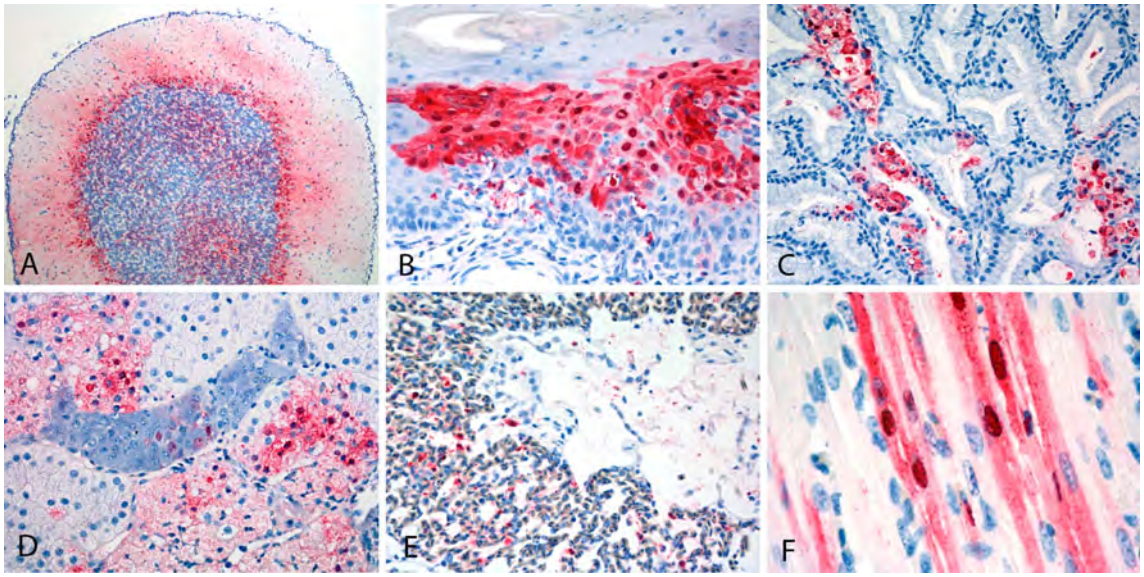

Fig. 2 Immunohistochemical staining at 2 dpi for avian influenza virus antigen in tissues of ducks infected with A/ck/Egypt/08124S-NLQP/2008. Viral antigen (in *red*) is seen in neurons and glial cells in the cerebellum (**a**, ×100), in tongue epithelial cells (**b**, ×400), in the Harderian gland epithelia (**c**, ×400), in adrenal cortical cells and medullary chromaffin cells (**d**, ×400), in phagocytic leukocytes within the pulmonary parenchyma (**e**, ×200), and in cardiac myocytes in the heart (**f**, ×400)

amino acid changes is found in Supplementary Table 1. A detailed analysis of the differences observed is found in the following sections.

Hemagglutinin genes

According to the standards established by WHO [76, 77], both Egypt/07 and Egypt/08 belong to clade 2.2 (Fig. 3) of the Qinghai lineage evolving from A/goose/Guangdong/1/96. The Egyptian isolates were further categorized into the recently named 2.2.1 clade [77] (formerly EMA clade 1 [1, 61] along with other H5N1 Egyptian viruses and African isolates as well as some European isolates and viruses from the Middle East (Fig. 3). This indicates that they were likely not separate introductions into Egypt, as was reported previously for recent Nigerian isolates

Table 3 Amino acid and nucleotide similarity between the viral proteins of A/ck/Egypt/9402-CLEVB213/2007 and A/ck/Egypt/08124S-NLQP/2008

Viral segment	No. of amino acid differences	Amino acid similarity (%)	No. of nucleotide differences	Nucleotide similarity (%)
PB2	3	99.6	31	98.6
PB1	5	99.3	31	98.6
PA	6	99.2	35	98.4
HA	16	97.0	32	98.1
NP	6	98.8	33	97.8
NA	4	99.1	22	98.4
M1	0	100	4	99.5
NS1	3	98.7	7	99.0
NS2	2	98.3	2	99.5
M2	1	99.0	2	99.3
PB1-F2	3	96.7	4	98.5

[11, 16, 17]. The Egypt/07 HA belongs to the recently identified variant cluster of Egyptian viruses containing isolates from 2007/2008 and contains 11 amino acid changes characteristic of this large group of viruses that contribute to its longer branch length [3]. The Egypt/08 virus HA clustered with other emergent viruses as part of a smaller cluster of viruses containing amino acid changes at positions 110 and 529, branching from the 2006 viruses [3]. Although there were 16 amino acid differences in the HA proteins of the Egypt/07 and Egypt/08 viruses (Table 3), both viruses contain multiple basic amino acids found to be associated with highly pathogenic avian influenza viruses [8, 36], and the GERRRKKR cleavage site found in the majority of the Egyptian viruses of 2006 was present in both [2].

The Egypt/08 virus had a unique 373R amino acid, while all the remaining viruses, including Egypt/07, had 373 K at this position. Amino acids 222Q and 224G (H5 numbering) were present, which is suggestive of a preference for avian α2,3 receptor binding in both viruses [25]. The Egypt/08 virus HA contained a 97D amino acid, which has been identified in other highly pathogenic AIVs [30]. Previously, P251S was determined to be a signature mutation in HA for the geographic region of Egypt [11]. However, the Egypt/08 virus maintained 251P, while Egypt/07 displayed the signature P251S change, as did the remaining Egyptian and Israeli viruses. The Egypt/08 virus HA also differed from most Egyptian and Nigerian viruses at position 553 (537 H5 numbering), where 553F was found to be predominant, while Egypt 08 had a 553S at that position [54]. Previously, it was reported that the majority of clade 2.2 HA proteins contain a 193R amino acid (H3 numbering) and have lost the glycosylation site at amino acid 158. Having 193R increased the affinity of HA for α2-6 sialosides when combined with the receptor-binding differences at amino acids 226 and 228 [68]. Both of the Egyptian isolates lost the 158 glycosylation site and have 193R, consistent with other clade 2.2 viruses. The S123P amino acid change found in Egypt/07 has also been shown to increase α-2,6 receptor binding when combined with other amino acid differences not present in the Egypt/07 virus [79]. Studies using escape mutants showed that amino acid positions 140 and 141 are involved in the antigenic recognition site [35] and have been identified as undergoing positive selection [66]. Egypt/07 had an amino acid change previously identified in an escape mutant (140G); however, both position 140 and 141 were different between these 2 viruses.

Of the 16 amino acid differences between the hemagglutinin proteins of the two Egyptian isolates, 2 amino acid differences could potentially change the number of N-linked glycosylation sites (N-X-S/T where X ≠ P). Egypt/08 lacked a potential glycosylation site (S74P; H5 numbering) and added a potential site (H165 N) [6, 47]. The two viruses have 5 other identical potential glycosylation sites (amino acids 11, 23, 286, 484, and 543). Changes in amino acid 184 (H5 numbering) correspond to the receptor-binding structural element the 190-helix, according to the numbering in Refs. [44, 67].

Neuraminidase genes

Phylogenetic analysis of the nucleotide sequences of the coding regions of the Egypt/07 and Egypt/08 viruses showed that the viral NA genes grouped with the other clade 2.2. viruses. The relationships seen in the NA phylogenetic tree (Fig. 4) were similar to those in the HA tree (Fig. 3). The Egypt/07 NA gene also contains the amino acid change at position 188 that corresponds to the residue present in the majority of the variant viruses classified by HA sequence analysis [3]. Analysis of the protein sequence showed that Egypt/07 and Egypt/08 both contained the 49-68 stalk deletion associated with adaptation to poultry [24, 47], as well as an isoleucine at position 223, which is associated with increased pathogenicity for mice [34].

There are 4 amino acid differences between Egypt/07 and Egypt/08, located at amino acids 46, 188, 241 and 339. Both viruses had a histidine at amino acid position 274, which is the susceptible genotype for oseltamivir resistance [31].

Internal genes

The NP, PB2, PB1, PA, MP and NS gene segments of the Egyptian isolates all cluster with other African viruses, with no evidence of reassortment (Figs. S1–S9). In the M2 protein, the only amino acid difference between the two Egyptian isolates was located at amino acid 31, which is associated with amantadine resistance, with Egypt/07 being resistant (31 N) and Egypt/08 (31S) having a sensitive phenotype [58, 62]. Other M2 drug resistance markers at positions 27 and 30 were not present.

The NS1 protein of the Egypt/07 and Egypt/08 viruses contains the five-amino-acid deletion (80–84) often associated with highly pathogenic avian influenza in poultry since 2001 [46]. The amino acid change of aspartic acid to glutamic acid at position 92 of NS1 that has been shown to be involved in cytokine resistance in pigs [63] was not present in either of the two Egyptian isolates studied here. However, the Egyptian viruses contained an alanine at amino acid 149 of NS1, which has been shown to be important for virulence in chickens [43]. The S42 NS1 mutation, which has also been associated with increased virulence in mice and has been shown to antagonize the interferon induction pathways [32], was found in both isolates.

The NP proteins have 6 amino acid differences, at positions 34, 109, 287, 350, 371, and 425. The Egypt/08 viral nucleoprotein contains a unique valine at amino acid position 109, which is a host marker typically associated more with human influenza viruses than with avian viruses [12]. Both Egyptian viruses, as well as the other Egyptian, Middle Eastern and African viruses, contained an isoleucine at amino acid position 33, which is also a persistent host marker associated with viruses that infect humans [64]. All of the amino acid differences in NP correspond to locations on the protein known to be involved in PB2 interactions. In addition, amino acids 34 and 109 are also involved in RNA binding, and 350, 371 and 425 are involved in the NP-NP interactions [57].

The Egypt/08 PA protein contains 5 out of 6 total amino acid changes that are unique to that virus when compared with the other 34 strains analyzed in this study. The T515A mutation in PA and the Y436H mutation in PB1, which both Egyptian viruses contain, have been shown to decrease pathogenicity of H5N1 viruses in mallard ducks [29]. However, since both viruses contain these mutations, it is unlikely to be a determining factor in the pathogenesis differences observed in this study. PA amino acids 669 and 716 have been shown to be involved in the interaction with PB1, while amino acid 642 of the PB1 protein has been shown to be involved in the interaction with PB2 and in RNA binding [22, 75].

Fig. 3 Phylogenetic analysis of the full nucleotide coding region of the hemagglutinin genes from 98 viruses. The tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 using the neighbor-joining method with 1,000 bootstrap replicates. Evolutionary distances were computed using the Kimura 2-parameter method and are given in units of base substitutions per site. The trees were rooted to the A/goose/Guangdong/1/1996 sequence, and the two Egyptian virus isolates referred to in this study are indicated in *bold* ▶

The PB2 protein sequence was well conserved, and there were only 3 amino acid differences between the Egyptian isolates. One of the differences was 473V in Egypt/08, which was unique compared with the other 34 viruses analyzed and shown to be in the region of one of the nuclear localization signals [48]. Another difference in Egypt/08 was an amino acid change at position 559, which involves the cap-binding region [27, 42]. Both Egyptian isolates contain 627K in PB2, which is an indicator of potential pathogenicity in mammals, including humans [26, 69]. The PB1-F2 protein is thought to be important for pathogenicity in mammals as well [14]. There is a distinct clustering of Egyptian and Israeli viruses and a single A/ck/Nigeria/228-5/2006 isolate in the PB1-F2 phylogenetic tree, and those viruses contain a 73E at that position. Previous reports show most avian viruses contain 73K at this position while human viruses tend to have 73R at this position [12].

Comparison with previous full-genome sequencing of viruses of different pathogenicity in ducks

Comparison of the current and previous duck pathogenesis experiments with full-genome sequence data of the viruses used identified a number of amino acids in the HA, NP and PB1 proteins that were consistently similar in the viruses that were more lethal for ducks (Table 4). However, no specific set of changes identified viruses of low, moderate or high pathogenicity in ducks.

Discussion

In the present study, two H5N1 HPAI viruses isolated in Egypt in 2007 and 2008, both belonging to HA clade 2.2.1, were shown to be moderately and highly pathogenic, respectively, in Pekin ducks. Both viruses produced clinical signs and mortality in ducks; however, the Egypt/08 virus was more virulent, inducing earlier onset of neurological signs and killing all ducks with a mean death time of 4.1 days. The Egypt/07 virus killed 3 of 8 ducks with a mean death time of 7 days, acting more similarly to

HA
A/chicken/Egypt/0815-NLQP/2008
A/chicken/Egypt/0855-NLQP/2008
A/chicken/Egypt/0869h3-NLQP/2008
A/chicken/Egypt/08194S-NLQP/2008
A/chicken/Egypt/07632S-NLQP/2007
A/chicken/Egypt/07202-NLQP/2007
A/chicken/Egypt/088S-NLQP/2008
A/chicken/Egypt/9402-CLEVB213/2007
A/chicken/Egypt/07201-NLQP/2007
A/chicken/Egypt/17NLQP-CLEVB241/2008
A/chicken/Egypt/10NLQP-CLEVB299/2008
A/chicken/Egypt/0859-NLQP/2008
A/chicken/Egypt/0837-NLQP/2008
A/chicken/Egypt/0879-NLQP/2008
A/Egypt/3300-NAMRU3/2008
A/chicken/Egypt/0876-NLQP/2008
A/chicken/Egypt/0880-NLQP/2008
A/chicken/Egypt/0831-NLQP/2008
A/chicken/Egypt/08139S-NLQP/2008
A/turkey/Egypt/07203-NLQP/2007
A/chicken/Egypt/1078-NAMRU3/2006
A/chicken/Egypt/0811-NLQP/2008
A/chicken/Egypt/0850-NLQP/2008
A/turkey/Egypt/07444S-NLQP/2007
A/duck/Egypt/0875-NLQP/2008
A/chicken/Egypt/0875S-NLQP/2008
A/duck/Egypt/0845S-NLQP/2008
A/chicken/Egypt/07665S-NLQP/2007
A/chicken/Egypt/0823-NLQP/2008
A/chicken/Egypt/15NLQP-CLEVB244/2008
A/chicken/Egypt/0836S-NLQP/2008
A/chicken/Egypt/08371S-NLQP/2008
A/chicken/Egypt/0870-NLQP/2008
A/chicken/Egypt/07181-NLQP/2007
A/chicken/Egypt/06959-NLQP/2006
A/chicken/Egypt/3045NAMRU3-CLEVB60/2007
A/peacock/Egypt/07667S-NLQP/2007
A/duck/Egypt/08425S-NLQP/2008
A/chicken/Egypt/08124S-NLQP/2008
A/duck/Egypt/07322S-NLQP/2007
A/goose/Egypt/07364S-NLQP/2007
A/turkey/Egypt/2253-2/2006
A/chicken/Egypt/06207-NLQP/2006
A/duck/Egypt/07264S-NLQP/2007
A/chicken/Egypt/07118-NLQP/2007
A/chicken/Egypt/1080-NAMRU3/2006
A/chicken/Egypt/07175-NLQP/2007
A/duck/Egypt/5169-1/2007
A/chicken/Egypt/960N3-004/2006
A/chicken/Egypt/2253-1/2006
A/duck/Egypt/2253-3/2006
A/duck/Gaza/760/2006
A/chicken/Egypt/06459-3-NLQP/2006
Egypt/06495-NLQP/2006
A/turkey/Israel/364/2006
A/chicken/Egypt/06553-NLQP/2006
A/turkey/Israel/345/2006
A/Egypt/2289-NAMRU3/2008
A/chicken/Egypt/0847-NLQP/2008
A/chicken/Egypt/0894-NLQP/2008
A/chicken/Egypt/0896-NLQP/2008
A/chicken/Egypt/07480S-NLQP/2007
A/chicken/Egypt/0838-NLQP/2008
A/chicken/Egypt/07125-NLQP/2007
A/duck/Egypt/0891-NLQP/2008
A/chicken/Egypt/22NLQP-CLEVB232/2008
A/chicken/Egypt/0813-NLQP/2008
A/chicken/Egypt/2628-1/2007
A/chicken/Egypt/0841-NLQP/2008
A/duck/Egypt/0877-NLQP/2008
A/chicken/Nigeria/228-5/2006
A/mallard/Italy/835/2006
A/goose/Hungary/14756/2006
A/chicken/Nigeria/1047-62/2006
A/chicken/Cote dIvorie/1787-35/2006
A/turkey/Cote dIvorie/4372-3/2006
A/guinea fowl/Burkina Faso/1347-20/2006
A/bar-headed Goose/Mongolia/1/2005
A/turkey/Saudi Arabia/6732-6/2007
A/falcon/Saudi Arabia/D1936/2007
A/chicken/Nigeria/1047-34/2006
A/chicken/Nigeria/1047-54/2006
A/duck/Niger/914/2006
A/Cygnus cygnus/Iran/754/2006
A/chicken/Rostov-on-Don/35/2007
A/chicken/Rawalakot/NARC2441A/2006
A/chicken/Kuwait/KISR2/2007
A/grebe/Tyva/Tyv06-2/06
A/whooper swan/Mongolia/2/2006
A/Chicken/Guangdong/174/2004
Clade 2.5
A/human/China/GD02/2006
Clade 2.3.4
A/whooper swan/Hokkaido/1/2008
Clade 2.3.2
A/duck/Viet Nam/201/2005
A/chicken/Indonesia/7/2003
Clade 2.1.1
A/Egret/Hong Kong/757.2/2003
Clade 1
A/tree sparrow/Henan/1/2004
Clade 7
A/Goose/Guangdong/xb/2001
A/Goose/Guangdong/1/1996
Clade 0
Clade 2.2
0.001

previously studied clade 2.2 viruses [38, 45]. The high virulence of Egypt/08 virus in ducks is similar to what has been shown with some H5N1 viruses from Vietnam, Lao PDR and China [7, 38, 56, 74], where continued exposure and circulation of viruses in ducks could have driven changes in H5N1 HPAI viruses that have made them more pathogenic.

The Egypt/08 virus belongs to the HA group of Egyptian viruses containing a common 2-amino-acid difference identified by Arafa et al. [3]. It is readily identified in a wide range of species, such as ducks, geese and peacocks (Fig. 3), and likely has origins in mixed backyard flocks and live-bird markets rather than vaccinated commercial poultry. Passage of the virus in multiple species, including ducks, may explain why the Egypt/08 virus has an increased pathogenicity in ducks compared to other clade 2.2 viruses. Egyptian viruses seem to be evolving separately and are not segregated based on geographic location within the country. No single sublineage has yet become predominant in the country. The variant sublineage to which the Egypt/07 virus belongs, is mainly identified in chickens in vaccinated flocks, meaning it probably has not passed to other bird species, which would explain the moderate pathogenicity seen with this virus in ducks.

The risk of the presence of HPAI H5N1 virus in countries like Thailand and Vietnam has been associated with the number of free-ranging ducks and the local abundance of both ducks and geese [20, 55] in addition to other risk factors such as number of chickens, human population, and topographical features [21]. This observation can be extended to other countries like Egypt, where duck production may also be an important driver of HPAI H5N1 virus persistence [21]. In Egypt, outbreaks of H5N1 HPAI, in some cases with high mortality, have been reported in ducks from farms, backyards, live-bird markets and rooftops, as well as wild ducks and even vaccinated ducks [50]. The virus has ample opportunity to circulate and, consequently, to change in domestic ducks, and this is probably what is contributing to the observed increase in pathogenicity. This increase in pathogenicity in ducks observed with certain H5N1 HPAI viruses has implications for the control of the disease, since vaccinated ducks infected with highly virulent strains shed viruses through the oropharyngeal and cloacal route for longer periods of time, perpetuating the virus in the environment and increasing the possibility of transmission to susceptible birds [56].

The cause of the increased pathogenicity of H5N1 HPAI viruses in ducks is still unknown. There are many molecular determinants of influenza virus pathogenicity, and they vary between mammals and avian species. Amino acid changes that are significant in one species do not necessarily correspond to pathogenicity of the virus in the other

Fig. 4 Phylogenetic analysis of the nucleotide coding region of the neuraminidase genes from 73 viruses. The tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 using the neighbor-joining method with 1,000 bootstrap replicates. Evolutionary distances were computed using the Kimura 2-parameter method and are given in units of base substitutions per site. The trees were rooted to the A/goose/Guangdong/1/96 sequence, and the two Egyptian virus isolates referred to in this study are indicated in *bold* ▶

[29]. In this study, the pathogenicity experiment was complemented with full-genome sequencing in order to identify molecular changes that might explain the increased virulence of some H5N1 HPAI viruses in ducks. Because of the many differences observed between the two viruses studied, it was not possible to narrow down specifically the amino acid changes responsible for the differences observed in pathogenicity. However, the full-genome sequence can be used as a reference for future studies aimed at understanding the pathogenicity of avian influenza viruses in avian species.

Glycosylation of the surface glycoproteins is thought to play an important, but ill-defined, role in avian influenza virus pathogenicity. The addition of glycosylation sites to HA has been shown to correlate with the shortened stalk of NA, and it is suggested that the additional glycosylation sites on HA allow the virus to compensate for the decreased NA-releasing activity resulting from the shortened NA stalk [4, 47, 49]. The Egyptian viruses contain the 20-amino-acid deletion in NA, located at amino acids (49–68), so the additional glycosylation site of Egypt/08 HA at the conserved 169 site (H3 numbering) may give the Egypt/08 virus an added replication advantage, given the shortened stalk region of NA. The loss of another glycosylation site by Egypt/08 at amino acid 72 may not be as significant as the more conserved 169 site. The potential HA glycosylation site at 165 and the lack of a glycosylation site at 72 are consistent with what has been observed in several previously identified Egyptian viruses from 2006 [2]. The Egypt/07 virus has a serine residue at amino acid 74 (H5 numbering), resulting in a glycosylation site that is not present in Egypt/08. Adjacent to this glycosylation site is amino acid 75, and changes in this amino acid have been found to be important in alpha-2,6 receptor binding [79]. It is possible that the additional glycosylation site of the Egypt/07 virus alters the binding capability of the virus, thus altering pathogenicity. More work on the importance of HA glycosylation in avian species is needed. Glycosylation of NA has also been studied [30]; however, both Egyptian viruses contained the same glycosylation sites in NA.

While the majority of the molecular markers in the two Egyptian viruses were avian, both viruses display some persistent host markers and drug-resistance markers

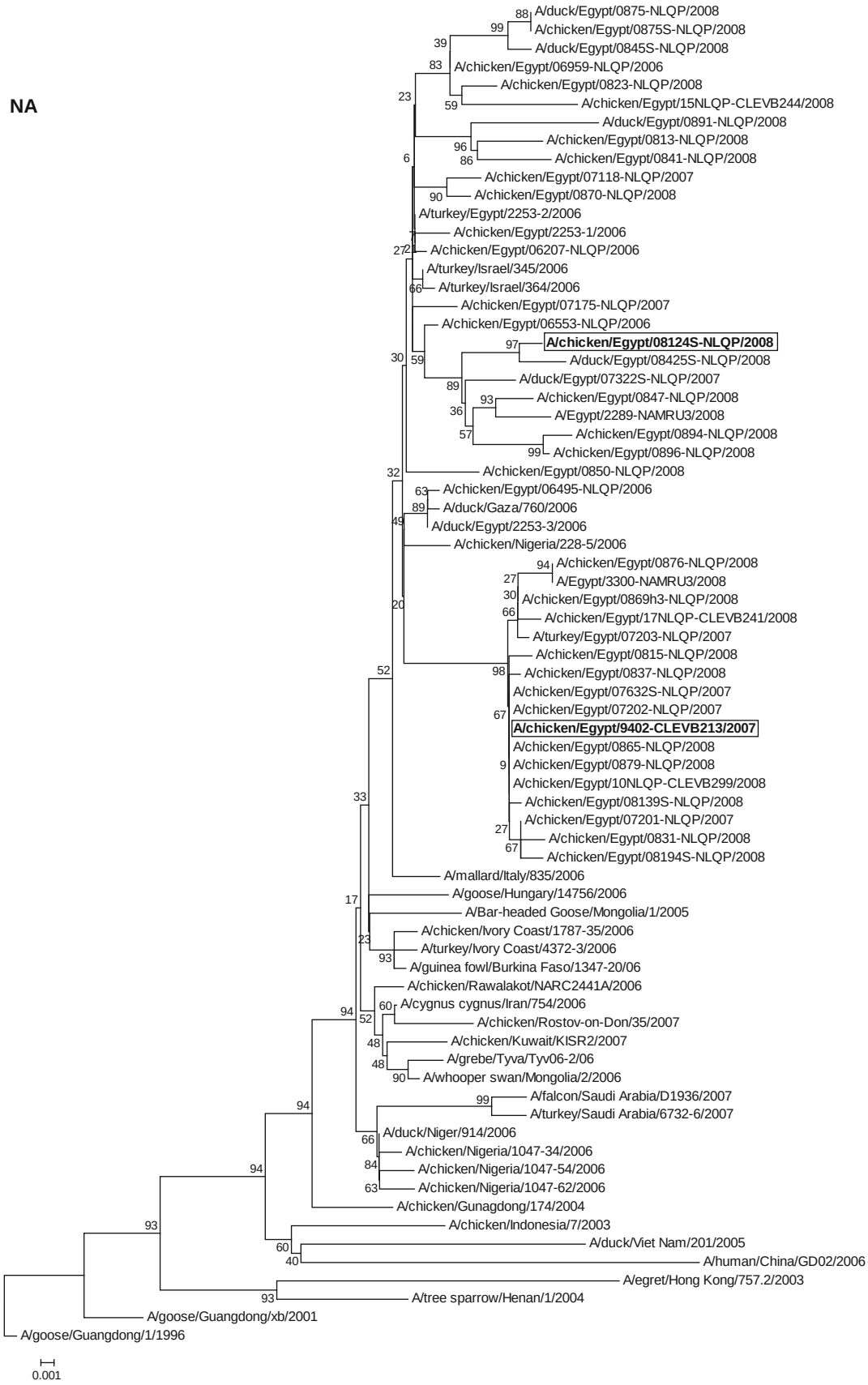
NA
A/duck/Egypt/0875-NLQP/2008
A/chicken/Egypt/0875S-NLQP/2008
A/duck/Egypt/0845S-NLQP/2008
A/chicken/Egypt/06959-NLQP/2006
A/chicken/Egypt/0823-NLQP/2008
A/chicken/Egypt/15NLQP-CLEVB244/2008
A/duck/Egypt/0891-NLQP/2008
A/chicken/Egypt/0813-NLQP/2008
A/chicken/Egypt/0841-NLQP/2008
A/chicken/Egypt/07118-NLQP/2007
A/chicken/Egypt/0870-NLQP/2008
A/turkey/Egypt/2253-2/2006
A/chicken/Egypt/2253-1/2006
A/chicken/Egypt/06207-NLQP/2006
A/turkey/Israel/345/2006
A/turkey/Israel/364/2006
A/chicken/Egypt/07175-NLQP/2007
A/chicken/Egypt/06553-NLQP/2006
A/chicken/Egypt/08124S-NLQP/2008
A/duck/Egypt/08425S-NLQP/2008
A/duck/Egypt/07322S-NLQP/2007
A/chicken/Egypt/0847-NLQP/2008
A/Egypt/2289-NAMRU3/2008
A/chicken/Egypt/0894-NLQP/2008
A/chicken/Egypt/0896-NLQP/2008
A/chicken/Egypt/0850-NLQP/2008
A/chicken/Egypt/06495-NLQP/2006
A/duck/Gaza/760/2006
A/duck/Egypt/2253-3/2006
A/chicken/Nigeria/228-5/2006
A/chicken/Egypt/0876-NLQP/2008
A/Egypt/3300-NAMRU3/2008
A/chicken/Egypt/0869h3-NLQP/2008
A/chicken/Egypt/17NLQP-CLEVB241/2008
A/turkey/Egypt/07203-NLQP/2007
A/chicken/Egypt/0815-NLQP/2008
A/chicken/Egypt/0837-NLQP/2008
A/chicken/Egypt/07632S-NLQP/2007
A/chicken/Egypt/07202-NLQP/2007
A/chicken/Egypt/9402-CLEVB213/2007
A/chicken/Egypt/0865-NLQP/2008
A/chicken/Egypt/0879-NLQP/2008
A/chicken/Egypt/10NLQP-CLEVB299/2008
A/chicken/Egypt/08139S-NLQP/2008
A/chicken/Egypt/07201-NLQP/2007
A/chicken/Egypt/0831-NLQP/2008
A/chicken/Egypt/08194S-NLQP/2008
A/mallard/Italy/835/2006
A/goose/Hungary/14756/2006
A/Bar-headed Goose/Mongolia/1/2005
A/chicken/Ivory Coast/1787-35/2006
A/turkey/Ivory Coast/4372-3/2006
A/guinea fowl/Burkina Faso/1347-20/06
A/chicken/Rawalakot/NARC2441A/2006
A/cygnus cygnus/Iran/754/2006
A/chicken/Rostov-on-Don/35/2007
A/chicken/Kuwait/KISR2/2007
A/grebe/Tyva/Tyv06-2/06
A/whooper swan/Mongolia/2/2006
A/falcon/Saudi Arabia/D1936/2007
A/turkey/Saudi Arabia/6732-6/2007
A/duck/Niger/914/2006
A/chicken/Nigeria/1047-34/2006
A/chicken/Nigeria/1047-54/2006
A/chicken/Nigeria/1047-62/2006
A/chicken/Gunagdong/174/2004
A/chicken/Indonesia/7/2003
A/duck/Viet Nam/201/2005
A/human/China/GD02/2006
A/egret/Hong Kong/757.2/2003
A/tree sparrow/Henan/1/2004
A/goose/Guangdong/xb/2001
A/goose/Guangdong/1/1996
0.001

Table 4 Comparison of genes of H5N1 HPAI viruses with different pathogenicity in 2-week-old domestic ducks

Isolate	Lethality in ducks	Mortality	HA	NA	NP	M	NS	PB2	PB1
A/chicken/Egypt/08	H	8/8	2.2.1[a]	[b]	[c]		[e]	[f]	[g]
A/chicken/Egypt/07	M	3/8	2.2.1[a]	[b]	[c]	[d]	[e]	[f]	[g]
A/duck/Vietnam/10/07	H	8/8	1[a]	[b]	[c]	[d]	[e]		[g]
A/duck/Vietnam/218/05	H	8/8	2.3.2[a]	[b]	[c]		[e]		[g]
A/duck/Vietnam/203/05	H	8/8	2.3.4[a]	[b]	[c]		[e]		[g]
A/VietNam/1203/04	H	7/8	1[a]	[b]	[c]	[d]	[e]	[f]	[g]
A/Crow/Thailand/04	H	8/8	1[a]	[b]	[c]	[d]	[e]		[g]
A/Egret/HK/757.2/02	H	7/8	1[a]		[c]		[e]		[g]
A/Goose/HK/739.2/02	H	7/8	1[a]		[c]		[e]		[g]
A/Thailand/PB/6231/04	M	3/8	1[a]	[b]	[c]	[d]	[e]	[f]	[g]
A/Chicken/Korea/ES/03	M	2/8	2[a]	[b]	[c]		[e]		[g]
A/Chicken/Indonesia/7/03	M	4/8	2[a]	[b]	[c]	[d]	[e]		[g]
A/Duck/Anyang/ALV-1/01	L (systemic infection)	0/8	1[a]	[b]					
A/Goose/VietNam/113/01	L (systemic infection)	0/8	1[a]				[e]		
A/Chicken/HK/317.5/01	L	0/8	0[a]						
Env./HK/437.6/99	L	0/8	0[a]						
A/Chicken/HK/220/97	L	0/8	0[a]	[b]					

Mortality data were obtained from this study and previously published studies [52, 56]

[a] Numbers indicate different genetic clades. A few amino acid changes distinguished the more pathogenic viruses and included amino acids T320S, K483R, N499-K, and M511I

[b] 20-amino-acid stalk deletion (positions 49–68)

[c] 3 amino acid differences were observed when compared to non-lethal A or I/353V, S377N and S482N

[d] M1; R27-K; M2 S31-N consistent with amantadine resistance

[e] 5-amino-acid deletion (80–84)

[f] Lysine at position 627 (associated with replication and virulence in humans)

[g] 3 amino acid differences were observed when compared to non-lethal A14V, S384L and R386K

associated with viruses that can infect humans. Both viruses contain the PB2 627K mutation, which increases pathogenicity in mice [26, 69], as well as 33I in NP, which is associated with human-infectious viruses [64]. A 109V in the NP of the Egypt/08 virus is also a human host marker [12]. While both viruses contain 226Q and 228G (H3 numbering), which are associated with an avian α2,3 receptor binding preference, they have a 193R (H3 numbering), which, when combined with 226Q and 228G, results in increased affinity for α2,6 receptors [68]. The Egypt/07 virus also has the 31N mutation in M2, which is associated with amantadine resistance; however, neither virus has the additional 27- and 30-amino-acid resistance markers in M2 [70]. The S42 NS1 mutation, which has also been associated with increased virulence of avian influenza viruses in mice and has been shown to be involved in antagonizing the interferon induction pathways, was found in both Egyptian virus isolates [32].

The results of this and previous studies show that H5N1 HPAI viruses are continually evolving and changing their pathogenicity in ducks. These changes have resulted in viruses with increased virus replication and shedding, which consequently increases the risk of transmission to other animal species, including humans. In addition, some of these viruses are very virulent in ducks, inducing high mortality. Sequence analysis showed amino acid differences in most of the proteins of the two viruses studied, with the greatest number of differences present in the HA protein. When compared with sequences from other H5N1 HPAI viruses that are highly virulent for ducks, no common set of changes was observed, indicating that more studies are needed in order to understand the molecular determinants of virus pathogenesis in ducks.

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AVIAN DISEASES 54:345–349, 2010

Research Note—

Phylogenetic Analysis of Hemagglutinin and Neuraminidase Genes of Highly Pathogenic Avian Influenza H5N1 Egyptian Strains Isolated from 2006 to 2008 Indicates Heterogeneity with Multiple Distinct Sublineages

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SUMMARY. The Eurasian-lineage H5N1 highly pathogenic avian influenza (HPAI) virus caused widespread outbreaks in Egypt in 2006 and eventually become enzootic in poultry. Although outbreaks have a seasonal pattern, with most occurring during the cooler winter months, it remains unclear whether this seasonality reflects virus maintenance within Egypt or yearly introductions of the virus into the country. To evaluate the epidemiology of H5N1 HPAI in Egypt, sequence analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of selected Egyptian isolates from early 2006 to 2008 was conducted. The data from this study identifies distinct genetic markers in both HA and NA genes and suggests grouping Egyptian isolates into two major HA isolate sublineages from 2006 to 2008 and into three smaller, emergent subgroups. The NA phylogenetic and sequence analysis showed a similar pattern, except that two of the emergent groups from the HA phylogenetic tree clustered together, evidence of likely reassortment. The different subgroups did not appear to segregate by relation to the date of isolation, to the species of origin, nor to the geographic location of the viruses. The conclusion is that H5N1 is continuing to mutate with multiple heterogenic strains persisting in Egypt.

RESUMEN. *Nota de Investigación*—El análisis filogenético de los genes de la hemaglutinina y la neuraminidasa de cepas egipcias del virus de la influenza aviar de alta patogenicidad subtipo H5N1 aisladas del 2006 al 2008 indica heterogeneidad con múltiples diferentes sublinajes.

El linaje euroasiático del subtipo H5N1 de los virus de la influenza aviar de alta patogenicidad ha causado brotes diseminados en Egipto en el año 2006 y existe la posibilidad de que se vuelva enzoótico. Aunque los brotes tienen un patrón estacional y la mayoría de los brotes ocurren en invierno, aún no se ha determinado si la estacionalidad refleja el mantenimiento del virus en Egipto o refleja introducciones del virus a este país cada año. Para determinar la epidemiología de los virus de la influenza aviar altamente patógenos subtipo H5N1 en Egipto, se realizaron análisis de los genes de la hemaglutinina (HA) y la neuraminidasa (NA) de aislamientos selectos en Egipto detectados desde el principio del año 2006 al 2008. Los datos de este estudio identificaron distintos marcadores genéticos de los genes Ha y NA y sugiere agrupamiento de los aislamientos egipcios en dos sublinajes principales del gene de la HA con los virus detectados del año 2006 al 2008 y en tres subgrupos pequeños emergentes. El análisis filogenético y los análisis de secuencias mostraron un patrón similar, excepto que dos de los grupos emergentes del árbol filogenético de la HA, se agruparon juntos, lo que posiblemente presenta evidencia de reacomodo genético. Los diferentes subgrupos no aparecen clasificar con relación a la fecha de aislamiento, a la especie de origen ni a la localización geográfica de los virus. Las conclusiones es que los virus subtipos H5N1 continúan mutando con múltiples cepas heterogénicas que persisten en Egipto.

Key words: highly pathogenic avian influenza, subtype H5N1 virus, phylogenetic analyses, hemagglutinin, neuraminidase

Abbreviations: EMA = European–Middle Eastern–African; HA = hemagglutinin; HPAI = highly pathogenic avian influenza; NA = neuraminidase; RRT = real-time RT-PCR; RT-PCR = reverse transcription–PCR; SPF = specific-pathogen-free; VLA = Veterinary Laboratories Agency

A unique highly pathogenic avian influenza (HPAI) H5N1 virus lineage first emerged in China in 1996 (3), and in the past 5 yr, it has become widespread, affecting poultry, wild birds, and humans in Asia, Europe, and Africa (2). Phylogenetic studies have identified, to date, nine major clades of H5N1 HPAIV of Asian origin (13). Clade 2 appears to be the most diversified, and members of subclade 2.2 have been responsible for the westward spread of H5N1 from Asia. Within subclade 2.2, three further subtypes have been distinguished and were designated with respect to their geographic origin, Europe, Middle East, or Africa (8). HPAI H5N1 virus was first recognized to cause serious disease outbreaks in Egypt in 2006, and the virus has become enzootic in poultry throughout Egypt (1). The aim of this work was to study the epidemiology of H5N1 HPAI in Egypt, based on the sequence analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of the Egyptian isolates from 2006–2008. This analysis will also aid in understanding which viruses circulating in Egypt are most important, assisting in the selection of viruses to be used for vaccine production and for challenge strains of existing vaccines.

MATERIALS AND METHODS

Clinical samples of pooled tracheal and cloacal swabs were tested by real-time reverse transcription-PCR (RRT-PCR) from poultry disease outbreaks in Egypt from 2006 to 2008, and representative, positive samples were inoculated in 10-day-old, SPF, embryonating chicken eggs

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Fig. 1. Phylogenetic tree of HA gene of Egyptian isolates compared with representative H5N1 Eurasian strains. The tree was produced with PAUP 4.0 software, using a bootstrap analysis with A/goose/Guangdong/1/1996 as an outgroup. All the Egyptian viruses are clade 2.2.

and were observed for up to 5 days at 37 C. The allantoic fluids were harvested and tested for HA activity, as previously described (12), and were also confirmed by RRT-PCR for H5N1 subtype. RNA was extracted, using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), then tested by RRT-PCR, using a One-Step real-time RT-PCR method according to Veterinary Laboratories Agency (VLA; Weybridge, Surrey, U.K.) (9) protocols for influenza A viruses. The samples positive for type A influenza were further tested using RRT-PCR for H5N1 kits (Roche, Mannheim, Germany). Twenty isolates were selected for the complete nucleotide-coding sequence for both the HA and NA genes. The amplified HA products were purified using a PCR purification kit (Qiagen, Valencia, CA) and was sequenced using internal specific primers to generate overlapping HA fragments (4) (primer sequences are available upon request), using BigDye Terminator

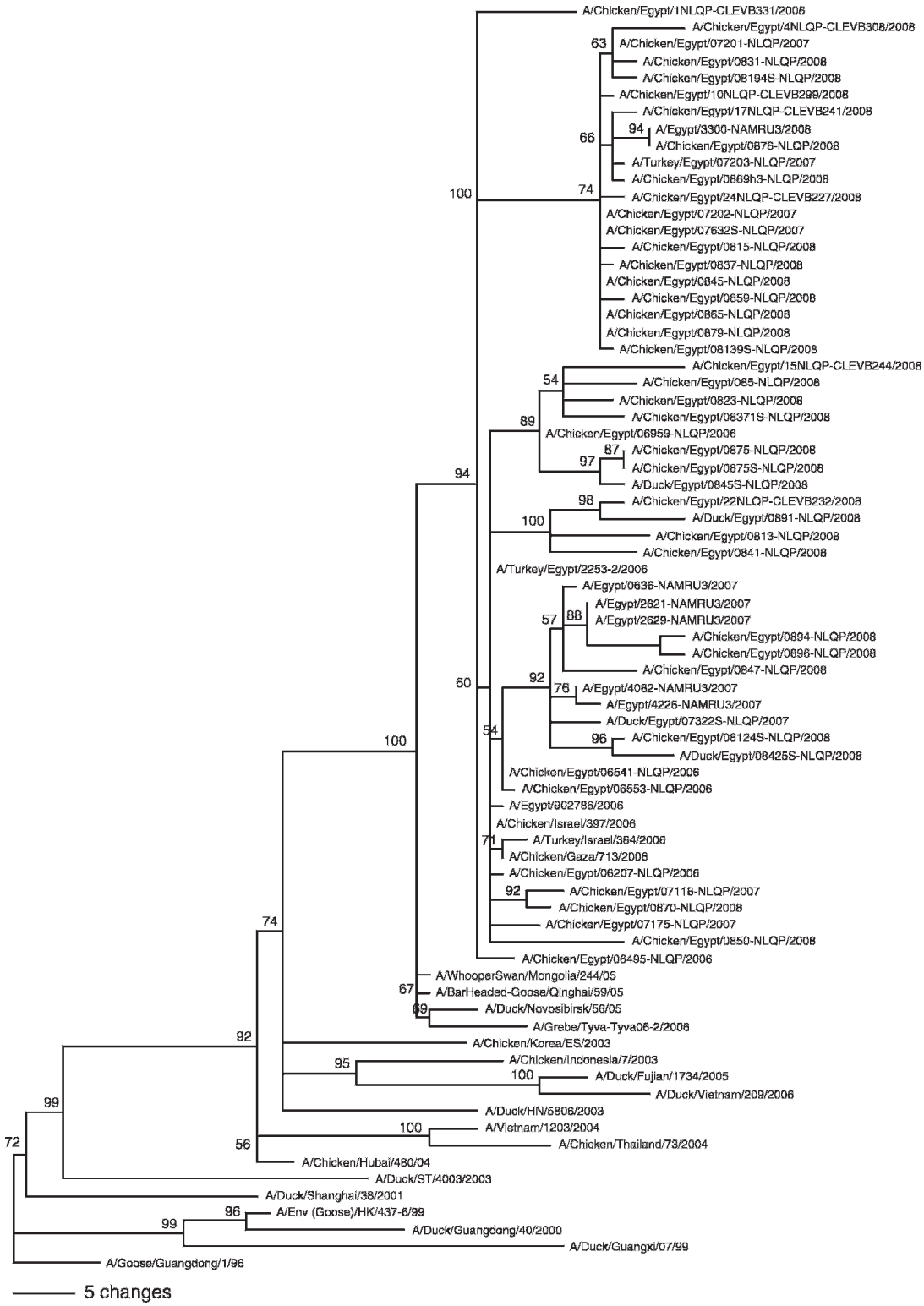

Fig. 2. Phylogenetic tree of NA gene of Egyptian isolates compared with representative H5N1 Eurasian strains. The tree was produced with PAUP 4.0 software, using a bootstrap analysis with A/goose/Guangdong/1/1996 as an outgroup.

v3.1 Cycle Sequencing Kit on an automatic sequencer (ABI-3130; Applied Biosystems, Foster City, CA).

Phylogenetic analysis was carried out on the full-length HA and NA genes of the Egyptian strains (Figs. 1, 2), where multiple and pairwise sequence alignments were constructed using the ClustalV algorithm of MegAlign program from LaserGene biocomputing software package (DNASTAR, Madison, WI). Phylogenetic relationships were estimated by the maximum parsimony method (PAUP software; version 4.0b10; Sinauer Associates, Inc., Sunderland, MA) using a bootstrap resampling method (100 bootstraps) with a heuristic search algorithm. The strain (A/goose/Guangdong/1/1996) was used as an outgroup for both the HA and NA trees.

Table. 1. Position of amino acid mutation changes in HA and NA proteins.

Cluster	HA[A]	NA[A]
Variant		188
	90, 113, 126, 139, 156, 157, 160, 178, 181, 200, 242	
Additional groups		46, 339
	110, 529	
		45, 80
	133, 242, 499	
	59, 136, 145 (deletion), 167	

[A]Numbered according to H5N1 (goose/Guangdong/96) and compared in relation to the original Egyptian strain in 2006 (A/chicken/Egypt/06207-NLQP/2006).

RESULTS

Clinical samples were tested and confirmed to be H5N1 avian influenza viruses by RRT-PCR. Selected positive samples were used for virus isolation. Positive samples had inoculated embryos that died usually within 2–3 days. HA titer of the isolated viruses ranged from 5 $\log_2$ to 9 $\log_2$.

The selected isolates in this study were confirmed positive by RRT-PCR for subtype H5N1 avian influenza virus. Twenty isolated viruses from 2006 and 2008 were sequenced for the HA and NA genes and compared with additional sequences available from GenBank. The HA gene sequences clustered with clade 2.2 H5N1 viruses that have been isolated in other Asian, European, and African countries (Fig. 1). The phylogenetic and amino acid sequence analysis also revealed that the Egyptian viruses from 2006 to 2008 could be divided into five and four sublineages, respectively, for both the HA and NA gene (Table 1; Figs. 1, 2). Most viruses isolates from 2006 and early 2007 were closely related to viruses isolated at the same time in the Middle East region, with no distinct phylogenetic sublineage of viruses or unique amino acid markers being observed. However, in late 2007, one variant cluster did emerge that was prominent based on both the number of amino acid changes in the HA and the high percentage of viruses that belonged to the group of those viruses isolated in 2007 and 2008. This variant group had 11 amino acid differences from other Egyptian viruses examined. Using the goose/Guangdong/96 amino acid numbering, these changes were at positions 90, 113, 126, 139, 156, 157, 160, 178, 181, 200, and 242 of the HA protein. The amino acid change at position N181H (HA1 position 165) results in a potential loss of the *N*-linked glycosylation site. The analysis of the NA gene showed a similar phylogenetic pattern with the same group of viruses, but, although phylogenetically distinct, this group of viruses differed by only a single amino acid from the other Egyptian viruses at position 188. This cluster of viruses, therefore, represents a distinctly different cluster of viruses, which may represent an important antigenic variant.

Phylogenetically, three other small clusters of emergent viruses were observed, branching from the original introduced group of viruses. For the HA protein, they had unique amino acid changes at only two (positions 110 and 529), three (positions 133, 242, and 499), or four (positions 59, 136, 145, and 167) from the main (original) branch of viruses. In the last group, the change at amino acid 145 was a deletion, not a substitution. The NA analysis had viruses from the original introduction, the large variant group described above, and two other small emergent groups. The emergent groups had amino acid differences at positions 46 and 339 and at positions 45 and 80. One of the emergent groups included isolates from two of the HA emergent groups, suggesting that reassortment had occurred between the two groups. Otherwise, the isolates in the NA tree clustered together in the same pattern as in the HA tree.

DISCUSSION

Genetic drift of the influenza virus is explained by random mutations that became dominant because of selection or adaptation, including vaccination pressure. The HA protein is one of the principal virulence determinants of the virus in poultry and is the primary target for neutralizing antibodies produced by vaccination or natural infection. The HA gene is variable in length, but most Egyptian H5N1 viruses are typically 1778 bp long with an open reading frame of 1704 bases (568 amino acid residues). Phylogenetic analysis of the HA gene showed that all viruses from Egypt from 2006 to 2008 analyzed in this study had a common origin in the European-Middle Eastern-Africa (EMA) clade 1 (Fig. 1) (8). This group of viruses is also classified as the H5N1 clade 2.2. The EMA clade 1 includes viruses isolated in 2006 from wild birds in Europe and from poultry in the Middle East (Israel and Gaza) and in Africa (Sudan, Côte d'Ivoire, and Nigeria) (7,8). All the viruses in Egypt appeared to be from a single origin, with the phylogenetic tree having a straight trunk with a number of small clusters branching from the trunk. Only a single group that has a longer branch length (the group of 25 isolates in the HA tree; Fig. 1) were clearly distinct from the other viruses with a longer branch. This sublineage cluster had viruses isolated from December 2007 to May 2008. The isolates that were on short branches from the main trunk included the viruses first isolated in 2006 to the last viruses included in the study of September 2008. Three other subgroups could also be identified from late 2007–2008, with several unique amino acid changes in both the HA and NA genes. This lack of a clear lineage suggests that a single lineage of virus has not become dominant in Egypt.

The phylogenetic analysis based on HA and NA genes in this study is consistent with previous experiments (5,6,10) that grouped all the H5N1 Egyptian viruses into clade 2.2 (13). Although all the viruses were clade 2.2, there is no primary sublineage that has become discernable from the 2006 original introduction, but three additional smaller sublineages are also, possibly, emerging (Figs. 1, 2). The lack of a single, clear lineage in Egypt is unusual because no geographic or political barriers are present to account for the emergence and persistence of so many different lineages. Preliminary analysis showed that origin of virus by species did not determine the lineage of the virus. Potentially poultry and wild birds can be in distinct compartments, i.e., backyard or commercial poultry that allow for the independent maintenance of virus within the country. An alternative hypothesis is that virus is evolving outside of Egypt and is periodically introduced into the country. However, more detailed epidemiologic and sequence information from Egypt and from surrounding countries is needed to understand the maintenance of virus in Egypt.

Temporally, viruses from all five HA groups, major and minor, were isolated in 2008 showing that a single, predominant lineage has not established itself in Egypt. Preliminary analysis also did not show viruses clustering based on the geographic locations from which the samples were taken. Additionally, isolates did not cluster based on the species of origin, and human viruses were present in four of five HA groups and three of four NA groups.

From the data in this study, we concluded that H5N1 in Egypt is continuously mutating and that multiple heterogenic strains persist inside Egypt. The original, introduced viruses appear to have emerged into one distinct HA sublineage, with three smaller groups

also having emerged. The NA data provide similar results, except that two of the HA emergent groups appear in the same branch on the NA tree, likely representing reassortment between viruses. This study also illustrates the important role of longitudinal gene analyses in endemic countries like Egypt, especially to help in the characterization of the newly emerged strains. Efforts to do whole-genome sequencing will aid in determining which are the most important emerging viruses in the country. Based on the available information, multiple lineages of viruses continue to circulate in Egypt, which can affect decisions on vaccination and control, but the reasons for the diversity within Egypt remain unclear.

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AVIAN DISEASES 54:483–495, 2010

Review Article—

Evolution of Highly Pathogenic Avian Influenza H5N1 Virus in Natural Ecosystems of Northern Eurasia (2005–08)

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SUMMARY. Fifty-four strains of H5N1 highly pathogenic avian influenza (HPAI) virus were isolated from wild birds in the ecosystems of northern Eurasia and from poultry in the south of western Siberia (July 2005), at the mouth of Volga River (November 2005), at Uvs-Nur Lake on the boundary of the Great Lakes Depression in western Mongolia and the Tyva Republic of Russia (June 2006), in the vicinity of Moscow (February 2007), in the southeastern part of the Russian Plain (September 2007 and December 2007), and in the far east (April 2008) of the Russian Federation and were phenotypically characterized and deposited into the Russian state collection of viruses. Complete genome nucleotide sequences for 24 strains were obtained and deposited into GenBank. In all cases when strains were isolated from both wild birds and poultry in the same outbreak these strains were genetically closely related to each other. Until 2008 all HPAI H5N1 strains isolated in northern Eurasia clustered genetically with the viruses from Kukunor Lake (Qinghai Province, China), known as genotype 2.2 or the ''Qinghai-Siberian'' genotype. The viruses from the Qinghai-Siberian genotype have continued to evolve from those initially introduced into western Siberia in 2005 into two genetic groups: ''Iran–North Caucasian'' and ''Tyva-Siberian.'' *In vitro* replication potential (50% tissue-culture infectious dose in porcine embryo kidney) of Qinghai-Siberian strains decreased over time, which could reflect decreasing virulence. Comparison of genome sequences with biological characteristics of the respective strains permitted us to identify point mutations in PB2, PB1, PA, HA, NP, NA, M2, NS1, and NS2 that possibly influenced the level of replication potential. The HPAI H5N1 virus, which penetrated into the south of the Russian Far East in spring 2008, belonged to genotype 2.3.2.

RESUMEN. *Estudio Recapitulativo*—Evolución de los virus de la influenza aviar de alta patogenicidad H5N1 en ecosistemas naturales del Norte de Eurasia (2005-08).

Cincuenta y cuatro cepas del virus de la influenza aviar de alta patogenicidad subtipo H5N1 fueron aisladas de aves silvestres en los ecosistemas del norte de Eurasia y de aves de corral del sur de Siberia occidental (julio de 2005), de la desembocadura del Río Volga (Noviembre de 2005), en el Lago Uvs al-Nur ubicado en el límite de la Depresión de los Grandes Lagos del Oeste de Mongolia y de la República de Tuvá en Rusia (Junio de 2006), de las cercanías de Moscú (Febrero del 2007), en la parte sureste de la Llanura Rusa (septiembre de 2007 y diciembre de 2007), y en el Extremo Oriente de Rusia (Abril de 2008). Las cepas fueron caracterizadas fenotípicamente y se depositaron en la colección estatal rusa de virus. Las secuencias completas de nucleótidos del genoma de 24 cepas fueron obtenidas y depositadas en el GenBank. En todos los casos, cuando las cepas fueron aisladas de aves silvestres y de aves de corral del mismo brote, estas cepas resultaron estar estrechamente relacionadas entre sí genéticamente. Hasta el año 2008, todas las cepas de influenza aviar altamente patógenas H5N1 aisladas en el norte de Eurasia se agruparon genéticamente con el virus del Lago Kukunor (provincia de Qinghai, China), conocido como genotipo 2.2 o el genotipo ''Qinghai-siberiano''. Los virus del genotipo Qinghai-siberiano han continuado su evolución a partir de los virus que inicialmente se introdujeron en Siberia en el 2005 resultando en dos grupos genéticos: el ''Irán-Cáucaso del Norte'' y el ''Tuvá-Siberiano''. El potencial de replicación *in vitro* de las cepas Qinghai-Siberianas (en dosis infectantes 50% en cultivo de tejidos de células de porcino embrionarias) disminuyó con el tiempo, lo que podría reflejar una disminución en la virulencia. La comparación de las secuencias del genoma con las características biológicas de las cepas respectivas ha permitido identificar mutaciones puntuales en PB2, PB1, PA, HA, NP, NA, M2, NS1 y NS2 que posiblemente influyeron en el nivel del potencial de replicación. El virus de la influenza aviar altamente patógena H5N1, que penetró en el sur del Extremo Oriente de Rusia en la primavera de 2008, pertenecían al genotipo 2.3.2.

Key words: virus, HPAI, H5N1, natural ecosystems, northern Eurasia, evolution

Abbreviations: AIV = avian influenza virus; BHK-21 = baby hamster kidney cell line; HA = hemagglutinin; HPAI = highly pathogenic avian influenza; LECH = human embryo lung cell line; LPAI = low pathogenicity avian influenza; MDCK = Madin-Darby canine kidney cell line; NA = neuraminidase; NLS = nuclear location signal; RNP = ribonucleoproteide; RSCV = Russian state collection of viruses; RT-PCR = reverse transcription followed by polymerase chain reaction; SPEV = porcine embryo kidney cell line; $TCID_{50}$ = 50% tissue-culture infectious dose; Vero E6 = clone E6 of vervet-obtained monkey kidney cell line

The richest gene pool of influenza A virus is among wild waterfowl, and the ability of influenza viruses to reassort provides a vast array of possible variants. Virus variants circulating in bird populations could be progenitors for the emerging and reemerging epizootic and pandemic viruses. It is why 45 yr ago we started doing surveillance of avian influenza virus (AIV) circulation in key points on the main migration routes of birds in northern Eurasia. During this time more than a thousand influenza A virus strains belonging to 15 of the 16 known HA subtypes were isolated in different ecosystems of northern Eurasia (17,18,20,21,30,31,35,38,39) (Fig. 1). The HA subtypes of the strains isolated from teal in 2001 in the Russian Far East and

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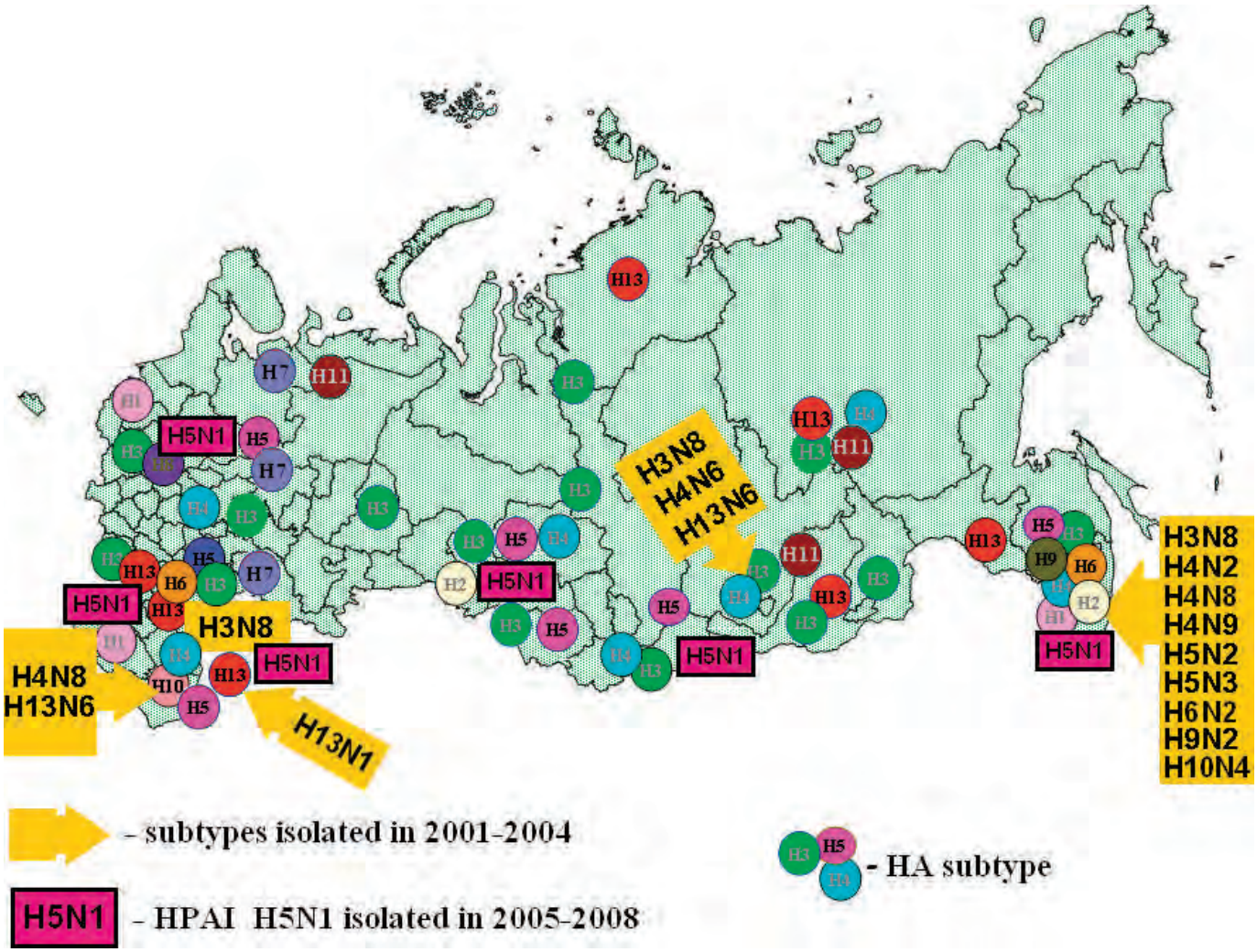

Fig. 1. Influenza A virus subtypes isolated from birds in natural and anthropogenic ecosystems of northern Eurasia (1962–2008).

eastern Siberia were shown to be genetically related to the H5 strains isolated from poultry in 1997 in Italy. The AIV strains from mallard isolated in 1991 in the Altai region of southwestern Siberia were similar to A/duck/Malaysia/F119-3/97 with a HA cleavage site that is characteristic of low pathogenicity avian influenza (LPAI) strains (18,30,31,33).

Development and integration of modern virologic, ecologic, and genetic techniques could allow early detection of viruses that are a threat to both veterinary and public health and promote efforts to mitigate new outbreaks. In the former USSR, there was a system for wide-scale monitoring of natural foci of viral infections that was coordinated by the Virus Ecology Center of the D. I. Ivanovsky Institute of Virology (Russian Academy of Medical Sciences, Moscow, Russia). This monitoring network has allowed us to document the introduction of the current H5N1 epizooty into northern Eurasia, and with modern methods we have biologically and molecularly characterized these newly introduced viruses. In the present article we discuss results obtained at the Influenza Ecology and Epidemiology Center as a result of studying HPAI H5N1 from the time of its introduction into the ecosystems of northern Eurasia in spring of 2005 until now.

MATERIALS AND METHODS

Collection of field materials (cloacal/tracheal swabs, brain, liver, lungs) from sick and dead birds was performed during epizootic outbreaks in northern Eurasia during 2005–08. Each sample was placed into a 2-ml tube with 40% glycerol in a phosphate buffer solution (pH 7.4), stored, and transported to the laboratory in liquid nitrogen in Dewar vessels.

Reverse transcription followed by polymerase chain reaction (RT-PCR) for the detection of RNA of NP, M, and HA genes from H5 of the influenza A virus was conducted according to the standard techniques using specific primers (NARVAC, Russia) (11).

Isolation and investigation of replication potential *in vitro* of virus strains was performed using either 9-to-10-day-old specific-pathogen-free chicken eggs or continuous cell culture lines: porcine embryo kidney (SPEV), Madin-Darby canine kidney (MDCK), baby hamster kidney (BHK-21), human embryo lung (LECH), or clone E6 of vervet-obtained monkey kidney (Vero E6) (6).

Decrease of the *in vitro* replication potential (50% tissue-culture infectious dose [$TCID_{50}$] in SPEV) over time was characterized by angulation of the best fit line calculated by the least squares method.

Identification of isolated virus strains was carried out by RT-PCR, hemagglutination inhibition testing, neutralization tests according to the standard techniques, and biologic microchips (Biochip-IMB Ltd., V.A. Engelhardt Institute of Molecular Biology, Moscow, Russia) according to the manufacturer's recommendations.

Nucleotide sequencing was performed using the automated sequencing apparatus ABI prism 3130 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations.

Comparative analysis of nucleotide sequences was carried out using the commercial software package DNASTAR (DNASTAR Inc., Madison, WI), as well as our own information techniques based on

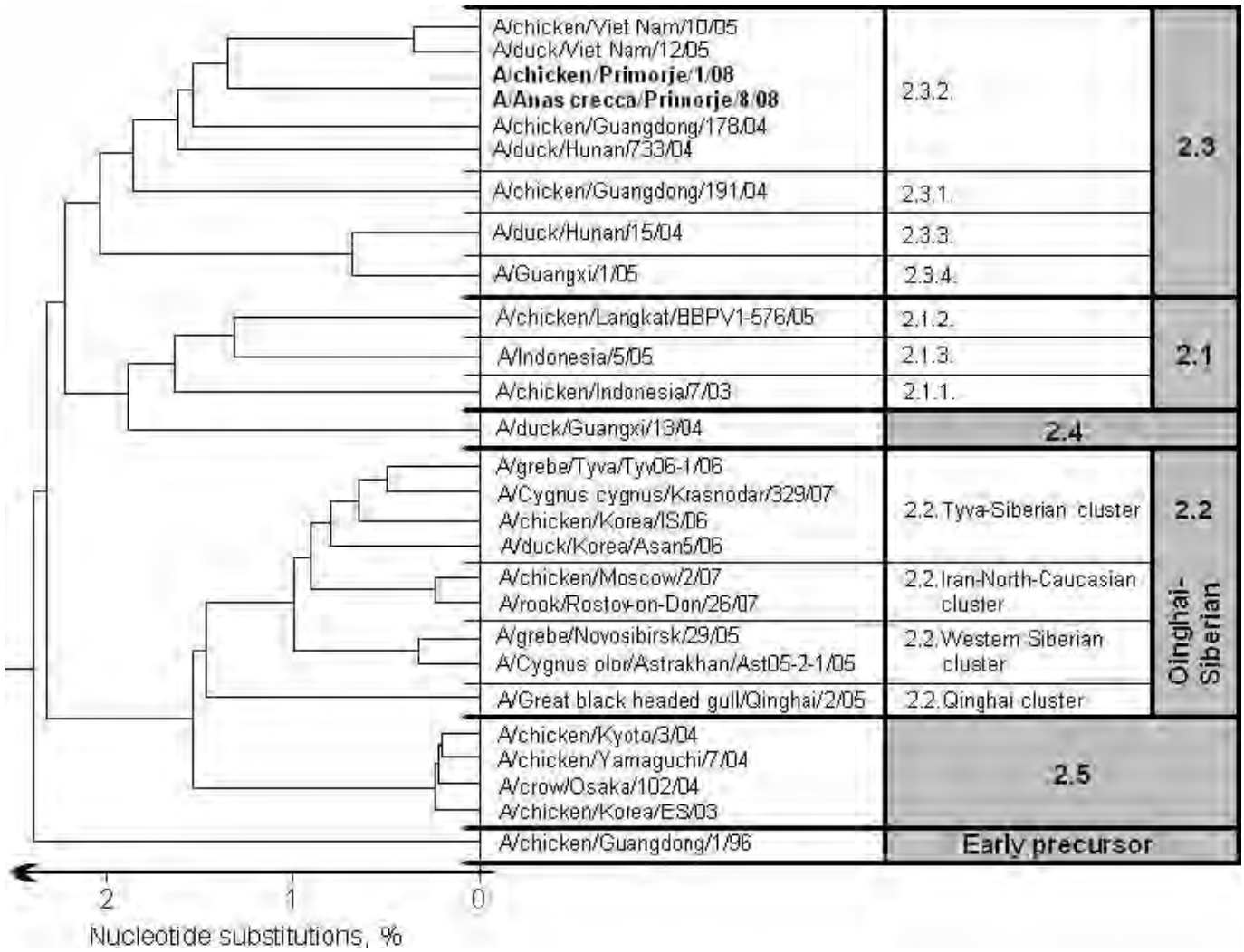

Fig. 2. Phylogenetic analysis of complete nucleotide sequences of open reading frame for HPAI H5N1 HA.

Statistics 6.0 (StatSoft Inc., Tulsa, OK), ArcView 3.2 (ESRI Inc., Redlands, CA), and MatLab 6.0 (Softline Inc., Berkeley, CA).

RESULTS

After the introduction of the HPAI H5N1 virus into northern Eurasia in the spring of 2005 until 2008, we have isolated 54 strains (27 from wild birds, 27 from poultry) of this virus from epizootic outbreaks in the following locations: in the south of western Siberia (July 2005) (22,25,36); at the mouth of the Volga River (November 2005) (23); at Uvs-Nur Lake on the boundary of the Great Lakes Depression in western Mongolia and the Tyva Republic of Russia (June 2006) (24); in the vicinity of Moscow (February 2007) (27); in the northeastern part of the Azov Sea basin (September 2007) (26); in the southwestern part of the Russian Plain (December 2007) (28); and in the Russian Far East (April 2008) (29). All strains were phenotypically characterized and deposited into Russian state collection of viruses (RSCV) (Table 1). The nucleotide sequences of all eight gene segments for 24 strains were deposited into GenBank (Table 2).

All isolated strains appeared to belong to the Asian H5N1 genotypic clade 2.2 with the exception of four strains belonging to genotype clade 2.3.2, which were isolated in the Far East in the spring of 2008 (Table 1; Fig. 2). The clade 2.2 avian influenza viruses that were introduced into northern Eurasia (22,25,36) were most closely related to viruses responsible for the mass mortality in wild birds—mainly among bar-headed geese (*Eulabeia indica*) and great black-headed gulls (*Larus ichthyaetus*)—at Kukunor Lake in April of 2005 (Qinghai Province, China) (5,14). The virus lineage appeared to move north with the wild bird migration defining the genotype 2.2 viruses as the "Qinghai-Siberian" clade.

The main genetic characteristics of Qinghai-Siberian clade (22) persisted as the virus lineage extended into the western part of northern Eurasia (1,2,12,22,23,24,25,26,27,28) and Africa (2005–08) (2,3,10). The other genes of the Qinghai-Siberian genotype are associated with group Z, which has dominated among poultry in southeastern Asia since 2003–04 (4). The group Z genotype has several unique genetic markers including a 20-mer amino acid fragment deletion C_{49}NQSIITYENNTWVNQTYVN$_{68}$ in the N1 gene compared to A/goose/Guangdong/1996. The HA cleavage site—P_{337}QGERRRKKRGLF$_{349}$—has multiple basic amino acid insertions. Three types of silent nucleotide substitutions are known in the coding region of the cleavage site among the Qinghai-Siberian clade: $G \overset{1020}{\rightarrow} A$ (A/chicken/Volgograd/236/2006), $G \overset{1028}{\rightarrow} A$ (A/pied magpie/Liaoning/7/2005), and $A \overset{1044}{\rightarrow} G$ (A/chicken/Crimea/04/2005), as well as four types of amino acid substitutions: $G \overset{339}{\rightarrow} R$ (A/bar-headed goose/Qinghai/{1,3}/2005, A/brown-headed gull/Qinghai/1/2005, A/great black-headed gull/Qinghai/3/2005, A/great cormorant/Qinghai/3/2005, A/whooper swan/Mongolia/

Table 1. Infection activity *in vitro* of HPAI H5N1 strains isolated in natural and anthropogenic ecosystems of northern Eurasia (2005–08).

Date	Region	Ecologic group of birds	Strain[A]	RSCV deposition number	Clinical features[B]	$\log_{10}$ $TCID_{50}$/ml for SPEV
July 2005	South of western Siberia (Novosibirsk region) (19,24,35)	Wild	**A/grebe/Novosibirsk/29/2005**	2372	∅	5.7
			Mean value:			**5.7**
		Poultry	**A/duck/Novosibirsk/56/2005**	2371	⊗	7.7
			A/duck/Novosibirsk/67/2005	2376	⊕	10.2
			A/chicken/Novosibirsk/64/2005	2373	⊕	11.2
			A/chicken/Novosibirsk/65/2005	2374	⊕	10.7
			A/chicken/Novosibirsk/66/2005	2375	⊕	10.7
			Mean value:			**10.1**
November 2005	Mouth of Volga River (Astrakhan region, Kalmyk Republic) (22)	Wild	**A/Cygnus olor/Astrakhan/Ast05-2-1/2005**	2379	⊗	3.7
			A/Cygnus olor/Astrakhan/Ast05-2-2/2005	2380	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-3/2005	2381	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-4/2005	2382	⊗	3.7
			A/Cygnus olor/Astrakhan/Ast05-2-5/2005	2383	⊗	5.2
			A/Cygnus olor/Astrakhan/Ast05-2-6/2005	2384	⊗	5.2
			A/Cygnus olor/Astrakhan/Ast05-2-7/2005	2385	⊗	5.7
			A/Cygnus olor/Astrakhan/Ast05-2-8/2005	2386	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-9/2005	2387	⊗	3.2
			A/Cygnus olor/Astrakhan/Ast05-2-10/2005	2388	⊗	4.7
			Mean value:			**4.4**
June 2006	Uvs-Nur Lake (Tyva Republic) (23)	Wild	**A/grebe/Tyva/Tyv06-1/2006**	2393	⊗	8.0
			A/grebe/Tyva/Tyv06-2/2006	2394	⊗	8.5
			A/cormorant/Tyva/Tyv06-4/2006	2396	∅	5.0
			A/coot/Tyva/Tyv06-6/2006	2397	⊗	5.0
			A/grebe/Tyva/Tyv06-8/2006	2395	⊕	8.0
			A/tern/Tyva/Tyv06-18/2006	2399	∅	5.0
			Mean value:			**6.6**
February 2007	Vicinity of Moscow (Moscow and Kaluga regions) (26)	Poultry	A/chicken/Moscow/1/2007	2403	⊕	4.0
			A/chicken/Moscow/2/2007	2404	⊕	4.5
			A/chicken/Moscow/3/2007	2405	⊕	4.0
			A/chicken/Moscow/4/2007	2406	⊕	4.0
			A/goose/Moscow/5/2007	2407	⊕	4.0
			A/chicken/Moscow/6/2007	2408	⊕	4.5
			A/chicken/Moscow/7/2007	2409	⊕	4.5
			A/chicken/Moscow/8/2007	2410	⊕	4.0
			A/chicken/Moscow/9/2007	2414	⊕	4.0
			Mean value:			**4.2**
September 2007	Northeastern part of Azov Sea basin (Krasnodar krai) (25)	Wild	**A/Cygnus cygnus/Krasnodar/329/2007**	2421	⊗	3.5
			Mean value:			**3.5**
		Poultry	**A/chicken/Krasnodar/300/2007**	2418	⊗	3.5
			A/chicken/Krasnodar/301/2007	2419	⊗	3.0
			A/chicken/Krasnodar/302/2007	2420	⊗	3.5
			Mean value:			**3.3**
December 2007	Southwestern part of Russian Plain (Rostov region) (27)	Wild	**A/pigeon/Rostov-on-Don/6/2007**	2423	∅	6.5
			A/pigeon/Rostov-on-Don/7/2007	2424	∅	5.5
			A/heron/Rostov-on-Don/11/2007	2425	∅	6.0
			A/pigeon/Rostov-on-Don/21/2007	2426	∅	6.0
			A/rook/Rostov-on-Don/26/2007	2427	∅	6.5
			A/rook/Rostov-on-Don/27/2007	2428	∅	6.0
			A/tree sparrow/Rostov-on-Don/28/2007	2429	∅	6.0
			A/starling/Rostov-on-Don/39/2007	2435	∅	6.0
			Mean value:			**6.1**
		Poultry	A/chicken/Rostov-on-Don/31/2007	2430	⊗	7.5
			A/chicken/Rostov-on-Don/32/2007	2431	⊗	7.0

Table 1. Continued.

Date	Region	Ecologic group of birds	Strain[A]	RSCV deposition number	Clinical features[B]	$\log_{10}$ $TCID_{50}$/ml for SPEV
			A/chicken/Rostov-on-Don/33/2007	2432	⊕	7.0
			A/chicken/Rostov-on-Don/34/2007	2433	⊕	7.5
			A/chicken/Rostov-on-Don/35/2007	2434	⊕	7.0
			A/muscovy duck/Rostov-on-Don/51/2007	2436	⊕	7.0
			A/chicken/Rostov-on-Don/52/2007	2437	⊕	7.5
			Mean value:			**7.2**
April 2008	Suifun-Khanka Lowland (Primorsky krai) (28)	Wild	A/Anas crecca/Primorje/8/2008	2441	∅	4.0
			Mean value:			**4.0**
		Poultry	**A/chicken/Primorje/1/2008**	2440	⊕	4.5
			A/chicken/Primorje/11/2008	2442	⊕	4.0
			A/chicken/Primorje/12/2008	2443	⊕	4.5
			Mean value:			**4.3**

[A]Strains with complete genome sequences (Table 2) are marked by bold font.
[B]∅ = birds without clinical features; ⊗ = birds with clinical features; ⊕ = dead birds.

13/2005); $R \overset{341}{\rightarrow} G$ (A/chicken/Sudan/1784-{7,10}/2006); $R \overset{343}{\rightarrow} K$ (A/pied magpie/Liaoning/7/2005); $K \overset{344}{\rightarrow} R$ (A/whooper swan/Mongolia/7/2005). The presence of consensus G_{339} makes the Qinghai-Siberian clade different from other HPAI H5N1 variants from southeastern Asia containing consensus R_{339}. The highest portion of R_{339}—5/15 (33.3%)—was detected in the initial outbreak at Kukunor Lake (Qinghai, China). This suggests that the Qinghai-Siberian strains that originated from southeastern Asia were under a "bottleneck" selection at the early stage of their evolution. The NS1 protein has E_{92}, instead of the D_{92} that is common for virus variants from birds (13,15), and a five–amino acid deletion.

The Qinghai-Siberian clade includes viruses that have infected and caused severe disease and mortality in humans, but currently the virus does not appear to transmit efficiently in humans. We analyzed representative viruses in our collection for their potential to replicate in mammals. We found that isolated strains replicated effectively in mammalian cell culture lines BHK-21, LECH, Vero E6, MDCK, and SPEV (6,25). The PB2 has a consensus K_{627} that promotes virulence in mammalian cells (4,16). Six representative isolates from the Qinghai-Siberian clade have E_{627}, including A/bar-headed goose/Qinghai/2/2005, A/ruddy shelduck/Qinghai/1/2005, A/duck/Novosibirsk/02/2005, A/duck/Kurgan/08/2005, A/Cygnus olor/Astrakhan/Ast05-2-4/2005, and A/Cygnus olor/Italy/808/2006. These strains are uniformly distributed through time and territory as the result of stochastic nature of E_{627} and the absence of a tendency for K_{627} elimination. Substitutions that are correlated with virus tropism in mammals have been identified, including $D \overset{701}{\rightarrow} N$ in PB2 (4,10), $S \overset{714}{\rightarrow} R$ in PB2, $L \overset{13}{\rightarrow} P$ in PB1, $S \overset{678}{\rightarrow} N$ in PB1, and $K \overset{615}{\rightarrow} N$ in PA (8). In the viruses of the Qinghai-Siberian clade, proline is present in all the genomes in the 13th aa position of PB1, and asparagine at position 701 of the PB2 protein was found only in A/ruddy shelduck/Qinghai/1/2005.

Based on the amino acid sequence of HA receptor-binding sites of Qinghai-Siberian isolates we predict its affinity for α2′-3′-sialic acids common for intestinal avian cells (*vs.* α2′-6′-sialic acids of cells in the human respiratory tract) (9,34) containing E_{202}, Q_{238}, and G_{240} using aa numbering from the beginning of the protein including the leader sequence. Double mutation, $Q \overset{238}{\rightarrow} L$ and $G \overset{240}{\rightarrow} S$, or just the single mutation of $E \overset{202}{\rightarrow} D$ could switch HA receptor-binding affinity from avian to human receptors (34).

All the Qinghai-Siberian isolates are sensitive to amantadine, rimantadine, and oseltamivir, which has been confirmed by both direct biological experiments *in vitro* (19) and the presence (22,23,24,25,26,27,28) of marker substitutions (16,32,40) in M and NA virus proteins.

Decrease of *in vitro* reproduction potential of isolated strains (Fig. 3) is more evident for poultry ($TCID_{50} = 11.847 - 0.272 \times t$) in respect to wild birds ($TCID_{50} = 6.185 - 0.066 \times t$), where t is time expressed in months starting from the beginning of 2005. Table 3 facilitates comparison of phenotypic changes with point mutations in the proteins of HPAI H5N1/2.2 strains isolated in northern Eurasia.

Strains isolated from wild birds and poultry (Table 1) in Primorje (Far East) in April 2008 were found to be different from the clade 2.2 (Qinghai-Siberian) genotype and belonged to the clade 2.3.2. The A/chicken/Primorje/1/2008 and A/Anas crecca/Primorje/8/2008 were identical. The closest neighbor of Primorje 2008 strains are A/chicken/Viet Nam/10/2005 (nucleotide sequence similarity for HA gene is 97.5%), A/chicken/Guandong/178/2004 (97.3%), and A/duck/Viet Nam/12/2005 (97.2%). The cleavage site of HA—P_{337}QRERRRKRGLF_{348}—contained multiple basic amino acid motifs, which is typical for HPAI, but differs from the Qinghai-Siberian HA cleavage site. The 2008 isolates also belonged to group Z for the internal genes, had avian-type receptor specificity, were sensitive for M2-channel formation, and were neuraminidase inhibitors. Nevertheless, in contrast with HPAI H5N1/2.2 strains, which contained K_{627} (which promotes virulence in mammalian cells), the Far Eastern 2008 isolates contained E_{627} typical for avian-adaptive variants. Reduced tropism for mammalian cell lines was also verified by direct experiments *in vitro* (29).

A comparison of the biologic biochip of a representative isolate from the clade 2.2 and from 2.3.2 isolates show a clear difference in hybridization pattern in the hemagglutinin and neuraminidase genes (Fig. 4). The sequence similarity between A/chicken/Primorje/1/2008 and A/Anas crecca/Primorje/8/2008 *vs.* HA H5/2.2 strains are 92.9%–95.3% for HA and 94.1%–95.3% for NA nucleotide sequences. These differences are reflected by nucleotide probes of the biologic microchip that leads to different hybridization pattern for HA H5/2.2 and HA H5/2.3.2 in Fig. 4.

DISCUSSION

Northern Eurasia has the world's largest nesting area for migratory birds and it connects with Southeast Asia, Middle Asia, India, the Middle East, Africa, North America, and the Pacific islands by migration pathways (20). Every year large populations of nonim-

Table 2. Identification numbers of GenBank for HPAI H5N1 strains isolated in natural and anthropogenic ecosystems of northern Eurasia (2005–08) with complete nucleotide sequences of genomes (marked by bold font in Table 1).

Strain	HA genotype	Source of isolation	Complete nucleotide sequences							
			PB2	PB1	PA	HA	NP	NA	M	NS
A/grebe/Novosibirsk/29/05	2.2	Great crested grebe (*Podiceps cristatus*)	DQ232607	DQ232605	DQ234075	DQ230521	DQ232609	DQ230523	DQ234077	DQ234073
A/duck/Novosibirsk/56/05	2.2	Domestic duck (*Anas platyrhynchos domesticus*)	DQ232608	DQ232606	DQ234076	DQ230522	DQ232610	DQ230524	DQ234078	DQ234074
A/Cygnus olor/Astrakhan/Ast05-2-1/05	2.2	Mute swan (*Cygnus olor*)	DQ389161	DQ394578	DQ394579	DQ389158	DQ394577	DQ389159	DQ394576	DQ389160
A/Cygnus olor/Astrakhan/Ast05-2-2/05	2.2	Mute swan (*Cygnus olor*)	DQ343506	DQ343505	DQ343504	DQ343502	DQ359694	DQ343503	DQ359692	DQ359693
A/Cygnus olor/Astrakhan/Ast05-2-3/05	2.2	Mute swan (*Cygnus olor*)	DQ358750	DQ358749	DQ358748	DQ358746	DQ358751	DQ358747	DQ358739	DQ358752
A/Cygnus olor/Astrakhan/Ast05-2-4/05	2.2	Mute swan (*Cygnus olor*)	DQ363916	DQ363915	DQ363917	DQ363918	DQ363929	DQ363919	DQ363925	DQ363926
A/Cygnus olor/Astrakhan/Ast05-2-5/05	2.2	Mute swan (*Cygnus olor*)	DQ365011	DQ365008	DQ365007	DQ365004	DQ365006	DQ365005	DQ365009	DQ365010
A/Cygnus olor/Astrakhan/Ast05-2-6/05	2.2	Mute swan (*Cygnus olor*)	DQ365001	DQ365000	DQ364999	DQ364996	DQ364998	DQ364997	DQ365002	DQ365003
A/Cygnus olor/Astrakhan/Ast05-2-7/05	2.2	Mute swan (*Cygnus olor*)	DQ363921	DQ363920	DQ363922	DQ363923	DQ363930	DQ363924	DQ363928	DQ363927
A/Cygnus olor/Astrakhan/Ast05-2-8/05	2.2	Mute swan (*Cygnus olor*)	DQ386305	DQ386304	DQ399537	DQ399540	DQ399539	DQ399541	DQ399542	DQ399538
A/Cygnus olor/Astrakhan/Ast05-2-9/05	2.2	Mute swan (*Cygnus olor*)	DQ399543	DQ406738	DQ406737	DQ399547	DQ399545	DQ399546	DQ400912	DQ399544
A/Cygnus olor/Astrakhan/Ast05-2-10/05	2.2	Mute swan (*Cygnus olor*)	DQ434890	DQ423612	DQ434891	DQ434889	DQ440580	DQ440579	DQ434888	DQ434887
A/grebe/Tyva/Tyv06-1/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ914807	DQ914810	DQ978999	DQ914808	DQ916293	DQ914809	DQ914805	DQ914806
A/grebe/Tyva/Tyv06-2/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ852607	DQ852606	DQ852603	DQ852600	DQ852602	DQ852601	DQ852604	DQ852605
A/grebe/Tyva/Tyv06-8/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ863510	DQ863509	DQ863508	DQ863503	DQ863506	DQ863507	DQ863504	DQ863505
A/chicken/Moscow/2/07	2.2	Chicken (*Gallus gallus domesticus*)	EF474443	EF474444	EF474445	EF474450	EF474447	EF474448	EF474449	EF474446
A/chicken/Krasnodar/300/07	2.2	Chicken (*Gallus gallus domesticus*)	EU163436	EU163435	EU163434	EU163431	EU163432	EU163433	EU163429	EU163430
A/Cygnus cygnus/Krasnodar/329/07	2.2	Whooper swan (*Cygnus cygnus*)	EU257707	EU257636	EU257637	EU257631	EU257635	EU257632	EU257633	EU257634
A/pigeon/Rostov-on-Don/6/07	2.2	Rock dove (*Columba livia*)	EU441930	EU441931	EU441932	EU441937	EU441933	EU441936	EU441935	EU441934
A/rook/Rostov-on-Don/26/07	2.2	Rook (*Corvus frugilegus*)	EU814510	EU814509	EU814508	EU814503	EU814506	EU814505	EU814504	EU814507
A/starling/Rostov-on-Don/39/07	2.2	Starling (*Sturnus vulgaris*)	EU486848	EU486849	EU486850	EU486855	EU486851	EU486854	EU486853	EU486852
A/chicken/Rostov-on-Don/35/07	2.2	Chicken (*Gallus gallus domesticus*)	EU414265	EU420032	EU408333	EU401751	EU401754	EU401753	EU401752	EU401755
A/muscovy duck/Rostov-on-Don/51/07	2.2	Muscovy duck (*Cairina moschata*)	EU441922	EU441923	EU441924	EU441929	EU441925	EU441928	EU441927	EU441926
A/chicken/Primorje/1/08	2.3.2	Chicken (*Gallus gallus domesticus*)	EU672455	EU672456	EU672457	EU676174	EU672458	EU672460	EU676173	EU672459

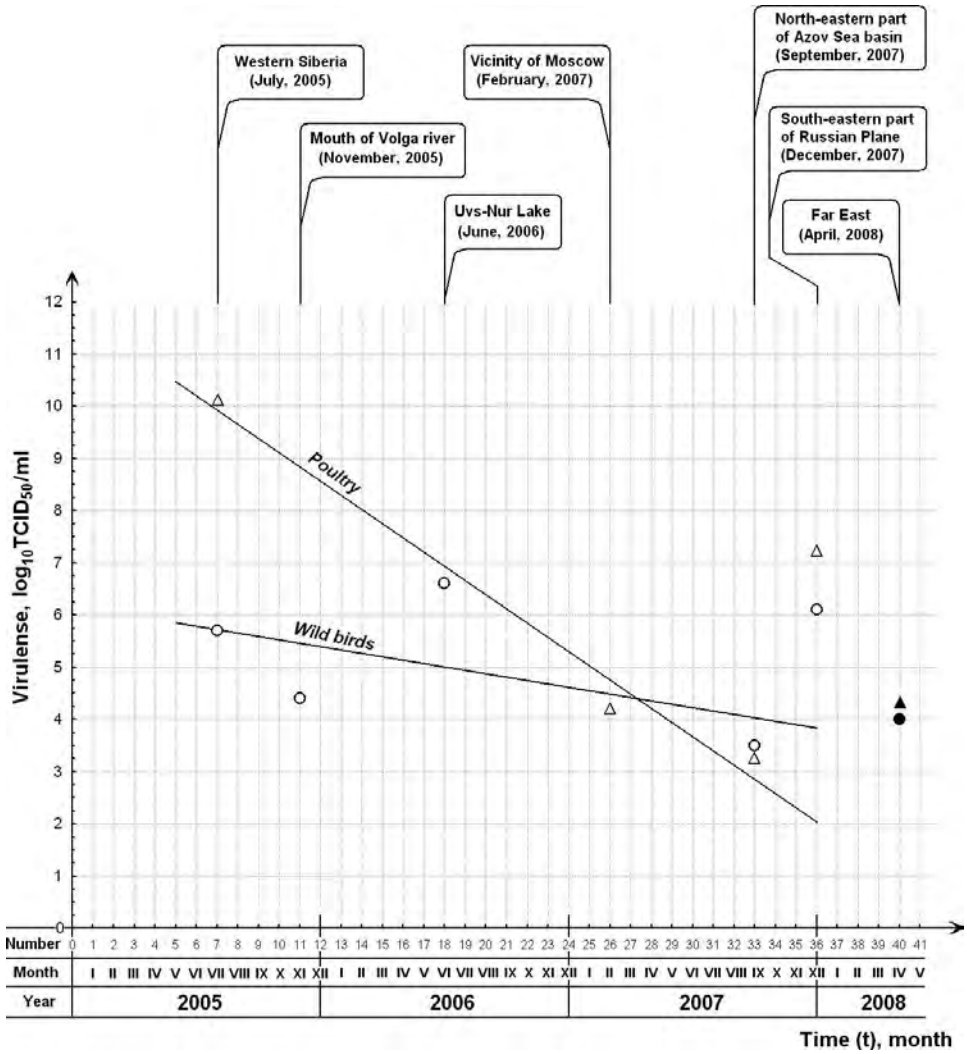

Fig. 3. Virulence dynamics of HPAI H5N1 strains isolated in northern Eurasia (2005–08): ○ = wild birds; △ = poultry; ● = genotype HA/H5/2.2; ▲ = genotype HA/H5/2.3.2. Epizooty in December 2007 was excluded from the trend calculation because of distinctive ecologic features described in the text.

mune juvenile birds are infected with one or more different influenza viruses with the potential generation of new virus variants, which are under intensive selective pressure in overwintering areas. On the eve of HPAI H5N1 epizooty starting in the autumn of 2003 we warned about the likelihood for outbreaks with the Asian lineage HPAI H5N1 at the International Conference ''Options for the Control of Influenza V'' (Okinawa, Japan, October 4–13, 2003) (31).

A second prediction was that overwintering migrating birds could transmit the HPAI virus into northern Eurasia during the spring migration. We discussed two possible scenarios of this introduction: through Dzhungarian (crossing with the Indian-Asian migration route) and via the Asian-Pacific migration routes. Preparing for these possible events we started doing increased surveillance in the south of western Siberia (Russian Foundation for Basic Research Project 03-a04-49158) and in Primorje (International Science-Technical Center Project 2800) in the spring of 2004. In April of 2005 a wide epizootic outbreak emerged at Kukunor Lake (Qinghai Province, China) and from this location we predicted the virus could spread at the Dzhungarian Gate, which links northwestern spurs of Tibet and West Siberian Lowland. The HPAI H5N1 epizooty, which emerged initially in the Chany Lake Depression in July 2005 and then spread to the south of western Siberia in August 2005, verified our prediction. This epizooty was likely introduced through the movements of infected wild birds and spread to poultry as a result of direct or indirect (water contamination) interactions. This was facilitated by the location of villages and poultry production facilities surrounded by wide marshes. Sick poultry (see Fig. 5A) had extremely high temperatures, depression, diarrhea, plumage shedding, corneal opacity, conjunctivitis, cerebral hemorrhages, lesions on viscera, and hemorrhages in mucosa, viscera and muscles (25,36). The viruses isolated in Siberia from wild birds and poultry (July 2005; Tables 1, 2) together with Qinghai strains (May 2005) (5,14) formed a separate clade in the Asian H5N1 lineage and demonstrated how quickly this lineage of virus could move within the region. The last process was facilitated by the presence of large populations of naive waterfowl that were going to migrate throughout overwintering areas.

The third prediction was that the virus would move with the migrating birds to the overwintering locations. In accordance with this prediction epizootic outbreaks occurred along the main migration routes in the Urals, the Russian Plain, Europe, Africa, Middle Asia, and India (1,2,3,10,12,23,37). In November 2005, the epizooty with mass deaths emerged in the downstream part of the mouth of the Volga River among the local population of mute swans

Table 3. Point mutations in the proteins of HPAI H5N1/2.2 strains isolated in northern Eurasia (2005–08).

A

Strains *	Amino acid substitutions **														
	NP					NA				M1	M2	NS1		NS2	
A/{*}/05	Y_{10}		N_{397}									D_{202}		M_{50}	I_{60}
↓	↓		↓			no				no	no	↓		↓	↓
A/{*}/{06–07}	**H_{10}**		**S_{397}**									**G_{202}**		**V_{50}**	**T_{60}**
A/duck/Novosibirsk/56/05			T_{403}			M_{29}			L_{143}						
↓			↓			↓			↓	no	no	no		no	
A/grebe/Novosibirsk/29/05			A_{403}			I_{29}			V_{143}						
A/duck/Novosibirsk/56/05			**T_{403}**					**L_{143}**							
↓			↓					↓		no	no	no		no	
A/Cygnus olor/Astrakhan/Ast05-2-{1-10}/05			A_{403}					V_{143}							
A/duck/Novosibirsk/56/05	Y_{10}		N_{397}		**T_{403}**	**L_{143}**			P_{320}			D_{202}		M_{50}	I_{60}
↓	▼		▼		↓	↓			▼	no	no	▼		▼	▼
A/grebe/Tyva/Tyv06-{1,2,8}/06	H_{10}		S_{397}		A_{403}	V_{143}			L_{320}			G_{202}		V_{50}	T_{60}
A/duck/Novosibirsk/56/05	Y_{10}	K_{90}	A_{373}	N_{397}	**T_{403}**	R_{44}			**L_{143}**			D_{202}		M_{50}	I_{60}
↓	▼	▼	▼	▼	↓	↓			↓	no	no	▼		▼	▼
A/chicken/Moscow/2/07	**H_{10}**	R_{90}	T_{373}	**S_{397}**	A_{403}	C_{44}			V_{143}			**G_{202}**		**V_{50}**	**T_{60}**
A/duck/Novosibirsk/56/05	Y_{10}		N_{397}		**T_{403}**	Q_{39}	G_{41}	**L_{143}**	P_{320}	K_{412}		I_{64}	D_{202}	M_{50}	I_{60}
↓	▼		▼		↓	↓	↓	↓	▼	↓	no	↓	▼	▼	▼
A/{*}/Krasnodar/{*}/07	**H_{10}**		**S_{397}**		A_{403}	L_{39}	R_{41}	V_{143}	L_{320}	E_{412}		M_{64}	**G_{202}**	**V_{50}**	**T_{60}**
A/duck/Novosibirsk/56/05	Y_{10}	K_{90}	A_{323}	A_{373}	N_{397}	**T_{403}**	A_{46}	V_{63}	I_{102}	**L_{143}**	Q_{288}	V_{68}	Q_{81}	D_{202}	M_{50}	I_{60}
↓	▼	▼	↓	▼	▼	↓	↓	↓	↓	↓	↓	↓	↓	▼	▼	▼
A/{*}/Rostov-on-Don/{*}/07	**H_{10}**	**R_{90}**	T_{323}	**T_{373}**	**S_{397}**	A_{403}	V_{46}	L_{63}	V_{102}	V_{143}	R_{288}	I_{68}	R_{81}	**G_{202}**	**V_{50}**	**T_{60}**

B

Strains *	Amino acid substitutions **																
	PB2							PB1					PB1-F2	PA		HA	
A/{*}/05																N_{171}	
↓			no						no				no	no		↓	
A/{*}/{06–07}																**D_{171}**	
A/duck/Novosibirsk/56/05	A_{69}	T_{103}	A_{221}	K_{718}			V_{200}				Q_{294}			T_{618}		H_{371}	
↓	↓	↓	↓	↓			↓				↓		no			↓	
A/grebe/Novosibirsk/29/05	E_{69}	F_{103}	T_{221}	R_{718}			I_{200}				H_{294}			A_{618}		R_{371}	
A/duck/Novosibirsk/56/05		**A_{69}**													L_{8}		N_{170}
↓		↓							no				no	no	↓		↓
A/Cygnus olor/Astrakhan/Ast05-2-{1-10}/05		E_{69}													F_{8}		D_{170}
A/duck/Novosibirsk/56/05	**A_{69}**		M_{473}				T_{182}				V_{451}				V_{11}	A_{512}	N_{171}
↓	↓		▼				▼				▼		no	no	▼	▼	▼
A/grebe/Tyva/Tyv06-{1,2,8}/06	E_{69}		T_{473}				I_{182}				L_{451}				I_{11}	S_{512}	D_{171}
A/duck/Novosibirsk/56/05	M_{50}	I_{64}	**A_{69}**	K_{126}	V_{338}	Y_{38}	I_{113}	L_{212}	E_{618}	M_{646}	S_{654}	S_{678}	A_{741}	V_{554}	T_{52}	N_{171}	L_{545}
↓	↓	▼	↓	▼	▼	↓	▼	↓	▼	↓	↓	↓	↓	▼	▼	▼	↓
A/chicken/Moscow/2/07	I_{50}	M_{64}	E_{69}	R_{126}	I_{338}	H_{38}	T_{113}	M_{212}	K_{618}	I_{646}	N_{654}	G_{678}	T_{741}	I_{554}	A_{52}	D_{171}	M_{545}
A/duck/Novosibirsk/56/05	**A_{69}**	I_{354}	M_{473}	V_{495}			T_{182}				V_{451}			R_{213}	D_{272}	V_{11} S_{12} A_{102} N_{171} R_{178} A_{512}	
↓	↓	↓	▼	↓			▼				▼		no	↓	↓	▼ ↓ ↓ ▼ ↓ ▼	
A/{*}/Krasnodar/{*}/07	E_{69}	V_{354}	T_{473}	I_{495}			I_{182}				L_{451}			K_{213}	E_{272}	I_{11} N_{12} S_{102} D_{171} I_{178} S_{512}	
A/duck/Novosibirsk/56/05	I_{64}	**A_{69}**	Q_{73}	K_{126}	V_{338}		I_{113}				E_{618}			R_{269}	S_{451} V_{554} Q_{51} T_{52} K_{56} I_{99} N_{171}		
↓	▼	↓	↓	▼	▼		▼				▼		no	↓	↓ ▼ ↓ ▼ ↓ ↓ ▼		
A/{*}/Rostov-on-Don/{*}/07	**M_{64}**	E_{69}	R_{73}	R_{126}	I_{338}		T_{113}				K_{618}			K_{269}	A_{451} I_{554} H_{51} A_{52} R_{56} N_{99} D_{171}		

* Group of strains is shown using braces: designations common for all strains in the given group are shown outside the braces; variable part of designations is cited inside the braces; the asterisk "*" means "any designation". Only mutations that are found in all the strains of the given group are listed in the table.

** **Bold font** indicates substitutions in respect to HPAI / H5N1 / 2.2 consensus; the frame – substitutions unique for Northern Eurasian strains (Tables 1–2) i.e. did not occur among Northern Eurasian strains previously; the frame with grey background – substitutions unique for all HPAI / H5N1 / 2.2 genotype (strains isolated in both Northern Eurasia and other places); «↓» – substitution that takes place in the strains of the given epizootic outbreak only; «▼» – substitution that takes place in the strains of both the given and late/previous epizootic outbreaks.

(*Cygnus olor*) (23). Swans had neurological disorders including inability to keep the neck or head raised (Fig. 5B), paralysis of the extremities (mainly legs), and depression. Because there are no human settlements in this part of the Volga River, no infections were detected among poultry. Migrating tufted ducks (*Aythya fuligula*), among which clinical features were not detected, are suspected to be the source of infection because the start of the epizooty coincided with tufted duck appearance. The sequences of the swan viruses, including A/Cygnus olor/Astrakhan/Ast05-2/2005, were closely related to the western Siberian strains (23) (Fig. 2) indicating distribution of virus through the Eastern European flyway of birds connecting western Siberia, the Russian Plain, Eastern Europe, the Middle East, and Africa (20).

Our fourth prediction was the return of virus in migrating birds from their overwintering places to northern Eurasia in the spring of 2006 with widening of the epizootic. Dramatic events occurred June 10–28, 2006, at the Uvs-Nur Lake, which is situated on the boundary of the Great Lakes Depression of Mongolia and Tyva

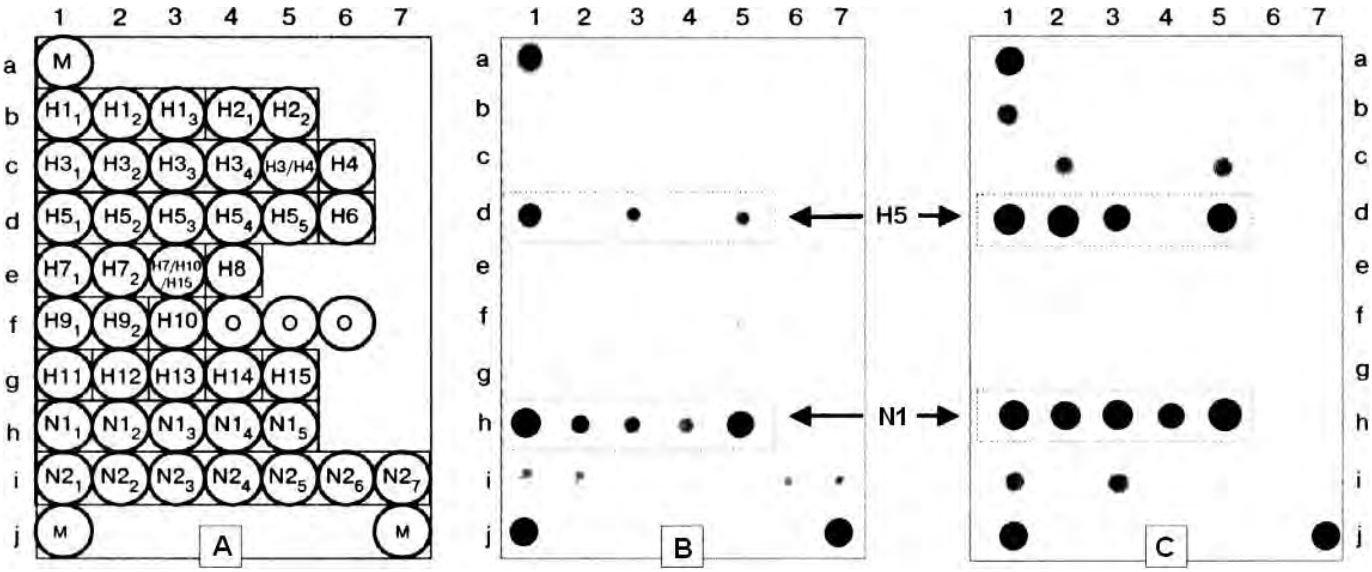

Fig. 4. Subtyping of HPAI H5/2.2 and 2.3.2 virus strains using biological microchips. (A) Biochip structure. (B) Hybridization pattern for A/chicken/Novosibirsk/64/2005 belonging to HA H5/2.2 (Qinghai-Siberian) genotype. (C) Hybridization pattern for A/chicken/Primorje/1/2008 belonging to HA H5/2.3.2 genotype.

Republic of Russia (24), where more than an estimated 3000 birds died in the Russian part of this lake, which is only about 1% of total area of the lake. The most affected species was crested grebes (*Podiceps cristatus*; Fig. 5C) as well as coots (*Fulica atra*; Fig. 5D) and cormorants (*Phalacrocorax carbo*). Terns and gulls were involved in the epizootic to significantly lesser extent. Absence of poultry farms in the vicinity of the Uvs-Nur Lake excluded outbreaks among poultry. The Tyva strains appeared to be the beginning of a new genetic lineage in the Qinghai-Siberian genotype that was designated as the ''Tyva-Siberian'' subgroup (Fig. 2), which was isolated not only in Siberia, but also in the Europe. It is believed that the Tyva-Siberian subgroup emerged in 2006 on nesting grounds of wild ducks in western Siberia. These birds were thought to have been infected in 2005 at the Great Lake Depression and at overwintering grounds in India. After the virus was introduced into the nesting place of northern Eurasia it was amplified.

A set of nine outbreaks occurred in the outskirts of Moscow beginning in February 2007. The occurrence of the outbreak at this time of year seems to preclude the participation of wild waterfowl in virus introduction or spread, and terrestrial wild birds were negative according to RT-PCR. Virus was isolated (see Table 1) from dead and sick poultry, and all the isolates were identified as HPAI H5N1/2.2 with high level of sequence similarity to the Qinghai-Siberian subgroup. This implied a common source of infection for all the outbreaks, and subsequent epidemiologic investigation demonstrated a link to the live bird markets in Moscow, where the affected farmers had purchased poultry several days before. The complete genome analysis of the prototype A/chicken/Moscow/2/2007 (27) revealed that the highest similarity occurred for the strains isolated in the Caucasian region during 2005–06 winter: A/cat/Dagestan/87/2006, A/Cygnus cygnus/Iran/754/2006, A/chicken/Krasnodar/01/2006, A/chicken/Adygea/203/2006 (similarity for nucleotide sequences of PB2 was 99.5%; PB1, 99.3%; PA, 99.7%; HA, 99.1%–99.4%; NP, 99.4%; NA, 99.1%–99.6%; M1, 99.5%–99.9%; NS1, 99.9%). The closest neighbor for A/chicken/Moscow/2/2007 was found to be A/Cygnus cygnus/Iran/754/2006. Later, this genetic subgroup of HPAI H5N1/2.2 was designated as ''Iran–North Caucasian'' (Fig. 2). Nevertheless, A/chicken/Moscow/2/2007 significantly differed from other Qinghai-Siberian strains. In 4 genes—PB2, PB1, HA, and NP—there were 12 unique amino acid substitutions (Table 3). Moreover, all eight amino acid substitutions in PB1 were unique at that time. This was the evidence of active circulation of virus before 2007. However, the specific origin of A/chicken/Moscow/2/2007 has not been officially identified, and it is suspected that virus was circulating in a small intermountain valley ecosystem in the North or South Caucasus in the winter of 2007 and that virus was introduced in the live bird market through contaminated poultry cages or grain.

In September 2007, an outbreak was detected on a chicken farm ''Lebyazhje-Chepiginskaya'' in the Krasnodar region (northeastern part of the Azov Sea basin). The virus isolates, A/chicken/Krasnodar/300/2007 from poultry and A/Cygnus cygnus/Krasnodar/329/2007 from sick whooper swan (*Cygnus cygnus*), found in the liman (shallow gulf) near the farm were closely related to each other (two synonymous nucleotide substitutions in PB1; two synonymous substitutions in PB2; one nonsynonymous substitution in M1; two nonsynonymous substitutions in NA; one nonsynonymous substitution in NS1) and belonged to the Iran–North Caucasian subgroup of the Qinghai-Siberian genotype (Fig. 2). Isolated strains contained 10 unique amino acid substitutions with respect to the Qinghai-Siberian consensus in PB2, PA, HA, NA, and NS1, which suggested that regional variants were continuing to emerge.

In December, 2007 infection of a poultry farm, Gulyai-Borisovskaya, in the Rostov region took place. Unfortunately, the infection was not reported in time and infected poultry manure was spread on adjacent fields, where wild terrestrial birds could be infected (28). This exposure is thought to have contributed to the infection of a number of species including (Fig. 5E) rooks (*Corvus frugilegus*), jackdaws (*Corvus monedula*), rock doves (*Columba livia*), common starlings (*Sturnus vulgaris*), tree (*Passer montanus*) and house (*Passer domesticus*) sparrows, and others. Surveillance of these species detected H5 virus by RT-PCR in 60% of pigeons and crows, in around 20% of starlings, and in 10% of tree sparrows without clinical features. These results were confirmed by virus isolation from wild birds and poultry (Table 1). Birds whose infection was confirmed by RT-PCR and virus isolation seemed reluctant to move and had ruffled feathers. On necropsy the birds were observed to have conjunctivitis and hemorrhages on lower extremities;

Fig. 5. Sick birds as the result of HPAI H5/2.2 virus infection. (A) Sick domestic duck (*Anas platyrhynchos domesticus*; south of western Siberia; July 2005). (B) Sick mute swan (*Cygnus olor*; mouth of Volga River; November 2005). (C) Sick great crested grebe (*Podiceps cristatus*; Uvs-Nur Lake; June 2006). (D) Sick coot (*Fulica atra*; Uvs-Nur Lake; June 2006). (E) Rooks (*Corvus frugilegus*) on the mixed fodder ground at the poultry farm Gulyai-Borisovskaya (Rostov region; December 2007). (F) Intestinal vessel plethora and changes in pancreas structure of infected rook (*Corvus frugilegus*) from the poultry farm Gulyai-Borisovskaya (Rostov region; December 2007).

hemorrhage in muscle, adipose tissue, intestine, mesentery, and brain; and changes in pancreas and liver structure (Fig. 5F). Wide involvement of wild terrestrial birds in virus circulation, presumably from the exposure to infected chicken manure, distinguished this outbreak from others (in particular virulence data of this outbreak are excluded from virulence trend analysis presented on Fig. 3). Genome analysis revealed the isolated strains belonged to the Iran–North Caucasian subgroup of the Qinghai-Siberian genotype (Fig. 2). They were phylogenetic similar to A/chicken/Moscow/2/2007 and had 13 unique amino acid substitutions with respect to Qinghai-Siberian consensus in PB2, PA, HA, NP, NA, and M2 (see Table 3).

Genetic stratification of the Qinghai-Siberian (2.2) genotype of HPAI H5N1 virus in northern Eurasia appeared to occur on the following ecologic model. In summer 2005, western Siberian cluster variants selected during epizootic outbreak at Kukunor Lake (Qinghai Province, China) were amplified in the summer nesting places. In winter 2005–06, HPAI H5N1/2.2 was under selection in two main overwintering areas: Africa, Transcaucasia, and Middle East (penetrating along Eastern and Western European flyways) as

Table 4. Possible influence of amino acid substitutions on their virulence (see Table 1, Fig. 3).[A]

Protein	Position	Virulence		Functional region of the protein	Influenced process of virus life cycle
		Increased	Decreased		
PB2	69	E[B]	A[B]	Domain of noncovalent binding with C-terminus of PB1	Formation and functioning of polymerase complex PB2-PB1-PA
	73	Q	R		
	221	A	T		
	473	M	T	NLS	Import of RNP into the nucleus
PB1	212	L	M	NLS	Import of RNP into the nucleus
	294	Q	H	Enzyme polymerase center	Functioning of polymerase complex PB2-PB1-PA
	451	V	L		
	618	E	K	Domain of noncovalent binding with N-terminus of PB2	Formation and functioning of polymerase complex PB2-PB1-PA
	678	S	G		
	741	A	N		
PA	213	R	K	NLS	Import of RNP into the nucleus
HA	512	A	S	Elongation fragment during fusion peptide disengagement	Virion and endosomal membrane fusion
	545	L	M	C-terminus transmembrane domain	
NP	10	Y	H	NLS	Import of RNP into the nucleus
	323	A	T		
	373	A	T		
	397	N	S		
	403	T	A		
NA	29	M	I	N-terminus transmembrane domain	New virion assembling
M2	68	I	V	C-terminus cytoplasm domain	Interaction between M2 and M1
	81	R	Q		
NS1	64	I	M	Unknown	Interaction with signal systems of infected cell
	202	D	G		
NS2	50	M	V	Terminus of α-helix N2	Export of RNP from the nucleus
	60	I	T	Base of α-helix C1	

[A]NLS = nuclear location signal; RNP = ribonucleoproteide.
[B]Letters are abbreviations for amino acids.

well as India and Central Asia (penetrating along Indian-Asian flyway) (20). The first overwintering area could be the source for the Iran–North Caucasian subgroup whereas the second could be the source for the Tyva-Siberian subgroup. Returning back to nesting areas in northern Eurasia in the spring of 2006, wild birds started the amplification of the variant subgroups, which were naturally mixed in the south of the Russian Plain, on the crossroad of different flyways.

In April 2008, the second breach of HPAI H5N1 into northern Eurasia emerged in Primorje (in the Far East) and was linked with another genotype 2.3.2 (29). The epizootic originated from unvaccinated poultry in outermost backyard of Vozdvizhenka village, located by a small river and watery meadow, where poultry often interacted with wild waterfowl. One initial theory of introduction to poultry was from exposure to hunted ducks, but the direct interaction of wild birds with poultry seems more likely. The isolates (Table 1) from dead chickens and common teal (*Anas crecca*) collected in the vicinity of epizootic farms were identical and indicated a direct role of migrating birds in virus introduction. Common teal, which appeared to be the most likely source of infection for poultry, had no obvious behavior changes but on necropsy they did have hemorrhagic lesions on the intestines. It is interesting to underline that it was common teals that were the source of isolation of H5 strains in Primorje in autumn 2001. Common teals migrate for a long distances, so the hypothesis is that HPAI H5N1/2.3.2 variants migrated from the Far East, possibly southern China, Vietnam, or Laos. The HPAI H5N1 virus was widely distributed in Primorje in spring of 2008; according to RT-PCR, 26% of wild ducks in the Suifun River–Khanka Lake lowland were infected (29). However, during monitoring in autumn of 2008 we were not able to find HPAI H5N1. Nevertheless, emergence of HPAI H5N1 virus in Primorje creates a new type of HPAI stratification in northern Eurasia (genotype 2.2 in the eastern areas and 2.3.2 in the western sectors of this subcontinent) as well as a threat of HPAI introduction into North America.

Comparison of data from Table 3 and Fig. 3 allows localization amino acid substitutions that are possibly associated with the level of *in vitro* replication potential. Possible functional roles of such amino acid substitutions are suggested in Table 4.

Thus, using the example of HPAI H5N1 evolution in ecosystems of northern Eurasia (2005–08) we have attempted to illustrate the concept that emerging and reemerging infections such as HPAI can not be completely eliminated in the near future. Nevertheless, ecologic monitoring and adequate data analysis could lead to well-timed predictions, which can provide opportunities to mitigate disease outbreak consequences.

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Research Note—

Biologic Characterization of Chicken-Derived H6N2 Low Pathogenic Avian Influenza Viruses in Chickens and Ducks

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SUMMARY. Low pathogenic avian influenza H6N2 viruses were biologically characterized by infecting chickens and ducks in order to compare adaptation of these viruses in these species. We examined the clinical signs, virus shedding, and immune response to infection in 4-wk-old white leghorn chickens and in 2-wk-old Pekin ducks. Five H6N2 viruses isolated between 2000 and 2004 from chickens in California, and one H6N2 virus isolated from chickens in New York in 1998, were given intrachoanally at a dose of 1×10^6 50% embryo infectious dose per bird. Oral–pharyngeal and cloacal swabs were taken at 2, 4, and 7 days postinoculation (PI) and tested by real-time reverse-transcriptase polymerase chain reaction for presence of virus. Serum was collected at 7, 14, and 21 days PI and examined for avian influenza virus antibodies by commercial enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) testing. Virus shedding for all of the viruses was detected in the oral–pharyngeal swabs from chickens at 2 and 4 days PI, but only three of the five viruses were detected at 7 days PI. Only two viruses were detected in the cloacal swabs from the chickens. Virus shedding for four of the five viruses was detected in the oral–pharyngeal cavity of the ducks, and fecal shedding was detected for three of the viruses (including the virus not shed by the oral–pharyngeal route) in ducks at 4 and 7 days PI. All other fecal swabs from the ducks were negative. Fewer ducks shed virus compared to chickens. Both the chickens and the ducks developed antibodies, as evidenced by HI and ELISA titers. The data indicate that the H6N2 viruses can infect both chickens and ducks, but based on the number of birds shedding virus and on histopathology, the viruses appear to be more adapted to chickens. Virus shedding, which could go unnoticed in the absence of clinical signs in commercial chickens, can lead to transmission of the virus among poultry. However, the viruses isolated in 2004 did not appear to replicate or cause more disease than earlier virus isolates.

RESUMEN. *Nota de Investigación*—Caracterización biológica en pollos y patos de virus de la influenza aviar de baja patogenicidad H6N2 derivados de pollos.

Se caracterizaron de manera biológica los virus de la influenza aviar de baja patogenicidad H6N2 mediante la infección de pollos y patos para comparar la adaptación de estos virus en estas especies. Se examinaron los signos clínicos, la eliminación viral y la respuesta inmune a la infección en aves leghorn de cuatro semanas de edad y en patos Pekín de dos semanas de edad. Cinco virus H6N2 que fueron aislados entre los años 2000 y 2004 en pollos de California y un virus H6N2 aislado de pollos en Nueva York en el año 1998, fueron administrados por vía intracoanal en una dosis de 1×10^6 dosis infectantes 50% por ave. Se recolectaron hisopos orofaríngeos y cloacales a los 2, 4 y 7 días postinoculación y se analizaron por la prueba de transcripción reversa y reacción en cadena de la polimerasa en tiempo real para detectar la presencia del virus. Se recolectaron muestras de sueros a los días 7, 14 y 21 después de la inoculación y se examinaron para detectar anticuerpos mediante la prueba de inmunoabsorción con enzimas ligadas (ELISA) y por la prueba de inhibición de la hemaglutinación (HI). Se detectó eliminación viral con todos los virus en los hisopos orofaríngeos a los días 2 y 4 después de la inoculación, pero solo tres de los cinco virus se detectaron a los 7 días post inoculación. Solo dos virus se detectaron en los hisopos cloacales de los pollos. Se detectó eliminación viral en la cavidad orofaríngea de los patos con cuatro de los cinco virus y la eliminación fecal se detectó con tres virus (incluyendo los virus no eliminados por la ruta orofaríngea) en los patos a los 4 y 7 días postinoculación. Todos los otros hisopos fecales de los patos fueron negativos. Un número menor de patos eliminaron virus en comparación con los pollos. Los pollos y los patos desarrollaron anticuerpos, tal como se hizo evidente por los títulos de HI y ELISA. Estos datos indican que los virus H6N2 pueden infectar pollos y patos, pero de acuerdo con el número de aves que eliminaban virus y a la histopatología, estos virus parecen estar más adaptados a los pollos. La eliminación viral, que puede pasar desapercibida por la ausencia de signos clínicos en pollos comerciales puede facilitar la transmisión viral en la avicultura comercial. Sin embargo, los virus aislados en el año 2004 no parecen poseer una replicación mayor o causar enfermedad más severa que los virus aislados previamente.

Key words: avian influenza virus, H6N2, chickens, ducks, California live bird market, biologic characterization, pathogenicity

Abbreviations: AIV = avian influenza virus; Ct = cycle threshold; EID_{50} = 50% embryo infectious dose; ELISA = enzyme-linked immunosorbant assay; HI = hemagglutination inhibition; LPAI = low pathogenicity avian influenza viruses; PBS = phosphate-buffered saline; PI = postinoculation; RT-PCR = reverse transcriptase-polymerase chain reaction; SPF = specific-pathogen-free

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Avian influenza viruses (AIV) are worldwide in distribution and continue to be a threat to commercial poultry as well as to human health. Wild aquatic birds (Charadriiformes and Anseriformes), which include shorebirds, ducks, geese, and swans, are the natural reservoirs of AIV and play an important role in the ecology of the virus. Sometimes, these AIVs transmit from wild aquatic to domestic birds, producing subclinical infections and, occasionally, respiratory disease and drops in egg production. These viruses are typically classified as low pathogenicity avian influenza viruses (LPAI) in standard intravenous pathogenicity studies in chickens. The viruses in wild birds include at least 16 antigenically distinct hemagglutinin subtypes, and most have been isolated from poultry. Only a few H5 and H7 LPAI viruses have mutated to produce highly pathogenic avian influenza viruses after circulating in domestic poultry (11). Although little or no disease is usually associated with LPAI viruses in commercial chickens, they can infect, and are shed by, the birds (10). Some LPAI viruses have adapted to efficient replication in poultry, causing more prominent clinical signs and mortality (2).

The H6 subtype viruses are present in ducks and presumably can transmit to commercial chickens (4,8,13). In February 2000, an outbreak of H6N2 LPAI occurred in California, and 12 separate incidences were reported which primarily involved layer-type birds, but viruses were also isolated from backyard chickens and a primary broiler breeder (3,15). A drop in egg production and an increase in mortality were among the clinical signs reported in the layer flocks; however, the pathologic changes observed in theses earlier cases were primarily associated with mild respiratory infections. Yolk peritonitis was a feature later described for cases in 2001 and 2002 (15). The H6N2 subtype LPAI viruses continued to be detected in commercial chickens in California in 2002, 2003, and 2004, and an autogenous killed vaccine was developed to eradicate the virus (4).

The LPAI California H6N2 viruses, isolated from 2000 to 2002, likely had a common ancestor based on the close sequence similarity of the hemagglutinin gene, but analysis of all eight gene segments demonstrated different constellations of genes, likely from reassortment viruses between aquatic bird and chicken AIVs (14). In addition, those LPAI H6N2 viruses circulating between 2000 and 2002 were shown to have an 18 amino acid deletion in the neuraminidase protein, which is associated with adaptation to growth in chickens; however, the isolates were reported to be nonpathogenic in chickens by standard intravenous pathotyping studies (1,13,14).

In this study, we were interested in biologically characterizing H6N2 LPAI viruses isolated from chickens in California in 2000, 2002, and 2004 in order to determine adaptation of these viruses for chickens by examining infection and replication, and also to see if, by adaptation to chickens, these viruses are less adapted to ducks. One H6N2 LPAI virus isolate from New York was also studied for comparison purposes. We examined the clinical signs, virus shedding, adaptive immune response, and microscopic lesions in 4-wk-old white leghorn chickens and in 2-wk-old Pekin ducks challenged with the viruses.

MATERIALS AND METHODS

Viruses. All of the viruses used in this study were LPAI viruses. The virus designations and titers are presented in Table 1. Isolate A/CK/NY/14677-13/98 was the only virus examined from outside of California (6). Isolates from California include A/CK/CA/431/00 (15), isolated from a rooster in a back yard flock (thought to be the initial case); A/CK/CA/139/01 (14,15), isolated from a chicken in a mixed flock that included ducks and squabs; A/CK/CA/1255/02 (15), isolated from 118 1-wk-old layers; A/CK/CA/6028/04 from a bird at a live-bird-market custom slaughter plant; and A/CK/CA/7211/04 from a bird in a feed and pet supply store in Los Angeles that was in close proximity to the custom slaughter plant where A/CK/CA/6028/04 was isolated (Woolcock, pers. comm.).

Table 1. Viruses examined in this study.

Virus isolate	GenBank accession no.	Titer (EID_{50}/ml)
CK/NY/14677-13/98[A]	DQ021663	$1 \times 10^{5.9}$
CK/CA/431/00	AF474039	$1 \times 10^{8.0}$
CK/CA/139/01	AF457711	$1 \times 10^{8.0}$
CK/CA/1255/02[B]	GQ358535	$1 \times 10^{5.5}$
CK/CA/6028/04	GQ358533	$1 \times 10^{8.3}$
CK/CA/7211/04	GQ358535	$1 \times 10^{7.9}$

[A]Only ducks were exposed to this isolate.
[B]Only chickens were exposed to this isolate.

Experimental design. A minimum of eight, 4-wk-old SPF white leghorn chickens (Merial Select, Gainesville, GA) and 2-wk-old Pekin ducks (Metzer Farms, Gonzalas, CA) per group were used in this study. All experiments were conducted in a biosafety level 2 Ag+ facility at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, the University of Georgia (Athens, GA) in accordance with U.S. Department of Agriculture's Animal and Plant Health Inspection Service permit number 103372. Birds were housed in stainless steel and polycarbonate, negative pressure, HEPA filtered, isolator units with internal dimensions of 42'' L × 24'' W × 31'' H.

Viruses were diluted with sterile phosphate-buffered saline (PBS, pH 7.4) to adjust the amount of inoculum to 1×10^6 50% embryo infectious dose (EID_{50}) per 0.1 ml per bird. For viruses below 1×10^6 EID_{50} per 0.1 ml concentration, 0.1 ml of undiluted virus per bird was given. The viruses were administered via the oropharyngeal (intrachoanal) route. For each experiment, one group of birds was not inoculated and served as negative controls. After inoculation, all birds were observed for clinical signs of disease and mortality twice daily for 21 days. Clinical signs of disease were scored and recorded as follows: 0 = no signs; 1 = mild to moderate respiratory signs; 2 = moderate respiratory signs, depressed or not eating; 3 = moderate to severe respiratory signs, depressed, not eating, and neurologic signs; 4 = moribund birds (were removed and necropsied immediately).

Oropharyngeal and cloacal swabs were collected in 1 ml of sterile PBS from each bird at 2, 4, and 7 days postinoculation (PI), stored at −80 C, and thawed only once for RNA extraction. Serum was also collected from each bird at 7, 14, and 21 days PI and stored at −20 C. Tissue samples for histopathology were collected from three birds per group at 3 days PI.

RNA extraction and quantitative, real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Viral RNA was extracted from swab samples using the MagMax-96 Total RNA isolation kit (Ambion Inc., Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's recommendations.

The Ambion Ag Path ID One Step RT-PCR kit (Ambion Inc.) was used for nucleic acid amplification with a 25-μl reaction mixture containing the following reagents: 12.5 μl of kit-supplied 2× RT-PCR buffer, 1 μl of kit-supplied 25× RT-PCR enzyme mix, and 10 μl of extracted viral RNA. Each reaction mixture utilized 10 picomoles of matrix gene primers (forward and reverse) and probe sequences (all three using 0.5 μl each), following the protocol of the real time RT-PCR assay developed for type A influenza virus (5). Real-time RT-PCR was carried out in a Smart Cycler thermocycler machine (Cepheid, Sunnyvale, CA) with the following conditions for the RT step (50 C for 30 min and 94 C for 15 min) and the PCR cycling protocol (94 C for 15 sec and 60 C for 20 sec for 45 cycles). Data were reported as the average cycle threshold (Ct) value.

Histopathology. Microscopic examination was conducted on the following tissues: heart, lung, liver, spleen, pancreas, duodenum, jejunum, cecum, cecal tonsils, ileum, bursa of Fabricius, breast and thigh muscle, thymus, nasal cavity, and brain. Tissue samples were fixed

Table 2. Virus detection in chickens presented as real-time RT-PCR cycle threshold values.[A]

	Days postchallenge					
	Day 2		Day 4		Day 7	
Virus isolate	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
A/CK/CA/431/00	31.6/5.7 (8/10)[B]	Neg (0/10)	28.7/6.6 (7/7)	Neg (0/7)	32.3/5.6 (2/7)	31.2/5.9 (1/7)
A/CK/CA/139/01	28.4/6.68 (10/10)	37.3/4.2 (1/10)	29.5/6.4 (7/7)	34.4/5.0 (1/7)	Neg (0/7)	Neg (0/7)
A/CK/CA/1255/02	30.25/6.2 (10/10)	Neg (0/10)	33.8/5.2 (7/7)	Neg (0/7)	35.8/4.6 (1/7)	Neg (0/7)
A/CK/CA/6028/04	32.1/5.6 (10/10)	Neg (0/10)	33.2/5.3 (7/7)	Neg (0/7)	33.7/5.2 (2/7)	Neg (0/7)
A/CK/CA/7211/04	33.1/5.4 (9/10)	Neg (0/10)	34.1/5.1 (7/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)
Controls	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)

[A]Average Ct value for the positive birds (values greater than 38.5 were considered negative [Neg])/relative viral equivalents ($\log_{10}$)/ml calculated from the average Ct value and a standard curve for the H6 LPAI viruses ($y = -0.278x + 14.58$).
[B]Number positive per total examined.

in 10% neutral-buffered formalin, routinely processed, and embedded into paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin and examined by light microscopy.

Serologic testing. Chicken sera were tested for antibodies to AIV using a commercial enzyme-linked immunosorbant assay (ELISA) kit, the FlockChek™ avian influenza virus antibody test (IDEXX, Portland, ME). Duck sera were tested using the avian influenza MultiS-Screen ELISA kit (IDEXX). In addition, samples were tested for antibodies by the hemagglutination inhibition (HI) test using 4 HA units of the CK/CA/431/00 virus (11).

Sequencing. The HA1 region of the influenza HA genes were amplified by RT-PCR as previously described (9). The RT-PCR products were purified by agarose gel extraction with the Qiaquick gel extraction kit (Qiagen, Inc., Valencia, CA) and were directly sequenced. The BigDye terminator kit (Applied Biosystems, Foster City, CA) was used for cycle sequencing and subsequently run on an ABI 3730 sequencer (Applied Biosystems).

Phylogenetic and sequence analysis. The HA1 coding region of the HA genes (nucleotides: 1–1035) were aligned with Clustal V (Lasergene, V. 8.0.2 DNAStar, Inc., Madison WI). The phylogenetic tree was generated using the neighbor-joining distance method (DNAStar, Inc.) and confirmed by the maximum parsimony method with 1000 bootstrap replicates in a heuristic search using the PAUP 3.1 software program (MacDNASIS, Hatachi Software Engineering America, Ltd., San Bruno, CA).

RESULTS AND DISCUSSION

The titers of the viruses used to challenge the chickens and ducks are presented in Table 1. For comparison purposes, we wanted to use the same titered virus stock for both chickens and ducks. Unfortunately, two of the viruses, CK/NY/14677-13/98 and CK/CA/1255/02, grew to low titers in eggs and, thus, were only given to ducks and chickens, respectively, because there was not enough virus from the same stock to infect both species. All of the other viruses were given to both chickens and ducks. No clinical signs were observed in either species during the experiment, which is typical of LPAI viruses (7,10). Two of the viruses (CK/CA/431/00 and CK/CA/139/01) were previously shown to be infectious in experimentally exposed chickens, but did not cause any overt clinical signs (14).

The virus detection data is presented in Table 2 (chickens) and Table 3 (ducks). No virus was detected in the nonchallenged control birds. All of the viruses given to the chickens were detected in the oropharyngeal swabs. With the exception of two birds given CK/CA/431/00 and one bird given CK/CA/7211/04, both necropsied on day 2 PI, all of the oropharyngeal swabs from the chickens were positive on days 2 and 4 PI. Virus was not detected in the oropharyngeal swabs from most of the chickens by day 7 PI. Previously, the CK/CA/139/01 virus was reported to have been recovered from the cloacae of an infected chicken after an intravenous challenge (14). We found CK/CA/139/01 in the cloacal swab from one chicken on day 2 PI and from one chicken on day 4 PI. We also detected virus in the cloacal swab from one chicken given CK/CA/431/00 at day 7 PI. Virus was not detected in the cloacal swabs from any of the other chickens.

In the ducks, virus was detected in the oropharyngeal swabs for four of the five viruses, and three viruses, CK/NY/14677-13/98, CK/CA/139/01, and CK/CA/6028/04, induced shedding from the cloaca, which was detected at day 4 postchallenge for the CK/NY/14677-13/98 virus and at day 7 postchallenge for the CK/CA/139/01 and CK/CA/6028/04 viruses. In general, the Ct values for virus samples from chickens were lower (indicating relatively more virus) than the samples from the ducks, and a greater number of chickens were infected, compared to ducks, for the viruses that were given to both species. Taken together, these data indicate that the viruses

Table 3. Virus detection in ducks presented as real-time RT-PCR cycle threshold values.[A]

	Days postchallenge					
	Day 2		Day 4		Day 7	
Virus isolate	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
A/CK/NY/14677-13/98	27.4/7.0 (3/10)[B]	Neg (0/10)	30.2/6.2 (7/7)	19.0/9.29 (7/7)	34.5/5.0 (4/7)	Neg (0/7)
A/CK/CA/431/00	29.0/6.5 (3/10)	Neg (0/10)	32.6/5.5 (1/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)
A/CK/CA/139/01	33.3/5.3 (2/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	32.5/5.5 (1/7)
A/CK/CA/6028/04	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	27.9/6.8 (5/7)
A/CK/CA/7211/04	33.4/5.3 (4/10)	Neg (0/10)	29.3/6.4 (1/7)	Neg (0/7)	37.4/4.2 (1/7)	Neg (0/7)
Controls	Neg (0/10)	Neg (0/10)	Neg (0/7)	Neg (0/7)	Neg (0/7)	Neg (0/7)

[A]Average Ct value for the positive birds (values greater than 38.5 were considered negative [Neg])/relative viral equivalents ($\log_{10}$)/ml calculated from the average Ct value and a standard curve for the H6 LPAI viruses ($y = -0.278x + 14.58$).
[B]Number positive per total examined.

Table 4. Hemagglutination inhibition (HI) antibody titers against avian influenza virus and the number of serologically positive chickens by ELISA.

	Days postchallenge					
	Day 7		Day 14		Day 21	
Virus isolate	HI[A]	ELISA[B]	HI	ELISA	HI	ELISA
A/CK/CA/431/00	64.6 (5/7)	1/7	476.9 (7/7)	5/7	749.7 (7/7)	2/7
A/CK/CA/139/01	71.6 (7/7)	3/7	50.6 (7/7)	2/7	72.3 (7/7)	4/7
A/CK/CA/1255/02	40.3 (7/7)	2/7	41.1 (7/7)	5/7	67.8 (7/7)	5/7
A/CK/CA/6028/04	126.8 (7/7)	4/7	87.1 (6/7)	1/7	78.0 (6/7)	5/7
A/CK/CA/7211/04	40.7 (6/7)	2/7	82.3 (7/7)	4/7	121.1 (7/7)	4/7
Controls	Neg (0/7)	0/7	Neg (0/7)	0/7	Neg (0/7)	0/7

[A]Mean HI titer of the positive samples (number positive/total). HI testing was done with 4 HA units of the CK/CA/431/00 virus and positives were considered to be ≥8. Neg = negative.

[B]Number of positive birds/total examined. Positive samples were determined from the sample-to-positive ratio, as calculated by the manufacturer's software (IDEXX Laboratories).

Table 5. Hemagglutination inhibition (HI) antibody titers against avian influenza virus and the number of serologically positive ducks by ELISA.

	Serology					
	Day 7		Day 14		Day 21	
Virus isolate	HI[A]	ELISA[B]	HI	ELISA	HI	ELISA
A/CK/NY/14677-13/98	45.7 (3/7)	6/7	31.6 (7/7)	7/7	31.6 (5/7)	7/7
A/CK/CA/431/00	54.8 (7/7)	5/7	70.8 (7/7)	4/7	69.3 (5/7)	5/7
A/CK/CA/139/01	80.0 (6/7)	5/7	31.6 (6/7)	3/7	27.4 (6/7)	4/7
A/CK/CA/6028/04	16.4 (2/7)	3/7	20.0 (2/7)	0/7	12.0 (2/7)	0/7
A/CK/CA/7211/04	71.6 (6/7)	7/7	19.3 (4/7)	2/7	48.2 (6/7)	2/7
Controls	Neg (0/7)	0/7	Neg (0/7)	0/7	Neg (0/7)	0/7

[A]Mean HI titer of the positive samples (number positive/total). HI testing was done with 4 HA units of the CK/CA/431/00 virus and positives were considered to be ≥8. Neg = negative.

[B]Number of positive birds/total examined. Positive samples were determined from the sample-to-positive ratio, as calculated by the manufacturer's software (IDEXX Laboratories).

were infectious and shed at higher levels in chickens than in ducks. In addition, it appears that virus replication and shedding from the oropharynx, rather than from the cloaca, is a characteristic of theses viruses in chickens and ducks.

Although the true LPAI virus dose that can sustain transmissibility is unknown, the mean intranasal bird infectious dose for selected LPAI viruses was previously reported to range from 3.0 to 7.7 in chickens and from 1.9 to 3.3 in ducks, and the dose varied with the virus isolates (12). Calculated relative viral equivalents, based on Ct values, indicated that an infectious dose was being shed by some of the birds in our experiments (Tables 2, 3).

Serology results are presented in Tables 4 and 5, for the chickens and ducks, respectively. None of the nonchallenged birds had detectable antibody titers against AIV. Although the HI titers for the viruses given to both species were, on average, higher in chickens than in ducks, more ducks seroconverted based on ELISA testing. It appears that the ducks were more susceptible to infection with these viruses, but virus replication in the ducks was lower than in the

Table 6. Severity of microscopic lesions found in tissues of chickens infected with H6N2 LPAI viruses.[A]

	Virus				
Tissue	A/CK/CA/431/00	A/CK/CA/139/01	A/CK/CA/1255/02	A/CK/CA/6028/04	A/CK/CA/7211/04
Nasal cavity	++[B]	++	+	++	++
Trachea	+	++	++	+	+
Lung	+	+	+	+	++
Heart	+/−	−	−	+/−	+
Brain	−	−	−	−	−
Enteric tract	+	+	+	++	+
Pancreas	+	+	+/−	+	+/−
Liver	++	++	+	+	+
Kidney	−	−	−	−	−
Spleen	−	−	−	−	−
Bursa	+/−	+/−	+/−	+	+/−
Thymus	+/−	+	−	+/−	+/−
Muscle	−	−	−	−	−
Gonads	+	+	+/−	+/−	+

[A]Tissues collected from three birds at day 3 PI.

[B]Average severity of lesions; − = no lesions; +/− = minimal; + = mild; ++ = moderate; +++ = severe lesions.

Table 7. Severity of microscopic lesions found in tissues of ducks infected with H6N2 LPAI viruses.[A]

Tissue	Virus					
	A/CK/NY/14677/89	A/CK/CA/431/00	A/CK/CA/139/01	A/CK/CA/1255/02	A/CK/CA/6028/04	A/CK/CA/7211/04
Nasal cavity	+[B]	+	+	+	+	++
Trachea	++	++	++	++	+	++
Lung	+/−	+/−	−	−	+	+/−

[A]Tissues collected from three birds at day 3 PI.
[B]Average severity of lesions; − = no lesions; +/− = minimal; + = mild; ++ = moderate; +++ = severe lesions.

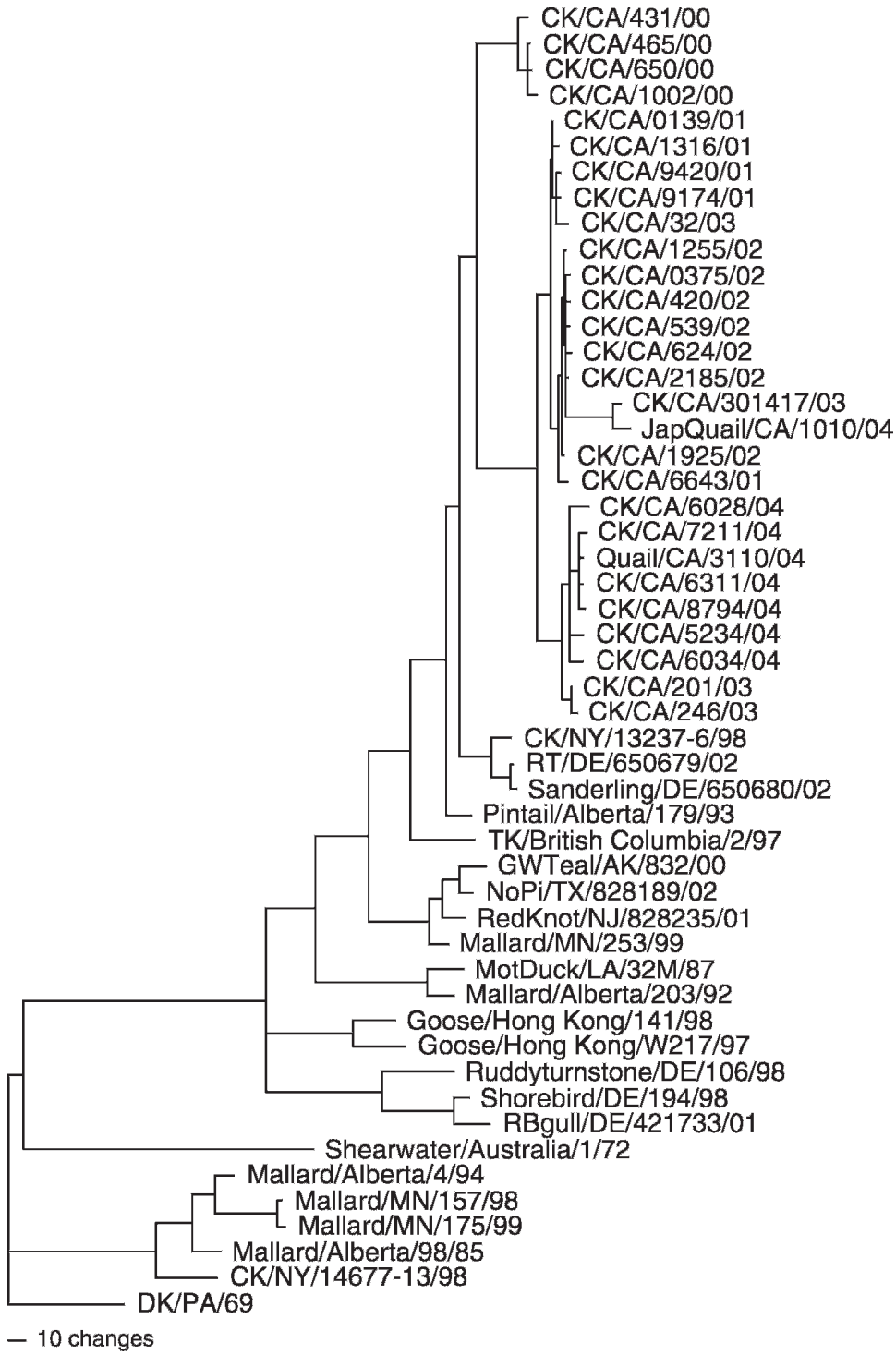

Fig. 1. Phylogenetic analysis of the HA1 hemagglutinin gene segment based on nucleotide sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method with the PAUP 4.0b10 program. The outgroup used is DK/PA/69. Abbreviations used for identifying isolates: CK = chicken; DK = duck; JapQuail = Japanese quail; RT = ruddy turnstone; MotDuck = mottled duck; GWTeal = green-winged teal; RBGull = ring-billed gull.

chickens that became infected. One virus, CK/CA/6028/04, not only induced higher HI titers in chickens than in ducks, but more chickens seroconverted to that virus, indicating that it was more adapted to the chickens.

Most of the microscopic lesions observed in virus-infected chickens and ducks were confined to the respiratory tract (Tables 6, 7). All of the chickens examined (that were given the viruses studied) presented lesions in the nasal cavity, trachea, and lungs. In the nasal cavity, mild to moderate catarrhal or lymphocytic rhinitis and sinusitis, with mucocellular exudates containing sloughed epithelial cells, submucosal edema, and glandular hyperplasia were observed. The trachea presented mild to moderate degenerative changes of the overlying epithelium and mild lymphocytic infiltration in the submucosa, with mild edema. The lesions present in the lung consisted of mild congestion, mild interstitial inflammation with mixed mononuclear cells, mild to moderate catarrhal bronchitis, and mild proliferation of bronchiole-associated lymphoid tissues. Moderate, multifocal interstitial pneumonia was present in one of the chickens infected with CK/CA/431/00 and in two of the chickens infected with CK/CA/7211/04.

Minimal lymphocytic infiltration was present in the heart of two chickens infected with CK/CA/431/00 and CK/CA/6028/04, and one bird infected with CK/CA/7211/04 had moderate, diffuse lymphocytic pericarditis. All of the infected chickens examined had mild to moderate hyperplasia of the duodenum epithelium and mild proliferation of gut-associated lymphoid tissues. A mild to moderate increase in lymphocytic infiltrates in the periportal regions of the liver was observed in all infected chickens. Mild lymphocytic infiltration was also observed in the pancreas and gonads of some birds in all virus-inoculated groups. Also, minimal to mild lymphoid atrophy was observed in the bursa of Fabricius and thymus in some chickens in each group. Remaining organs lacked significant histopathologic lesions. No significant lesions were found in noninoculated chickens.

In ducks, similar to the chickens, most of the microscopic lesions were found in the upper respiratory tract (nasal cavity, trachea); however, different than seen in chickens, minimal or no lesions were observed in any other tissues including the enteric tract. Mild to moderate lymphocytic rhinitis and sinusitis, and mild to moderate tracheitis, was noted in all ducks inoculated with any of the viruses. Loss of trachea epithelium, with areas of squamous metaplasia, was commonly observed. Lesions in the lung were mild or nonexistent. Some ducks had mild congestion and minimal to mild mononuclear cell infiltration. One duck inoculated with CK/CA/6028/04 had moderate interstitial pneumonia. Negative control birds did not have any appreciable lesions.

Phylogenetically, the hemagglutinin gene of the H6N2 viruses from California all appeared to be part of a unique lineage with a common origin (Fig. 1). However, as previously described, comparison of the other gene segments showed multiple different lineages, providing evidence of reassortment with other influenza viruses (13). However, from a common origin of the H6 gene, genetic drift of the virus into three main groups can be observed. The viruses used in this study include representatives from all three groups. The earliest group of viruses, all isolated in 2000, is separated from the other isolates by a 2–4% sequence difference. The two other genetic groups are separated from each other by 3–7% nucleotide differences. The CK/CA/6028/04 isolate from a bird at a live bird market custom slaughter plant, and CK/CA/7211/04 from a bird in a nearby feed and pet supply store in Los Angeles, were closely related. The earliest isolate from California CK/CA/431/00 was more distant from the other California isolate. The CK/NY/14677-13/98 virus is genetically distinct from the California isolates, with approximately a 25% nucleotide sequence divergence. A previous study places CK/CA/139/01 and CK/CA/431/00 in sublineage II along with other H6N2 isolates from California and with one H6N1 isolate (A/pintail/Alberta/179/93) from Alberta (1).

In summary, these viruses can infect both chickens and ducks but, based on virus detection and histopathology, they are more adapted to infection and replication in chickens. No clear difference in pathology or virus shedding was observed between the H6N1 isolates from different years. Most infected chickens shed virus, but not all of the birds seroconverted. This is important because virus shedding in commercial chickens can lead to transmission of the virus among poultry, which could go unnoticed in the absence of clinical signs or detectable antibodies. In contrast, few of the ducks given the H6N1 viruses from California shed virus, but many of the birds seroconverted.

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Research Note—

Rapid Detection of Eurasian and American H7 Subtype Influenza A Viruses Using a Single TaqManMGB Real-Time RT-PCR

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SUMMARY. A real-time reverse transcription PCR (RRT-PCR) targeting a highly conserved HA2 H7 region was developed for the detection of all H7 subtype avian influenza viruses (PanH7). The wide phylogenetic scope and analytical sensitivity and specificity were validated with the use of a panel of 56 diverse influenza A viruses. The detection limit was determined with the use of serial dilutions of Eurasian isolates A/Ck/BE/06775/2003 and A/Ck/It/1067/v99 and North American isolates A/CK/PA/143586/2001 and A/Quail/PA/20304/1998, to be 1 $\log_{10}$ higher than the detection limit of the generic influenza A matrix RRT-PCR (about 2.5 EID_{50}/reaction compared to 0.25 EID_{50}/reaction for matrix). Diagnostic test properties of PanH7 were determined with the use of 102 swabs from A/Ck/It/1067/v99 experimentally infected chickens, and were not affected by the increased detection limit of PanH7. In comparison to matrix RRT-PCR and virus isolation in embryonated chicken eggs (VI), the PanH7 detected more weakly positive oropharyngeal swabs at the onset of the infection. PanH7 diagnostic sensitivity compared to virus isolation (VI) was 83.3% (compared to 72.2% for matrix RRT-PCR); and diagnostic specificity was 88.1% (94.0% for matrix). The PanH7 test can also be tailored to detect only American (AmH7) or only Eurasian (EurH7) strains by changing the mix of forward and reverse primers used in combination with the unique probe. Overall, this new test is a valuable tool for the detection and identification of H7 subtype influenza A.

RESUMEN. *Nota de Investigación*—Detección rápida de virus de la influenza aviar euroasiáticos y americanos subtipo H7 utilizando un método de RT-PCR en tiempo real con una sonda TaqManMGB sencilla.

Se desarrolló un método de transcripción reversa y reacción en cadena de la polimerasa en tiempo real (RRT-PCR) que está dirigido a una región altamente conservada de la hemaglutinina 2 (HA2) del subtipo H7 para detectar todos los subtipos del virus de la influenza aviar H7 (PanH7). Se validó el alcance filogenético, así como la sensibilidad y la especificidad analíticas mediante el uso de un panel de 56 virus diferentes de la influenza A. El límite de detección fue determinado con el uso de diluciones seriadas de los aislamientos euroasiáticos A/Ck/BE/06775/2003 y A/Ck/It/1067/v99 y de los aislamientos norteamericanos A/CK/PA/143586/2001 y A/Quail/PA/20304/1998, para ser un logaritmo base 10 más alto que el nivel de detección del método de RRT-PCR genérico dirigido a la matriz de los virus de la influenza A (aproximadamente 2.5 dosis infectantes para embrión de pollo 50% por reacción en comparación con 0.25 dosis infectantes embrión de pollo 50% por reacción para el gen de la matriz). Las propiedades como prueba diagnóstica del método PanH7 fueron determinadas mediante 102 hisopos recolectados de pollos infectados experimentalmente con el virus A/Ck/It/1067/v99 y no resultaron afectadas por el incremento en el límite de detección del método PanH7. En comparación a la técnica de RRT-PCR dirigida a la matriz y al aislamiento en embriones de pollo, el método PanH7 detectó muestras de hisopos orofaríngeos que eran positivas débiles al inicio de la infección. La sensibilidad diagnóstica del método PanH7 en comparación con el aislamiento viral fue del 83.3% (en comparación con el 72.2% -observado con el método RRT-PCR dirigido a la matriz). La especificidad diagnóstica fue del 88.1% (94.0% con el método para la matriz). El método PanH7 también puede adaptarse para detectar solamente cepas americanas (AmH7) o solo euroasiáticas (EurH7) cambiando la mezcla de los iniciadores de avance y de reversa, usados en combinación con la sonda única. En conclusión, este método es un recurso valioso para la detección e identificación de virus de la influenza A subtipo H7.

Key words: avian influenza, real-time RT-PCR, molecular diagnosis, H7 subtype

Abbreviations: AI = avian influenza; CT = cycle threshold; dpi = days postinfection; EID_{50} = egg infectious dose 50; HP = highly pathogenic; LPAI = low pathogenicity avian influenza; MGB = minor groove binder; NDV = Newcastle disease virus; RRT-PCR = real-time reverse transcriptase–polymerase chain reaction; PBS = phosphate-buffered saline; SPF = specific-pathogen free; VI = virus isolation

Wild waterfowl, mainly Anseriformes and Charadriiformes, constitute the natural reservoir of low pathogenicity avian influenza (LPAI) viruses (43), and transmission events of LPAI viruses from wild birds to domesticated species with subsequent mutation to highly pathogenic (HP) viruses have been reported (37). Real-time RT-PCR (RRT-PCR) is becoming the primary screening technique for the rapid diagnosis of avian influenza (AI) viruses in poultry and wild birds (5,33). Well-validated generic influenza A assays targeting conserved regions of the influenza genome have been documented (11,29). Following positive identification of a sample as Type A influenza, rapid subtyping of the sample to identify H5 and H7 viruses is essential as H5 and H7 subtype viruses have the potential to be highly pathogenic (25). As a consequence, recent changes in international legislation define all H5 and H7 viruses as *notifiable avian influenza* (45). Rapid detection and characterization of these strains before extensive circulation in poultry is therefore crucial. Given the spreading epizootic occurrence of HPAI H5N1 viruses throughout Asia, Europe, and Africa in recent years, more attention has been given to the development of H5 subtyping assays. However, the H7 subtype viruses represent an equally important threat of highly pathogenic avian influenza for poultry (6,18,32),

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and some H7 viruses have also been documented to have zoonotic potential (3,12,13,14,16,22,24,26,27,38,44); therefore, the need for H7 subtyping is obvious. Because of the variable nature of the hemagglutinin gene (30), currently available H7 tests are restricted to the detection of American (28,29) or Eurasian (21) (M. Slomka and I. Brown, pers. comm.) H7 viruses. Moreover, excessive mutations in probe target sequences may result in failure to detect isolates (46). Current data support sequence separation between H7 viruses from the American and Eurasian flyways, and even divisions within a flyway like North American and South American or Europe from Australian isolates (4,34,35). Viruses with a mixed genetic signature containing RNA segments originating from different continents have been described (10,15,19,41,42), showing a possibility of intercontinental contact between virus lineages. It is prudent to have validated diagnostic tests available for all H7 viruses, rather than sublineage specific tests.

This article describes the development of a new RRT-PCR test using minor groove binder (MGB) technology that provides improved interaction between the target sequence and the probe, making the use of shorter oligonucleotides possible without compromising test specificity (17). A PanH7 RRT-PCR assay was developed detecting isolates with a wide range of geographic origin.

MATERIALS AND METHODS

Oligonucleotide design. A total of 210 H7 hemagglutinin sequences, including 107 Eurasian isolates and 91 American isolates, were downloaded from public databases (accession numbers available on request) and aligned with the use of the ClustalW algorithm (7). Conserved regions were identified in the HA2 coding sequence and a TaqManMGB probe and primers (Table 1) were defined with the help of Primer Express™ software (Applied Biosystems, Foster City, CA). The specificity of these oligonucleotides was confirmed with the use of a BLASTn search (2).

Reference material and RNA purification. A panel of 56 reference viruses (Table 2) including 20 American H7 and 9 Eurasian H7 subtype viruses was used to confirm the analytical specificity and sensitivity of the test. Viral RNA was purified from reference viruses with the use of the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Brussels, Belgium) or the MagMAX AI/ND Viral RNA Isolation Kit (Ambion Corp., Austin, TX) according to the manufacturer's instructions. Equivalence of both RNA purification methods was previously demonstrated (SVB, unpubl. data).

Real-time RT-PCR. RRT-PCRs were carried out with the use of the QuantiTect® Probe RT-PCR Kit (Qiagen, Venlo, Netherlands) in a total reaction volume of 25 µl including 2 µl of purified RNA. The matrix RRT-PCRs (M) included 900 nM of each primer, 200 nM of TaqMan probe (29), and 4 U per reaction of Qiagen RNase Inhibitor (Qiagen). Primers and TaqMan probes were supplied by Eurogentec (Liège, Belgium), TaqManMGB probe by Applied Biosystems. The PanH7 reactions included a final concentration of 200 nM MGB probe and 900 nM of each primer FeurH7, ReurH7, FameH7, and RameH7 (Table 1). Alternatively, sublineage specific RRT-PCRs were set up with the use of 900 nM of each primer FeurH7 and ReurH7 for identification of Eurasian isolates (EurH7 test) on the one hand and using 900 nM of each primer FameH7 and RameH7 on the other hand for the identification of American isolates (AmH7 test), in combination with 200 nM of the unique PanH7 MGB probe. Because of logistical reasons, RRT-PCR tests had to be run in two different laboratories. However, all H7 RRT-PCRs were amplified and detected on an Applied Biosystems 7500 real-time PCR cycler with the use of the same reagents and an identical protocol: 30 min at 50 C, 10 min at 95 C, followed by 45 cycles of 15-sec denaturation at 95 C, 33-sec annealing at 54 C (this step included fluorescence detection), 30-sec extension at 72 C. Matrix RRT-PCRs were amplified and detected with the use of an identical protocol on an Applied Biosystems 7500 real-time PCR cycler, or alternatively on a Cepheid Smartcycler (Sunnyvale, CA).

Detection limit. The detection limit of this new test (PanH7) was determined with the use of serial 10-fold dilutions in phosphate-buffered saline solution (PBS) of Eurasian isolates A/Ck/BE/06775/2003 (H7N7, HPAI) and A/Ck/It/1067/v99 (H7N1, LPAI), and North American isolates A/CK/PA/143586/2001 (H7N2) and A/Quail/PA/20304/1998 (H7N2) and compared with the detection limit of the generic influenza A matrix RRT-PCR (29). RNA was extracted from each virus dilution with the High Pure Viral Nucleic Acid Kit (Roche Applied Science) or the MagMAX AI/ND viral RNA isolation kit (Ambion Corp.; Table 3). Viral titers per milliliter of diluted sample and per RRT-PCR reaction (2 µl of RNA extract) are specified in Table 3.

Validation on clinical samples. Twelve 4-wk-old specific-pathogen-free (SPF) white leghorn chickens were inoculated oculonasally with 5 $\log_{10}$ EID_{50} of A/Ck/It/1067/v99 LPAI H7N1. A negative control group of five 4-wk-old SPF chickens received 100 µl of PBS oculonasally. All animals were housed in BSL3+ isolators, and their treatment complied with all relevant national guidelines and institutional ethical and biosafety policies. Cloacal and oropharyngeal swabs were taken at 2, 4, and 10 days postinfection (dpi) as previously described (31). RNA from swabs was purified with the use of the Magmax AI/ND 96 viral RNA kit (Ambion Corp.) on a KingFisher Magnetic Particle Processor (Thermo Fisher Scientific, Zellik, Belgium) following the manufacturer's instructions. A negative extraction control was processed with each extraction run and RNA extraction efficiency was monitored for each individual sample with the use of a beta actin RRT-PCR as previously described (39). Samples with a beta actin cycle threshold (CT) value above 40 were excluded from the analysis. Matrix and H7MGB (primer mix *EA*) RRT-PCRs were carried out as described above with the use of 2 µl of purified RNA in a total reaction volume of 25 µl. For virological confirmation, each sample was inoculated in a series of four 9-to-11-day-old embryonated chicken eggs as previously described (8,36).

RESULTS AND DISCUSSION

Because H7 subtype avian influenza isolates potentially represent highly pathogenic viruses or may mutate to the highly pathogenic form of the virus, their timely identification is critically important. Classical virological techniques (virus isolation and hemagglutination inhibition) are limited primarily because of time to diagnosis. This critical delay can result in the transmission of the virus to other farms making control more difficult. Molecular diagnostic alternatives have the advantage of being fast and potentially to be as or more sensitive than virus isolation. We have developed a new RRT-PCR test based on TaqManMGB technology that is able to detect

Table 1. Primer and probe sequences used in the PanH7, EurH7, and AmH7 RRT-PCR tests.

Primer	Target sequences	Sequence (5′–3′)
FeurH7	Eurasian (EA) H7	GYAGYGGYTACAAAGATGTG
ReurH7	Eurasian (EA) H7	GAAGACAAGGCCCATTGCAA
H7MGB	Universal H7	FAM-TGGTTTAGCTTCGGGGC-MGB
FameH7	American (NoA) H7	GYRGYGGRTACAARGACATA
RameH7	American (NoA) H7	GAARACCARYCCCATTRCAA

Table 2. Analytical specificity of four RRT-PCRs based on a panel of 56 influenza A viruses, 1 influenza B virus, and 1 NDV. The detection limit was set at CT 35 for the matrix test and at CT 39 for the other tests. ND = not done.

Subtype	Virus	Matrix	EurH7	AmH7	PanH7
H1N1	A/New Caledonia/20/99	+	−	−	−
H2N3	A/dk/Germ/1215/1973	+	−	−	−
H3N1	A/Ck/Belgium/380VB/1978	+	−	−	−
H3N1	A/duck/Belgium/02216/2006	+	−	−	−
H3N2	A/Wyoming/3/2003	+	−	−	−
H3N6	A/Ch/Belgium/06639/2006	+	−	−	−
H4N6	A/Ck/Belgium/909,1514VB/1984	+	−	−	−
H5N1	A/Duck/Vietnam/tg−24−01/2005	+	−	ND	−
H5N1	A/crested eagle/Belgium/01/2004	+	−	−	−
H5N1	A/turkey/Turkey/2005	+	−	−	−
H5N2	A/TKY/ENG/N28/1973	+	−	−	−
H5N2	A/Ch/Belgium/150VB/1999	+	−	−	−
H5N2	A/mallard/Denmark/2006	+	−	−	−
H5N2	A/ck/Italy/8/A98	+	−	−	−
H5N3	A/teal/Eng/2006	+	−	−	−
H5N7	A/Dk/Denmark/64650/2003	+	−	−	−
H5N9	A/chicken/Italy/22A/98PD	+	−	−	−
H7N1	A/ty/It/4580/V99	+	+	−	+
H7N1	A/Africa Starling/ Eng/983/1979	+	+	−	+
H7N1	A/Flycatcher/CA−Quarantine/14875−1/1994[A]	+	+	−	+
H7N1	A/softbill/Quarantine station/33445−136/1992[A]	+	+	−	+
H7N2	A/CK/NJ/294508−12/2004	+	+	+	+
H7N2	A/CK/FL/90348−4/2001	+	+	+	+
H7N2	A/CK/PA/143586/2001	+	+	+	+
H7N2	A/Quail/PA/20304/1998	+	+	+	+
H7N2	A/TK/PA/7975/1997	+	+	+	+
H7N2	A/CK/NY/13412−5/1994	+	+	+	+
H7N3	A/CK/Chile/184240−4/2002	+	−	+	+
H7N3	A/ty/It/9289/V02	+	+	−	+
H7N3	A/chicken/Eng/2006	+	+	−	+
H7N3	A/CK/Pakistan/1369−CR2/1995	+	+	−	+
H7N3	A/Mallard/US/423/2001	+	+	+	+
H7N3	A/Black Duck/US/415/2001	+	+	+	+
H7N3	A/Black Duck/US/424/2001	+	+	+	+
H7N3	A/Blue Winged Teal/US/658/2004	+	+	+	+
H7N3	A/Blue Winged Teal/US/648/2004	+	+	+	+
H7N3	A/Quail/AR/16309−2/1994	+	+	+	+
H7N3	A/DK/PA/143585/2001	+	+	+	+
H7N3	A/TK/OR/1971	+	+	+	+
H7N3	A/CK/NY/14714−2/1999	+	+	+	+
H7N4	A/mallard/Italy/2004	+	+	ND	+
H7N4	A/CK/NSW/1688/1997	+	+	−	+
H7N7	A/Ck/Belgium/06775/2003	+	+	−	+
H7N7	A/Seal/MA/1/1980	+	+	+	+
H7N7	A/equine/Prague/1956	+	+	−	+
H7N8	A/Mallard/OH/421/1987	+	+	+	+
H8N4	A/teal/Eng/2006	+	−	−	−
H9N2	A/ty/wis/1966	+	−	−	−
H9N2	A/Ck/Belgium/150VB/1983	+	−	−	−
H9N2	A/ck/Belgium/818VB/1978	+	−	−	−
H10N7	A/mallard/Eng/2006	+	−	−	−
H10N7	A/Green Winged Teal/LA/1696W/1988	+	−	−	−
H10N7	A/TK/VA/31409/1991	+	−	−	−
H11N9	A/duck/Belgium/1266/2007	+	−	−	−
H15N6	A/Shearwater/Western Australia/2576/1979	+	−	−	−
H16N3	A/Black Headed Gull/Mongolia/1756/2006	+	−	−	−
	B/Jiangsu/10/2003	−	−	−	−
	NDV La Sota	−	−	−	−

[A]Isolate from Asian bird in American quarantine station.

both Eurasian and American H7 subtype influenza A viruses. The MGB probe targeting the HA2 region was 100% conserved for the publicly available H7 sequences. Because of the variability in sequence among H7 isolates, separate primers were needed to target the American and Eurasian viruses, and even with different primer designs, degenerate primers were needed to cover all publicly available H7 sequences. As a rule, no degeneracies were allowed in the first three 3′ nucleotides of a primer, and maximum one

Table 3. Detection limit and reproducibility of three influenza A RRT-PCRs depends on the virus strain showing only limiting dilutions. A/Ck/Belgium/06775/2003 (H7N7), A/Ck/Italy/1067/v99 (H7N1), A/CK/PA/143586/2001 (H7N2), and A/Quail/PA/20304/1998 (H7N2). N = number of repeated RRT-PCR reactions, n = number of positive results, ND: not done, U: undetected. Calculation of average and SD based on positives only. RNA from Eurasian strains was extracted with the Roche High Pure Viral NA kit with a starting volume of 200 µl of sample, RNA from American strains with the Ambion Magmax 96 AI/ND viral RNA kit with a starting volume of 50 µl of sample.

			Matrix gene RRT-PCR			PanH7 RRT-PCR		
Virus	EID_{50}/ml sample	EID_{50}/ reaction	n/N	Average CT	SD CT	n/N	Average CT	SD CT
A/Ck/Belgium/06775/2003	3.63×10^4	2.90×10^2	3/3	24.10	0.05	3/3	28.8	0.39
A/Ck/Belgium/06775/2003	3.63×10^3	29.0	3/3	27.67	0.20	3/3	32.48	0.70
A/Ck/Belgium/06775/2003	3.63×10^2	2.90	3/3	30.92	0.31	3/3	37.85	0.69
A/Ck/Belgium/06775/2003	36.3	0.29	3/3	33.79	0.43	0/3	U	U
A/Ck/Belgium/06775/2003	3.63	2.90×10^{-2}	0/3	>35		0/3	U	U
A/Ck/Italy/1067/v99	2.88×10^4	2.30×10^2	3/3	23.56	0.18	3/3	28.36	0.13
A/Ck/Italy/1067/v99	2.88×10^3	23.0	3/3	26.94	0.05	3/3	31.44	0.20
A/Ck/Italy/1067/v99	2.88×10^2	2.30	3/3	30.23	0.22	3/3	35.67	0.52
A/Ck/Italy/1067/v99	28.8	0.23	3/3	33.3	0.51	1/3	38.25	
A/Ck/Italy/1067/v99	2.88	2.30×10^{-2}	0/3	>35		0/3	U	U
A/CK/PA/143586/2001	3.16×10^5	6.32×10^2	ND	ND	ND	2/2	28.98	0.04
A/CK/PA/143586/2001	3.16×10^4	63.2	ND	ND	ND	2/2	33.70	0.27
A/CK/PA/143586/2001	3.16×10^3	6.32	ND	ND	ND	2/2	38.88	0.18
A/CK/PA/143586/2001	3.16×10^2	6.32×10^{-1}	ND	ND	ND	0/2	U	U
A/CK/PA/143586/2001	31.6	6.32×10^{-3}	ND	ND	ND	0/2	U	U
A/Quail/PA/20304/1998	5.01×10^6	1.002×10^4	ND	ND	ND	2/2	27.36	0.01
A/Quail/PA/20304/1998	5.01×10^5	1.002×10^3	ND	ND	ND	2/2	31.11	0.07
A/Quail/PA/20304/1998	5.01×10^4	1.002×10^2	ND	ND	ND	2/2	37.13	0.26
A/Quail/PA/20304/1998	5.01×10^3	10.02	ND	ND	ND	2/2	>39	0.72
A/Quail/PA/20304/1998	5.01×10^2	1.002	ND	ND	ND	0/2	U	U

degeneracy was allowed in the first 10 3′ nucleotides. The probe, however, also shows a 100% match with some older H10 and H15 isolates, but the specificity is guaranteed with primer mismatches to these strains (at least three mismatches in a primer, including one mismatch in the first three 3′ nucleotides). Taking the combined effect of RNA extraction (RNA was extracted from each virus dilution), RT and PCR into account, the resulting correlation coefficient (R^2 = 0.983 based on four replicate virus dilution series) and extraction + RT + PCR efficiency (103.18%) demonstrates near theoretical test properties over a linear dynamic range of seven 10-fold dilutions matching a typical range of virus load in AI infected samples (10–10^7 EID_{50}/RRT-PCR reaction).

The PanH7 test (all primers FeurH7, ReurH7, FameH7, and RameH7) allowed the specific detection of all Eurasian or American sequences as shown, with the use of a panel of 56 diverse influenza A viruses. All H7 strains in the test panel (from diverse geographic sources and times: North American, European, Asian, South American, Australian) were detected, whereas no signal was detected in any of the other HA subtype strains in the panel or for influenza B or Newcastle disease virus (NDV; Table 2). When the primer mix was limited to FeurH7 and ReurH7, all Eurasian H7 strains were detected at comparable CT levels to the PanH7 test, whereas amplification of American isolates could be seen at higher CT values (>5 CTs higher than PanH7). When only primers FameH7 and RameH7 were used, on the other hand, all American isolates were detected, including a South American isolate from Chile. The latter primer combination did not detect any Eurasian isolates. The combination of both regional sublineage specific primer sets thus allows a differentiation between Eurasian and American lineage viruses on a real-time PCR scale (hours). Later confirmation by (partial) sequencing of an isolates HA gene could then follow (time scale: days) with the added advantage of identifying the HA cleavage site sequence. A/softbill/Quarantine station/33445-136/1992 (Table 2) is a good example of the full potential of the newly developed H7MGB tests. This isolate, coming from a Eurasian bird imported into the United States, shows a clear positive result for the PanH7 test, as well as for the Eurasian specific test, although negative for the American specific test, identifying the strain as a Eurasian lineage H7 influenza. Other examples of the discriminating power of these tests are A/CK/Pakistan/1369-CR2/1995 and A/equine/Prague/1956 (Table 2). In the current global situation bird migration is not the only potential route of introduction of influenza viruses. Legal trade in poultry and pet birds presents frequent intercontinental contacts, but strict national and international regulations and controls are imposed at least in developed countries. Illegal trade of live birds (40), poultry products, bioterrorism/agroterrorism, and global traveling present other opportunities for movement of viruses, the importance of which is difficult to estimate. In this context the added value of influenza A assays with a wide phylogenetic scope for the notifiable subtypes H5 and H7, and with the potential to identify the geographic origin of an isolate, is clear.

The detection limit of PanH7, as well as of the M RRT-PCR, depended on the virus strain used (Table 3). Based on the data in Table 3, we set a CT threshold for positivity at 39. The detection limit was determined as the last dilution giving a CT <39 in all replicates. The threshold for the M RRT-PCR was set at 35 based on previous experience. Depending on the virus strain, the detection limit of M RRT-PCR ranged from 0.14–2.3 EID_{50}/reaction (17.6–288 EID_{50}/ml sample). PanH7 was less sensitive, detecting 6.32–100 EID_{50}/reaction (3.16×10^3 to 5.01×10^4 EID_{50}/ml sample). The detection limit of the regional clade–specific tests was at least as good as, and for the European test 1 $\log_{10}$ better than, the detection limit of PanH7 (data not shown). The range of detection limits of the new PanH7 test using replicate serial dilutions of multiple viruses was thus roughly about 1 $\log_{10}$ higher than the detection limit of matrix RRT-PCR. The broad specificity realized with a degenerate oligonucleotide mix has a sensitivity cost. This difference in detection limit compared to matrix RRT-PCR, and the variable detection limit in function of the strain should be taken into account when interpreting, e.g., matrix weak positive, H7 negative results.

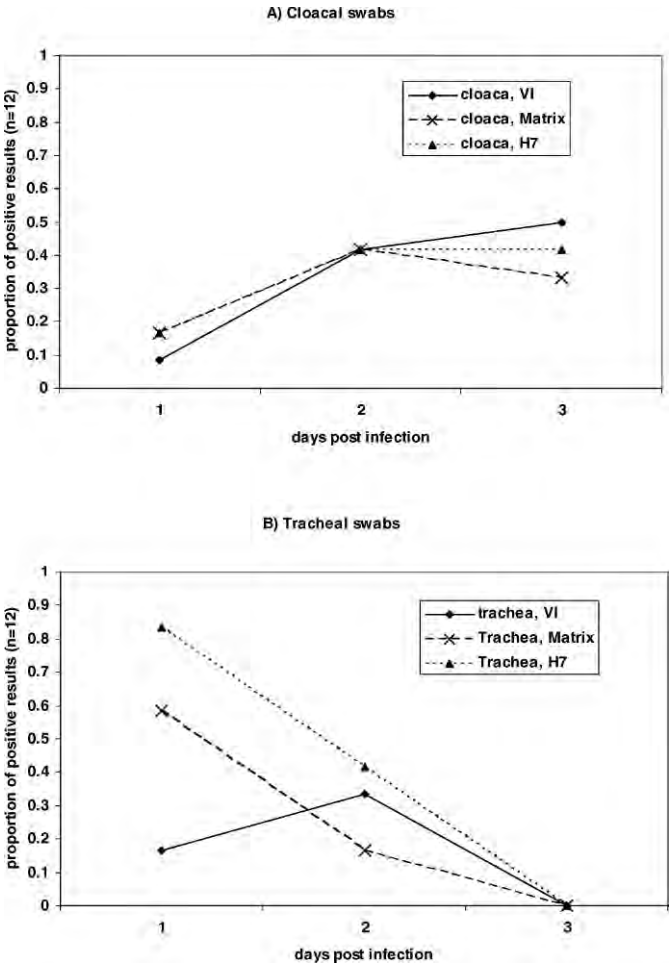

Fig. 1. Detection of A/Ck/Italy/1067/v99 excretion in experimentally infected chickens using virus isolation and two different RRT-PCR methods (matrix RRT-PCR and PanH7 RRT-PCR) and VI.

However, the detection limit also seems variable for the routinely used M gene RRT-PCR.

Although the analytical sensitivity seems equivalent or 1 log10 lower for PanH7 compared to matrix RRT-PCR, the new test seems to detect more weakly positive oropharyngeal swab samples at the onset of a LP experimental H7 infection than the matrix test. The beta actin endogenous control demonstrated efficient RNA extraction for all swab samples (CT values ranging from 28.59 to 39.03). In a total of 102 swab samples, PanH7 detected 6 more positive oropharyngeal samples than matrix RRT-PCR (Fig. 1). All of these are weak positive samples at the onset (3 dpi–6 dpi) of the excretion period (PanH7 CT $>$ 33). At the onset of excretion (3 dpi), no virus was detected with the use of virus isolation (VI), but both RRT-PCRs detect oropharyngeal excretion. At 6 dpi VI and H7MGB RRT-PCR detect the same positive swab samples, where the matrix RRT-PCR fails to detect some positives. It is unlikely that the additional positive results from using the H7MGB compared to matrix RRT-PCR represent false positives, as none of the 30 analyzed samples from negative control chickens (treated under identical conditions) tested positive. Moreover, the correlation of PanH7 with VI was better than that of matrix. Two swabs (one oropharyngeal 6 dpi and one cloacal 6 dpi) were positive in VI but failed to amplify in both RRT-PCRs. There was no indication from the beta actin results (CT values 33.06 and 36.01) that PCR inhibition or poor RNA extraction efficiency may be involved. Based on our data, the trend of reduced analytical sensitivity (1 $\log_{10}$ for the A/Ck/Italy/1067/v99 challenge strain used in the experiment) does not seem to implicate a reduced diagnostic sensitivity on swab samples. With VI used in embryonated chicken eggs as the gold standard method, the diagnostic sensitivity of PanH7 is 83.3% and diagnostic specificity is 88.1% compared to a sensitivity of 72.2% and specificity of 94.0% for the matrix RRT-PCR. However, none of the swabs from negative control chickens tested positive in PanH7, and the higher number of positive RRT-PCR results compared to VI is most likely due to increased sensitivity in this set of clinical samples. The relative sensitivity and specificity of H7MGB compared to matrix RRT-PCR are 100% and 91.4%, respectively. Our data on oropharyngeal swabs from experimentally infected chickens shows that at the onset of excretion (3 dpi), both PCR tests (M and PanH7) outperform virus isolation. The PanH7 provides better performance for this virus isolate, detecting three additional positive oropharyngeal swabs compared to M.

MGB technology has been previously applied to influenza type A detection targeting the matrix gene (9). Unfortunately, the limited validation on clinical swab samples presented in that study does not allow conclusions about decreased sensitivity to PCR inhibitors compared to regular TaqMan RRT-PCR. The major advantage of MGB oligonucleotide modifications is that shorter sequences can be targeted without compromising specificity. This has been applied to other influenza A subtyping (H5, N1) RRT-PCRs, where conserved sequences are scarce and limited in length (1,20,23).

In summary, PanH7 presents a specific test for all H7 influenza A viruses. Its broad reactivity comes at a cost of reduced analytical sensitivity (1 $\log_{10}$) compared to matrix gene RRT-PCR, but the experimental data in infected chickens indicate that this does not affect the diagnostic sensitivity of the test from clinical samples. Using alternative primer combinations, a rapid differentiation between American and Eurasian lineage viruses is also possible. Further validation on clinical samples in different diagnostic laboratories is needed before the test can be widely adopted for clinical use.

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Development and Evaluation of an Avian Influenza, Neuraminidase Subtype 1, Indirect Enzyme-Linked Immunosorbent Assay for Poultry Using the Differentiation of Infected from Vaccinated Animals Control Strategy

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SUMMARY. An indirect enzyme-linked immunosorbent assay (ELISA) was developed using baculovirus, purified, recombinant N1 protein from A/chicken/Indonesia/PA7/2003 (H5N1) virus. The N1-ELISA showed high selectivity for detection of N1 antibodies, with no cross-reactivity with other neuraminidase subtypes, and broad reactivity with sera to N1 subtype isolates from North American and Eurasian lineages. Sensitivity of the N1-ELISA to detect N1 antibodies in turkey sera, collected 3 wk after H1N1 vaccination, was comparable to detection of avian influenza antibodies by the commercial, indirect ELISAs ProFLOK® AIV *Plus* ELISA Kit (Synbiotics, Kansas City, MO) and Avian Influenza Virus Antibody Test Kit (IDEXX, Westbrook, ME). However, 6 wk after vaccination, the Synbiotics ELISA kit performed better than the N1-ELISA and the IDEXX ELISA kit. An evaluation was made of the ability of the N1-ELISA to discriminate vaccinated chickens from subsequently challenged chickens. Two experiments were conducted, chickens were vaccinated with inactivated H5N2 and H5N9 viruses and challenged with highly pathogenic H5N1 virus, and chickens were vaccinated with recombinant poxvirus vaccine encoding H7 and challenged with highly pathogenic H7N1 virus. Serum samples were collected at 14 days postchallenge and tested by hemagglutination inhibition (HI), quantitative neuraminidase inhibition (NI), and N1-ELISA. At 2 days postchallenge, oropharyngeal swabs were collected for virus isolation (VI) to confirm infection. The N1-ELISA was in fair agreement with VI and HI results. Although the N1-ELISA showed a lower sensitivity than the NI assay, it was demonstrated that detection of N1 antibodies by ELISA was an effective and rapid assay to identify exposure to the challenge virus in vaccinated chickens. Therefore, N1-ELISA can facilitate a vaccination strategy with differentiation of infected from vaccinated animals using a neuraminidase heterologous approach.

RESUMEN. Desarrollo y evaluación de un ensayo indirecto de inmunoabsorción con enzimas ligadas para detectar la neuraminidasa subtipo 1 para ser aplicado dentro de una estrategia de control mediante la diferenciación entre los animales infectados de los vacunados.

Se desarrolló un ensayo indirecto de inmunoabsorción con enzimas ligadas (ELISA) utilizando como antígeno a una proteína N1 recombinante expresada en un baculovirus y purificada, que provenía del virus A/pollo/Indonesia/PA7/2003 (H5N1) virus. Esta prueba de ELISA (N1-ELISA) mostró alta selectividad en la detección de anticuerpos contra N1, sin desarrollar reacciones cruzadas con otros subtipos de neuraminidasas, además demostró poseer una amplia sensibilidad con sueros contra aislamientos subtipo N1 de los linajes norteamericano y euroasiático. La sensibilidad de la prueba N1-ELISA para detectar anticuerpos contra el subtipo N1 en sueros de pavos recolectados a las tres semanas después de la vacunación con un subtipo H1N1, resultó comparable a la detección observada con los estuches de ELISA tipo indirecto disponibles comercialmente, como el estuche ProFLOK® AIV Plus ELISA Kit (Synbiotics, Kansas City, MO) y el estuche Avian Influenza Virus Antibody Test Kit (IDEXX, Westbrook, ME). Sin embargo, a las seis semanas después de la vacunación, el estuche de ELISA de Synbiotics, mostró mejores resultados que el método N1-ELISA y que el estuche ELISA de IDEXX. Se realizó una evaluación de la capacidad del método N1 ELISA para discriminar pollos vacunados y subsecuentemente infectados. Se realizaron dos experimentos, en el primero, los pollos fueron vacunados con vacunas inactivadas de los subtipos H5N9 y H5N2 seguido por un desafío con un virus de alta patogenicidad de subtipo H5N1. En el segundo experimento, los pollos fueron vacunados con una vacuna recombinante con un poxvirus como vector que codifica la proteína H7 y posteriormente desafiados con un virus H7N1 de alta patogenicidad. Se recolectaron muestras de suero a los 14 días después del desafío y se analizaron mediante las pruebas de inhibición de la hemaglutinación, por la inhibición cuantitativa de la neuraminidasa y con el método N1-ELISA. A los dos días después del desafío, se recolectaron hisopos orofaríngeos para el aislamiento viral para confirmar la infección. El método N1-ELISA mostró una concordancia aceptable con los resultados obtenidos por aislamiento viral y por la inhibición de la hemaglutinación. Aunque el método N1-ELISA mostró una sensibilidad más baja que el ensayo de inhibición de la neuraminidasa, se demostró que la detección de anticuerpos contra N1 mediante N1-ELISA fue un método rápido y efectivo para identificar la exposición al virus de desafío en pollos vacunados. Por lo tanto, el método N1-ELISA puede facilitar la estrategia de diferenciación entre los animales infectados y los vacunados utilizando el enfoque de neuraminidasas heterólogas.

Key words: avian influenza, heterologous neuraminidase, ELISA, DIVA

Abbreviations: AGID = agar gel immunodiffusion; AGPT = agar gel precipitation test; AI = avian influenza; APHIS = Animal and Plant Health Inspection Service; BSA = bovine serum albumin; DIVA = differentiation of infected from vaccinated animals; EID_{50} = 50% embryo infective dose; ELD_{50} = 50% embryo lethal dose; ELISA = enzyme-linked immunosorbent assay; FPV = fowl poxvirus; GMT = geometric mean titer; HA = hemagglutinin; HI = hemagglutinin inhibition; HP = high pathogenicity; iIFA = indirect immunofluorescent antibody; IBV = infectious bronchitis virus; ILTV = infectious laryngotracheitis virus; IMAC = immobilized metal affinity chromatography; LP = low pathogenicity; LsNPV = *Leucania separata Nucleopolyhedrovirus*; M1 = matrix; MG = *Mycoplasma gallisepticum*; MS = *Mycoplasma synoviae*; MUN = 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate; NDV = Newcastle disease virus; NP = nucleoprotein; N1 = neuraminidase subtype 1;

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NA = neuraminidase; NI = neuraminidase inhibition; OD = optical density; SDS10%PAGE = sodium dodecyl sulfate and 10% polyacrylamide gel electrophoresis; Sf9 = *Spodoptera frugiperda*; S:P = sample to positive ratio; SPF = specific-pathogen-free; $TCID_{50}$ = 50% tissue culture infective dose; VI = virus isolation

Low pathogenicity (LP) avian influenza (AI) viruses have the potential to mutate into high pathogenicity (HP) viruses with the possibility of causing devastating disease in commercial poultry. AI control programs that combine increased biosecurity with vaccination have been shown to be successful in the control of LP and HP AI by reducing virus shedding and, consequently, decreasing viral transmission and increasing resistance to field challenge (3,4,15,21). A key element of this strategy involves a differentiation of infected from vaccinated animals (DIVA), combined with an appropriate detection system to monitor large numbers of flocks. Several DIVA vaccination strategies have been proposed to identify vaccinated and subsequently exposed flocks (14). The use of sentinel birds to detect exposure to field challenge is the simplest and older methodology; however, it introduces new management and biosecurity risks that may compromise the overall efficacy of the AI control program. A second alternative is the use of AI subunit vaccines encoding the AI hemagglutinin (HA) gene alone or in combination with the neuraminidase (NA) gene. This strategy allows the use of existing serologic surveillance tools including either the ELISA or agar gel immunodiffusion (AGID) tests that target the matrix (M1) or nucleoprotein (NP) genes (18). Vaccinated/noninfected birds will only develop antibodies against the surface glycoproteins (HA, NA), but antibodies against the internal, structural, viral proteins will not be generated. However, vaccinated/infected birds will develop antibodies against the virus, internal, structural proteins (NP, M1). For example, recombinant fowlpox (2) and avian paramyxovirus vaccines (13) carrying H5 and H7 AI genes are available, and a DIVA vaccination strategy can be used, but it has not been exploited in the field.

A third DIVA vaccination strategy involves the monitoring of the antibody response against the NS1 protein, which is produced in high levels in infected cells, but is not found in the viral particle, which is the main component of killed, adjuvanted vaccines. Therefore, infected birds should generate antibodies to the NS1 protein, but vaccinated-only birds should not. The NS1 DIVA vaccination strategy is appealing because it can be coupled with any killed vaccine, despite the AI subtype. Under experimental conditions, detection of NS1 antibodies in chickens by indirect enzyme-linked immunosorbant assay (ELISA) has been successful; antibodies to the NS1 protein were detected only in chickens experimentally infected, not in the sera of chickens immunized with purified, killed vaccine (20,25). However, the interpretation of the NS1-ELISA during a DIVA vaccination strategy can be hampered when residual NS1 protein, carried over by multiple vaccinations, elicits an NS1 antibody response in vaccinated birds (20). Furthermore, the NS1 antibody response declines at a significantly faster rate than the antibody response to the NP because of the weak, antigenic nature of the NS1 protein (23). The recent development of attenuated, NS1-deletion mutants open the possibility to more effectively use an NS1 DIVA approach to detect exposed birds (22).

A fourth DIVA vaccination strategy that has been considered requires the use of killed vaccines with homologous HA and heterologous NA to the challenge virus. Although the heterologous NA DIVA strategy was first proposed more than 20 yr ago (1), the concept was applied successfully only recently to control an HPAI H7N1 outbreak in Italy (3,6). Capua *et al.* (6) used an indirect immunofluorescent antibody (iIFA) test with baculovirus-infected cells expressing N1 antigen as a discriminatory test to differentiate H7N1-infected, commercial turkeys from H7N3-vaccinated turkeys. A similar approach was applied in Italy during the LP H7N3 outbreak in chickens and turkeys. An N3-iIFA test was developed to differentiate H7N3-infected birds from H7N1-vaccinated birds (8), and using that assay, it was possible to detect vaccinated and subsequently infected birds. Although the N3-iIFA (8) and N1-iIFA (6) assays were successful in the detection of infected birds in vaccinated flocks, they are not suitable for automation and high-throughput screening of flocks and are dependent on individual interpretation skills. To overcome the disadvantages of the neuraminidase antibody detection by iIFA, indirect and competitive ELISA systems for the detection of N1, N2, and N3 antibodies (9,12) have been developed. The competitive N1, N2, and N3 ELISAs were evaluated with samples from experimentally and naturally infected flocks (12), and the indirect N2 ELISA system was evaluated with serum samples from experimentally vaccinated (H9N8) and subsequently infected (low-pathogenic H9N2) chickens (10). The specific objectives of this study were 1) to develop an N1 ELISA using recombinant purified N1 protein, 2) to compare the sensitivity of N1-ELISA to ELISA systems commercially available in the United States, and 3) to evaluate the ability of the N1-ELISA system to discriminate vaccinated chickens from vaccinated and subsequently challenged chickens under experimental conditions.

MATERIALS AND METHODS

Cloning, expression, and propagation of recombinant N1-baculovirus. A plasmid coding for the N1 gene of isolate A/CK/Indonesia/PA7/2003 (H5N1; accession AY651433) was used for the development of a baculovirus transfer plasmid by PCR, amplifying the gene with primers 5′-GAATTCATGGACATTTTTAGAATTTGCTCTCTAATCGTGATCGCGATGTGCGCGAACGGTAGCGACACGTTACAAATTGGGAACATGATCTC-3′ and 5′-GGAAGCTTTTAATGGTGATGGTGATGGTGCTTGTCAATGGTG AATGGCAACTCA-3′. The forward primer replaced the natural signal peptide of the N1 protein by a signal peptide from the structural efp protein from *Leucania separata Nucleopolyhedrovirus* (LsNPV), and the reverse primer introduced a coding sequence for a C-terminal 6xHis oligopeptide. *EcoRI* and *HindIII* restriction sites facilitated cloning in the corresponding restriction enzyme cleavage sites of pFastBacDual (Invitrogen, Carlsbad, CA) and placed the manipulated N1 gene downstream of the baculovirus polyhedrin promoter. Inserts of recombinant plasmids were sequenced to confirm identity (pFAST-N1). The transfer N1 plasmid DNA was used to generate recombinant baculovirus using the Bac-to-Bac system (Invitrogen) following the instructions of the manufacturer. The recombinant N1-bacmid was then amplified and purified and used to transfect *Spodoptera frugiperda* (Sf9) insect cells in serum-free medium using Cellfectin (Invitrogen). Recombinant baculovirus (N1-Bac) was harvested at 6 days after transfection. To generate a viral stock, the recombinant virus was plaque purified and subsequently propagated in Sf9-cell suspension cultures (5.0×10^5 Sf9 cells/ml) for 72–96 hr incubation at 28 C under agitation. The viral stock was titrated by plaque assay following the procedure provided by the manufacturer.

Western blot analysis. Expression of recombinant N1-His was analyzed by western blot using an anti-6xHis monoclonal antibody (BD Bioscience, Franklin, Lakes, NJ). Briefly, 1-ml aliquots of infected Sf9 cultures were rapidly lysed in 110 μl of 10× FastBreak cell-lysis buffer

(BD Bioscience) with 1× protease inhibitor cocktail (Roche, Florence, SC). Then, 20 µl of lysate was separated with a sodium dodecyl sulfate and 10% polyacrylamide gel electrophoresis (SDS10%PAGE) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane was blocked overnight in a Tris-buffered saline (TBS; Thermo Scientific, Rockford, IL) containing 5% bovine serum albumin (BSA) at 4 C. Subsequently, the membrane was incubated at 37 C for 1 hr with anti-6xHis monoclonal antibody (Clontech Laboratories Inc., Mountain View, CA) at a 1:10,000 dilution in a 3% BSA-TBS buffer. The membrane was washed three times with TBS–0.05% Tween 20. After washes, the blot was incubated for 1 hr at 37 C with a 1:1000 dilution of horseradish peroxidase (HRP)–conjugated, anti-mouse antibody (KPL, Gaithersburg, MD). After washing, the membrane-bound antibodies were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Biotechnology, Rockford, IL [now Thermo Scientific]), following the manufacturer's recommendations, and were documented by exposure to medical x-ray film (Amersham Hyperfilm, GE Healthcare, Buckinghamshire, U.K.). In addition, western blots that used purified N1-His protein were also incubated with sera from chickens raised against commercial vaccine a/ck/Guangdong/96 (H5N1). Chicken sera were diluted 1:1000, 1:2000, and 1:4000, and secondary HPR-conjugated anti-chicken antibody (KPL) was used at a 1:10,000 dilution following the procedure described above.

N1-His protein purification. Several batches of N1-His protein were purified from the supernatant of Sf9-cell suspension cultures (60–100 ml) infected with N1-Bac virus, at a multiplicity of infection of five, at a density of 1×10^6 cells/ml. At 48 hr after infection, culture supernatants were collected and centrifuged at $850 \times g$ for 5 min to remove the cells, followed by $17{,}000 \times g$ for 45 min to remove the cell debris. The N1-His was purified from the clarified supernatants by immobilized metal affinity chromatography (IMAC), following the protocol recommended by the manufacturer (Clontech). Briefly, 10 ml of the clarified supernatant was loaded onto the TALON resin column (Clontech) and mixed. The column was washed with 1× washing buffer three times. N1-His protein was eluted with 300 mM of imidazole elution buffer provided by the manufacturer. The purity and yield of the N1-His protein was evaluated in Coomassie (Pierce)–stained SDS10%PAGE gels; the concentration of N1-His protein was measured using the bicinchoninic acid protein assay (Pierce).

N1-ELISA optimization. Different antigen concentrations were tested against serial dilutions of positive and negative control sera in a checkerboard titration fashion. Several twofold, serial dilutions of the N1-His antigen, ranging from 500 ng to 4 ng, were performed in polystyrene, flat-bottom, 96-well plates (FisherBrand, Fisher Scientific, Pittsburgh, PA) with 1× coating buffer (KPL). The twofold, serial dilutions (1:50 to 1:51,200) of chicken serum raised against A/TK/Kansas/80 (H1N1) and negative chicken sera were used for assay optimization. Briefly, diluted N1-His antigen was incubated with 1× coating buffer at 4 C overnight. The plate was washed three times with 1× wash buffer (KPL), incubated with 1% BSA buffer, and incubated with serial dilutions of the positive and negative control sera at 37 C for 1 hr, followed by three washes. The plate was then incubated with HRP-goat, anti-chicken or anti-turkey, antibody conjugate at a 1:1000 dilution (KPL) followed by three washes. The colorimetric reaction was developed with peroxidase substrate solution for 15 min and terminated with a stop solution (KPL). The reactions were read at 405 nm in an ELx808 plate reader (Bio-Tek, Winooski, VT). The optical density (OD) values obtained were compared to determine the optimal antigen concentration that produced the lowest background and the best positive-control titration curve. Using the optimal antigen concentration and serum dilution, a total of 210 chicken and 200 turkey serum samples confirmed as negative for AI by agar gel precipitation tests (AGPT) were tested in triplicates by the N1-ELISA. A negative cutoff value was determined as the mean OD + 2 SD. All serum samples tested by the N1-ELISA were run in triplicate. To account for the assay variability, the sample-to-positive ratio (S:P) was calculated for all sera tested. Samples with average S:P values larger than the cutoff OD value were considered positive for N1 antibodies.

N1-ELISA selectivity of reactions. Two panels of reference chicken sera raised against N1–N9 AI neuraminidase subtypes produced in the National Veterinary Service Laboratory (Ames, IA) and the Southeast Poultry Research Laboratory (Athens, GA) were used to determine the N1-ELISA selectivity of reaction among the different neuraminidase subtypes. In addition, antisera raised against different pathogens of poultry, such as infectious bronchitis virus (IBV) serotypes (Arkansas, DPI, Del-072, GA98), Newcastle disease virus (NDV), avian adenovirus type 1, avian reovirus, infectious laryngotracheitis virus (ILTV), *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS), were tested by N1-ELISA.

Vaccination with H5N2, H5N9, H5N1 and challenge with H5N1. A total of 30, 3-wk-old, specific-pathogen-free (SPF), white leghorn chickens were immunized subcutaneously in the neck nape with 0.3–0.5 ml of commercially available, oil-emulsion vaccine preparations. Ten birds were immunized with H5N2 (A/turkey/California/208651/02, Animal and Plant Health Inspection Service [APHIS] Avian Influenza Vaccine Bank, Riverdale, MD) vaccine, 10 with H5N9 (A/turkey/Wisconsin/68, APHIS Avian Influenza Vaccine Bank) vaccine, and the remaining birds with a commercial, oil-emulsified vaccine based on rgA/chicken/Guangdong/1/1996 (H5N1; Yebio Bioengineering Co., Qingdao, China). All challenge experiments were conducted at the biosafety level 3 facility at the Southeast Poultry Research Laboratory (Athens, GA). At 6 wk of age, H5N2 and H5N9 vaccinated chickens were challenged with approximately 10^6 virus of 50% embryo infective dose (EID_{50}) A/chicken/Suphanburi/2(1)/2004 (H5N1) per bird via the nasal cleft, and chickens vaccinated with the rgA/chicken/Guangdong/1/1996 (H5N1) were challenged with A/chicken/Egypt/9402NAMRU3-HK213/2007 (H5N1) virus in a similar fashion and dose. Oropharyngeal and cloacal swabs were collected at day 2 postchallenge for virus isolation (VI) and titration in 10-day, embryonating, SPF chicken eggs, using standard procedures (16). Titers were expressed as the $\log_{10}$ 50% embryo lethal dose (ELD_{50})/ml. Sera samples were collected from each bird on the day of vaccination, the day of challenge, and 14 days postchallenge. Sera samples were tested by hemagglutination inhibition (HI) as previously described (16,17). Viral strains A/turkey/Wisconsin/1968 (H5N9), A/turkey/Italy/4580/99 (H7N1), and A/chicken/Hong Kong/220/1997 (H5N1) were used as HI antigens depending on the vaccine and challenge dose. The HI titers before and after challenge were expressed as the geometric mean titer (GMT); GMT that were ≤8 were considered positive. Sera samples collected before and after challenge were also tested for the presence of N1 antibodies by the N1-ELISA and NI titers using the quantitative NI assay described below.

Vaccination with avian influenza–fowlpox vector H7 vaccine and challenge with H7N1. A total of 26, 1-day-old, SPF chickens were vaccinated with three H7 fowl poxvirus (FPV) constructs carrying Eurasian-Australian and North American H7 lineage HA genes (A/chicken/Victoria/1/85, A/turkey/Italy/4426/2000, A/turkey/Virginia/66/2002). One-day-old chickens were vaccinated subcutaneously in the neck nape with 0.2 ml containing $10^{3.0}$ $TCID_{50}$ of FPV-H7 constructs. At 3 wk after vaccination, the 26 chickens were challenged intranasally via the nasal cleft with 10^6 EID_{50} of HP A/turkey/Italy/4580/99 (H7N1). Oropharyngeal and cloacal swabs were collected at day 2 postchallenge for VI and titrated as indicated above. Sera samples were collected from each bird on the day of vaccination, the day of challenge, and 14 days postchallenge and were tested for HI and N1-ELISA as indicated above.

Quantitative NI assay. The NI titers on sera collected from experimentally vaccinated and subsequently challenged chickens were measured using a quantitative assay (19). The sera were serially (twofold) diluted and incubated with a predetermined amount of live virus. For the FPV-H7/H7N2 experiment, the viral strain A/turkey/Italy/4580/99 (H7N1) was propagated and used as viral antigen; for experiments H5N2/H5N1 and H5N9/H5N1, the viral strain A/chicken/Suphanburi/2/2004 (H5N1) was used as antigen in the NI assay. After incubating at room temperature for 30 min, a fluorescent NA substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MUN), was added to the plate, loaded into a fluorometer, and heated to 37 C. The kinetic production of the fluorescent

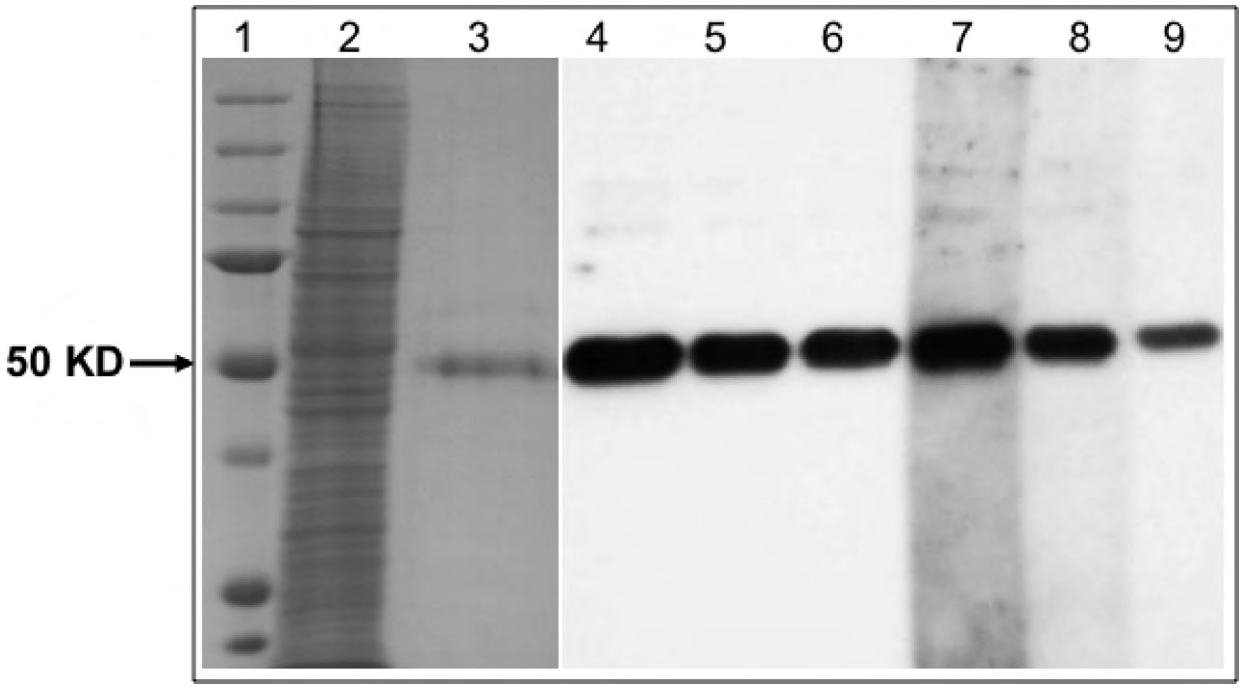

Fig. 1. Expression of N1 gene (A/CK/Indonesia/PA/2003 [H5N1]) in the supernatant of SF9-infected cells infected with N1-baculovirus by SDS-PAGE and western blot analysis. (Lanes 1–3) 10% SDS PAGE gel. (Lane 1) Molecular weight marker. (Lane 2) Supernatant of SF9 cells infected with N1-baculovirus. (Lane 3) Purified N1 protein from 10 ml of N1-baculovirus SF9-infected cells. (Lanes 4 to 9) Western blot analysis with (Lane 4) 500 ng, (Lane 5) 250 ng, and (Lane 6) 125 ng of purified N1 protein loaded per well and reacted with monoclonal antibody specific to the 6-histidine protein tag. (Lanes 7 to 9) 250 ng of purified N1 protein loaded per well and reacted with (Lane 7) 1:1000, (Lane 8) 1:2000, and (Lane 9) 1:4000 dilutions of H5N1 chicken polyclonal serum against vaccine strain A/goose/guandong/1/96 (H5N1).

byproduct (4-methylumbelliferone) was assayed every 3 min, for a total of 30 min, using a Synergy HT Multi-Detection microplate reader (BioTek) with an excitation and emission wavelength of 360 nm and 440 nm, respectively. If there are no antibodies in the serum to block the activity of NA, then a fluorescent moiety (4-methylumbelliferone) is cleaved off the MUN molecule and is detected. Conversely, if antibodies block the function of NA, then MUN is poorly cleaved, and little fluorescence is detected. Samples with little to no fluorescence were determined to be positive for NI activity, as compared with the positive control serum. The NI titer was determined as the $\log_2$ of the dilution below which the fluorescence reading began to achieve levels similar to that of the negative control serum.

N1-ELISA as compared with commercial ELISAs. The N1-ELISA effectiveness to detect an antibody response in commercial turkeys that were vaccinated with the bivalent, inactivated vaccine composed of A/turkey/North Carolina/05 (H3N2) and A/turkey/North Carolina/88 (H1N1) isolates, was compared with Avian Influenza Virus Antibody Test kit (IDEXX, Westbrook, ME), which uses whole virus as antigen, and ProFLOK® AIV *Plus* ELISA kit (Synbiotics, Kansas City, MO), which uses purified NP as antigen; both of these systems detect antibodies against avian influenza, irrespective of the virus subtype. A total of 45 serum samples, 15 samples collected at 3 and 6 wk after the first vaccination and 15 samples collected at 3 wk after the second vaccination, were tested by the three ELISA systems. As indicated by the manufacturers, serum samples with S:P $\geq$ 0.500 were considered positives for the IDEXX kit, and serum samples with S:P $\geq$ 0.350 were considered positive for the Synbiotics kit.

Statistical analysis. A one-way ANOVA with Tukey-Kramer posttest was used to analyze N1-ELISA as compared with the commercial ELISA systems, which were analyzed using Prism v4.03 Software package (GraphPad Software Inc., San Diego, CA).

The sensitivities of the N1-ELISA and the NI tests relative to both VI and HI testing were calculated as the proportion of test-positive birds out of those that had been challenged with a virus containing the N1 antigen and were subsequently confirmed to be VI-positive or HI-positive, respectively. Overall agreement between tests was calculated as the proportion of samples with concordant results out of the total number tested, and κ statistics were calculated to estimate the agreement between tests beyond that which would be expected to occur by chance alone.

RESULTS

N1 protein expression and purification. Earlier attempts to purify membrane-bound, recombinant N1-His from baculovirus-infected cells had resulted in a partially purified protein, which we considered unsuitable for establishment of a specific NA ELISA. To produce a secreted N1-protein, the authentic NA signal peptide sequence was exchanged for the signal peptide sequence of the structural protein gene *efp* from LsNPV (24), which was known to be cleaved by insect cell-signal peptidase during passage through the secretory pathway. As expected, the manipulated N1 was secreted into the supernatant of N1-Bac infected Sf9 cultures and could be purified by IMAC. The purified protein appeared as a single band with a relative molecular weight of approximately 50 kD, as estimated from migration in SDS-PAGE relative to molecular weight standards (Fig. 1, lane 3). Absence of other protein species besides purified N1-His in Coomassie-stained gels ensured sufficient purity of the preparation. In western blots, purified N1-His was detected by the anti-6xHis monoclonal (Fig. 1, lanes 4–6), as well as by polyclonal sera from chickens vaccinated with an inactivated H5N1 vaccine (Fig. 1, lanes 7–9).

N1-ELISA optimization. An N1 protein concentration of 25 ng/well and a sera dilution of 1:100 were determined to provide the optimal OD ratios between positive and negative controls and the lower background readings (Fig. 2a). In the protocol adopted for the N1-ELISA, the antigen was coated in the plate, incubated at 4 C overnight, washed three times with 1× washing buffer (KPL),

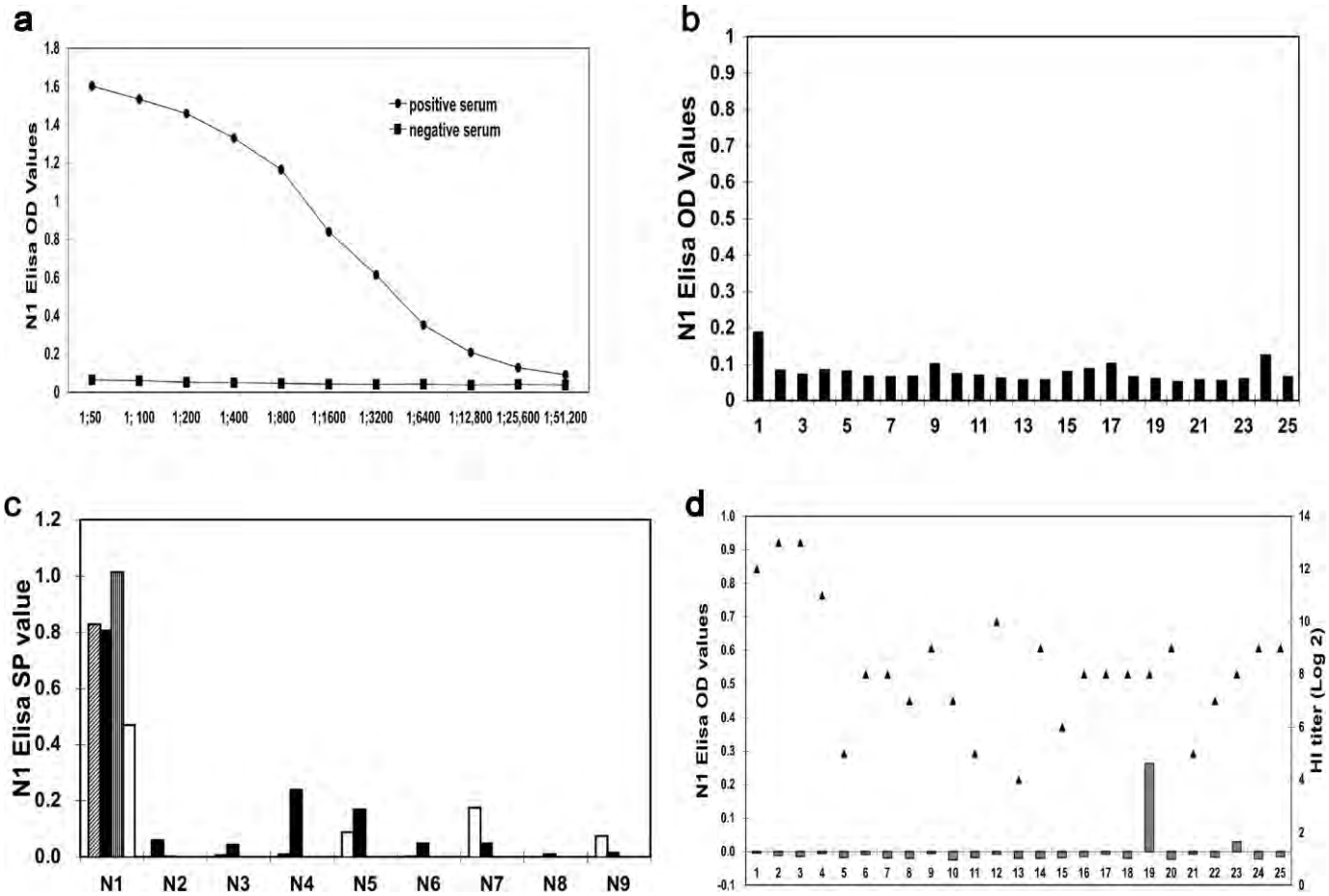

Fig. 2. N1-ELISA optimization. (a) N1-ELISA optimal antigen and serum dilution. Twofold, serial dilutions of the N1 antigen ranging from 500 ng to 4 ng were coated and reacted to twofold, serial dilutions (1:50 to 1:51,200) of chicken polyclonal antibody raised against A/TK/Kansas/80 (H1N1) and negative AI sera. (b) Determination of cutoff OD value of N1-ELISA. Individual OD values on AI negative samples from commercial poultry were used to estimate the N1-ELISA cutoff value (25 samples). (c) N1-ELISA selectivity against N1–N9 serotypes. N1: A/CK/NY/94 (H1N1), A/EQ/Tenn/76 (H7N1), A/TK/KS/80 (H1N1); N2: A/MA/NY/78 (H2N2), A/TK/MA/65 (H6N2), A/TK/DE (H7N2); N3: A/TK/OR/71 (H7N3), A/TK/England (H0N3); N4: A/TK/ONT/67; N5: A/DK/ALB/76 (H12N5), A/Nws/England (H0N5), MA/Gurjev/82 (H14N5); N6: A/GU/MD/77 (H13N6), A/DK/England (H0N6), A/SH/WA/79 (H15N6); N7: A/CK/Germ/49 (H10N7), A/CK/Germ/49 (H10N7), A/CK/Germ/49 (H10N7); N8: A/CK/ALB/78 (H4N8), A/EQ/England (H0N8), N9: TK/WI/68 (H5N9), A/DK/Nws/546 (H0N9), A/DK/Memphis/74 (H11N9). (d) OD values (bars) on N1-ELISA and HI titers (triangles) for 25 serum samples from chickens vaccinated with TK/WI/68 (H5N9; samples 1–5), Env/DE/1346/01 (H5N7; samples 6–10), AV/NY/31588-3/00 (H5N2; samples 11–15), Emu/NY/12716/94 (H5N9; samples 16–20), and Mallard/OH/421/87 (H7N8; samples 21–25).

incubated with 1% BSA buffer followed by incubation of the unknown samples, diluted 1:100 at 37 C for 1 hr, and washed three times. The plate was then incubated with HRP-goat, anti-chicken or anti-turkey, antibody conjugate at a 1:1000 dilution (KPL) followed by three washes. The colorimetric reaction was developed with peroxidase substrate solution for 15 min and terminated with stop solution (KPL). The reactions were read at 405 nm in an ELx808 plate reader (Bio-Tek). Using the established protocol, a total of 210 chicken and 200 turkey AI negative serum samples from commercial flocks were tested by the N1-ELISA, and a OD $\geq$0.222 (2 SD) was determined as the cutoff value of the N1-ELISA assay (Fig. 2b). The selectivity of the N1-ELISA was also determined by using the established protocol and by testing three panels of chicken sera raised against N1–N9 virus neuraminidase subtypes (Fig. 2c). With the exception of one N4 antisera, all the sample samples tested had OD values below the cutoff of value of 0.222. OD values ranging from 0.45 to 0.92 were only obtained for antisera raised against N1 viral strains (Fig. 2c). The selectivity of the N1-ELISA was also tested against sera from chickens vaccinated with inactivated avian influenza vaccines A/turkey/Wisconsin/1968 (H5N9), A/environment/Delaware/1346/2001 (H5N7), A/avian/New York/31588-3/2000 (H5N2), A/Emu/New York/12716/1994 (H5N9), and A/Mallard/Ohio/421/1987 (H7N8; Fig. 2d). All sera samples tested from vaccinated birds had HI titers $\geq$8, and OD values for the N1-ELISA of $<$0.222 (Fig. 2d). The IBV, NDV, ILTV, adenovirus, reovirus, and MG/MS antisera showed OD readings below the determined N1-ELISA cutoff value of $<$0.222 (Table 1).

N1-ELISA in DIVA vaccination strategy. Three vaccination/challenge studies were conducted using vaccines with heterologous NA to the H7N1 and H5N1 HP challenge strains, whereas one vaccination/challenge experiment was conducted using homologous H5N1 vaccine and challenge virus. Serum samples, collected postvaccination and postchallenge, were tested by NI, N1-ELISA, and HI, and VI was also performed to confirm infection. The number of positive samples obtained by the N1-ELISA, HI, NI, and VI are shown in Table 2. In vaccination/challenge experiments H5N2/H5N1 and H5N9/H5N1, N1 antibodies were not detected in samples collected after vaccination by N1-ELISA or the NI assay,

Table 1. N1-ELISA tested against anti-sera from other poultry pathogens.[AB]

Antiserum	Average OD
IBV (ST2)	0.105
IBV (Variant E)	0.120
IBV (DEL 072)	0.054
IBV (GA98)	0.071
IBV (ARK)	0.047
IBV (DPI)	0.062
NDV (B1B1)	0.043
NDV (La Sota)	0.046
Adenovirus	0.047
MG/MS	0.121
ILTV	0.082
Reovirus	0.037

[A]IBV = infectious bronchitis virus (serotypes); NDV = Newcastle disease virus; MG/MS = *Mycoplasma gallisepticum/Mycoplasma synoviae*; ILTV = infectious laryngotracheitis virus.
[B]The cutoff OD for a positive sample was >0.222.

whereas N1 and H5 antibodies were detected in 100% (10 of 10) of the samples collected after vaccination and after challenge from the H5N1-vaccinated and H5N1-challenged chickens by N1-ELISA and HI assay (Table 2). In the vaccination/challenge experiment FPVH7/H7N1, H7 and N1 antibodies were detected only after challenge by HI (26 of 26; 100%), N1-ELISA (7 of 26; 27%), and the NI assay (16 of 26; 62%). Replication of the challenge virus was confirmed by VI in 81% (21 of 26) of the oropharyngeal swabs collected. In the H5N2/H5N1 experiment, H5 antibodies were detected by HI after vaccination (8 of 10; 80%) and after challenge (10 of 10; 100%). N1 antibodies were detected in serum samples collected after challenge by N1-ELISA (4 of 10; 40%), and by NI assay (10 of 10; 100%). Viral replication was confirmed by VI in 90% (9 of 10) of the chickens. In the H5N9/H5N1 experiment, H5 antibodies were detected by HI after vaccination (10 of 10; 100%) and after challenge (10 of 10; 100%). N1 antibodies were detected after challenge by N1-ELISA (6 of 9; 67%) and by the NI assay (6 of 9; 67%). Viral replication was confirmed by VI in all the samples tested (9 of 9; 100%). Table 3 shows the overall agreement, the κ value, and the relative sensitivity of the N1-ELISA and NI assays as compared with VI and HI for sera samples collected in FPV-H7/H7N1, H5N2/H5N1, and H5N9/H5N1 experiments after challenge. The overall agreement and κ value for the N1-ELISA with HI assay and VI was 60.0% ($\kappa = 0.274$; $P < 0.05$) and 62.2% ($\kappa = 0.334$; $P < 0.05$), respectively. The overall agreement of the NI assay with HI and VI was 64.4% ($\kappa = 0.037$; $P > 0.05$) and 55.6% ($\kappa = -0.20$; $P > 0.05$), respectively. The N1-ELISA sensitivity to detecting chickens exposed to the challenge virus was 0.53, compared with HI, and 0.49, compared with VI. The NI assay sensitivity to detecting exposed chickens as compared with HI was 1.0 and 0.94 as compared with VI.

N1-ELISA as compared with commercial AI ELISA systems. Results on antibody responses elicited after vaccination of turkey breeders with the bivalent-inactivated (A/turkey/North Carolina/05 [H3N2]-A/turkey/North Carolina/88 [H1N1] vaccine are presented in Fig. 3. At 3 wk after the first vaccination, the OD values for IDEXX and Synbiotics ELISA systems were significantly higher ($P < 0.05$) than the OD values obtained with the N1-ELISA (Fig. 3a); for the N1-ELISA, 80% (12 of 15) of the samples had OD values ≥0.222, indicating the presence of N1 antibodies, 73% (11 of 15) of the samples were positive for AI antibodies with the IDEXX ELISA, and 87% (13 of 15) were positive for the Synbiotics ELISA, detecting antibodies against the viral NP (Fig. 3d). At 6 wk after the

Table 2. Comparison of serologic assays and virus isolation on experimentally vaccinated and infected SPF chickens.

Vaccine/challenge	HI		N1-ELISA		NI		Virus isolation (2 days postinoculation)	
							Oropharyngeal	Cloacal
	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Postchallenge	Postchallenge
FPVH7/H7N1	0[A]/26 (0.6)[B]	26/26 (229.5)[B]	0[A]/26 (0.01)[C]	7 /26 (0.14)[C]	0 [A]/26 (2.8)[B]	16 /26 (16)[B]	19[A]/26 (2.40)[D]	7/26 (1.2)
H5N2/H5N1	8/10 (13)	10/10 (238.9)	0/10 (0.02)	4/10 (0.107)	0/10 (4)	10/10 (64)	7/10 (2.78)	0/10
H5N9/H5N1	10/10 (101.6)	10/10 (645.1)	0/10 (0.01)	6/10 (0.36)	0/10 (3.7)	7/9 (43.6)	8/9 (3.37)	4/9 (0.9)
H5N1/H5N1	10/10 (158)	10/10 (588.0)	10/10 (1.14)	10/10 (1.18)	ND[E]	ND	10/10 (2.60)	5/10 (1.58)

[A]Number of positive samples by HI, N1-ELISA, NI, and VI.
[B]Geometric mean titer detected by HI and NI.
[C]Mean S:P detected by N1-ELISA.
[D]Mean virus titer ($\log_{10}$ EID_{50}/ml) from positive samples.
[E]ND = not determined.

Table 3. Overall agreement (%), κ value, and relative sensitivity of N1-ELISA and NI assay as compared with HI and VI in samples collected from experimentally vaccinated (H5N2, H5N9, and FPV-H7) and infected (H5N1 and H7N1) chickens.

	N1-ELISA			Quantitative NI		
	% Agreement	κ (*P*)[A]	Relative sensitivity	% Agreement	κ (*P*)	Relative sensitivity
HI[B]	60.0	0.274 (0.016)	0.53	64.4	0.037 (0.802)	1.0
VI[C]	62.2	0.334 (0.003)	0.49	55.6	−0.200 (0.177)	0.94

[A]Probability (P) ≤ 0.05 indicates that agreement is not due to chance alone.
[B]Hemagglutinin inhibition assay using homologous antigen.
[C]Virus isolation on oropharyngeal swabs collected 2 days postchallenge.

first vaccination, OD values for the Synbiotics ELISA were significantly higher ($P < 0.001$) than the IDEXX ELISA and the N1-ELISA OD values (Fig. 3b); 47% (7 of 15) of the samples were positive by the N1-ELISA, 40% (6 of 15) of the samples were positive by the IDEXX ELISA, and 80% (12 of 15) of the samples were positive by the Synbiotics ELISA (Fig. 3d). For samples collected 3 wk after the second H1N1/H3N2 vaccination, OD values for the Synbiotics ELISA were significantly higher ($P < 0.001$) than the N1-ELISA OD values but not significantly different from the IDEXX ELISA (Fig. 3c); 100% (15 of 15) of the samples had OD values ≥0.222, ranging from 0.248 to 0.930 for the N1-ELISA (Fig. 3c), and 87% (13 of 15) of the samples were positive for AI antibodies with both the IDEXX and the Synbiotics commercial ELISA systems (Fig. 3d).

DISCUSSION

The use of vaccination as a tool to control AI in commercial poultry is becoming more relevant as outbreaks of LP AIV and HP AIV continue to be a threat to the poultry industry worldwide. A key element for a successful vaccination outcome involves DIVA with sensitive and specific assays and high-throughput capability for routine screening of vaccinated flocks (5,14). The use of heterologous,

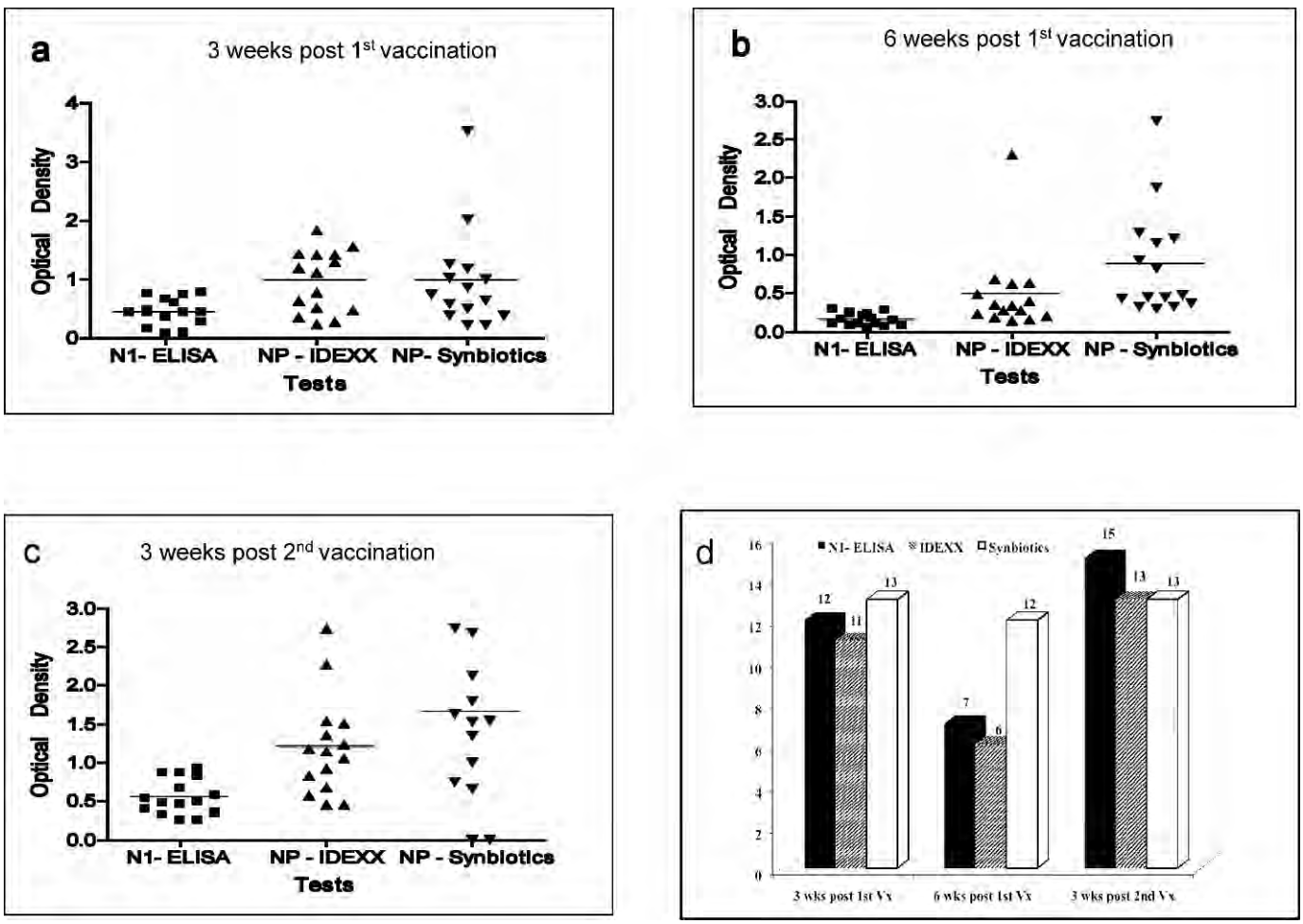

Fig. 3. Comparison of commercial AI ELISA systems and the N1-ELISA. (a) Serum samples from H1N1-vaccinated turkey-breeder samples collected 3 wk after the first vaccination. (b) Serum samples collected 6 wk after the first vaccination. (c) Serum samples collected 3 wk after the second vaccination. Serum samples tested by commercial ELISA systems from Synbiotics with OD > 0.350 and from IDEXX with OD > 0.500 were considered positive. Serum samples tested by N1-ELISA with OD > 0.222 were considered positive. (d) Number of samples positive at 3 and 6 wk after the first H1N1 vaccination and 3 wk after the second H1N1 vaccination.

neuraminidase ELISA systems to monitor infected flocks during a DIVA vaccination campaign has been addressed by several laboratories (9,12). The objective of this study was to develop an N1-ELISA using purified, recombinant N1 protein, to evaluate the reactivity, sensitivity, and ability of the assay to identify vaccinated and subsequently infected birds under controlled conditions. A truncated, recombinant N1-His protein was constructed, based on the N1 gene of A/CK/Indonesia/PA7/2003 (H5N1), expressed, and purified for the ELISA antigen. The N1-ELISA showed a high selectivity for detection of N1 antibodies with no cross-reactivity with other neuraminidase subtypes. In addition, the N1-ELISA showed a broad spectrum of reactivity with sera from N1-subtype North American isolates, A/chicken/NewYork/94 (H1N1), A/turkey/Kansas/1980 (H1N1), A/turkey/North Carolina/88 (H1N1), as well as sera from Eurasian isolates, A/turkey/Italy/4580/1999 (H7N1), A/chicken/Suphanburi/2(1)/2004 (H5N1), and A/chicken/Egypt/9402NAMRU3-HK213/2007 (H5N1). The percentage of the amino acid identity between the clone used for expression of the truncated, recombinant N1 protein, as compared with A/CK/Indonesia/PA7/2003 (H5N1), A/CK/Suphanburi/2(1)/2004 (H5N1), A/turkey/Italy/4580/1999 (H7N1), and A/turkey/NorthCarolina/88 (H1N1) neuraminidase-predicted amino acid sequences, was 99.5%, 96.2%, 92.7%, and 84.7%, respectively. As expected, the isolate A/turkey/NorthCarolina/88 (H1N1) shared the lowest amino acid sequence identity; however, a significant N1 antibody response was detected by the N1-ELISA in commercial turkeys vaccinated with A/turkey/North Carolina/88 (H1N1; Fig. 3a–d). The N1 antibody response at 3 wk after vaccination was comparable to the NP antibody response detected by the Synbiotics ELISA and to the antibody response detected by the whole-virus IDEXX ELISA. However, at 6 weeks after vaccination, the antibody response to the NP (Synbiotics ELISA) and the HA (tested by HI, data not shown) was significantly higher than the antibody responses to the N1 and to the whole-virus ELISA (Fig. 3d). The reduced reactors observed at 6 wks after vaccination with the N1-ELISA and IDEXX-ELISA, as compared with the Synbiotics ELISA, reflects the differences in sensitivity among these assays. In particular, the Synbiotics test is an indirect ELISA that uses a monoclonal-NP complex as antigen, which greatly increases the specificity and sensitivity of the assay. Furthermore, it has been shown that the antibody response to the NP protein is stronger and is maintained for longer periods than the antibody response to other nonenveloped, viral proteins as the matrix and nonstructural proteins (23). At 3 wk after the second H1N1 vaccination, a uniform anamnestic response to N1, NP, and HA (data not shown) was detected by the N1-ELISA, Synbiotics ELISA, IDEXX ELISA, and HI, respectively.

The ability of the N1-ELISA to detect exposed birds after vaccination was evaluated under controlled conditions. Antibody response to N1 was detected in samples collected after vaccination only in the group of chickens vaccinated with inactivated H5N1 vaccine, although only HA, and not N1, antibody response was detected in serum samples collected from chickens vaccinated with H5N2, H5N9, and recombinant FPV-H7 vaccines. As expected, postchallenge, the antibody response to HA, measured by HI, significantly increased. More relevant was the detection of N1 antibodies in vaccinated (H5N2, H5N9, and FPV-H7) and subsequently challenged chickens. To confirm infection in vaccinated chickens, VI from oropharyngeal swabs was performed at 2 days after the challenge, which was previously determined to be the peak of viral shedding (16). Because the goal of a DIVA vaccination strategy is to detect infected birds within a population of vaccinated flocks, the ability of the N1-ELISA and NI assay to detect vaccinated/exposed chickens was compared with VI, as a measure of infected chickens within a vaccinated population, and to HI, as an standard serologic assay used in the diagnosis of AI. Based on the scale recommended by Landis and Koch (10), κ values indicated that the N1-ELISA results were in fair agreement with both VI and HI results. On the other hand, κ values comparing the NI assay with HI and VI indicated poor agreement. This is due, in part, to the significantly higher sensitivity of the NI assay (HI = 1.0, VI = 0.94) as compared with the N1-ELISA (HI = 0.53, VI = 0.49). One factor that may contribute to the increased sensitivity of the quantitative NI assay is the fluorescence substrate used for the detection of the viral neuraminidase enzymatic activity; this fluorescence-based detection is probably more sensitive than the conventional, colorimetric detection system used by the N1-ELISA (19). Despite its increased sensitivity, the NI quantitative assay requires the propagation of virus and the handling of infectious, HP AI viruses and may not, therefore, be applicable in laboratories worldwide. Furthermore, the NI assay cannot be used for screening of a high number of sera samples. In similar fashion, several studies have suggested that the IFA methods are probably more sensitive than indirect ELISA for detecting NA antibodies in vaccinated-exposed poultry populations (5,8). However, IFA methods are not suitable for automation and high-throughput screening of flocks and are dependent on individual interpretation skills.

Several studies have demonstrated that vaccinated and subsequently infected flocks sustain low-grade infections with insufficient replication of the challenge virus, which, consequently, will affect the antibody responses to viral antigens (7,11,16). Therefore, the possibly weaker antibody response to the NA antigen of the challenge virus presents a potential problem for the heterologous NA DIVA vaccination strategy (12) and emphasizes the need to design serologic assays characterized for their increased sensitivity.

Overall, the N1-ELISA was able to specifically detect N1 antibodies in vaccinated and subsequently infected chickens. Although the N1-ELISA showed lower sensitivity than the NI assay, it was demonstrated that screening for N1 antibodies by N1-ELISA was an effective, rapid, and easy-to-interpret assay, capable to identify exposure to challenge viruses within a vaccinated population of birds. In its current form, the application of the N1-ELISA in the field will require the collection of a larger-than-usual volume of samples per flock to account for the assay sensitivity.

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A Heterologous Neuraminidase Subtype Strategy for the Differentiation of Infected and Vaccinated Animals (DIVA) for Avian Influenza Virus Using an Alternative Neuraminidase Inhibition Test

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SUMMARY. The option of vaccinating poultry against avian influenza (AI) as a control tool is gaining greater acceptance by governments and the poultry industry worldwide. One disadvantage about vaccination with killed whole-virus vaccines is the resulting inability to use common serologic diagnostic tests for surveillance to identify infected flocks. There has been considerable effort to develop a reliable test for the differentiation of infected from vaccinated animals (DIVA). The heterologous neuraminidase (NA) subtype DIVA approach has been used with some success in the field accompanied by an *ad hoc* serologic test. The traditional NA inhibition (NI) test can be used for all nine NA subtypes, but it is time consuming, and it is not designed to screen large numbers of samples. In this study, a quantitative NI test using MUN (2′-[4-methylumbelliferyl]-α-D-Nacetylneuraminic acid sodium salt hydrate) as an NA substrate was investigated as an alternative to the traditional fetuin-based NI test in a heterologous neuraminidase DIVA strategy. Serum NI activity was determined in chickens administered different vaccines containing different H5 and NA subtypes and challenged with a highly pathogenic avian influenza (HPAI) H5N2 virus. Prior to challenge, the NI DIVA test clearly discriminated between chickens receiving vaccines containing different antigens (e.g., N8 or N9) from control birds that had no NA antibody. Some birds began to seroconvert 1 wk postchallenge, and 100% of the vaccinated birds had significant levels of N2 NI activity. This activity did not interfere with the presence of vaccine-induced NI activity against N8 or N9 subtypes. The level of N2-specific NI activity continued to increase to the last sampling date, 4 wk postchallenge, indicating the potential use for the heterologous NA-based DIVA strategy in the field.

RESUMEN. Utilización de un subtipo heterólogo de neuraminidasa dentro de una estrategia para la diferenciación de animales infectados y vacunados (DIVA) para el virus de la influenza aviar mediante una prueba alternativa de inhibición de la neuraminidasa.

La opción de vacunar contra la influenza aviar en la avicultura como una herramienta de control está ganando mayor aceptación por los gobiernos y por la industria avícola a nivel mundial. Una desventaja acerca de la vacunación con vacunas inactivadas con virus completos es la consecuente incapacidad para utilizar pruebas de diagnóstico serológico comunes para identificar parvadas infectadas. Se han desarrollado esfuerzos considerables para desarrollar pruebas confiables para la diferenciación entre animales infectados y vacunados (DIVA). La estrategia DIVA basada en una neuraminidasa heteróloga se ha utilizado con éxito en el campo acompañada de una prueba serológica para este propósito. La prueba tradicional de inhibición de la neuraminidasa puede ser usada para los nueve diferentes subtipos, pero es una prueba tardada y que no está designada para analizar cantidades grandes de muestras. En este estudio, se realizó un estudio cuantitativo de inhibición de la neuraminidasa utilizando MUN (2′-[4-metilumbeliferil]-α-D ácido-N Acetilneuramínico hidrato) como el sustrato para la neuraminidasa, como una alternativa para la prueba de neuraminidasa basada en fenitoína en una estrategia DIVA utilizando una neuraminidasa heteróloga. La actividad inhibidora de la neuraminidasa en el suero fue determinada en pollos a los que se les administró diferentes vacunas que contenían diferentes subtipos de H5 y NA y que fueron desafiados con el virus de la influenza aviar altamente patogénica subtipo H5N2. Antes del desafío, la prueba de inhibición de la neuraminidasa dentro de la estrategia DIVA, claramente discriminó entre pollos que recibieron vacunas con diferentes antígenos (por ejemplo N8 o N9) de la aves control que no tenían anticuerpos contra la neuraminidasa. Algunas aves empezaron a seroconvertir una semana después del desafío y 100% de las aves vacunadas tenían niveles significativos de actividad inhibidora de la neuraminidasa N2. Esta actividad no interfirió con la presencia de la actividad inhibidora de la neuraminidasa contra los subtipos N8 y N9. El nivel de actividad inhibitoria de la neuraminidasa específica contra N2 continuó incrementándose hasta la ultima fecha de muestreo, cuatro semanas después del desafío, indicando el uso potencial en el campo de la estrategia DIVA basada en el uso de una neuraminidasa heteróloga.

Key words: influenza A virus, DIVA strategy, neuraminidase inhibition test, diagnostic, chickens

Abbreviations: A/CK/Queretaro = A/CK/Queretaro/14588-19/95 (H5N2); AI = avian influenza; ANOVA = analysis of variance; BPL = β-propiolactone; DIVA = differentiating infected from vaccinated animals; HPAI = high pathogenic avian influenza; LPAI = low pathogenic avian influenza; NA = neuraminidase; NI = neuraminidase inhibition; MUN = 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid

Vaccination against avian influenza (AI) has been a controversial issue for many years, in large part because of effects on trade of vaccinated poultry (4,13). One of the major issues for the use of vaccination is the inability of the current surveillance serologic tests such as the agar gel immunodiffusion assay or commercially available antibody enzyme-linked immunosorbent assay (ELISA) tests that detect type A influenza antigens to discriminate between the immune responses produced to vaccination and to infection (17). This diagnostic limitation affects not only surveillance programs but

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also international commerce because trading countries are not willing to accept poultry or poultry products from vaccinated birds because of the assumption that they are from non–virus-free areas and could potentially be infected (17). After the emergence of highly pathogenic avian influenza (HPAI) H5N1 virus in Southeast Asia in 1996 and its spread across Asia and into Europe and Africa (1,2,28), vaccination has gained interest as a more cost effective control option, and its restriction has even been lifted in some countries (5,6,8,17). There have been several research studies evaluating different differentiation of infected from vaccinated animals (DIVA) testing approaches for avian influenza virus (AIV) (10,11,15,16,36,38). However, there is no readily available DIVA test that allows the detection of field virus-infected birds within a vaccinated population, especially in the context of effectively matching the vaccine and the uncertainty of the infecting AIV subtype. The heterologous neuraminidase (hNA) approach was evaluated under field conditions in Italy during the H7N1 avian influenza epidemic (1999–2000) using an *ad hoc* indirect fluorescent antibody assay (IFA) and baculovirus-derived recombinant NA protein as antigen for the detection of specific anti-N1 antibodies (8,10). Additional studies have also evaluated an anti-N3 immunofluorescent antibody test with laboratory and field samples (11). Although antibodies directed against the NA protein can provide some protection to AIV challenge, antibodies targeting the hemagglutinin protein are critical for protection (16,18,33,35). However, the heterologous NA strategy potentially requires antibody tests to each of the nine NA subtypes, and it presumes that only a single NA subtype of virus is circulating in poultry. If more than one AIV isolate with different NA subtype genes is circulating at the same time, then results from this test can be difficult to interpret. Similarly, the IFA test used to detect seroconversion against different NA subtypes can be difficult to interpret because the results are qualitative (9,10,26). The NA inhibition (NI) test is a potential alternative test for use in a DIVA strategy; it uses fetuin as a substrate and is primarily performed at reference laboratories for NA virus subtyping. However, the NI test is a more labor intensive, time consuming technique, and it is impractical for screening large numbers of samples. In these studies, a quantitative NI assay using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUN) as a fluorescent substrate was evaluated as a companion test in the heterologous neuraminidase DIVA approach (35). Using this quantitative NI assay approach, two objectives were pursued: 1) to quantitatively determine the onset of the NI activity to the challenge virus, and 2) to determine if this assay will clearly discriminate the serum NI activity produced to the NA protein of the infected virus from the NI activity produced to the NA protein from the vaccine virus.

MATERIALS AND METHODS

Viruses. The HPAI virus A/Chicken/Queretaro/14588-19/95 (H5N2)(CK/Queretaro/95) (12) and A/Turkey/Wisconsin/68 (H5N9) (TK/WI/68) used in this study were obtained from the virus repository at the Southeast Poultry Research Laboratory (SEPRL).

Titration of the virus. Tenfold dilutions of the viruses were performed using brain heart infusion medium (BHI; Difco, Detroit, MI). Five 10-day-old chicken embryos per dilution were inoculated with 100 μl via allantoic cavity and incubated for 7 days at 37 C. Eggs were candled daily to detect deaths, and they were chilled overnight at 4 C before harvesting allantoic fluid and testing for hemagglutination. Samples where death of the embryo occurred during the first 24 hr after inoculation were discarded. The egg 50% infectious dose (EID_{50}) was calculated using the method of Reed and Muench (23).

Recombinant vaccine viruses. RNA from the isolates used for the vaccine preparation was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) from infected allantoic fluid. Next, hemagglutinin (HA) genes from A/Chicken/Pennsylvania/13609/93 (H5) virus, neuraminidase (NA) genes from A/Duck/New York/191255-59/02 (N8), and full-length NS1 genes from A/Turkey/Oregon/71 viruses were amplified by one-step reverse transcriptase (RT)-PCR with HA, NA, and NS1 segment-specific primers as described previously (16). The RT-PCR products were reamplified by PCR with primers containing *Bsm*BI sites, digested with *Bsm*BI restriction enzyme, and cloned into pHH21 vectors between the promoter and terminator sequences of RNA polymerase I. Primer sequences will be provided upon request. RNA polymerase I constructs that contain the other five internal genes of A/WSN/33 virus and four expression plasmids (pCAGGS-WSN-NP, pcDNA774-PB1, pcDNA762-PB2, and pcDNA787-PA; provided by Dr. Yoshihiro Kawaoka at the University of Wisconsin) (20), and the H5, N8, and NS1 plasmids were transfected using Lipofectamine 2000 (Invitrogen, San Diego, CA) in 293T cells to create the reassortant virus used as a vaccine as previously described (20).

Vaccine preparation. The viruses generated by reverse genetics were grown in 10-day-old embryonating chicken eggs (ECE), and the allantoic fluid was pooled for each virus. Infectious titers were determined prior to inactivation with 0.1% betapropiolactone (BPL; Sigma, St. Louis, MO). The procedure to produce oil-emulsion vaccines was performed as previously described (25). Briefly, one part aqueous antigen was emulsified in four parts oil phase (Drakeol 6 VR mineral oil, Penreco, Burler, PA) containing 7.5% sorbitan monooleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 2.5% polysorbate 80 (Tween 80, ICI US).

Vaccination, challenge, and determination of virus shedding. Groups of ten 2-wk-old white leghorn chickens obtained from specific-pathogen-free (SPF) flocks maintained at the Southeast Poultry Research Laboratory (SEPRL) were immunized subcutaneously with the equivalent of $10^{7.5}$ 50% egg infective doses (EID_{50}) per 0.5 ml of rH5N8 inactivated vaccine or with 0.5 ml of commercial avian influenza vaccine TK/WI/68 subtype H5N9. A nonvaccinated group was included as a challenge control. Two weeks later, vaccination chickens were challenged intranasally via the choanal cleft with 10^5 EID_{50}/0.2 ml of highly pathogenic CK/Queretaro/95 (H5N2). Blood samples were obtained from each bird via the brachial vein before challenge and every week after challenge for 4 wk, and the serum was harvested and stored at −20 C for serologic testing. Tracheal swabs were taken from infected birds at days 3 and 5 after infection, suspended in 1 ml of BHI broth, and stored at −70 C. Total RNA was extracted using an RNeasy mini kit (Qiagen), and real-time RT-PCR was performed with primers and probes specific for type A AI virus matrix RNA (24). The fluorescence data were taken at the end of each annealing step using a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine. Food and water were provided *at libitum*, and management was provided as required by the Institutional Animal Care and Use Committee.

Quantitative neuraminidase inhibition (NI) assay. A fluorescent substrate MUN (Sigma) was used to measure the NA enzymatic inhibitory activity in serum samples, as previously described (35). Briefly, 25 μl of stock viruses (e.g., A/CK/Queretaro/95 [H5N2], rH5N8 [H5N8], or TK/WI/68 [H5N9]) were diluted 1:8 in a calcium saline buffer (CaS; 20 mM sodium borate, 7 mM calcium chloride, 154 mM sodium chloride, 12.5 mM sodium acetate; pH 7.2), and 25 μl were added per well of a 96-well, clear, round-bottom plate. Next, 25 μl of each serum sample were twofold diluted, in the respective diluted stock virus, to produce a range of 1:2 to 1:4096. Virus and serum were incubated at room temperature for 15 min, and 15 μl of 0.5 mM MUN were added per well. Uninfected allantoic fluid or MUN alone served as negative controls. The kinetic production of fluorescent by-product 4-methylumbelliferone was assayed after 15 min of incubation at 37 C using a Synergy HT Multi-Detection microplate reader (BioTek, Winooski, VT) with an excitation and emission wavelength of 360 and 440 nm, respectively. The linear range of NA activity was determined by plotting fluorescence data *vs.* the inverse of viral dilution ($1/x$, where $x = \log_2$ dilution factor). Samples with little to

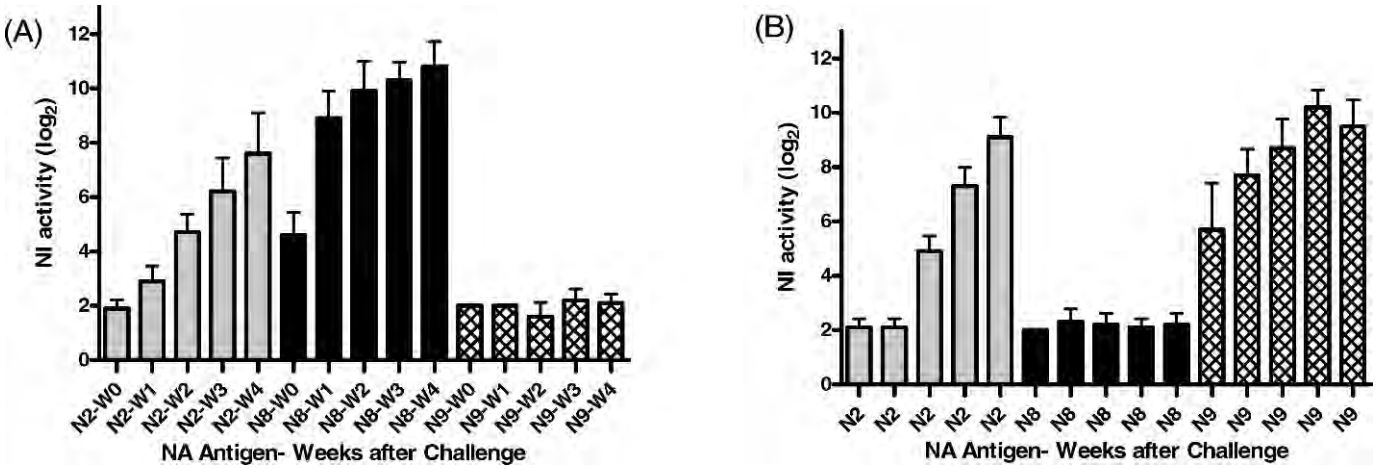

Fig. 1. Two groups of ten 2-wk-old SPF chickens were immunized with inactivated oil-emulsion vaccine rH5N8 (A) or commercial vaccine H5N9 (B) and challenged 2 wk later with 10^5 EID_{50} of HPAI virus CK/Queretaro/95 (H5N2). Serum samples taken at 2 wk after vaccination right before challenge (W-0), and every week for four consecutive weeks after challenge (W-1, W-2, W-3, W-4) were analyzed for NI activity with inactivated antigens produced with CK/Queretaro/95 (N2), TK/WI/68 (N9), and rH5N8 (N8) viruses as described in Materials and Methods. Data represent mean ± standard deviation $\log_2$ NI titer of ten chickens per vaccination group.

no fluorescence were determined to be positive for NI activity, as compared to control chicken serum. The NI titer was determined as one $\log_2$ dilution below which fluorescence began to achieve levels similar to that of SPF control serum for each antigen. NI values $\leq \log_2 3$ were considered negative.

Statistical analysis. Data were analyzed using Prism v4.03 software package (GraphPad Software Inc., San Diego, CA), and are expressed as mean ± standard deviation from chickens per experimental group. One-way analysis of variance (ANOVA) with Tukey's post-test were used to analyze NI and HI data.

RESULTS

Neuraminidase inhibition activity in birds immunized with H5 subtype oil-emulsion vaccines and challenged with an H5 HPAI virus containing a heterologous neuraminidase. To evaluate the enzymatic NI assay as a companion test in the heterologous NA DIVA approach, groups of chickens that were vaccinated with inactivated rH5N8 or commercial vaccine H5N9 were challenged with 10^5 EID_{50}/bird of the HPAI virus CK/Queretaro/95. The serum samples were taken at 2 wk after vaccination shortly before challenge (W-0), and every week for four consecutive weeks after challenge (W-1, W-2, W-3, W-4), and they were evaluated for NI activity. The serum NI activity was assayed against three inactivated antigens CK/Queretaro/95 (H5N2), rH5N8 (H5N8), and TK/WI/68 (H5N9; Fig. 1).

At wk 0, both vaccinated groups showed positive NI results only to the homologous NA antigen contained in the vaccine. The chickens vaccinated with the recombinant vaccine had NI titers of 4.6 $\log_2$ ± 0.84 (against the N8 antigen), and those vaccinated with the commercial vaccine had a mean titer of 5.7 $\log_2$ ± 1.7 (against the N9 antigen). The NI results of both vaccinated groups were significantly higher ($P < 0.001$) when compared to NI titer to the N8 or N9 antigens obtained in the sham-vaccinated group (Table 1). After challenge in the rH5N8 group, the NI activity against the NA protein of the challenge strains (N2) was detected at 1 wk postchallenge in only one of 10 birds (NI titer of 4 and mean group NI titer 2.8 $\log_2$ ± 0.84), but at 2 wk postchallenge, all the birds in this group had detectable titers against the challenge N2, and $\log_2$ titer ranged from 4 to 6 $\log_2$ (group NI titer of 4.7 $\log_2$ ±

Table 1. NI activity in serum samples from chickens immunized with inactivated oil-emulsion vaccines and challenged with HPAI virus containing heterologous neuraminidase.

Weeks after challenge[A]	Sham vaccine			Recombinant vaccine (H5N8)			Commercial vaccine (H5N9)		
	NI test antigen								
	N2	N8	N9	N2	N8	N9	N2	N8	N9
W-0	2.1^{1C} (0.56)	2^{1} (0.0)	3^{1} (0.0)	1.9^{a1B} (0.32)	4.6^{a2} (0.84)	2^{a1} (0.0)	2.1^{a1} (0.32)	2^{a1} (0.0)	5.7^{a2} (1.7)
W-1				2.8^{b1} (0.63)	8.9^{b2} (0.89)	2^{a1} (0.0)	2.1^{a1} (0.32)	2.3^{a1} (0.48)	7.7^{b2} (0.95)
W-2				4.7^{c1} (0.67)	9.9^{c2} (1.1)	1.6^{a3} (0.5)	4.9^{b1} (0.57)	2.2^{a2} (0.42)	8.7^{c3} (1.1)
W-3				6.2^{d1} (1.2)	10^{c2} (0.67)	2.2^{a3} (0.4)	7.3^{c1} (0.67)	2.1^{a2} (0.32)	10^{d3} (0.63)
W-4				7.6^{e1} (1.5)	11^{c2} (0.92)	2.1^{a3} (0.3)	9.1^{c1} (0.7)	2.1^{a2} (0.32)	9.5^{d3} (0.97)

[A]Groups of ten 2-wk-old birds were immunized with recombinant (rH5N8) or commercial (H5N9) inactivated vaccines or were sham vaccinated and challenged with HPAI A/CK/Queretaro/14588-19/95 (H5N2) virus 2 wk after vaccination. Serum samples were collected after vaccination right before challenge (W-0) and every week after challenge for 4 wk, and samples were tested for NI activity against three different NA proteins: N2 (challenge), N8 (recombinant vaccine), and N9 commercial vaccine. Values represent NI activity mean $\log_2$ ± (standard deviation).

[B]NI values in each column denoted by different lowercase letters (a–d) are statistically significantly different from each other ($P < 0.050$) as determined by Tukey test All Pairwise Multiple comparison procedure.

[C]NI values under each row denoted by numbers (1–3) are statistically significantly different from each other ($P < 0.050$) as determined by Tukey test All Pairwise Multiple comparison procedure.

Table 2. HI activity in serum samples from chickens immunized with inactivated vaccines and challenged with HPAI virus containing heterologous neuraminidase.

	HI test[B]					Virus shedding data	
	Weeks after challenge					After challenge	
Vaccine treatment[A]	W-0	W-1	W-2	W-3	W-4	3 days	5 days
Sham vaccine	$\pm 3^{1}$						
H5N8-SEPNS1 inactivated vaccine	5.4^{a2} (1.6)	10.7^{b1} (1.6)	11.0^{b1} (0.7)	11.3^{b1} (0.7)	10.9^{b1} (0.8)	3/10	1/10
Commercial inactivated H5N9 vaccine	8.0^{a3} (0.8)	12.3^{b1} (1.3)	11.7^{b2} (1.6)	13.4^{b2} (1.1)	12.9^{b2} (1.1)	0/10	0/10
Negative control group	$\leq 3^{1}$	$\leq 3^{2}$	$\leq 3^{3}$	$\leq 3^{3}$	$\leq 3^{3}$	0/10	0/10

[A]Serum samples from birds vaccinated with recombinant (rH5N8) or commercial (H5N9) inactivated vaccines as well as the sham-vaccinated birds were tested for HI activity with BPL inactivated homologous antigen to each vaccine strain. Values represent mean ± standard deviation $\log_2$ HI activity.

[B]HI values in each row denoted by different lowercase letters (a–b) are statistically significantly different from each other ($P < 0.050$), and HI values under each column denoted by different numbers (1–3) are statistically significantly different from each other ($P < 0.050$) as determined by Tukey test All Pairwise Multiple comparison procedure.

0.63). In the group immunized with the commercial vaccine, none was antibody positive 1 wk postchallenge, but all the chickens responded to the challenge NA 2 wk after challenge, and individual $\log_2$ NI titers ranged between 4 and 6 $\log_2$ (mean group NI titer $\log_2$ 4.9 ± 0.57). The initial NI titers in both groups were significantly ($P < 0.001$) higher when compared with the NI activity to N2 observed in the same groups before challenge or to the N2 activity observed in the sham-vaccinated group (Table 1). At 3 wk after challenge, the NI titers against the N2 subtype increased in both vaccine groups, and NI $\log_2$ levels for the recombinant vaccinated group ranged between 5 and 9 (mean group NI titer of 6.2 $\log_2$ ± 1.2), and between 6 and 8 (group NI titer of 7.3 $\log_2$ ± 0.67) for the commercially vaccinated group. The NI titers were significantly higher ($P < 0.001$) at 4 wk postchallenge as compared to NI activity at 1 and 2 wk postchallenge for both groups. Both vaccinated groups were tested for NI activity against the NA subtype contained in the opposite group vaccine, and results demonstrated no cross-reactivity and indicated good specificity of this test.

The vaccine immunogenicity was evaluated with the HI test using BPL-inactivated HI antigen homologous to each vaccine strain. The HI data showed a strong antibody response to the vaccine in both immunized groups at day of challenge with an HI $\log_2$ titer of 5.4 and 8.0 for the group immunized with the recombinant and commercial vaccine, respectively (Table 2). The HI titers in both groups increased significantly ($P < 0.001$) after the challenge and stayed high during the testing period.

Virus shedding was detected in 3 of 10 tracheal swabs taken 3 days after challenge and 1 of 10 tracheal swabs taken 5 days after challenge in the group immunized with the rH5N8 vaccine. No virus shedding was recorded in the group vaccinated with the commercial vaccine or in the negative control that was not vaccinated or challenged. All the birds in the sham-vaccinated and challenged group died by day 3 after the challenge, and serology was not performed on these birds.

DISCUSSION

The results obtained using the NI test in the present work indicate that the DIVA strategy based on the immune response to NA is a rapid and sensitive approach that effectively discriminates between vaccinated and HPAI-challenged chickens. Using our protocol, we could quantitatively determine the NI titer of 100 serum samples, against a single NA antigen, in approximately 3 hr. This technique may be made even more rapid using a high-throughput robotic approach. In contrast, the standard fetuin-based NI assay takes approximately 6 hr per plate and yields only qualitative data (3). These findings confirm previous observations (7,16,18,33) where the heterologous neuraminidase DIVA strategy has been able to discriminate infection within a vaccinated population, and has been used successfully in the field in the control of AIV (10,11). The NI test described here offers a quantitative approach that does not just identify the antibody response to different NA subtypes but also enables the measurement of the antibody responses over time. This quantitative property may allow a better characterization of both the immune response as well as evaluate protectiveness of the vaccine in field situations. The antibody response to the NA protein can provide protection to influenza infection if present in sufficient quantities, and the availability of a quantitative NA antibody test can help correlate levels of protection (35). Antigenic drift occurs in the NA protein as well as the HA protein, and a poorly matched antigen may affect the sensitivity for detecting NA antibody. The NI test used in this method can use the field strain to provide maximal sensitivity (14,19,22,35). Thus, this method is quite flexible and can be applied to all nine NA subtypes.

Because of the unpredictability of the field challenge, there is a concern that the circulation of a virus with a homologous NA subtype to the vaccine may prevent this method from working as planned. The option of genetically engineering vaccines with a rare NA from the challenge virus as demonstrated in this work with the recombinant vaccine will be a useful option once the regulatory and intellectual property issues are overcome. Alternative methods of producing viruses with a rare NA subtype, such as classical reassortment methods, can potentially overcome some of the intellectual property issues.

Previous studies have shown that high levels of antibody to the NA protein can provide at least partial protection from virulent challenge (35), and this additional protection may be lost with the heterologous NA DIVA strategy. However, several experimental studies have shown that matching of the HA protein may by itself provide excellent protection from clinical disease and reduce virus shedding. For example one study on the protection of heterologous NAs containing vaccines against HPAI H5N1 challenge indicated that increasing the HA unit per dose of heterologous H5N3 vaccine could provide 100% protection from mortality and could prevent detectable viral shedding. Another study, using a low pathogenic avian influenza (LPAI) challenge, compared homologous and heterologous NA vaccines with the same HA protein, and it showed

no additional protection with the inclusion of homologous NA (16). However in this study, the HA gene of vaccine and challenge were closely matched, and it is not clear if high levels of specific NA antibody would provide measurable protection in this experimental model if the HA genes were poorly matched.

The quantitative NI assay detected inhibitory antibodies against the NA component of the challenge virus in 100% of chickens immunized with two different vaccines containing viruses with heterologous NA. The earlier detection of antibodies to the challenge NA in the rH5N8-vaccinated group probably indicates higher levels of AIV replication of the challenge, although the difference was not significant because just 1 of 10 birds seroconverted at 1 wk after challenge. Although the sequence similarity between the two vaccines and the challenge strain were high, the rH5N8 vaccine induced lower levels of antibody, contributing to more birds shedding virus (Table 2). The difference in the antibody response between the vaccines is not clear, although it is possible that the proprietary adjuvant in the commercial vaccine may be superior to that used for the vaccine prepared in house. However, differences in antigenicity of the viruses and differences in the concentration of HA protein can also influence the quality of the immune response (31).

The stronger immunity observed in the commercial-vaccinated group seems to delay the onset of the antibodies against the NA protein of the challenge virus. These results are expected since protective immunity is known to decrease virus shedding, and the immune system requires a minimal level of replication to provide antigen to stimulate an antibody response (30,32,33). In this study, we used a, HPAI challenge model that was 100% lethal to the sham-vaccinated controls, showing that the virus in unprotected birds replicated to high titer. Although few of the vaccinated birds had detectable virus, the RT-PCR test used for quantification may not have been sensitive enough to detect low levels of viral shedding.

A potential drawback to any serologic DIVA strategy is that protection from the vaccine may provide too much protection, and that infected birds may not have enough viral replication and antigen stimulation for a strong antibody response for serologic detection. The heterologous DIVA strategy needs to be investigated to further understand how differences between flocks and outbreak viruses may affect the sensitivity of the test. Differences that should be evaluated include vaccines are well matched *vs.* poorly matched to the challenge strain, high *vs.* low levels of HA antibody, high *vs.* low challenge dose, and highly pathogenic *vs.* low pathogenic challenge. This research will provide an understanding of how robust the NA DIVA vaccine strategy is, and it will provide data to suggest the number of birds that need to be sampled per flock to ensure a reasonable and reproducible level of detection.

The results of the present experiments also show that some variation is observed in the time it takes until initial detection of NI activity against the NA protein of the field challenge. Previous work in turkeys shows a similar antibody response, where most birds become seropositive between 7 and 10 days after experimental challenge (11). The study in turkeys also showed that unvaccinated control groups had more birds that seroconverted at 7 days compared to the two vaccinated groups, likely because the increased replication resulted in more antigen presentation and a more robust immune response. The 1- to 2-wk window is not unexpected for an antibody response to occur, but this delay to diagnosis provides a gap in detection that is present with all serology-based assays (27,29).

NI titers to the vaccine NA protein in each vaccinated group increased even after challenge with heterologous NA. This continued increase in antibody is likely because the antibody peak had not been reached because challenge was only 2 wk after vaccination. An alternative possibility is the concept of original antigenic sin that has been reported for influenza response in humans. The theory is that the original HA subtype a person is exposed to will influence the immune response for the rest of the person's life. This even extends to when a person is exposed to other HA subtypes, such that they will develop not only an immune response to the new subtype, but they often have a boosted response to the original HA subtype as well (22,37). Unfortunately, in this experiment, vaccinated-only controls were not included, so these different theories could not be tested.

The results obtained in this study confirm previous reports on the feasibility of DIVA approaches based on the detection of antibodies to the challenge virus heterologous NA (10,11,16,18,33). The NI test described in the present work clearly discriminates antibodies against the vaccine from antibodies against the challenge NA proteins with high specificity. In conclusion, the quantitative NI technique, using MUN as substrate, is a rapid and easy-to-use test for the detection of NA antibodies.

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Differentiation of Infected and Vaccinated Animals (DIVA) Using the NS1 Protein of Avian Influenza Virus

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SUMMARY. Vaccination against avian influenza (AI) virus, a powerful tool for control of the disease, may result in issues related to surveillance programs and international trade of poultry and poultry products. The use of AI vaccination in poultry would have greater worldwide acceptance if a reliable test were available that clearly discriminated between naturally infected and vaccinated-only animals (DIVA). Because the nonstructural protein (NS1) is expressed in influenza virus–infected cells, and it is not packaged in the virion, it is an attractive candidate for a DIVA differential diagnostic test. The aim of this work was to determine the onset of the antibody response to the NS1 protein in chickens infected with low pathogenic avian influenza (LPAI) virus, and to evaluate the diagnostic potential of a baculovirus-expressed purified NS1 protein in an indirect ELISA-based DIVA strategy. An antibody response against NS1 was first detected 3 wk after infection, but the antibody levels were decreasing rapidly by 5 wk after infection. However, most chickens did not have detectable antibodies in spite of high hemagglutination inhibition (HI) antibody titers in one group. In birds vaccinated with inactivated oil-emulsion vaccines, antibodies against NS1 were not detected before virulent challenge, and only a small percentage of birds seroconverted after homologous LPAI virus challenge. Vaccinated birds challenged with highly pathogenic AI showed a higher NS1 antibody response, but at most only 40% of birds seroconverted against NS1 protein by 3 wk after challenge. Because of the variability of seroconversion and the duration of the antibody response in chickens, the NS1 protein DIVA strategy did not perform as well as expected, and if this strategy were to be used, it would require sampling a higher number of birds to compensate for the lower seroconversion rate.

RESUMEN. Diferenciación de animales infectados y vacunados (DIVA) utilizando la proteína NS1 del virus de la influenza aviar.

La vacunación contra la influenza aviar, que es una herramienta poderosa para el control de la enfermedad, puede resultar en problemas relacionados con los programas de vigilancia y comercio internacional de aves y productos avícolas. El uso de la vacunación contra la influenza aviar tendría mayor aceptación mundialmente si hubiera disponible una prueba que discriminara claramente entre animales infectados de manera natural y animales solamente vacunados (DIVA). Debido a que la proteína no-estructural NS1 se expresa en la células infectadas con el virus de la influenza, y no se encuentra como parte del virión, es una excelente candidata para una prueba de diagnóstico diferencial basada en el concepto DIVA. El objetivo de este trabajo fue determinar el comienzo de la respuesta por anticuerpos hacia la proteína NS1 en pollos infectados con un virus de influenza de baja patogenicidad y evaluar el potencial diagnóstico de la proteína purificada NS1 expresada en baculovirus dentro de una estrategia DIVA basada en una prueba de ELISA indirecta. La respuesta de anticuerpos contra NS1 fue detectada tres semanas después de la infección, pero los niveles de anticuerpos decrecieron rápidamente alrededor de las cinco semanas después de la infección. Sin embargo, la mayoría de los pollos no tenían anticuerpos detectables a pesar de los títulos altos detectados con la prueba de inhibición de la hemaglutinación en un grupo. En las aves inmunizadas con vacunas inactivadas emulsionadas en aceite, los anticuerpos contra la proteína NS1 no fueron detectados antes del desafío virulento y solo un pequeño porcentaje de las aves mostraron seroconversión después del desafío con un virus de la influenza de baja patogenicidad homólogo. Las aves vacunadas y desafiadas con influenza aviar de alta patogenicidad mostraron una respuesta de anticuerpos alta contra la proteína NS1, pero solamente se observó como máximo un 40% de seroconversión contra la proteína NS1 alrededor de las tres semanas después del desafío. Debido a la variabilidad de la seroconversión y a la duración de la respuesta de anticuerpos en pollos, la estrategia DIVA utilizando la proteína NS1 no funcionó como se esperaba y si esta estrategia se utilizara, se requeriría muestrear un número mayor de aves para compensar el bajo nivel de seroconversión.

Key words: influenza, DIVA, NS1, ELISA, poultry

Abbreviations: A/CK/California = A/CK/California/K0301417Ct/03 (H6N2); A/CK/Queretaro = A/CK/Queretaro/14588-19/95 (H5N2); AI = avian influenza; AIV = avian influenza virus; A/TK/Wisconsin/68 = A/TK/Wisconsin/68 (H5N9); BPL = β-propiolactone; DIVA = differentiating infected from vaccinated animals; ELISA = enzyme-linked immunosorbent assay; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza

Influenza viruses are segmented, negative-strand RNA viruses belonging to the family *Orthomyxoviridae*, and they are divided in three types, A, B, and C (35). All three types of influenza are found in humans, but only the type A viruses are found in birds and are referred to as avian influenza (AI) viruses. Type A influenza viruses are divided into subtypes based on the antigenic properties of the major surface glycoproteins: the hemagglutinin (HA) and neuraminidase (NA) proteins. In total, 16 HA subtypes (H1–H16) and nine neuraminidase subtypes (N1–N9) are described currently (35). AI viruses can be further divided into two distinct virulence groups based on the severity of the disease that they cause in chickens in standard pathotyping studies (35). Highly pathogenic avian influenza (HPAI) results in mortality of 75% or greater in chickens after intravenous inoculation and, in the field, typically results in flock mortality in susceptible species that may be as high as 100%

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(32,34). Only the H5 and H7 subtypes are known to have the highly pathogenic phenotype, but most viruses of these subtypes are not highly pathogenic (32,34). Low pathogenic avian influenza (LPAI) viruses typically cause mild clinical disease but no mortality in experimentally infected chickens and include the majority of AI virus subtypes isolated from poultry. Some of these viruses in the field however may cause a mild to severe respiratory disease, depression, and reproductive disease in a variety of domestic species, particularly when complicated by concurrent infection or poor environmental conditions (34).

Although AI vaccines can be successful in controlling the clinical signs of the disease, they do not prevent infection of vaccinated flocks if they are exposed to high levels of virus (31). Vaccination has historically not been commonly used as part of the control programs for the eradication of LPAI or HPAI because the most commonly used AI vaccines, killed whole virus adjuvanted vaccines, interfere with the traditional serologic tests used for surveillance of influenza infection such as the agar gel immunodiffusion and nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) tests (3,4,26,27). Vaccinated birds cannot be differentiated from naturally infected birds using the commonly available serologic tests, severely restricting the use of serology as a surveillance tool. Vaccination however is growing in acceptance as an alternative to the stamping out approach (4,6,26,27), where all infected and exposed flocks are euthanized because it is viewed as a less costly control strategy when infection is widespread in a region or country. However, vaccination may have a negative impact on international trade because of the limitations of the current serologic tests. Therefore, efforts have been focused on developing new serologic tools to allow differentiation of infected from vaccinated animals, commonly known as the DIVA strategy. For international commerce of vaccinated birds, the DIVA strategy needs to not only discriminate between infected and vaccinated birds, but also to identify vaccinated birds that become infected with AIV. Several different DIVA strategies have been proposed (26), some of which require the use of appropriate vaccines and specific companion serologic discriminatory tests (3,7,9,16,33). Such a strategy is important to provide assurance to trading partners that the poultry product is free of influenza virus contamination and can be safely traded (3–6). The DIVA strategy has been accepted by some countries in the European Union as a way to monitor and provide assurance on the infection-free status of vaccinated poultry during an LPAI outbreak (4,26).

One potential target for the DIVA strategy for avian influenza is the nonstructural (NS1) protein. The NS1 protein is encoded by the smallest gene segment of influenza virus, segment 8, and it has a critical role in preventing the induction of interferon in the host to allow efficient virus replication (13). The NS1 protein is a conserved protein amongst type A influenza viruses, but it exists as two major subtypes, A and B, that differ by about 35% in nucleotide sequence (28). The NS1 protein is produced during influenza virus replication in infected cells (2,33), but it is not packaged into the infectious viral particle, being a true nonstructural protein. Because most AIV vaccines are primarily chemically inactivated whole virus, vaccinated animals theoretically should not have an antibody response to the NS1 protein, but infected poultry should have an NS1 antibody response because it is produced at high levels in infected cells. This NS1 DIVA approach was first proposed for equine influenza viruses, and purified NS1 protein was used to detect antibodies to NS1 in serum samples from ponies and horses experimentally infected with influenza virus (2,22). Serologic studies on chickens and turkeys using an ELISA with AIV NS1 protein produced in *Escherichia coli* as antigen indicated that infected birds showed higher levels of NS1 antibody as compared to vaccinated birds (33,36). Since available data on baseline production of NS1 antibodies after natural infection with influenza virus is limited, this work was undertaken to determine the kinetics of the induction of antibodies against NS1 protein after infection with LPAI virus, and to determine whether a baculovirus-derived NS1-ELISA approach is a reliable DIVA diagnostic test.

MATERIALS AND METHODS

Viruses. The HPAI virus A/CK/Queretaro/14588-19/95 (H5N2) and the low pathogenic avian influenza viruses A/CK/Pennsylvania/13609/93 (H5N2), A/CK/California/K0301417Ct/03 (H6N2), A/TK/Oregon/1971 (H7N3), and A/TK/Wisconsin/68 (H5N9) used in this study were obtained from the virus repository at Southeast Poultry Research Laboratory (SEPRL). Viruses were propagated in 10-day-old embryonating chicken eggs (ECE) for 3–4 days. The harvested allantoic fluid was tested for hemagglutination using 0.5% chicken erythrocytes following standard procedure. The 50% egg infectious dose (EID_{50}) titers for all the viruses used in this study were determined using the method of Reed and Muench (24).

Recombinant vaccine viruses. RNA from the AIV isolates used for the vaccine preparation was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) from allantoic fluid of infected ECE. Next, HA genes from A/CK/PA/13609/93 (H5) virus, NA genes A/DK/NY/191255-59/02 (N8), and NS1 genes from A/TK/OR/71-SEPRL viruses were amplified by one-step reverse transcriptase (RT)-PCR with HA, NA, and NS1 segment-specific primers (HA 5′-CUACUACUACUA-CTCTTCG-AGCAAAAGCAGGGGA, HA 3′-CAUCAUCAUCU-TAATACGACTCACTATAAGTAGAAACAAGGGTG, NA 5′-CUACUACUACUA-CTCTTCG-AGCAAAAGCAGG, NA 3′-CAUCAUCAUCAU-TAATACGACTCACTATA-AGTAGAAACAAGG, NS 5′-AGCAAAAGCAGGGTGACAA, NS 3′-AGTAGAAACAAGGGTGTT) as described previously (29). The RT-PCR products were reamplified by PCR with primers containing *Bsm*BI sites (5′-TATTCGTCTCAGGG-AGCAAAAGCAGG, 3′-ATATCGTCTCGTATT-AGTAGAAACAAGG), digested with *Bsm*BI restriction enzyme, and cloned into pHH21 vector between the RNA polymerase I promoter and terminator sequences. Primer sequences will be provided upon request. RNA polymerase I constructs that contain the remaining five internal genes of A/WSN/33 virus and four expression plasmids (pCAGGS-WSN-NP, pcDNA774-PB1, pcDNA762-PB2, and pcDNA787-PA) were provided by Dr. Yoshihiro Kawaoka at the University of Wisconsin (21). Reassortant viruses were generated by DNA transfection as described previously (16). Briefly, 293T cells were transfected with 12 plasmids with the use of Lipofectamine 2000 reagent (Invitrogen, San Diego, CA). Supernatant was collected after 72 hr of transfection and subsequently inoculated into 9- to 10-day-old ECEs. After 48 hr of incubation, allantoic fluid containing the reassortant virus was harvested and stored at −70 C for additional experiments.

Vaccine preparation. The virus generated by reverse genetics was propagated in 10-day-old ECEs, and the allantoic fluid was pooled and stored at −70 C until inactivation. Egg infectious titers (EID_{50}) were determined prior to inactivation with 0.1% betapropiolactone (Sigma, St. Louis, MO). Oil-emulsion vaccines were prepared as previously described (25). Briefly, one part aqueous antigen was emulsified in four parts oil phase (Drakeol 6 VR mineral oil, Penreco, Burler, PA) containing 7.5% sorbitan monooleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 2.5% polysorbate 80 (Tween 80, ICI United States, Inc.). The inactivated vaccine prepared with H5 reassortant for this study was designated as rH5N8 inactivated vaccine.

Hemagglutination inhibition (HI) test. All serum samples were tested for antibodies against hemagglutinin by HI test using homologous antigens. For this purpose, A/turkey/Wisconsin/68 (H5N9), A/CK/California/K0301417Ct/03 (H6N2), and the experimental rH5N8 were inactivated with 0.1% β-propiolactone (BPL) and used for the HI test as previously described (16,31). All HI titers were reported both as $\log_2$

titers and as geometric mean titers (GMT), with $3\log_2$ or eight being the minimum positive titer.

Infection and vaccination studies. For the kinetics study of antibodies against NS1, 38-day-old chickens were infected with LPAI virus and housed in biosafety level 3 Ag facilities. Groups of eight 38-day-old birds were infected by the intranasal route with LPAI virus CK/CA/03 (H6N2) or TK/WI/68 (H5N9) at 10^5 EID_{50} and 10^6 EID_{50}/0.2 ml, respectively. Serum samples were taken for serologic analysis before the infection and every week after the infection for five consecutive weeks. Food and water were provided *ad libitum* in all the experiments, and management was provided as required by the Institutional Animal Care and Use Committee.

In two different experiments, groups of ten 2-wk-old birds were immunized subcutaneously with the equivalent of $10^{7.5}$ EID_{50}/0.5 ml of rH5N8 inactivated vaccine or with 0.5 ml of commercial avian influenza vaccine subtype H5N9. In each experiment, a sham-vaccinated group was included as a challenge control, as well as one group that served as a negative control. Chickens were challenged intranasally via the choanal cleft 2 wk after vaccination with 10^5 EID_{50}/0.2 ml of LPAI CK/PA (H5N2) LPAI virus (experiment 1) or 10^5 EID_{50}/0.2 ml of highly pathogenic A/CK/Queretaro (H5N2) HPAI virus (experiment 2) (12). Morbidity and mortality were monitored daily. In both experiments, blood samples were obtained via the brachial vein right before challenge and every week after challenge for four weeks. Serum was harvested from each sample and stored at −20 C for serologic testing. Tracheal or oropharyngeal swabs were taken from experimental and control birds at days 3 and 5 after infection, suspended in 1 ml of BHI broth, and stored at −70 C. Total RNA was extracted by using an RNeasy mini kit (Qiagen), and quantitative real-time RT-PCR was performed with primers and probes specific for type-A AI virus matrix RNA using a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine (17). The amount of virus (number of copies of viral RNA recovered from swab/ml of BHI) was interpolated from the sample cycle thresholds by using a standard curve generated from known amounts of control A/CK/Queretaro/95 RNA titers at 10^1–10^6 EID_{50}/ml (17) (data not shown).

Generation of recombinant baculovirus encoding viral NS1. Two recombinant NS1 proteins were generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA). Viral RNA was extracted from allantoic fluid of ECEs infected with A/TK/Oregon/71-SEPRL using Trizol LS (Invitrogen) as described by the manufacturer. The NS1 gene was amplified by one-step RT-PCR as described previously (29). RT-PCR products were cloned into the pCR 2.1-TOPO (Invitrogen) and transformed into the competent *E. coli* cells DH5α (Invitrogen). Prepared plasmid DNA from selected colonies was screened by PCR for the presence of the NS1 genes with NS1 specific primers. DNA extracted from a confirmed positive colony was digested with BamHI and Hind III (NS1-amino terminus, NS1-N) or with *Eco*RI and *Hin*dIII (NS1-carboxy terminus, NS1-C) and cloned into the corresponding sites of the plasmid's pFastBac HT A or pFastBac Dual (Invitrogen), respectively. To confirm, the appropriate DNA fragment sequence analysis was performed. The generation of recombinant baculoviruses was performed as described in the manufacturer's protocols. *Spodoptera frugiperda* (Sf9) cells (Invitrogen) were cultivated in all experiments with serum-free medium (SF900 II, Invitrogen). The viral titers ($TCID_{50}$) of the obtained supernatants were determined by immunofluorescence using the baculovirus-specific monoclonal antibody (mAb) AcV5 (Sigma) with tenfold diluted virus in SF900 II medium, and 100 μl of each dilution was fourfold transferred to a 96-well tissue culture plate. Samples of 100 μl of Sf9 cells, at a density of 3×10^5 cells/ml were added to each well. The cells were incubated for 72 hr, and after removal of the medium, the cells were fixed with ice-cold ethanol for 10 min. Immunofluorescence was performed following standard procedures using the mAb AcV5 (Sigma) and goat anti-mouse fluorescein isothiocyanate (FITC) conjugated antibodies (Jackson Immunoresearch, West Grove, PA).

Production and purification of recombinant NS1. Sf9 cells were infected with recombinant baculovirus at multiplicity of infection (MOI) of at least 5 and harvested 3 days postinfection by centrifugation at 700 × *g* for 10 min. The NS1 proteins were purified using a TALON resin (Clontech, Mountain View, CA) as described previously (18). Briefly, pelleted cells of one T150 tissue culture flask were suspended in 5 ml of lysis buffer containing 1× protease inhibitor Complete ethylene diamine tetraacetic acid-free (Roche, Mannheim, Germany) and 100 mg IGEPAL CA-630 (Sigma). After 30 min incubation on ice, insoluble proteins were sedimented by centrifugation at 45,000 × *g* for 45 min at 4 C using the tabletop ultracentrifuge Optima TLX (Beckman, Fullerton, CA). The obtained supernatants were used for purification as described previously (18). The recombinant proteins were eluted using 3 ml of elution buffer containing 300 mM Imidazol (BD-Biosciences, San Jose, CA). The eluted proteins were collected in fractions of 500 μl and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by either Coomassie brilliant blue staining or Western blot analysis using a mouse monoclonal anti-polyhistidine peroxidase conjugate (Sigma) following standard procedures. Fractions containing the highest amount of eluted NS1 were pooled, and the protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

Development of NS1 DIVA ELISA. The optimum dilution of the NS1 protein for the ELISA test was obtained by checkerboard titration on a 96-well ELISA plate against known positive and negative samples. The optimum dilution considered was the highest dilution of antigen that still saturated the plate and gave maximum contrast in terms of optical density (OD) between known positive and known negative sera. A dilution of 1:50 for the experimental serum samples was determined to produce an optimal level of sensitivity for detection of positive serum samples with low background in negative samples. A cutoff point of 0.223 represents the mean of the OD of 120 negative serum samples tested at the 1:50 dilution plus 3 standard deviations.

Indirect ELISA was performed using the following procedure. Ninety-six well ELISA plates (Thermo Fisher Scientific, Rochester, NY) were coated with 150–200 ng/well purified NS1-his tag protein diluted in carbonate buffer, pH 9.6, in a 50 μl volume and incubated overnight at 4 C. All washing steps were performed using an automated ELISA washer (Biotek, Winooski, VT) three times with 1× Wash Solution (KPL, Gaithersburg, MD). Incubations of serum samples and conjugate were performed at 37 C for 1 hr.

After overnight incubation, the plates were washed, and the wells were blocked with 100 μl/well of 1× bovine serum albumin/Blocking solution (KPL) and incubated for 45 min at 37 C, followed by another washing step. Each test sample was diluted 1:50 in Sample Dilution Buffer (Synbiotics, San Diego, CA) and 50 μl were added to the ELISA plate. Horseradish peroxidase–conjugated goat anti-chicken (KPL) antibodies were diluted 1:2000 in Sample Dilution Buffer, and 50 μl were added to each well. After a last washing step, 50 μl of ABTS (2, 2′-azino diethylbenzothiazoline sulfuric acid) 2-Component Microwell Peroxidase Substrate (KPL) were added followed by 15 min of incubation time at room temperature, and the reaction was stopped by the addition ABTS Peroxidase Stop Solution (KPL). The OD of each well was read at 405 nm using a microplate reader (Biotek, Winooski, VT).

Positive and negative chicken serum sample controls for NS1-ELISA. Positive control serum was obtained from a nonvaccinated bird that survived challenge with HPAI A/chicken/Chile/184240-1/02 (H7N3) in an ongoing experiment at SEPRL. As a negative control, normal chicken serum (Bethyl Laboratories, Montgomery, TX) was used. The initial ODs of 1.261 for the positive and 0.097 for negative control sera were determined by NS1-ELISA at a dilution of 1:50. The determined HI titer of the positive control serum sample was 128.

RESULTS

Purification of the NS1 protein. Two recombinant baculoviruses were generated. One recombinant baculovirus was encoded for a NS1 protein containing 6× His tag sequence at its N-terminus (NS1-N), whereas the second recombinant baculovirus was encoded

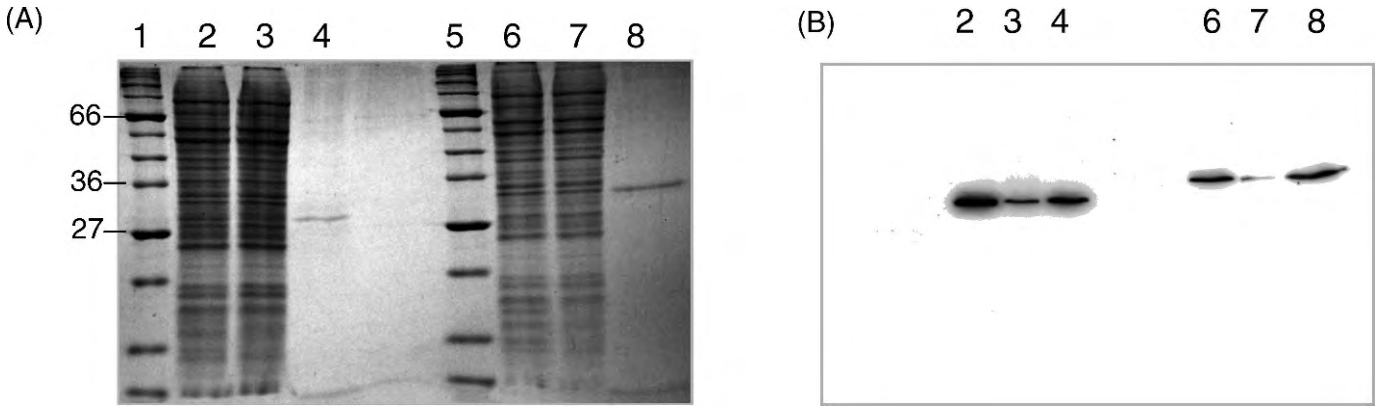


Fig. 1. Purification of recombinant NS1 proteins from infected Sf9 cells. Obtained protein samples were separated on a 12% polyacrylamide gel using SDS polyacrylamide gel electrophoresis. After electrophoresis, the gel was (A) stained with Coomassie brilliant blue and (B) the presence of recombinant antigen was detected using anti-polyhistidine monoclonal antibody (mAb). The presence of mAb binding was visualized using a goat anti-mouse peroxydase labeled conjugate by enhanced chemoluminescence. Protein samples after lysis (lanes 2, 6), supernatant after centrifugation (lanes 3, 7), and eluate (lanes 4, 8) of NS-C (lanes 2–4) and NS1-N (lanes 6–8) are shown. Protein markers (lanes 1 and 5) indicate the apparent molecular weight of the samples.

for NS1 containing a 6× His tag sequence at its C-terminus (NS1-C). Both proteins were purified following a protocol as described previously (18). After purification (Fig. 1), one single band was visible after the separation of the protein samples on a 12% SDS PAGE gel. The presence of the 6× His sequence was verified by Western blot using anti-His monoclonal antibody.

The identity of the purified N-terminal NS1 protein was confirmed by direct protein sequencing using MALDI-TOF MS/MS analysis (data not shown) and a protein sample separated on a 12% SDS-PAGE and processed for trypsin cleavage (Proteomics Resource Facility and Integrated Biotechnology Laboratories at the University of Georgia, Athens, GA).

Comparison of NS1-N and NS1-C in ELISA. Two different NS1 proteins, NS1-N and NS1-C, were evaluated to determine if the location of the tag affected the sensitivity of the ELISA test. The ELISA tests with either protein were performed with AIV-positive serum samples. The obtained results showed that the location of the 6× His tag did not influence the sensitivity of the ELISA (data not shown). In subsequent experiments, the NS1-N protein was chosen as antigen for the ELISA test for analysis of serum samples.

Antibody response to NS1 protein produced in birds after infection with LPAI virus. To test the hypothesis that infection with AIV in chickens produces a humoral immune response to the NS1 protein, groups of eight birds at 38 days of age were inoculated with two different LPAI viruses, CK/CA/03 and TK/WI/68. The serologic response to these viruses is summarized in Table 1. Infection with 10^5 EID_{50} of CK/CA/03 induced detectable levels of NS1 antibody in only 25% of birds. The level of antibodies, as measured by the NS1-ELISA in the serum of the two NS1-antibody-positive birds, was highest 3 wk postinfection. By the end of the experiment at 5 wk postinfection, the NS1 antibody titers had decreased but were still detectable by ELISA (Fig. 2). In contrast to the low NS1 antibody response, the HI antibody response to infection with CK/CA/03 was high, showing a geometric mean titer (GMT) of 194 at wk 1 after infection, and reaching a peak at wk 3, with a GMT of 891 with all challenged birds seroconverting. No NS1 antibodies were detected by ELISA in the birds inoculated with TK/WI/68, but only 50% of these birds seroconverted by HI during the course of the experiment.

To analyze the level of NS1 antibodies present in the seroconverted birds, 3 wk postinfection samples from the group infected with the CK/CA/03 AIV were selected for further analysis. Series of twofold dilutions beginning at 1:5 were tested with the ELISA. The obtained data revealed a titration curve for the two positive samples similar to the titration curve obtained with the known positive serum sample, suggesting high levels of antibodies to NS1, while the negative samples showed a titration curve similar to the negative control (Fig. 3).

Western blot analysis. Western blot analysis with NS1-N protein was performed using serum samples from birds that had tested positive or negative on the NS1-ELISA. The serum samples were tested at two different dilutions (1:50 and 1:10) to evaluate sensitivity using an alternative detection method. Bands with the predicted molecular weight for the NS1-N protein were considered positive for the presence of NS1 antibodies. Results from western blot analysis corroborated the results from the NS1-ELISA. Only serum samples showing reactivity on ELISA above the established threshold (OD 223) showed binding of antibodies to the NS1-N protein in the Western blot analysis (Fig. 4).

NS1 antibody detection in birds immunized with H5 subtype oil-emulsion vaccine and challenged with homologous LPAI virus. To determine if the antibody response to NS1 after infection with LPAI could be detected in birds vaccinated with a whole-virus killed oil adjuvanted vaccine, groups of 10 birds were vaccinated with inactivated rH5N8 (H5N8) vaccine or inactivated commercial H5N9 vaccine and challenged with the LPAI virus A/CK/PA/93 (H5N2) at a low challenge dose (10^5 EID_{50}/bird) to simulate a typical infection seen in the field.

NS1-ELISA results showed that only one bird developed a detectable NS1 antibody response 1 wk after challenge in birds vaccinated with inactivated rH5N8, and no birds immunized with the commercial vaccine showed a detectable NS1 antibody response (Table 2). The serum samples of this single bird showed positive reactivity on the ELISA test from wk 1 until wk 3 after challenge, but by wk 4, the measured OD value was below the cutoff point (0.223). Interestingly, in the sham-vaccinated and subsequently challenged group, no NS1 antibody response was detected, and the HI test in this group was positive only in 20% of the birds starting at 2 wk postinfection, suggesting that the virus titer used for the challenge was not sufficient to productively infect all the birds.

Viral RNA was detected in oropharyngeal swabs at 3 and 5 days after challenge from the sham-vaccinated group in the same two

Table 1. Antibody response to NS1 protein as detected by an indirect NS1-ELISA after infection with LPAI virus during a period of 5 wk.

Virus[A]	Weeks after infection											
	0		1		2		3		4		5	
	HI[B]	ELISA[C]	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
TK/WI/68 (H5N9)	0/8	0/8	2/8 4.5 ± 0.71 (22)	0/8	4/8 5.1 ± 1.5 (56)	0/8	4/8 6 ± 1.8 (64)	0/8	4/8 6 ± 1.8 (64)	0/8	4/8 6 ± 1.8 (64)	0/8
CK/CA/03 (H6N2)	0/8	0/8	5/8 5.6 ± 1.8 (194)	0/8	7/8 8 ± 2.4 (512)	0/8	8/8 9.8 ± 1.4 (891)	2/8	8/8 9.8 ± 1.4 (891)	2/8	8/8 9.8 ± 1.4 (891)	2/8

[A]Groups of eight 38-day-old SPF chickens were inoculated in the choanal cleft with 10^5 EID50 A/chicken/CA/K0301417Ct/03 (H6N2) or 10^6 EID50 A/Turkey/Wisconsin/68 (H5N9). Serum samples were taken before infection (W-0) and every week after infection for 5 wk (W-1, W-2, W-3, W-4, and W-5).

[B]HI serologic response was measured using homologous BPL-inactivated AI virus strains as HI antigen. The results represent total number of positives/total number tested, the average HI titer including standard deviation, and the geometric mean titer (GMT).

[C]NS1-ELISA results represent number of serum samples with an OD higher than 0.223 (405 nm)/total tested samples.

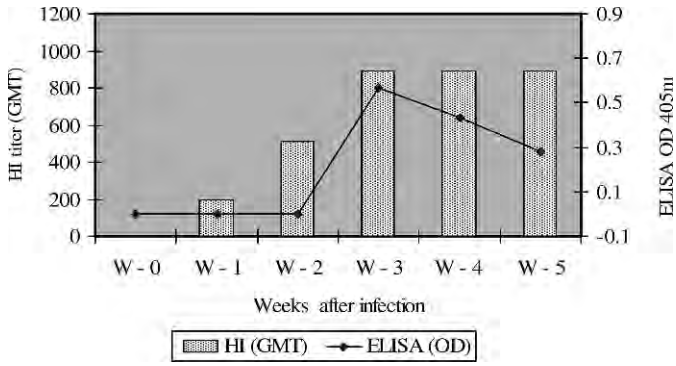


Fig. 2. Antibody response to HA and to NS1 proteins after infection with A/CK/California/K0301417Ct/03. The average geometric mean of the HI titers (shown as a column) obtained from all the birds ($n = 8$) and the average OD reading of the two birds that had detectable antibodies to the NS1 protein (shown as a curve) are shown starting with the serum sample taken before infection (W-0). The values for the HI titer are plotted at the left axis, whereas the OD values for the ELISA are plotted at the right axis.

birds (out of 10) that were positive for HI antibodies (Table 2). In the group vaccinated with rH5N8, three birds were positive for viral RNA at 3 days after challenge, and two birds were positive at 5 days after challenge. No viral RNA was detected in the group vaccinated with the commercial vaccine, which also had higher levels of HI antibody before challenge.

NS1 antibody detection in birds immunized with H5 subtype oil-emulsion vaccine and challenged with HPAI virus. To determine if vaccinated birds would develop a detectable NS1 antibody response after challenge with HPAI H5 virus, chickens were also vaccinated with inactivated rH5N8 or commercial vaccine H5N9 subtype vaccine and challenged with 10^5 EID_{50}/bird of the HPAI virus CK/Que/95. The NS1-ELISA results before challenge (2 wk after vaccination) were negative, with the exception of one positive serum sample showing an OD value (0.237) close to the

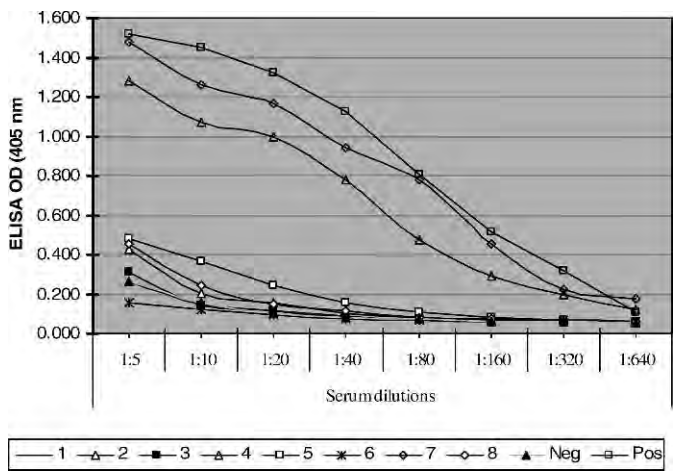
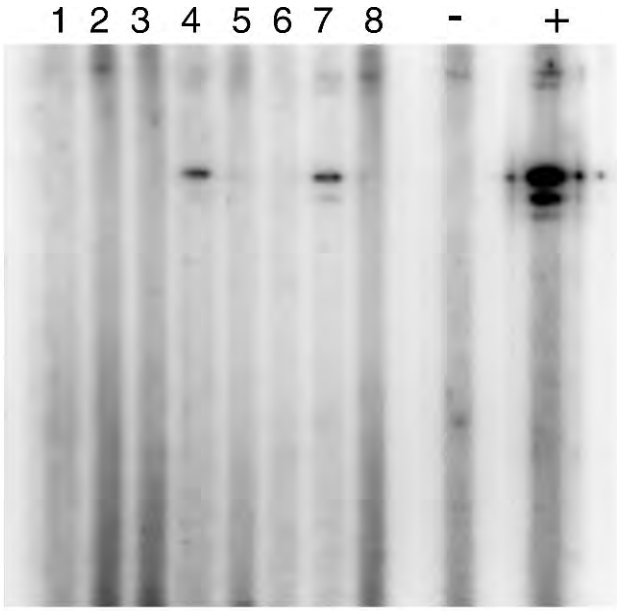


Fig. 3. Dilution of NS1-antibody-positive and NS1-antibody-negative serum samples. Data show the titration of antibodies to NS1 in chicken serum samples taken 3 wk after infection with A/CK/California/K0301417Ct/03. The positive serum and negative serum were taken as controls. The two serum samples that tested positive in ELISA (birds 7 and 4) and the sera that tested negative in ELISA (birds 1, 2, 3, 5, 6, and 8) were diluted starting with 1:5 in a twofold series against constant NS1 protein concentration. The OD values are plotted at the horizontal axis, whereas the serum dilutions are shown at the vertical axis.



A)

Bird ID	3 Weeks Post-Infection	
	HI*	NS1-ELISA†
1	9	0.068
2	9	0.090
3	10	0.074
4	≥11	0.490
5	≥11	0.114
6	7	0.069
7	≥11	0.643
8	10	0.090

B)

Fig. 4. Western blot analysis of serum samples. Eight birds (Nos. 1–8) were bled at 3 wk after infection with A/CK/California/K0301417Ct/03. (A) The obtained serum samples were tested by Western blot. Purified NS1-N antigen was separated on a 15% gel in a SDS polyacrylamide gel electrophoresis. The membrane was incubated with serum samples of the birds (Nos. 1–8) or appropriate control sera (negative −, positive +). (B) In parallel, the same serum samples were tested by HI and the NS1-ELISA. The obtained HI titer and ELISA titer are shown in the table.

ELISA threshold (0.223) in the group vaccinated with the commercial vaccine. All the sham-vaccinated birds died within the first week after challenge. In contrast, both vaccines prevented mortality. In the group vaccinated with rH5N8, NS1 antibodies were first detected 3 wk after challenge from 1 of 10 birds (OD 0.377). The same bird was positive at wk 4, but it had a lower ELISA OD value (0.266). Furthermore, one NS1-antibody-positive bird was found at 4 wk after challenge for a total of 2/10 birds positive for NS1 antibodies. In the group vaccinated with the commercial H5N9 vaccine, NS1 antibodies after challenge were detected at wk 2 in 1 of 10 chickens (OD 0.330), at wk 3 in 1 of 10 birds (OD 0.332), and at wk 4 in 3 of 10 chickens (OD 0.290, OD 0.385, OD 0.333). One bird was positive during 2 consecutive weeks, while two birds appeared positive at 4 wk after challenge (Table 3).

The HI test results using homologous antigen to each vaccine were positive in the groups immunized with rH5N8 and commercial H5N9 vaccines, showing a GMT of 42 and 416, respectively. Both groups experienced an increase in the HI titers until wk 3 (GMT 2521 and 10,809, respectively) after challenge. At wk 4 post-challenge, the HI antibodies started to decline in both groups. Similar to the previous experiment (Table 2), the commercial H5N9 induced higher HI levels than those vaccinated with rH5N8 vaccine.

Analysis of the virus shedding showed the presence of viral RNA in 3 out of 10 swab samples at 3 days after challenge and 1 out of 10 swab samples at 5 days after challenge in the rH5N8-vaccinated group. As with the previous experiment, no viral RNA was detected in swab samples taken from birds vaccinated with the commercial vaccine.

DISCUSSION

The primary objective of this study was to determine whether or not the NS1 antibody response in chickens after challenge with AIV could be reliably used as a DIVA strategy.

The kinetics of the antibody response to the NS1 protein after infection of SPF chickens with AIV virus demonstrate a low number of responders (2/8) in the group infected with CK/CA/03 LPAI, although all the birds had high levels of HI antibody titer, indicating a poor diagnostic correlation between these two tests. The challenge with another LPAI virus, TK/WI/68, showed similar results, although the virus likely did not infect all the animals as concluded by the data obtained from the HI tests.

These results were confirmed by Western blot (Fig. 4) for the CK/CA/03-infected chickens, showing the sensitivity of the NS1-ELISA test to be at least comparable to that of the Western blot analysis when serum samples were tested at low dilutions (1:10 and 1:50). Thus, the low detection of the antibodies directed against NS1 after infection of SPF chickens with AIV in this study is more likely due to the poor immune response to the NS1 protein rather than to the lack of sensitivity of the NS1-ELISA test. Previous work on NS1 protein–based DIVA strategy development for influenza virus in horses showed a similar pattern of response (2). Infection with equine influenza virus did not produce antibodies to NS1 in all infected horses as detected by NS1-ELISA.

Although a similar challenge dose was used for two different LPAI viruses in the present studies, the CK/CA/03 virus produced a higher HI antibody response than TK/WI/68 based on the number of birds that tested positive in the HI assay, and the levels of HI antibody titer. These data support the observation that the CK/CA/03 virus is better adapted to chickens than TK/WI/68, which could be anticipated because the CK/CA lineage of virus circulated for several years in chicken flocks in California, while the TK/WI/68 virus appears to be a recent introduction in turkeys from wild birds (8).

The detectable immune response to the NS1 protein in chickens appears to develop later than the response to HA, and it showed a rapid decrease after the initial detection (Fig. 2). These results are in disagreement with previous reports (36) where positive correlation was obtained in measured immune response with HI test and NS1-

Table 2. Summary of experimental data obtained in vaccinated birds with oil-emulsion inactivated vaccines after homologous challenge with LPAI virus.

	Time after challenge[B]											
	Virus shedding[C]		Week 0		Week 1		Week 2		Week 3		Week 4	
Treatment groups[A]	3 days	5 days	HI[D]	ELISA[E]	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
Sham vaccine	2/10	2/10	0/10	0/10	0/10	0/10	2/10 2.5 ± 0.7 (22.6)	0/10	2/10 4.5 ± 2.1 (90.5)	0/10	2/10 5 ± 1.4 (128)	0/10
H5N8-SEPNS1 inactivated vaccine	3/10	2/10	10/10 4.1 ± 2.5 (68.6)	0/10	10/10 6 ± 2.5 (256)	0/10	10/10 7.4 ± 2.1 (675)	1/10 (0.339)	10/10 6 ± 1.1 (256)	1/10 (0.451)	10/10 5.6 ± 1.3 (194)	1/10 (0.236)
Commercial H5N9 inactivated vaccine	0/10	0/10	10/10 8.8 ± 1.3 (1783)	0/10	10/10 9.6 ± 2.2 (3104)		9/9 11.1 ± 0.9 (5042)	0/10	10/10 11.1 ± 0.6 (8780)	0/10	10/10 11.7 ± 0.5 (13,307)	0/10
Negative control	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7

[A]SPF birds were vaccinated at 2 wk of age, either with an experimental vaccine (rH5N8) or a commercially available vaccine (H5N9). Both vaccines were based on inactivated virus and oil emulsion formulated.

[B]Birds received a challenge dose of 10^5 EID_{50} LPAI A/Chicken/PA/13609/93 (H5N2) via the intrachoanal cleft 2 wk after vaccination.

[C]Oropharyngeal swabs were collected from each bird at 3 and 5 days after challenge, and RNA was extracted and assayed for the presence of viral RNA using real time RT-PCR.

[D]HI serologic response was measured using the homologous BPL-inactivated AI virus strains as antigen. The results represent total number of positives/total number, the average HI titer along with the appropriate standard deviation, and the geometric mean titer (GMT).

[E]NS1-ELISA numbers represent: number of positive samples/total of serum samples tested (OD average of positive samples).

Table 3. Summary of experimental data obtained in vaccinated birds with oil-emulsion inactivated vaccines after homologous challenge with HPAI virus.

	Time after challenge[B]											
	Virus shedding[C]		Week 0		Week 1		Week 2		Week 3		Week 4	
Treatment groups[A]	3 days	5 days	HI[D]	ELISA[E]	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
Sham vaccine	0/10	0/10	0/10	0/10								
H5N8-SEPNS1 inactivated vaccine	3/10	1/10	9/10 3.8 ± 0.8 (42)	0/10	10/10 8.7 ± 1.3 (416)	0/10	10/10 9.0 ± 1.6 (2048)	0/10	10/10 9.3 ± 1.1 (2521)	1/10 (0.330)	10/10 8.9 ± 1.1 (1911)	2/10 (0.252)
Commercial H5N9 inactivated vaccine	0/10	0/10	10/10 6 ± 1.6 (256)	1/10	10/10 10.5 ± 1.8 (1448)	0/10	10/10 10.8 ± 0.6 (7131)	1/10 (0.333)	10/10 11.4 ± 0.7 (10,809)	2/10 (0.276)	10/10 10.9 ± 0.7 (7132)	3/10 (0.336)
Negative	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[A]SPF birds were vaccinated at 2 wk, either with an experimental vaccine (rH5N8) or a commercially available vaccine (H5N9). Both vaccines were based on inactivated virus and oil emulsion formulated.

[B]Birds received a challenge dose of 10^5 EID_{50} HPAI A/chicken/Queretaro/14588-19/95 (H5N2) via the intrachoanal cleft 2 wk after vaccination.

[C]Oropharyngeal swabs were collected from each bird 3 and 5 days after challenge, and RNA was extracted and assayed for the presence of viral RNA using real time RT-PCR.

[D]HI serologic response was measured using homologous BPL-inactivated AI virus strains as antigen. The results represent total number of positives/total number tested, mean ± standard deviation, $\log_2$ HI activity, and geometric mean titer (GMT).

[E]NS1-ELISA numbers represent number of positive samples/total of serum samples tested (OD average of positive samples).

ELISA after infection with AIV in chickens. The challenge model used in the previously mentioned studies differed from the present study. For example, one previous study used 10-mo-old chickens and challenged them two times with 10^6 EID_{50} of LPAI by subcutaneous and intramuscular routes. In the present study, a lower viral challenge dose and the mucosal route of inoculation were used to simulate the natural exposure to AIV. Previous studies (33,36) also showed successful discrimination of AIV-infected from vaccinated turkeys using an NS1-ELISA. Although we did not attempt studies in turkeys, recent studies (11) indicate that the immune response to AIV NS1 differs between turkeys and chickens under experimental infections with a H9N2 subtype virus. No antibodies to a peptide corresponding to the C-terminus of the NS1 protein were detected in chickens by ELISA, whereas the opposite was observed in turkeys as early as day 3 postinfection (11). The observations of these studies, compared with works previously published (11,33), suggest that the response to the NS1 protein after infection with AIV in chickens is different from turkeys, and each species must be evaluated separately to determine the immune response.

The immune response observed to the NS1 protein during AIV challenge in the described experiments was unexpected and appeared to vary from bird to bird. These results might be related to complex interactions of each individual bird's immune response and the specific strain of virus and NS1 protein, which is designed to help evade the host's innate immune response (14,15). Differences with regard to the immune response to nonstructural proteins from individual animals and species have been reported with other viruses (1,19,20). For example, researchers have tried to develop a DIVA test based on several NS proteins as antigen for foot and mouth disease (FMD) virus in cattle using an ELISA test (20). The variation of the results was so high that convalescent animals could not be differentiated from carrier animals on the basis of their antibody response to any of the NS proteins examined (20). Currently, only the 3ABC-based ELISA test appears to successfully detect the majority of infected animals within a vaccinated population, but even antibodies to this protein did not detect the carrier state, which has to be demonstrated by virus isolation or nucleic acid detection (23). In pigs, however, the ELISA test based on the nonstructural protein 3AB of FMD virus has allowed the differentiation of vaccinated from infected pigs (10). These various studies highlight the differences in immune response seen between different species, and they require each diagnostic test to be validated separately in each species.

The NS1-ELISA–based DIVA strategy as presented in this study detected infection better after HPAI virus challenge than for the LPAI virus challenge. However, the duration of the immune response appears to be brief and may not provide a sufficient time window for a viable approach under field conditions. Additionally, in vaccinated animals, the replication of a challenge virus is expected to be lower than in unvaccinated controls, and this decreased level of replication may not produce sufficient antigen to generate a detectable immune response. This issue is a concern for most DIVA strategies, including the NS1 DIVA strategy. For the LPAI virus, a closely matched vaccine and challenge strain was also used, which provided specific protection that helped reduce virus replication and likely contributed to the poor NS1 antibody response of chickens in this study (30,31).

The NS1 approach in chickens in this study seems to be a less reliable approach than has been seen in earlier studies (33,36). However, as previously mentioned, this study used different protocols, viruses, vaccines, and routes of challenge from previous studies. This included the use of the NS1 antigen expressed and purified in a baculovirus system, where the earlier studies used NS1 antigen expressed in *E. coli* (33,36). The baculovirus-expressed proteins were used to reduce the background that was described when using the *E. coli*–expressed protein as antigen. Because of the differences between studies, it is not clear if the results are clearly different, or if they are just a reflection of the protocols and methods used.

The data in this study confirm that the NS1 protein can be used as a DIVA strategy for chickens, but the antibody response appears to be inconsistent both in respect to the number of seroconverted birds, and the duration of immunity as compared with the HI test. The NS1 test could potentially be used as a DIVA strategy on a flock basis if the sampling size were increased to compensate for these inconsistencies. However, other potential DIVA strategies like the heterologous neuraminidase DIVA strategy appear to be more reliable, but all the proposed DIVA strategies still have serious limitations that have prevented them from routine use.

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Research Note—

New Approach to Delist Highly Pathogenic Avian Influenza Viruses from BSL3+ Select Agents to BSL2 Non-Select Status for Diagnostics and Vaccines

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SUMMARY. Highly pathogenic avian influenza viruses (AIVs) are Select Agents in the United States and are required to be handled in bio-containment level-3 enhanced (BSL3+) facilities. Using a reverse genetics system, we attenuated a highly pathogenic virus, with the goal of making it low pathogenic and having it delisted as a Select Agent so that it could be handled in a bio-containment level-2 facility for diagnostic or vaccine production applications. We utilized two approaches to attenuate the target AIV by mutating the highly pathogenic hemagglutinin (HA) cleavage site to be low pathogenic and by replacing the full-length NS gene segment with a naturally truncated 124–amino acid NS1 coding gene from A/turkey/Oregon/73 (H7N3) virus (tkOR71 trNS1). To delist an AIV so that it can be handled in a BSL2 facility, the amino acid sequence of the HA cleavage site of the rescued virus must be confirmed to be compatible with a low-pathogenic AIV; it should not plaque in cell culture without supplementation of exogenous trypsin; and intravenous pathotyping in 4–6-wk-old specific-pathogen-free chickens must confirm that the virus is low pathogenic. The candidate A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus with five PR8 internal genes, tkOR71 trNS1 gene, and A/chicken/Indonesia/7/03 N1 neuraminidase gene was constructed. The virus was shown to not plaque in cell culture without addition of trypsin. The virus was low pathogenic in the standard intravenous pathotyping test (IVPI = 0) and also caused no disease in a separate intranasal inoculation test in 4-wk-old specific-pathogen-free chickens, thus demonstrating that the virus is suitable for deselection.

RESUMEN. *Nota de Investigación*—Enfoque nuevo para la eliminación de un virus de influenza aviar de alta patogenicidad de la lista de agentes selectos BSL3+ para ser incluido en el estatus no selecto BSL2 con fines de diagnóstico y elaboración de vacunas.

Los virus de alta patogenicidad de influenza aviar (AIVs, por su siglas en inglés) son Agentes Infecciosos Selectos en los Estados Unidos y requieren para su manejo de instalaciones con un nivel de biocontención tipo 3 extra (BSL3+, por sus siglas en inglés). Usando un sistema de reversión genética, se atenuó un virus de alta patogenicidad con el objetivo de hacerlo de baja patogenicidad y lograr que se eliminara de la lista de agentes selectos para de esta manera, poder manejarlo con fines diagnósticos o para la producción de vacunas en instalaciones de un nivel de biocontención 2 (BSL2, por sus siglas en inglés). Se utilizaron dos enfoques para la atenuación del virus seleccionado, mediante mutación en el sitio de disociación de la hemaglutinina (HA, por sus siglas en inglés) de alta patogenicidad, para convertirlo en un virus de baja patogenicidad, o por el reemplazo del segmento completo del gene NS por un gene truncado de manera natural que codifica para una proteína NS1 de 124 aminoácidos y que pertenece al virus A/pavo/Oregon/73 (H7N3), (gene tkOR71 trNS1). Para retirar de la lista de agentes selectos a un virus de influenza aviar y de esta manera poder manejarlo en instalaciones tipo BSL2, la secuencia de aminoácidos del sitio de disociación de la hemaglutinina del virus rescatado debe ser confirmada como compatible con un virus de influenza aviar de baja patogenicidad; éste no debe formar placas en cultivo celular sin la suplementación de tripsina exógena y la tipificación de la patogenicidad mediante inoculación intravenosa en aves libres de patógenos específicos de 4 a 6 semanas debe confirmar que el virus es de baja patogenicidad. Se construyó un virus candidato, el virus A/pato/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) con cinco genes internos de PR8, con el gene tkOR71 trNS1, y el gene de la neuraminidasa N1 del virus A/pollo/Indonesia/7/03. El virus no produjo placas en los cultivos celulares sin la adición de tripsina. El virus se comportó como un virus de baja patogenicidad en la prueba estándar de tipificación de la patogenicidad por inoculación intravenosa mostrando un índice de patogenicidad intravenosa (IVPI, por sus siglas en inglés) de cero y no causó enfermedad después de la inoculación intranasal en pollos libres de patógenos específicos de cuatro semanas de edad, lo que demostró que el virus era apto para ser deseleccionado.

Key words: avian influenza virus, reverse genetics, NS1 gene, avian influenza vaccine, select agents

Abbreviations: AIV = avian influenza virus; bp = base pair; BSL-3+ = bio-containment level-3; ECE = embryonated chicken eggs; EID_{50} = 50% egg infectivity; HA = hemagglutinin; MDCK = Madin Darby Canine Kidney; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase–PCR; SPF = specific pathogen free

Highly pathogenic H5N1 avian influenza viruses (AIVs) have become endemic in poultry in several Asian and at least one African country, resulting in unprecedented economic loss to poultry farmers and zoonotic infections in people, leading to fatal illnesses (1,2,3,5,7,9,10,11,12,16,20,22,23,27,28,29). Under these circumstances, control and prevention of avian influenza in poultry is a high priority. In addition to the traditional culling approach, preventive vaccination for avian influenza in poultry has gained importance because it has yielded some success in controlling the disease and because of the increasing acceptance of vaccination as a control tool in some affected Asian countries. The success of preventive vaccination programs depends in part on the availability of vaccine strains that are antigenically matched to the circulating highly

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pathogenic AIV field strains (14,25). Because naturally circulating low pathogenic AIVs provide a poor antigenic match against the circulating Asian H5N1 lineage of viruses, the use of plasmid-based reverse genetics and site-directed mutagenesis has become indispensable as a way to produce antigenically matched vaccine strains. A vaccine seed strain can be attenuated by mutating the hemagglutinin (HA) cleavage site so that it has a low pathogenic cleavage site, and this approach allows the virus to remain antigenically matched to the parent strain, because the cleavage site is distantly located in relation to neutralizing sites on the HA protein (17,24). Alternative methods of attenuation have been described, including changes to the NS1 protein. One study characterized a variant of A/turkey/Oregon/71 (H7N3) that had a naturally truncated 124–amino acid NS1 protein, compared with the usual 230–amino acid NS1 protein. The variant virus with the truncated NS1 protein did not efficiently block the induction of type I interferon, which the parent virus with the full-length NS1 gene did, and it exhibited an attenuated pathotype in chickens and poorly transmitted to in-contact sentinel chickens (6,26).

Highly pathogenic AIVs are considered Select Agents in the United States, and the live virus is required to be handled in an enhanced bio-containment level-3 (BSL-3+) facility. Although an HPAI virus can be attenuated to be low pathogenic using reverse genetics, these viruses are still considered Select Agents and must be handled under BSL-3 conditions, which limits how they can be used. A virus can be delisted as a Select Agent in the United States if it meets certain requirements. In brief, the HA cleavage site must be changed so that it is compatible with a low pathogenic virus; it must be shown that the virus can no longer grow in cell culture without supplementation of exogenous trypsin in cell culture medium; and, finally, the virus must be tested in the standard intravenous pathotyping test in chickens, per World Organization for Animal Health guidelines. If a virus meets all of these conditions, it may be considered for deselection. In addition to the change at the HA cleavage, we considered it beneficial to include additional attenuation factors in the virus to increase the safety of working with live virus in BSL-2 facilities. The use of a truncated NS1 gene of A/turkey/Oregon/71 (H7N3) was selected as a way to attenuate the virus in animals; this selection will still allow the virus to be grown to high titer in 9-to-10-day-old embryonating chicken eggs. Based on this hypothesis, we used a reverse genetics approach to incorporate both changes: the H5 HA cleavage site mutation and a truncated NS1 protein in the recombinant H5N1 virus. Further, we evaluated the rescued virus for its ability to replicate in chickens in an attempt to have the virus deselected.

MATERIALS AND METHODS

Reverse transcriptase–PCR (RT-PCR) and nucleotide sequencing. The AIVs used in this study were obtained from the Southeast Poultry Research Laboratory (USDA-ARS, Athens, GA). The H5 HA gene of A/duck/Vietnam/Baclieu/09/07 (H5N1) virus was reverse transcribed into complementary DNA using influenza A virus 12 mer primer AGCAAAAGCAGG and AMV reverse transcription system (Promega, Inc., Madison, WI). Full-length H5 HA was PCR amplified using *Bsm*BI HA forward 5′-TATTCGTCTCAGGGAGCAAAAGCAGG-GG-3′ and *Bsm*BI HA reverse 5′-TATTCGTCTCGCTGTAGTA-GAAACAAGGGTGTTTT-3′ primers and PfuUltra® II Fusion HS DNA Polymerase (Stratagene, Inc., Cedar Creek, TX). The H5 HA gene amplicon was restriction digested with *Bsm*BI enzyme and cloned into a bi-directional 425–base pair (bp) promoter chicken RNA polymerase I reverse genetics vector (Jadhao and Suarez, unpubl. data). Automated nucleotide sequencing of the H5 HA gene was performed using gene-specific primers and a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PerkinElmer, Foster City, CA) on a 3730 PerkinElmer machine. Using site-directed mutagenesis PCR, the highly pathogenic H5 HA cleavage sequence PQREGRRKKR/GLF was mutated to low pathogenic PQRETR/GLF cleavage site. The mutated H5 HA cleavage site sequence was also confirmed by nucleotide sequencing. The AIV A/turkey/Oregon/71 (H7N3), with a truncated NS1 protein of only 124 amino acids, was used as the source to clone the NS gene. As was the case with the H5 HA gene, the NS gene segment of A/turkey/Oregon/71 (H7N3) virus variant was amplified using primers *Bsm*BI NS forward 5′-TATTCGTCTCAGGGAGCAAAAGCAGGGTG-3′ and *Bsm*BI NS reverse 5′-TATTCGTCTCGCTGTAGTAGAAACAAGGGTGTTTT-3′ and cloned in the reverse genetic vector. The NS segment was sequenced to confirm the truncated NS1 124–amino acid length. The NS gene segment-specific primers were used to perform one-step RT-PCR to demonstrate truncated NS segment in the recombinant H5N1 (rH5N1/PR8/trNS1) virus.

Generation of recombinant AIV using reverse genetics. The chicken RNA polymerase I 425 promoter-based bi-directional plasmid-based reverse genetics system was constructed for A/Puerto Rico/8/34 (H1N1) strain (Jadhao and Suarez, unpubl. data), similar to the 250–bp chicken RNA polymerase I promoter-based bi-directional plasmid-based reverse genetics system described previously by Massin *et al.* (18). To generate a recombinant virus, the mutated low pathogenic RETR cleavage site coding H5-subtype HA gene was derived from A/duck/Vietnam/Baclieu/09/07 (H5N1) virus, the N1-subtype neuraminidase gene was from A/chicken/Indonesia/7/03 (H5N1) virus, and the internal genes (PB2, PB1, PA, NP, and M), with the exception of the NS gene, originated from the A/Puerto Rico/8/34 (H1N1) strain. The NS gene segment was derived from the A/turkey/Oregon/71 (H7N3) virus variant coding a truncated 124–amino acid NS1 protein. The DF-1 cell line, procured from American Type Culture Collection and maintained at Southeast Poultry Research Laboratory (USDA-ARS, Athens, GA), was used for transfection. The recombinant rH5N1/PR8/trNS1 AIV was rescued in chicken fibroblast (DF-1) cells using an 8 bi-directional chicken RNA polymerase I promoter-driven plasmid-based reverse genetics system. The transfection procedure was similar to previously described protocols (18,19), but with a few modifications. Briefly, 1 μg each of the bi-directional plasmid and 16 μl of Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA) was used to transfect a six-well plate dish of chicken fibroblast (DF-1) cells. After incubation for 72 hr in 37 C/5% CO_2 incubator, the transfection supernatants were inoculated in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The allantoic fluids were harvested after 3–4 days from the eggs and tested for virus rescue by hemagglutination and egg infectivity assay (13). The sterility of the allantoic fluid with HA-positive test was ascertained by inoculation of thioglycolate broth tubes at 37 C for 1 wk. The stock virus was produced by a second limiting dilution passage in the SPF embryonated chicken eggs (ECE).

Confirmation of low pathogenic HA cleavage site and truncated NS1 gene. The H5 HA and NS gene segment of the reverse genetics rescued H5N1 virus was amplified using gene-specific primers (primer sequence available upon request) in a one-step RT-PCR (Qiagen, Inc., Valencia, CA). The H5 HA cleavage site low pathogenic RETR sequence was confirmed by direct nucleotide sequencing, as described earlier in this paper. The truncated NS segment of 700-bp size was confirmed by 1% agarose gel electrophoresis.

Growth properties in Madin Darby Canine Kidney (MDCK) cell culture. The plaque-forming ability of the recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus strain with mutated low pathogenic RETR cleavage site sequence and truncated NS1 gene from A/turkey/Oregon/71 (H7N3) virus variant was tested in MDCK cell line in the presence and absence of exogenous L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. The confluent MDCK cell cultures in the six-well culture plates were infected with 10-fold dilutions of the virus in Dulbecco's Minimum Essential Medium at 37 C/5% CO_2 atmosphere. After 1 hr, medium was removed and overlaid with 0.9% agar prepared in phenol red free

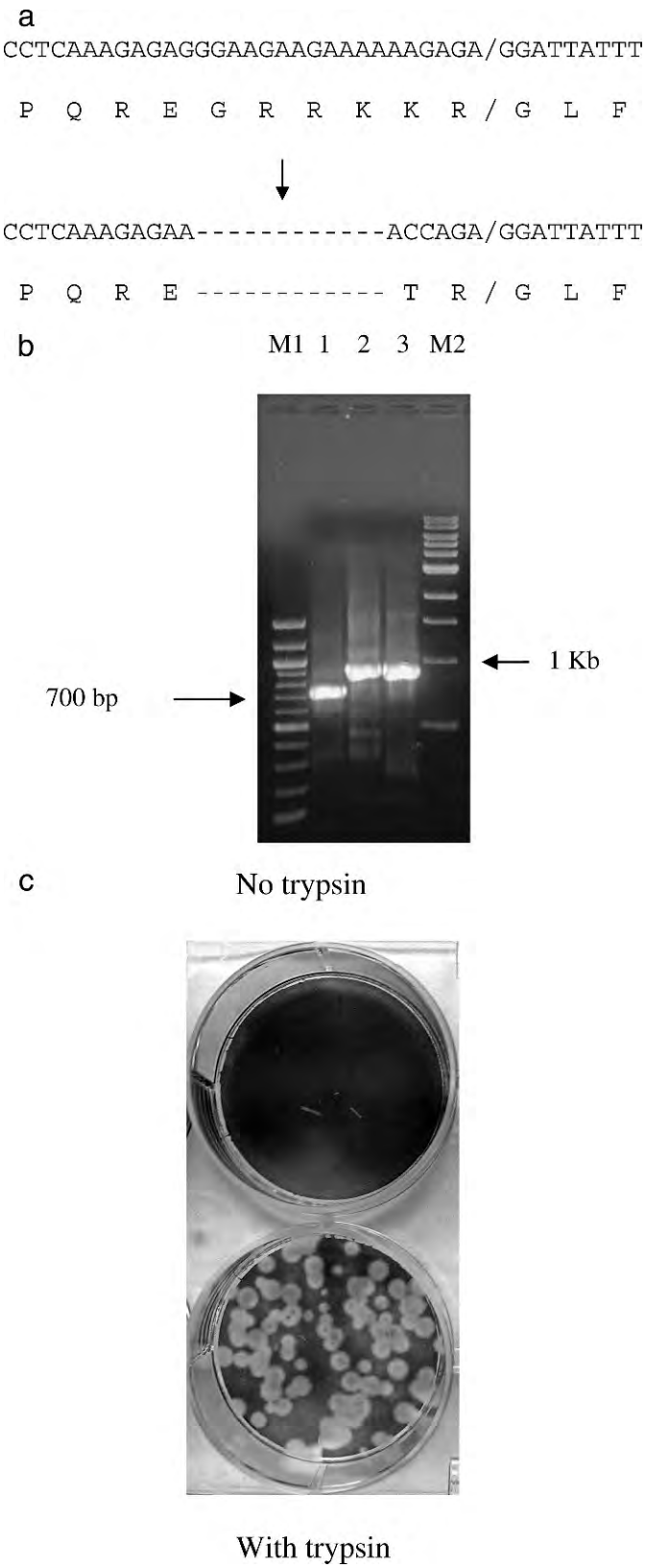

Fig. 1. (a) Mutagenesis of highly pathogenic H5 HA cleavage site of A/duck/Vietnam/Baclieu/09/07 (H5N1) virus to characteristic low pathogenic RETR/GLF HA cleavage site amino acid sequence. Using site-directed mutagenesis, amino acid sequence GRRK coding codons were deleted from the HP H5 HA cleavage site; at −2 position AAG coding for basic amino acid lysine (K) was mutated to ACC to code for threonine (T); and at −7 GAG coding for glutamic acid (E) was mutated to alternate codon GAA coding for glutamic acid (E) to obtain characteristic low pathogenic RETR cleavage site. (b) Demonstration of truncated 700-bp NS gene segment (lane 1) in recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus by one-step RT-PCR, as compared to the wild-type A/Puerto Rico/8/34 (H1N1) NS segment (lanes 2 and 3). M1 = 100-bp ladder; M2 = 1-Kb ladder. (c) Demonstration of inability to form plaques in MDCK cell culture in absence of exogenous trypsin (top) and ability to form plaques in presence of exogenous trypsin 4 days postinfection (bottom) with A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) H5N1 virus containing characteristic low pathogenic H5 HA RETR/GLF cleavage site amino acid sequence and truncated NS1 gene from A/turkey/Oregon/71 (H7N3) virus.

MEM medium with or without 1 μg/ml TPCK-treated trypsin. The plates were incubated at 37 C/5% CO_2 atmosphere for 4 days and fixed with 1% neutral formalin for at least 24 hr. Following removal of agar, the fixed cell cultures were stained with 0.05% neutral red to better visualize the plaques.

Growth properties in ECE. The recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus was diluted 10-fold in phosphate-buffered saline (PBS) with 1× antibiotic and antimycotic solution and inoculated at 0.1 ml via chorio-allantoic cavity route in 9-day-old SPF-ECE (n = 5/virus dilution) and incubated for 3–4 days. The allantoic fluids were harvested from the chilled eggs and tested by HA assay using 0.5% (v/v) chicken erythrocytes. Fifty percent egg infectivity (EID_{50}/ml) virus titers were determined by the Reed and Muench method (21).

Pathogenicity of the recombinant virus in chickens. The animal experiments were conducted with the approval and oversight of the Southeast Poultry Research Laboratory Institutional Animal Care and Use Committee. Eight 4-wk-old white leghorn SPF chickens were inoculated by intravenous route or intranasal route with 0.2 ml of 1:10 dilution of the A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus strain prepared in PBS (pH 7.2). The birds were housed in negative-pressure Horsfal units fitted with HEPA filters in an enhanced BSL-3 animal building. Water and feed were provided to the birds *ad libitum*. The chickens were monitored for 10 days for the presence of clinical signs or mortality, as required for AIV standard pathotyping. Oro-pharyngeal swabs were collected in 1-ml PBS/antibiotic-antimycotic solution (pH 7.2) on day 2 and day 4 postchallenge. RNA was extracted from the oro-pharyngeal swabs using the Trizol and magnetic bead–based extraction protocol (8). Influenza A virus matrix gene-specific real-time RT-PCR was performed to assess the extent of the oro-pharyngeal virus shedding (15). A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus aliquot with predetermined EID_{50} titer was used for RNA extraction and was included to generate a standard curve to determine the virus shedding in the oro-pharyngeal swabs of chickens in the pathotyping experiment (15). To assess the seroconversion, serum samples were harvested on day 14 postchallenge, and an agar gel precipitation test was performed using reagents procured from the National Veterinary Services Laboratories in Ames, IA (4).

RESULTS AND DISCUSSION

The goal of this work was to produce an attenuated virus from a highly pathogenic H5N1 virus using a protocol that would potentially allow the virus to be deselected so that it could be safely handled under BSL-2 conditions. Using a chicken RNA polymerase I 425-bp promoter plasmid-based reverse genetics system, a virus was rescued that contained an H5 HA gene from A/duck/Vietnam/Baclieu/09/07 that had a mutated cleavage site characteristic of a low pathogenic RETR sequence, a truncated NS1 gene from A/turkey/

Oregon/71 (H7N3) virus variant, the N1 gene from chicken/Indonesia/07/03, and the remaining genes (PB2, PB1, PA, NP, and M) from A/Puerto Rico/34 (H1N1) strain in the gene constellation. To clearly identify the recombinant virus, a list of the origins of all gene segments was included in the virus name, viz. A/duck/Vietnam/Baclieu/09/07 H5 HA × A/chicken/Indonesia/7/03 N1 NA × A/Puerto Rico/8/34 MA, NP, PA, PB1, PB2 × A/turkey/Oregon/71 NS truncated.

The recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus H5 HA gene was confirmed to have a low pathogenic cleavage site of RETR/GLF (Fig. 1a) by sequencing. The RT-PCR test using NS segment-specific primers indicated the presence of a truncated NS segment of approximately 700 bp (Fig. 1b). The recombinant virus did not form plaques in MDCK cell culture in the absence of exogenous supplementation of TPCK-treated trypsin (Fig. 1c). However, the same virus formed clear plaques in MDCK cells in 4 days when the agar overlay was supplemented with exogenous TPCK-treated trypsin (Fig. 1c). The growth property of A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus in 9-day-old ECE indicated that it grows to infectivity titers of 10^6 EID_{50}/ml and HA titers as high as 512. Negative testing of the recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus RNA by real-time RT-PCR for Newcastle disease virus ensured that the viral aliquots were free of this viral pathogen.

The intravenous pathotyping test, required for deselection of the virus, and the intranasal infectivity test indicated that the virus did not cause clinical signs over a period of 14 days in inoculated chickens. No sero-conversion was found by the influenza A virus-specific agar gel immunodiffusion assay both in intravenous and intranasally inoculated chickens. The influenza A virus matrix gene real-time RT-PCR test on the oro-pharyngeal swabs did not detect viral shedding on days 2 or 4 postchallenge. The agar gel immunodiffusion assay and negative viral shedding data indicate that the recombinant A/duck/Vietnam/Baclieu/09/07 (rH5N1/PR8/trNS1) virus either did not replicate or replicated only poorly in chickens and would be a safer alternative from which to produce diagnostic antigens for handling in the laboratories using BSL-2 containment levels.

The data from this rescued virus demonstrate that the virus is attenuated and meets the standards for being deselected. Regulatory approval is still required before a virus can be deselected. Upon U.S. Department of Agriculture approval, the mutated nonpathogenic recombinant H5N1 viruses would be transferred to the BSL-2 laboratory for further work. This study describes a protocol to generate more attenuated exotic rH5N1/PR8/trNS1 AIVs to handle in the laboratories with lower or BSL-2 containment levels.

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Efficacy of Commercial Vaccines in Protecting Chickens and Ducks Against H5N1 Highly Pathogenic Avian Influenza Viruses from Vietnam

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SUMMARY. Highly pathogenic (HP) H5N1 avian influenza (AI) viruses continue to circulate in Asia and have spread to other regions of the world. Though attempts at eradication of the viruses during various outbreaks have been successful for short periods of time, new strains of H5N1 viruses continue to emerge and have become endemic in parts of Asia and Africa. Vaccination has been employed in Vietnam as part of AI control programs. Domestic ducks, which make up a large part of poultry in Vietnam, have been recognized as one of the primary factors in the spread of AI in this country. As a result, ducks have been included in the vaccination programs. Despite the effort to control AI in Vietnam, eradication of the disease has not been possible, due in part to the emergence and spread of new viruses. Here, we tested the abilities of avian influenza oil emulsion vaccines of different genetic origins to protect against disease and viral shedding in both 2-wk-old white leghorn chickens and 1-wk-old Pekin ducks. Seventy-five to 100% of vaccinated chickens were protected from mortality, but viral shedding occurred for at least 4 days post challenge. All but one vaccinated duck were protected from mortality; however, all groups shed virus up through at least 5 days postchallenge, depending on the vaccine and challenge virus used. Differences in levels of hemagglutination inhibition (HI) antibody titers induced by the vaccines were observed in both chickens and ducks. Although the vaccines tested were effective in protecting against disease and mortality, updated and more efficacious vaccines are likely needed to maintain optimal protection.

RESUMEN. Eficacia de vacunas comerciales para proteger a pollos y patos contra virus de la influenza aviar de alta patogenicidad H5N1 originarios de Vietnam.

Los virus de la influenza aviar H5N1 de alta patogenicidad continúan circulando en Asia y se han diseminado a otras regiones del mundo. Aunque los intentos para erradicar estos virus durante varios brotes han sido exitosos por periodos de tiempo cortos, nuevas cepas de los virus H5N1 continúan emergiendo y se han convertido en virus endémicos en partes de Asia y África. La vacunación ha sido utilizada en Vietnam como parte de programas de control. Los patos domésticos, que constituyen una gran parte de la avicultura en Vietnam, se han identificado como uno de los factores primarios en la diseminación de la influenza aviar en este país. Como resultado, los patos han sido incluidos en los programas de vacunación. A pesar de los esfuerzos para el control de influenza aviar en Vietnam, la erradicación de la enfermedad no ha sido posible, debido en parte a la aparición y diseminación de virus nuevos. En este trabajo, se estudió la capacidad de las vacunas contra la influenza aviar emulsionadas en aceite con diferentes orígenes genéticos para proteger contra la enfermedad y contra la eliminación del virus en aves Leghorn blancas de dos semanas de edad y en patos Pekín de una semana de edad. Del 75% al 100% de los pollos vacunados estuvieron protegidos contra la mortalidad, pero la eliminación viral se presentó por lo menos durante cuatro días después del desafío. Con excepción de un pato, casi todos los patos vacunados estuvieron protegidos contra la mortalidad, sin embargo, todos los grupos eliminaron al virus por lo menos durante cinco días después del desafío dependiendo de la vacuna y del virus de desafío aplicados. Se observaron diferencias en los niveles de anticuerpos inhibidores de la hemoaglutinación inducidos por las vacunas en los pollos y en los patos. Aunque las vacunas estudiadas fueron efectivas para proteger contra la enfermedad y mortalidad, probablemente se requieren vacunas más eficaces y actualizadas para mantener una protección óptima.

Key words: avian influenza, vaccine, chickens, ducks, H5N1, highly pathogenic

Abbreviations: Ag = antigen; AI = avian influenza; BHI = brain heart infusion; DPC = days postchallenge; EID_{50} = 50% embryo infectious dose; GMT = geometric mean titer; HA = hemagglutinin; HI = hemagglutination inhibition; HP = highly pathogenic; NA = neuraminidase; RRT-PCR = real-time RT-PCR (real-time reverse transcriptase–polymerase chain reaction); SPF = specific-pathogen free

The first case of Asian lineage highly pathogenic (HP) H5N1 avian influenza (AI) virus was isolated in 1996 from a goose in the Guangdong province of China (56,62). A similar virus proceeded to cause an outbreak among poultry in Hong Kong in 1997 (57). Massive culling of all poultry led to the eradication of these viruses, but the Goose/Guangdong-like viruses continued to circulate among ducks in China (44,56). Cauthen *et al.* also demonstrated that H5N1 viruses obtained from cages where geese were housed, in 1999, were nearly identical to the Goose/Guangdong/1/96 virus (5). Evidence of continued circulation of virus in the region included H5N1 from exported Chinese duck meat in 2001, and H5N1 viruses being isolated in live bird markets in Vietnam in 2001 and Hong Kong in 2002 (14,23,50). However, starting late in 2003, an unprecedented spread of the virus occurred in Southeast Asia that eventually moved to Europe, Africa, and the Indian subcontinent (56). These H5N1 viruses have become endemic in several countries in Asia and Africa and variant strains continue to emerge (6,20,35,54,60). Vaccination has been implemented and is still encouraged as part of a control program in poultry in parts of Asia including Vietnam, Indonesia, China, and Egypt (Thanhnien news; 7/15/05) (8).

In addition to preventing clinical disease, a major goal of vaccination against AI (particularly H5 and H7 subtypes), when

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used as part of a control program, is to reduce levels of virus shed into the environment (43,51). Influenza viruses tend to accumulate point mutations in their hemagglutinin (HA) and neuraminidase (NA) surface proteins over time, resulting in antigenic drift, which enhances the ability of the virus to evade the host immune response induced by vaccination or natural infection, allowing higher levels of replication (11,40,61). With the increased amount of virus replication, higher levels of virus shedding by infected birds increase the likelihood of transmission to other susceptible birds or flocks. Earlier studies have shown that the closer the HA amino acid sequence of the vaccine virus is to that of the challenge virus, the lower the levels of virus that are shed from the oropharynx (47). Therefore, it is important when selecting a vaccine virus to take into consideration the amino acid sequence similarity between the circulating viruses and the vaccine virus candidates.

For human influenza viruses, the importance of antigenic drift in vaccine seed strains has resulted in the development of the World Health Organization Global Influenza Surveillance Network, whose principal function is to recommend what vaccine seed strains should be included in commercial vaccines. The factors that they evaluate are genetic differences in circulating strains, the prevalence of important variants, and the antigenic differences these viruses have from the current vaccine strains. A fourfold difference in hemagglutination inhibition (HI) titers between antibody against the current vaccine viruses and antigen of reference strains is an indication that the vaccine seed strain needs to be changed to maintain optimal protection from the vaccine (http://www.who.int/csr/disease/influenza/surveillance/en/index.html). Avian influenza viruses at one time were thought to be less susceptible to antigenic drift as it related to vaccine efficacy, at least as measured by morbidity or mortality in vaccinated birds (55). Because cross-protection has been provided by vaccines produced from heterologous viruses (46,52), frequent changing of AI virus vaccine strains was not considered to be necessary (45,47). This difference in human and AI viruses was believed to be a combination of less selection pressure in the birds due to the infrequent vaccination, short production lives of the birds, and the use of strong adjuvants that were commonly used with poultry vaccines (51). However, long-term vaccination for AI in poultry has recently become more common, and in countries like Mexico, where long-term vaccination without eradication of the low-pathogenic H5N2 avian influenza circulating there, a similar degree of antigenic drift as in human influenza viruses seems to occur at the antigenic level in AI viruses (16). In the study by Lee *et al.* in 2004 (18) comparing different Mexican lineage viruses isolated before and during the vaccination campaign, up to a 16-fold difference was seen in HI activity between the vaccine seed strain and the more recent AI isolates. Additionally, amounts of virus shed by vaccinated birds that were challenged with distantly related viruses were comparable to birds that had not been vaccinated (17). The findings from this study underscore the need for frequent evaluation of AI vaccines in their abilities to control viral shedding. Furthermore, it emphasizes the importance of carefully matching vaccine strains with circulating viruses.

In Vietnam, customary duck management practices often allow exposure of domestic ducks to the wild waterfowl population, which enables ample opportunity for AI viruses to infect domestic ducks and potentially to proceed to infect chickens and other poultry (4,10). Because ducks can serve as silent carriers of AI and because they make up a large part of the poultry population in Vietnam, vaccination of ducks has been included in the vaccine regimen (36). Some concern that this increased vaccination pressure may result in faster antigenic drift away from the vaccine strains has been raised. It has been suggested to evaluate vaccine efficacy at least biennially (43) to ensure that optimal levels of protection against clinical disease and viral shedding are met. Although various vaccines' protective abilities against AI challenge in chickens is relatively well understood, less is known about how well AI vaccines perform in ducks. Here, we evaluated the abilities of three commercial vaccines and two experimental vaccines homologous to the challenge strain to protect both chickens and ducks against lethal exposure of highly pathogenic H5N1 viruses that were obtained from chickens and ducks in Vietnam. The challenge viruses selected were classified as clade 2.3.2 or 2.3.4 viruses and were unusually virulent for ducks, as well as being characteristically virulent for chickens (24,30,60).

Table 1. Sequence similarity[A] between commercial vaccines and challenge virus HA1 proteins.

	DK/VN/203/05	CK/VN/209/05	DK/VN/218/05
N28 (H5N2)	87.1	87.7	87.7
Re-1 (H5N1)	92.5	92.5	92.5
Mexican vaccine (H5N2)	83.4	84.9	84.9

[A]The MegAlign program (DNASTAR, Madison, WI) was used to compare amino acid sequences, using the Clustal V alignment algorithm. Percent values shown.

MATERIALS AND METHODS

Viruses. The H5N1 viruses A/Duck/Vietnam/203/05 (DK/VN/203), A/Chicken/Vietnam/209/05 (CK/VN/209), and A/Duck/Vietnam/218/05 (DK/VN/218) were isolated from either ducks or chickens in Vietnam and were obtained from the National Center for Veterinary Diagnosis, Hanoi, Vietnam. The DK/VN/203 virus is a clade 2.3.2 virus and the two latter viruses belong to clade 2.3.4 (24,30,60), and were isolated in northern Vietnam in December of 2005 (30). Isolates were inoculated into the allantoic cavity of embryonating chicken eggs and grown for 24–30 hr at 37 C. Allantoic fluid was harvested, titered as previously described (30), and frozen at −70 C until further use.

Vaccines. Three commercially available vaccines used in Vietnam to control AI were used in this study. These vaccines were generated from the following whole, killed viruses, and contained an oil adjuvant: 1) A/TK/England/N-28/73, subtype H5N2 (referred to as N28); 2) a genetically modified reassortant H5N1 low pathogenic virus, A/Harbin/Re-1/2003 (referred to as Re-1) (31); and 3) A/CK/Mexico/232/94 (H5N2, referred to as Mexican vaccine; Table 1). The first two vaccines were obtained from Vietnam and both have been used in chickens and ducks in all 64 provinces of the country. They were produced by Weike Biological Company, of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China). The third vaccine used was from Intervet International/Investigacion Aplicada S.S., Tehuacan, Puebla, Mexico, and its use has been permitted in chickens in Vietnam. The Re-1 vaccine was produced through reverse genetics and derived its HA and NA genes from A/Goose/Guangdong/96 (31). This virus was attenuated by removing the multiple basic amino acids at the HA cleavage site (49). The six internal genes of this recombinant virus were derived from the high-growth A/Puerto Rico/8/34 (PR8) virus.

Two experimental vaccines containing either DK/VN/203 or CK/VN/209 antigen (Ag) were prepared on site, as previously described (41). Briefly, viruses were grown in 10-day-old embryonating chicken eggs for 1 day. Allantoic fluid from eggs infected with one of each particular virus was harvested and pooled. Following inactivation of each virus with 0.1% β-propiolactone (Sigma, St. Louis, MO), HA titers were determined by the HA test to be the following: DK/VN/203: 256 HA units and CK/VN/209: 256 HA units. One part aqueous Ag

(10 ml) was emulsified in four parts (40 ml) oil phase. The oil phase consisted of 36 ml Drakeol 6 VR pharmaceutical grade mineral oil (Penreco, Butler, PA), 3 ml 7.5% sorbitan mono-oleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 1 ml 2.5% polysorbate (Tween 80, ICI United States, Inc.). Vaccines produced on site were prepared 5 or 6 days prior to administration, homogenized with the use of a Waring blender (Fisher Scientific International Inc., Hampton, NH) (42), and stored at 4 C.

Evaluation of sequence similarity. Amino acid sequence similarities between vaccine and challenge virus HA1 proteins were compared with the use of the MegAlign program (DNASTAR, Madison, WI). The Clustal V alignment algorithm was used.

Animal experiments. Two-week-old specific-pathogen-free (SPF) white leghorn chickens from our flock at Southeast Poultry Research Laboratory, either 8 or 10 per group, were vaccinated once, subcutaneously in the nape of the neck with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine), or with 0.5 ml of experimental vaccine (41). Normal allantoic fluid in the form of an oil emulsion vaccine was used as negative control. Two weeks postvaccination, all birds were bled via the wing vein. Three weeks postvaccination, all birds were challenged with 10^6 EID_{50} (50% embryo infectious dose) of either DK/VN/203 or CK/VN/209 viruses in a total volume of 0.2 ml brain heart infusion (BHI) broth per bird, via the choanal slit. Birds were evaluated for clinical signs for 10 days following challenge. Oropharyngeal swabs were taken at 2 and 4 days postchallenge (DPC) for determining viral shedding. Moribund birds that were in pain, or had stopped eating or drinking, were euthanized with 0.2 ml sodium pentobarbital (5 g/ml) per bird. At 10 DPC, all survivors were bled via the wing vein, and then euthanized as described.

One-week-old white Pekin ducks (*Anas platyrhynchos*) obtained from a commercial farm were divided into six groups of 10 birds. Blood samples for serology were collected from the saphenous vein of a representative number of ducks to ensure that the birds were serologically negative for AI, as determined by the agar gel precipitin test (2,26). Ducks were vaccinated once, subcutaneously in the nape of the neck, with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine). Two groups served as nonvaccinated controls, receiving allantoic fluid in the form of an oil emulsion vaccine. Two weeks postvaccination, blood samples were collected from all ducks for serology. At this same time, the ducks were challenged via the choanal slit with $10^{5.0}$ EID_{50} of DK/VN/203 or DK/VN/218 influenza virus in 0.1 ml. Ducks were observed daily for clinical signs of disease. Oropharyngeal and cloacal swabs were collected at 2, 3, 5, 7, and 11 DPC to determine viral shedding. One duck per group was euthanized with 0.2 ml sodium pentobarbital (5 g/ml) per bird at 3 DPC, and tissues collected for virus detection by quantitative real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) (37). Moribund ducks that were in pain, or that had stopped eating or drinking, were also euthanized. Blood samples were collected at 11 DPC from all surviving ducks. Ducks remaining at the end of the experiment were euthanized as previously described.

Hemagglutination inhibition (HI) test. Hemagglutination inhibition antibody titers against AI were determined by using the HI test (48). Either homologous or heterologous β-propiolactone-inactivated Ag was diluted in phosphate-buffered saline to make a concentration of four HA units. Homologous Ag refers to the same strain of virus used to produce the vaccine. Heterologous Ag refers to any of the three viruses used to produce the vaccines tested in this study and which were not identical to the vaccine virus administered. Fifty microliters of Ag were added per well of a 96-well plate, where test serum was twofold, serially diluted. Plates were incubated for 15 min at room temperature before 0.5% chicken red blood cells were added to each well. Plates were shaken for 15 sec and incubated for 45 min at room temperature. Results were interpreted as the reciprocal of the last well that had complete inhibition of hemagglutination activity.

Determination of viral shedding. Oropharyngeal swab samples from chickens and ducks, and cloacal swab samples from ducks were suspended in 2 ml sterile BHI broth (Sigma-Aldrich, St. Louis, MO) containing 1× antibiotic/antimycotic (Mediatech, Herndon, VA), and frozen at −70 C until RNA extraction. Total viral RNA was extracted using Trizol or MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol (38). The procedure for RNA isolation was carried out using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA).

RRT-PCR was performed using primers and probe specific for type A avian influenza matrix gene as previously described (37), but with modifications. Two and three nucleotide changes were detected between the DK/VN/203/05 and CK/VN/209/05 matrix genes and the reverse primer created by Spackman *et al.* (37), so new primers were designed specific for these changes. The primer sequences are as follows: DK/VN/203/05 MA-124: 5′-TGCAAAGACATCTTCAAGTTTCTG-3′ and CK/VN/209/05 MA -124: 5′-TGCAAAGACATCCTCAAGTTTCTG-3′. Qiagen (Valencia, CA) OneStep RT-PCR Kit was used under the following conditions: 1× buffer, 3.75 mM $MgCl_2$, 10 pmol each primer, 320 μM each dNTP, 0.12-μM probe, and 13 units Rnase Inhibitor (Promega, Madison, WI). Eight microliters of the RNA sample (mentioned above) and nuclease-free water were added to make a final volume of 25 μl. The reverse transcription reaction consisted of one cycle of 30 min at 50 C, followed by 15 min at 95 C. Forty-five cycles of 1-sec denaturation at 94 C, followed by annealing for 20 sec at 60 C were carried out in the PCR reaction. Both reactions were carried out in a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine. Standard curves were generated by taking known amounts of the viruses (as measured in EID_{50}) obtained from egg allantoic fluid, diluting the allantoic fluid serially 10-fold, and then extracting RNA from these virus dilutions. The EID_{50}s of virus from the swab samples were extrapolated from the cycle thresholds by using standard curves generated from the known amounts of RNA of the challenge viruses used (18) and the results presented as EID_{50} equivalents.

Statistical analysis. Hemagglutination inhibition and swab sample data were analyzed with the use of Prism v5 Software package (GraphPad Software Inc., San Diego, CA). One-way ANOVA with Tukey's *post hoc* test was used to analyze $\log_2$ HI titers and viral shedding data. Results with P values <0.05 were considered to be statistically significant. Detection limits of individual RRT-PCR reactions were calculated from the standard curve, setting the cycle threshold value equal to the number of cycles run (38). Samples that were RRT-PCR negative in this study were assigned titer values equal to the detection limit of the RRT-PCR run minus $10^{0.1}$ EID_{50}/ml, as previously described (38).

RESULTS

Vaccine efficacy experiments. Three commercial vaccines that have been used in Vietnam and two experimental vaccines containing viruses homologous to the challenge strains were used. Two of the commercial vaccines (N28 and Re-1) are of the Eurasian lineage of influenza and have sequence similarities to the challenge viruses ranging from 87.1% to 92.5% (Table 1). The third virus is of the North American lineage and is approximately 84% similar to the challenge strains (Table 1).

DK/VN/203/05 challenge in chickens. Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or the homologous vaccine and challenged 3 wk later with DK/VN/203, a clade 2.3.2 virus. Results are shown in Table 2A. All negative control birds died by 2 DPC. Five of eight birds vaccinated with N28 vaccine displayed signs of mild sinusitis, conjunctivitis, and were less active by 2 DPC, but all birds recovered. No clinical symptoms were noted in any vaccinates in the Re-1, Mexican, or homologous vaccine groups.

Total viral RNA was isolated from oropharyngeal swab samples and quantitative RRT-PCR was performed to compare levels of virus shed by birds from the different vaccine groups both 2 and 4 days

Table 2A. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 wk of age and intranasally challenged at 5 wk of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.

Vaccine group	Morbidity: number ill/total	Mortality: number dead/total (MDT)[D]	Viral RNA detection from O/P[A] swab samples number positive/total (log EID_{50}/ml[BC])	
			2 days postchallenge	4 days postchallenge
Negative control	8/8	8/8 (2)	8/8 (6.2)[a]	N/A
N28	5/8	0/8	5/8 (2.3)[b]	6/8 (2.8)[a]
Re-1	0/8	0/8	3/8 (1.8)[b]	6/8 (2.0)[a]
Mexican	0/8	0/8	6/8 (2.7)[b]	5/8 (2.6)[a]
DK/VN/203/05	0/8	0/8	4/8 (1.7)[b]	3/8 (1.1)[a]

[A]Swab samples were taken from all birds remaining at each time point postchallenge. O/P = oropharyngeal; NA = not applicable.
[B]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37). Numbers in parentheses are averages of viral titers shed from birds in each group.
[C]Different lowercase superscripts denote significance between treatment groups (within columns; $P < 0.05$) as determined by one-way ANOVA.
[D]MDT = mean death time denoted in days.

following challenge. At 2 DPC, virus shedding from negative control birds was significantly higher than that from all vaccinated birds. At 4 DPC, no significantly different levels of virus were shed between any of the vaccine groups.

We evaluated HI titers with the use of both homologous and heterologous Ag. At 2 wk postvaccination, all vaccinated birds had HI titers (Table 3A). The Mexican vaccine induced the highest HI titers, which averaged to be 70 at 2 wk postvaccination, when homologous Ag was used (Table 3A). Correspondingly, 100% of the birds in these groups did not show any clinical signs (Table 2A). The Re-1 vaccine group had the second-highest titers, with a geometric mean titer (GMT) of 58, 2 wk postvaccination with homologous Ag (Table 3A). The DK/VN/203 and N28 vaccine groups' HI titers were close to 30 at 2 wk postvaccination when homologous Ag was used (Table 3A). An HI titer of 40 is considered to provide consistent and reproducible protection after challenge with any virulent virus of the same subtype (29), but lower titers may also be protective. This was demonstrated in DK/VN/203 and the N28 vaccine groups, where most birds had no or only mild clinical disease (Table 2A). At 10 DPC, HI titers in all surviving birds were at least 40, whether the Ag was homologous or not (Table 3A). As seen with the 2-wk postvaccination sera, HI titers were highest with homologous Ag. Even though the group vaccinated with the Ag that was homologous to the challenge strain did not have the highest HI titers, all of these birds were protected from disease and death (Table 2A).

CK/VN/209/05 challenge in chickens. Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or with the homologous vaccine and challenged 3 wk later with CK/VN/209, a clade 2.3.4 virus. Results are shown in Table 2B. Similar to the DK/VN/203 challenge group, all control birds died by 2 DPC. Five out of 10 birds in the N28 vaccine group displayed signs of depression and conjunctivitis. At 5 DPC, one bird had died and one, which had displayed severe respiratory signs, was euthanized. In the Re-1 vaccine group, two birds died at 7 DPC. Two birds in the homologous vaccine group also died 2 and 3 DPC. None of the deaths from the latter three groups was statistically significant and all but one of the birds had HI titers below the protective level of 40, 2 wk following vaccination. All birds in the Mexican vaccine group were active and eating normally.

Two days following challenge, all vaccinated birds shed significantly less virus than negative control birds (Table 2B). At 4 DPC, birds that were vaccinated with the N28 or Re-1 vaccine shed significantly higher levels of virus than birds that had received the homologous vaccine (Table 2B). There was no correlation between oropharyngeal virus shedding and survival of the birds; not all birds that died were shedding detectable levels of virus, based on RRT-PCR. However, several birds that did shed detectable levels of virus, based on RRT-PCR, survived.

All vaccinated birds seroconverted to AIV at 2 wk following vaccination (Table 3B). The Mexican vaccine induced the highest HI titers (GMT: 108) and the Re-1 vaccine induced the second-highest titers (GMT: 45) 2 wk postvaccination, when homologous Ag was used (Table 3B). Two weeks following vaccination, the group vaccinated with the homologous vaccine had HI titers near 30. However, only one of these birds displayed clinical symptoms

Table 2B. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 wk of age and intranasally challenged at 5 wk of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus.

Vaccine group	Morbidity: number ill/total	Mortality: number dead/total (MDT[D])	Viral RNA detection from O/P[A] swab samples number positive/total (log EID_{50}/ml[BC])	
			2 days postchallenge	4 days postchallenge
Negative control	8/8	8/8 (2)	8/8 (7.2)[a]	N/A
N28	5/10	2/10 (5)	10/10 (4.4)[b]	9/10 (5.2)[a]
Re-1	2/10	2/10 (7)	9/10 (4.1)[b]	8/10 (4.2)[a]
Mexican	0/8	0/8	5/8 (3.0)[b]	6/8 (3.8)[ab]
CK/VN/209/05	2/8	2/8 (2.5)	4/8 (3.0)[b]	2/6 (1.7)[b]

[A]Swab samples were taken from all birds remaining at each time point postchallenge. O/P = oropharyngeal; NA = not applicable.
[B]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37). Numbers in parentheses are averages of viral titers shed from birds in each group.
[C]Different lowercase superscripts denote significance between treatment groups (within columns; $P < 0.05$) as determined by one-way ANOVA.
[D]MDT = mean death time denoted in days.

Table 3A. HI titers of chickens vaccinated at 2 wk of age and challenged intranasally at 5 wk of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titers[B]		Range of postchallenge HI titers[C]	
	Homologous Ag[D]	DK/VN/203/05 Ag	Homologous Ag[D]	DK/VN/203/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	16–64 (30)[b]	4–16 (10)[bc]	32–1,024 (304)[a]	32–512 (152)[a]
Re-1	32–128 (58)[bc]	4–32 (14)[bc]	128–2,048 (832)[a]	32–1,024 (165)[a]
Mexican	32–128 (70)[c]	0–16[E] (7)[b]	256–2,048 (1,323)[a]	64–1,024 (278)[ab]
DK/VN/203/05	8–64 (27)[b]	8–64 (27)[c]	512–2,048 (776)[a]	512–2,048 (776)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum was collected 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum was collected from all surviving birds 10 days following challenge. In parentheses: geometric mean of HI titers.
[D]Homologous virus refers to the same strain of virus used to generate the vaccine. Because Goose/Guangdong/1/96 was not available, Goose/Hong Kong/99 was used as homologous Ag for Re-1 group antisera.
[E]One of eight birds did not have detectable levels of HI antibodies against this antigen.

(Table 2B). Birds that had been vaccinated with N28 had significantly lower HI titers than birds vaccinated with the Mexican vaccine, when homologous antigen was used (Table 3B). Similar to the DK/VN/203 challenge study, all surviving birds had HI titers greater than 40 following challenge (Table 3B). At 10 DPC, birds in the Re-1 vaccine group had significantly higher HI titers than those in the homologous vaccine group (Table 3B). As mentioned, all but one of the six vaccinated birds that died following challenge had HI titers less than 40, when homologous Ag was used.

DK/VN/203/05 challenge in ducks. One-week-old Pekin ducks were vaccinated once with one of the three commercial vaccines and challenged 2 wk later with DK/VN/203. Results are shown in Table 4A. All control birds died by 4 DPC. One duck vaccinated with Re-1 presented mild neurological signs including head tilting and incoordination, but continued to eat and drink and appeared alert, and survived until the end of the experiment.

Virus shedding was detected from both the oropharyngeal and cloacal routes from all challenged groups at 2 and 3 DPC. The N28 and Re-1 vaccines induced similar responses in ducks on levels and duration of shedding after challenge. All but one of the ducks vaccinated with the Mexican vaccine stopped shedding by 5 DPC, at least 1 day earlier than with the other two vaccines. Virus was detected from all tissues collected from the ducks euthanized at 3 DPC, demonstrating systemic spread (data not shown).

All vaccinated ducks had seroconverted prior to challenge (Table 5A), when tested against homologous Ag. Birds in the Re-1 vaccine group had significantly higher prechallenge titers than those in the N28 or Mexican vaccine groups (Table 5A). All groups' HI antibody titers were undetectable, prior to challenge, when heterologous antigen was used (Table 5A). No significant differences were detected between vaccine groups when homologous antigen was used on serum collected 11 DPC (Table 5A). However, ducks vaccinated with the Mexican vaccine had significantly higher HI titers than those vaccinated with N28 when heterologous Ag was used (Table 5A).

DK/VN/218/05 challenge in ducks. One-week-old Pekin ducks were vaccinated once with one of the three commercial vaccines and challenged 2 wk later with DK/VN/218. Results are shown in Table 4B. Negative control birds died in less than 3 DPC. All but one of the immunized ducks were protected against mortality upon challenge. This duck was vaccinated with N28 and it died 6 days after infection. This duck presented neurological signs as described above.

Compared to ducks challenged with the DK/VN/203 vaccine, those challenged with DK/VN/218 generally shed virus for a longer time period. Viral shedding was detected from both oropharyngeal and cloacal swabs through at least 5 days, and many continued shedding virus at 11 DPC. In the DK/VN/218 challenge group, the Mexican vaccine curtailed oropharyngeal shedding by at least 5 days, compared to the other two vaccines. Similar to the DK/VN/203/05 challenge birds, virus was detected in all tissues collected from the ducks euthanized at 3 DPC (data not shown).

Prior to challenge, all vaccinated birds had HI titers (Table 5B). No significant differences in HI titers were seen between any of the vaccinated groups prior to challenge when homologous Ag was used. No HI antibodies were detectable when heterologous Ag to the vaccine virus was used (Table 5B). At 11 DPC, when homologous Ag was used, no significant differences were seen between vaccine

Table 3B. HI titers of chickens vaccinated at 2 wk of age and challenged intranasally at 5 wk of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titers[B]		Range of postchallenge HI titers[C]	
	Homologous Ag[D]	CK/VN/209/05 Ag	Homologous Ag[D]	CK/VN/209/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	8–128 (24)[b]	0–32[E] (13)[b]	128–2,048 (861)[ab]	128–256 (194)[a]
Re-1	2–256 (45)[bc]	4–64 (18)[b]	512–4,096 (1,722)[a]	64–512 (181)[a]
Mexican	32–512 (108)[c]	4–32 (13)[b]	512–4,096 (891)[ab]	64–1,024 (194)[a]
CK/VN/209/05	4–128 (35)[bc]	4–128 (35)[b]	256–512 (362)[b]	256–512 (362)[a]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum was collected 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum was collected from all surviving birds 10 days following challenge. In parentheses: geometric mean of HI titers.
[D]Homologous virus refers to the same strain of virus used to generate the vaccine. Because Goose/Guangdong/1/96 was not available, Goose/Hong Kong/99 was used as homologous Ag for Re-1 group antisera.
[E]One of 10 birds did not have detectable levels of HI antibodies against this antigen.

Table 4A. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 wk of age and intranasally challenged at 3 wk of age with 10^5 EID_{50} of DK/VN/203 H5N1 HPAI virus.

		Viral RNA detection from swab samples[A] (log EID_{50}/ml)									
	Mortality: number dead/	2 DPC		3 DPC		5 DPC		7 DPC		11 DPC	
Vaccine group	total (MDT)[B]	O/P[C]	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal
Negative control	10/10 (3.7)[D]	9/10 (4.4)	5/10 (2.8)	6/6 (4.6)	6/6 (3.2)	—	—	—	—	—	—
N28	0/10	4/10 (3.5)	2/10 (2.9)	5/10 (3.7)	1/10 (3.1)	9/9 (2.9)	4/9 (2.8)	0/9	0/9	0/9	0/9
Re-1	0/10	4/10 (3.1)	1/10 (2.5)	9/10 (3.2)	9/10 (3.1)	5/9 (2.8)	2/9 (3.1)	0/9	0/9	0/9	0/9
Mexican	0/10	3/10 (3.2)	2/10 (4.5)	10/10 (3.3)	9/10 (3.2)	1/9 (3.3)	0/9	0/9	0/9	0/9	0/9

[A]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37).
[B]MDT = mean death time denoted in days.
[C]O/P = oropharyngeal.
[D]Number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

groups. Ducks vaccinated with the Mexican vaccine had significantly higher HI titers than those vaccinated with Re-1 and then challenged with DK/VN/218/05, when challenge strain viral Ag was used (Table 5B).

DISCUSSION

When used as part of an effective control strategy against AI, vaccination should not only prevent clinical signs and illness, but also significantly reduce the amount of viral shedding that could be a source of infection for other birds (19,43). The Asian lineage of H5N1 AI first caused disease outbreaks in poultry in Vietnam at the start of 2004 (58), and it spread widely across the country. In 2005, a campaign to vaccinate 220 million domestic fowl with the commercial vaccines tested in this study was launched in an attempt to control the outbreak (http://www.globalsecurity.org/security/library/news/2009/02/sec-090211-irin01.htm) (25). Between 2005 and 2006, the two Chinese vaccines (N28 and Re-1) were used in both chickens and ducks, while the Mexican vaccine was used exclusively in chickens (personal communication). It appeared to be a success over the next year by greatly reducing the number of reported outbreaks and human infections in Vietnam (59). During the latter part of 2006, H5N1 viruses were reported in nonvaccinated, asymptomatic ducks, upon routine surveillance (59). Even though vaccination is still being used in Vietnam, poultry outbreaks as well as human cases continue to be reported (59). The factor of low immunity rate in poultry, because of the difficulty to vaccinate and booster poultry populations, is thought to contribute to the resurgence of the disease. In addition, the viruses in Vietnam continue to change both by antigenic drift as well as new variants being introduced from other countries in the region. One of the goals of this study was to evaluate if the change of the lineage of virus circulating in northern Vietnam, from clade 1 to clade 2.3, contributed to the increase of poultry outbreaks in spite of the continued efforts at vaccination. For this, we evaluated the level of protection obtained with three widely available commercial vaccines in chickens and ducks after challenge with viruses representative of clade 2.3 strains circulating in Vietnam.

Although H5N1 HPAI viruses display virulent phenotypes in chickens, ducks may become infected and shed virus without presenting any signs of illness (1,7,15,26,27,28,33). Therefore, ducks have been linked to transmitting AI to poultry (34) by "silently" spreading virus, contributing to its circulation and further propagation among poultry. As a result, the H5N1 HPAI viruses continue to threaten both human and veterinary/poultry health. On the other hand, some HPAI viruses circulating in Vietnam have shown to produce high mortality in domestic ducks (30), directly affecting this important segment of this country's poultry industry. The duck-raising practices in Vietnam include the production of free-range ducks, which, because of the low biosecurity inherent with this production practice, poses a high risk of spread and maintenance of H5N1 in the country (21). Consequently, outbreaks continue to occur in nonvaccinated ducks (9,59). If efficacious vaccines could be given to ducks in this production system, it could significantly improve the control of AI.

Because of AI's tendency to drift antigenically, AI vaccines should be tested periodically to ensure sufficient protection from clinical disease and virus shedding (32,43). The challenge viruses for this study were not only highly pathogenic in chickens, but unusually

Table 4B. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 wk of age and intranasally challenged at 3 wk of age with 10^5 EID_{50} of DK/VN/218 H5N1 HPAI virus.

		Viral RNA detection from swab samples[A]									
	Mortality: number dead/	2 DPC		3 DPC		5 DPC		7 DPC		11 DPC	
Vaccine group	total (MDT)[B]	O/P[C]	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal
Negative control	10/10 (2.1)[D]	10/10 (3.4)	9/10 (4.9)	1/1 (4.8)	1/1 (2.9)	—	—	—	—	—	—
N28	1/10 (6)	5/10 (3.2)	1/10 (3.6)	4/10 (3.1)	2/10 (3.4)	4/9 (2.8)	7/9 (2.8)	4/8 (2.9)	8/8 (2.9)	5/8 (2.7)	8/8 (2.7)
Re-1	0/10	4/10 (2.8)	1/10 (2.6)	3/10 (3.7)	2/10 (2.5)	8/9 (2.9)	5/9 (2.6)	5/9 (2.8)	0/9	6/9 (2.8)	0/9
Mexican	0/10	3/10 (2.9)	1/10 (5.0)	4/10 (3.0)	5/10 (3.2)	4/9 (2.7)	7/9 (2.7)	0/9	0/9	0/9	0/9

[A]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37).
[B]MDT = mean death time denoted in days.
[C]O/P = oropharyngeal.
[D]Number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

Table 5A. Hemagglutination inhibition (HI) titers of ducks vaccinated at 1 wk of age and challenged intranasally at 3 wk of age with 10^5 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titer[B]		Range of postchallenge HI titer[C]	
	Homologous Ag[D]	DK/VN203/05 Ag	Homologous Ag[D]	DK/VN/203/05 Ag
Negative control	0[a]	0	N/A[E]	N/A
N28	16–32 (17)[b]	0	64–256 (166)[a]	0–16[F] (3)[a]
Re–1	32–128 (42)[c]	0	512–1,024 (446)[a]	0–32[G] (7)[ab]
Mexican	16–128 (23)[b]	0	128–1,024 (276)[a]	8–64 (16)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum samples were taken 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum samples were collected 11 days postinfection.
[D]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
[E]NA = not applicable.
[F]Four out of nine birds did not have detectable levels of HI antibodies against this antigen.
[G]Two out of eight birds did not have detectable levels of HI antibodies against this antigen.

virulent for ducks, as well (30). Specifically for ducks, an increase in tissue tropism, lesion severity, viral replication, and one of the shortest mean death times reported in both 2- and 5-wk-old Pekin ducks was observed with these viruses, as compared to previous H5N1 viruses tested in ducks (30). The level of protection rendered by the vaccines was influenced by the virus that served as the challenge strain. Though both of the groups of viruses were extremely virulent in chickens and ducks, the clade 2.3.4 viruses (CK/VN/209 and DK/VN/218) appeared more pathogenic than the clade 2.3.2 (DK/VN/203) virus (30), and caused higher morbidity and virus shedding in ducks.

Hemagglutination inhibition titers are commonly used to predict levels of protection against viral infection and disease in vaccinated birds. In chickens, the Mexican vaccine surpassed the others in its ability to induce high levels of HI antibodies (Table 3A,B) and to protect the birds from clinical disease (Table 2A,B). This is quite interesting because, compared to the other vaccine virus HA sequences, it has the lowest sequence similarity with the challenge viruses. Similar results were seen by Swayne *et al.* and Veits *et al.,* when testing the efficacy of H5N2 vaccines to protect chickens against HPAI viruses that were less than ideally matched to the vaccine viruses (46,53). Such remarkable immunogenicity could possibly be attributed to the proprietary adjuvant used in the formulation of the Mexican vaccine, the antigen mass used in the vaccine, or the inherent antigenicity of the hemagglutinin protein itself (45). With regards to shedding, chickens vaccinated with the CK/VN/209/05 vaccine (and challenged with the same virus) shed significantly lower amounts of virus from the respiratory tract, compared to the N28 vaccine, which was a low pathogenic turkey virus of Eurasian lineage isolated in 1973. In addition, several of the birds vaccinated with this older virus also displayed clinical symptoms. Another study tested the Re-1 vaccine in chickens and demonstrated, as did we, that all of the vaccinated birds were completely protected from disease and death, upon challenge with either homologous virus or heterologous viruses from 2004 (49). There was also virus detected in oropharyngeal swab samples from some of the vaccinated birds (49), similar to our findings.

Compilation of the data produced in chickens indicates that the sequence similarity is not the sole determining factor for predicting a vaccine's protective potential against disease or viral shedding. If the antibody titers are high enough to a subtype, protection from morbidity may be achieved, regardless of the differences in genetic relatedness of the vaccine and challenge viruses if the viruses are of the same subtype. It also appeared that even if the HI titers, using homologous Ag, prior to challenge were not quite at the typical protective level of 40, clinical protection was still observed. Additionally, there did not appear to be a clear correlation between HI titer and level of viral shedding.

In a previous duck study, a two-dose vaccination program starting in ducks at 1 day of age, followed by a booster at 4 wk of age, was used because of its compatibility with the duck husbandry practices in Asia, and was shown to be effective (3). It is important to vaccinate ducks at an early age to try to provide immunity as early as possible, but also because after the ducks are released into the fields, it becomes much more difficult to vaccinate them. We chose to use 1-wk-old ducks on a single-dose regimen to see if, when vaccinated at this age, they would obtain good protection and a reduction in virus shedding after challenge. We also chose to use 1-wk-old ducks because with increased vaccination of poultry in Vietnam, day-old birds may have maternal antibodies that could

Table 5B. Hemagglutination inhibition (HI) titers of ducks vaccinated at 1 wk of age and challenged intranasally at 3 wk of age with 10^5 EID_{50} of DK/VN/218/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titer[B]		Range of postchallenge HI titer[C]	
	Homologous Ag[D]	DK/VN218/05 Ag	Homologous Ag[D]	DK/VN/218/05 Ag
Negative control	0[a]	0	N/A[E]	N/A
N28	16–32 (20)[b]	0	256–1,024 (380)[a]	8–32 (17)[ab]
Re-1	16–128 (34)[b]	0	128–1,024 (474)[a]	0–32[F] (7)[a]
Mexican	16–256 (26)[b]	0	128–1,024 (406)[a]	16–128 (64)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum samples were taken 2 wk postvaccination. Titers are expressed as geometric mean titers.
[C]Serum samples were collected 11 days postinfection.
[D]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
[E]NA = not applicable.
[F]Three out of nine birds did not have detectable levels of HI antibodies against this antigen.

interfere with vaccine efficacy, and 1-wk vaccination is potentially a suitable compromise with ease of vaccination and less interference by maternal antibody.

Other duck studies involving various vaccination regimens followed by challenge have also demonstrated clinical protection and reduced virus shedding (12,22,39,40,52). However, these results were obtained, as ours, in laboratory settings. It is important to keep this in mind when applying experimental vaccine data to the field. Experimental data can not be directly extrapolated to the field setting because of differences in circumstances between the two. Unlike poultry raised in the field, the experimental animals do not have preexisting immunity to AI from maternal antibodies or prior AI infection and their immune systems are not compromised by other unrelated pathogens, which may be concomitantly circulating among flocks in a field setting. In any case, vaccination is not likely to prevent infection and provide sterilizing immunity. In a previous study, prevention of tracheal and cloacal shedding was achieved when a large dose (1 μg) of antigen was administered (13). Unfortunately, the large quantities of antigen or adjuvant required to induce such a potent immune response may be greater than could be realistically administered in the field for reasons of cost. With an appropriate vaccination program, however, shedding of infectious virus into the environment could be reduced to a minimum and consequently prevent transmission.

Kim *et al.* (13) tested vaccines that contained the HAs of either clade 1, clade 2.2, or clade 2.3.4 viruses, in their abilities to protect SPF white Pekin ducks from an extremely virulent H5N1 virus, Duck/Laos/25/06. Despite low or undetectable HI titers, all of the challenged, vaccinated birds were completely protected from morbidity and mortality after one vaccination (13). Regardless of the time point, the HI titers of vaccinated ducks in our study were much lower than those of chickens (Tables 3A,B, 5A,B). The results of their study support our findings that even if the humoral immune response to the vaccine viruses is not always detectable in ducks, the immune response may still be protective.

At 10 and 11 DPC, HI titers in all surviving birds, chickens and ducks alike, were at least 40, when homologous Ag was used (Tables 3A,B, 5A,B). However, compared to titers produced when heterologous Ag was used, the homologous HI titers were between 4- and 16-fold higher. This suggests that following challenge, the antibodies produced were not only against the challenge strain, but were the result of a memory response against the vaccine virus, as well.

Based on our results, the vaccines tested in this study provided both chickens and ducks protection from disease and reduced viral shedding, upon challenge with either of two different isolated clade 2.3 H5N1 highly pathogenic AI viruses from Vietnam. Though most birds vaccinated with the N28 vaccine did have clinical protection from virulent challenge, as compared to the nonvaccinated birds, the HI titers prechallenge were the lowest of the vaccines tested and the reduction of viral shedding was marginal. This vaccine would seem to be a poor option for a vaccine program, although the reasons for a poor response from this antigen were not fully investigated. The adjuvant used was assumed to be similar to the Re-1 vaccine because it was made by the same manufacturer, but differences in antigen mass, antigenicity of the hemagglutinin, or antigenic differences based both on HI data and sequence similarity may have all contributed to the poorer results. This vaccine would not be recommended for further use. The Re-1 and the Mexican lineage vaccines, which have continued to be used since 2007, still appear to provide good protection from challenge, but antigenic variability based on HI data and sequence similarity raises concern that vaccines made with these viruses will lose protectiveness as the field viruses continue to drift. The need to update vaccine seed strains is critical if optimal protection from vaccination is to be realized. However, good surveillance is needed to understand what viruses are circulating in a region or country. Since December 2005, in northern Vietnam, both clade 2.3.2 and 2.3.4 viruses were cocirculating, and therefore a single vaccine is unlikely to provide optimal protection. Availability of vaccines currently is problematic, not only because of antigenic drift, but also because of differential immune responses to vaccines in chickens as well as in individual duck species (Pantin-Jackwood and Suarez, unpubl. data). It will be useful to continue evaluating the current vaccines not only in chickens, but various species of ducks, also.

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Original Article

Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs

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Background There is a requirement to detect and differentiate pandemic (H1N1) 2009 (H1N1v) and established swine influenza A viruses (SIVs) by real time reverse transcription (RRT) PCR methods.

Objectives First, modify an existing matrix (M) gene RRT PCR for sensitive generic detection of H1N1v and other European SIVs. Second, design an H1 RRT PCR to specifically detect H1N1v infections.

Methods RRT PCR assays were used to test laboratory isolates of SIV (n = 51; 37 European and 14 North American), H1N1v (n = 5) and avian influenza virus (AIV; n = 43). Diagnostic sensitivity and specificity were calculated for swabs (n = 133) and tissues (n = 116) collected from field cases and pigs infected experimentally with SIVs and H1N1v.

Results The "perfect match" M gene RRT PCR was the most sensitive variant of this test for detection of established European SIVs and H1N1v. H1 RRT PCR specifically detected H1N1v but not European SIVs. Validation with clinical specimens included comparison with virus isolation (VI) as a "gold standard", while field infection with H1N1v in swine was independently confirmed by sequencing H1N1v amplified by conventional RT PCR. "Perfect match" M gene RRT PCR had 100% sensitivity and 95·2% specificity for swabs, 93·6% and 98·6% for tissues. H1 RRT PCR demonstrated sensitivity and specificity of 100% and 99·1%, respectively, for the swabs, and 100% and 100% for the tissues.

Conclusions Two RRT PCRs for the purposes of (i) generic detection of SIV and H1N1v infection in European pigs, and for (ii) specific detection of H1N1v (pandemic influenza) infection were validated.

Keywords H1N1v, Pandemic H1N1 2009 virus, RRT PCR, swine influenza virus (SIV), validation.

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Introduction

The pandemic (H1N1) 2009 influenza A virus (H1N1v) emerged in April 2009 and was first detected in a cluster of human respiratory cases in Mexico and the USA; initially, the virus could not be subtyped using available molecular tests.[1–3] Genetic and antigenic characterisation of the H1N1v virus showed it to be distinct from current seasonal H1N1 influenza A viruses circulating in humans.[4,5] Rapid spread of H1N1v to other continents and frequent escalating human transmission resulted in an official declaration of a new human influenza A pandemic by the World Health Organization (WHO) on 11 June 2009.[6,7]

Regardless of their host of origin, influenza A viruses usually have the potential to infect and, during dual infections, re-assort in other host species. Pigs are particularly susceptible to infections from other hosts because receptors for influenza A viruses of both human and avian origin are present in their upper respiratory tract.[8,9] Swine influenza viruses (SIVs) circulating in pig herds include H1N1, H3N2 and H1N2 subtypes and these have been observed globally.[10–13] H1N1 SIVs were first isolated in the 1930s,

and phylogenetic studies have shown that these ''classical swine'' isolates were originally closely related to the human H1N1 ''Spanish 'flu''' pandemic influenza virus of 1918, but subsequently these classical H1N1 SIVs evolved into a unique genetic lineage.[11] H3N2 SIVs have also evolved distinctly in pigs following the human ''Asian 'flu''' pandemic of 1968. During the early 1990s, ''avian-like'' H1N1 SIVs became common in European farmed pigs and have replaced classical swine H1N1.[10,11] However, classical swine H1N1 remained present in the USA and many parts of Asia.[10–13] Evolution of SIVs in North America has included emergence of a triple re-assortment H3N2 in 1998 that contained genes of human, avian, and classical H1N1 swine influenza origin genes.[12,13] These triple reassortant viruses have become endemic in North America, and, through additional reassortment events, triple reassortant variants of the H1N1 and H1N2 subtypes have been detected.[5,12,13] In the case of H1N2 SIVs isolated in Europe, the H1 gene is of human H1 seasonal influenza origin.[11,14]

The first instance of infection of pigs with H1N1v was reported in May 2009 in Canada where it was suspected that pigs were infected through contact with infected humans.[15,16] SIVs are known to infect humans[17,18] and poultry,[19–22] while human influenza A viruses are also known to transmit to pigs.[23] Continuing human cases of H1N1v during 2009 sustained veterinary concerns that this virus may become established in pigs, with a degree of concern that this host may then serve as a source for further influenza A reassortment and future zoonotic transmission.[24]

Although swine influenza is not listed as a notifiable disease by the World Organisation for Animal Health (OIE),[25,26] SIV surveillance programmes have been carried out in Europe and North America and have provided valuable epidemiological information.[10–13] These surveillance programmes have been based on conventional testing as recommended by the OIE,[27] using attempted virus isolation (VI) in cell culture and embryonated fowls' eggs (EFEs), followed by typing with defined antisera to identify the haemagglutinin (H) and neuraminidase (N) subtypes using well-established haemagglutination and neuraminidase inhibition tests (HI and NI), respectively.[27]

In recent years, highly sensitive and specific real time reverse transcription polymerase chain reaction (RRT PCR) technology has been exploited by veterinary institutes and reference laboratories to develop and validate appropriate tests for avian influenza viruses (AIVs).[28] These include validated RRT PCR tests for notifiable AI caused by H5 and H7 subtype viruses, following concerns resulting from the spread of H5N1 highly pathogenic (HP) AI in poultry in the Eastern Hemisphere, together with a number of accompanying zoonotic cases. Many veterinary laboratories have already embraced generic AI RRT PCRs that detect all sixteen H subtypes of influenza A, typically through amplifying within the highly conserved M gene, and also as specific assays for AIVs of H5 and H7 subtypes.[28] While public health institutions have recently described RRT PCRs for the detection of H1N1v in the context of the current human pandemic,[29–32] in this study, we describe and validate similar approaches for the detection of H1N1v in pigs. Full genome sequence analysis of the current H1N1v reveals the virus to be putatively of swine origin.[4,5] Segment 7, which includes the matrix (M) gene that encodes the matrix M1 and M2 proteins, appears to be of Eurasian SIV origin, while segment 4, which encodes the H1 haemagglutinin (HA), is related to American classical SIV H1 genes.[4,5] This study outlines the adaptation of an existing M gene RRT PCR assay for the generic detection of SIVs and H1N1v, plus a novel RRT PCR that amplifies within the H1 gene for the specific detection of H1N1v in European pigs.

Materials and methods

Viruses

Ninety-nine laboratory isolates of influenza A viruses were obtained from the influenza virus repository at the Veterinary Laboratories Agency (VLA-Weybridge), grown in 9- to 10-day-old specific-pathogen-free embryonated fowls' eggs (EFEs) and typed using standard protocols.[27,33] These included 51 swine influenza virus (SIV) isolates, 43 avian influenza virus (AIV) isolates plus five H1N1v isolates, which included two from humans and three from pigs (Table 1). These EFE-grown influenza A viruses were diluted at least 100- to 1000-fold prior to RNA extraction to give levels of virus that approximate to those present in clinical specimens.[34] Biological infectivity of EFE-grown influenza A viruses was determined as the median egg infectious dose (EID_{50}) per ml.[35] Fourteen laboratory isolates of other non-influenza A viruses known to infect swine were kindly provided by the Mammalian Virology group at VLA and included: Aujeszky's disease virus, border disease virus (isolate 137/4), bovine viral diarrhoea virus (C24V isolate), four isolates of classical swine fever virus (Alfort, Elsenburg 2006-07, Rilmser C and UK 2000), encephalomyocarditis virus (V12050), haemagglutinating encephalomyelitis virus, porcine circovirus type 2 (isolate 1010), porcine parvovirus (RC 6/06), porcine reproductive and respiratory syndrome virus (Euro strain), porcine respiratory coronavirus (isolate 135328) and Talfan virus.

Field clinical specimens from pigs

Ninety-seven frozen (−70°C) archived respiratory tissues were originally obtained during 1991–2009 from the routine swine influenza surveillance programme in United Kingdom, in which acute respiratory disease of a suspected

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Table 1. List of influenza A virus laboratory isolates (n = 99) used to assess (i) M gene RRT PCRs (original avian protocol and two variants) and (ii) H1-118 RRT PCR

			Ct values			
			M gene RRT PCR			"H1-118" RRT PCR
Influenza A category	Influenza A subtype	Isolate name	Original "avian"	"Perfect match"	"Combo"	
Pandemic (H1N1) 2009 influenza, human and swine isolates	H1N1v	A/California/07/09	30·86	20·96	21·03	20·98
	H1N1v	A/England/195/09	32·77	20·78	23·48	24·14
	H1N1v	A/swine/Singapore-Q/929/09	31·02	23·45	24·20	24·90
	H1N1v	A/swine/N Ireland/1012/09	No Ct	35·68	36·59	35·99
	H1N1v	A/swine/England/P0433/09	30·09	22·92	23·66	24·40
UK swine (classical) H1N1 isolates	H1N1	A/swine/England/117316/86	23·19	32·28	23·73	No ct
	H1N1	A/swine/England/604718/96	26·58	24·81	24·07	No ct
European swine (avian-like) H1N1 isolates	H1N1	A/swine/Finistere (France)/2899/82	23·94	22·69	23·56	No ct
	H1N1	A/swine/Eire/89/96	24·90	30·41	25·84	No ct
	H1N1	A/swine/England/195852/92	27·17	25·04	25·32	No ct
	H1N1	A/swine/England/452670/94	35·02	31·61	34·07	No ct
	H1N1	A/swine/England/600475/96	27·81	25·18	27·33	No ct
	H1N1	A/swine/England/604718/96	30·29	27·37	29·66	No ct
	H1N1	A/swine/England/95953/97	31·11	28·08	30·48	No ct
	H1N1	A/swine/England/101692/97	26·30	23·41	25·23	No ct
	H1N1	A/swine/England/108640/97	29·58	26·43	28·31	No ct
	H1N1	A/swine/England/147452/97	30·47	27·53	29·63	No ct
	H1N1	A/swine/England/159981/97	29·01	26·55	28·55	No ct
	H1N1	A/swine/England/167655/97	30·40	25·56	27·00	No ct
	H1N1	A/swine/England/706565/97	32·59	28·76	30·95	No ct
	H1N1	A/swine/Belgium/1/98	23·00	20·44	20·84	No ct
	H1N1	A/swine/Italy/1513-1/98	29·57	22·12	22·59	No ct
	H1N1	A/swine/Isles de Valles (France)/1455/99	24·54	21·33	22·01	No ct
	H1N1	A/swine/Brno (Czech Republic)/1/02	23·09	22·75	23·27	No ct
	H1N1	A/swine/England/1195/07	27·67	24·95	25·87	No ct
	H1N1	A/swine/England/589/2/07	28·23	26·03	27·02	No ct
	H1N1	A/swine/England/663/08	23·27	21·21	21·93	No ct
European swine H1N2 isolates	H1N2	A/swine/England/410440/94	27·30	24·69	26·55	No ct
	H1N2	A/swine/England/448813/94	29·33	26·77	28·72	No ct
	H1N2	A/swine/England/17394/96	26·35	23·91	26·03	No ct
	H1N2	A/swine/Cotes d'Armor (France)/790/97	24·36	23·12	23·88	No ct
	H1N2	A/swine/Italy/1521/98	23·83	22·55	23·25	No ct
	H1N2	A/swine/England/053307/00	27·07	24·17	25·98	No ct
	H1N2	A/swine/England/997/08	29·63	27·16	28·07	No ct
	H1N2	A/swine/England/3/09	23·62	21·26	21·87	No ct
European swine (human-like) H3N2 isolates	H3N2	A/swine/Cotes d'Armor (France)/3633/84	26·28	25·22	25·98	No ct
	H3N2	A/swine/England/285044/93	25·24	23·44	24·15	No ct
	H3N2	A/swine/England/502321/94	25·96	24·04	25·02	No ct
	H3N2	A/swine/England/742104/95	24·94	20·76	22·02	No ct
	H3N2	A/swine/Flanders (Belgium)/1/98	23·07	20·21	20·89	No ct
	H3N2	A/swine/Italy/1477/96	22·99	21·42	22·09	No ct
	H3N2	A/swine/Italy/1523/98	24·83	23·49	24·19	No ct
North American swine flu isolates	H1N1	A/swine/Indiana/1726/88	20·18	21·22	20·09	No ct*
	H1N1	A/swine/Wisconsin/H04YS2/04	23·09	31·80	22·50	28·18*
	H1N1	A/swine/Ontario/11112/04	23·80	34·88	22·97	No ct*
	H1N1	A/swine/Minnesota/02011/08	21·99	22·98	21·13	No ct*
	H1N1	A/swine/North Carolina/02084/08	23·58	24·25	22·81	No ct*
	H1N1	A/swine/Nebraska/02013/08	23·21	24·64	23·87	28·36*
	H1N1	A/swine/Minnesota/02093/08	22·57	23·70	21·77	No ct*
	H1N2	A/swine/Indiana/9K035/99	25·42	32·88	24·35	21·93*
	H1N2	A/swine/Ontario/48235/04	24·60	33·51	24·49	No ct*
	H3N2	A/swine/Iowa/8548-1/98	22·29	22·07	20·54	No ct
	H3N2	A/swine/Minnesota/593/99	28·22	35·98	27·00	No ct
	H3N2	A/swine/Wisconsin/H02AS8/02	26·17	34·09	25·09	No ct
	H3N2	A/swine/North Carolina/307408/04	22·78	23·62	21·69	No ct
	H3N2	A/swine/Ontario/33853/05	21·23	29·12	20·07	No ct

Table 1. (Continued)

Influenza A category	Influenza A subtype	Isolate name	Ct values			
			M gene RRT PCR			"H1-118" RRT PCR
			Original "avian"	"Perfect match"	"Combo"	
Avian influenza viruses, with highly pathogenic (HP) H5 and H7 isolates indicated	H1N1	A/duck/Alberta/35/76	26·71	*30·65*	*28·61*	No ct
	H1N1	A/turkey/Netherlands/07014290/07	22·93	*29·81*	*24·51*	No ct
	H1N1	A/turkey/Netherlands/07016245/07	22·68	*29·62*	*24·45*	No ct
	H1N1	A/mallard/Germany/R355/07	23·79	*27·88*	*25·85*	No ct
	H1N1	A/mallard/Italy/357-24/07/08	23·34	*26·74*	*25·29*	No ct
	H1N1	A/Egyptian goose/Germany/R1419/06/07	24·82	*28·76*	*26·69*	No ct
	H1N1	A/wild duck/Germany/R30/06/07	22·85	*27·13*	*24·39*	No ct
	H1N1	A/teal/N Ireland/784/07	24·43	*29·04*	*26·34*	No ct
	H1N2	A/wigeon/England/4/06	26·01	*30·90*	*28·25*	No ct
	H2N3	A/duck/Germany/1215/73	29·21	*32·77*	*31·23*	No ct
	H2N3	A/mallard/England/7277/06	32·27	*36·06*	*34·30*	No ct
	H3N2	A/turkey/England/69	27·35	*30·77*	*29·23*	No ct
	H3N2	A/duck/Malaysia/F11107/02	30·66	*33·66*	*33·05*	No ct
	H4N6	A/duck/Czechoslovakia/56	29·46	*32·90*	*31·20*	No ct
	H4N6	A/duck/Italy/473/07	22·52	*22·65*	*22·72*	No ct
	H5N2	A/wild birds/Denmark/04	33·77	*38·09*	*35·44*	No ct
	H5N1	A/turkey/England/614/07 HP	34·85	*37·23*	*35·39*	No ct
	H5N3	A/teal/England/06	36·52	*36·94*	*39·03*	No ct
	H5N1	A/turkey/England/50/92 HP	35·17	*39·10*	*35·93*	No ct
	H6N2	A/teal/England/7440/06	29·24	*33·30*	*30·86*	No ct
	H6N8	A/duck//Denmark/883/02	28·55	*32·73*	*30·56*	No ct
	H7N1	A/African starling/Q-England/983/79	27·31	*31·25*	*29·23*	No ct
	H7N2	A/psittacine/Italy/1384//91	25·10	*28·60*	*26·86*	No ct
	H7N7	A/chicken/England/08 HP	34·98	*No ct*	*36·70*	No ct
	H7N7	A/mallard/Sweden/08	36·86	*No ct*	*No ct*	No ct
	H7N1	A/chicken/Italy/99 HP	29·27	*32·18*	*30·50*	No ct
	H8N4	A/turkey/Ontario/6118/68	28·84	*32·60*	*30·71*	No ct
	H8N4	A/teal/England/06	33·74	*37·04*	*36·03*	No ct
	H9N2	A/chicken/Pakistan/99	29·13	*No ct*	*31·15*	No ct
	H9N1	A/teal/N Ireland/07	35·34	*No ct*	*36·34*	No ct
	H9N2	A/goose/England/07	20·58	*23·03*	*21·43*	No ct
	H10N7	A/chicken/England/279/01	30·67	*34·58*	*32·89*	No ct
	H10N7	A/mallard/England/England/7495/06	29·72	*34·03*	*31·95*	No ct
	H11N6	A/duck/England/56	28·87	*33·23*	*30·80*	No ct
	H11N3	A/duck broiler/Singapore/F107/05/02	29·09	*33·00*	*30·81*	No ct
	H12N5	A/duck/Alberta/60/76	26·84	*30·83*	*28·79*	No ct
	H12N2	A/duck/Belgium/10157/07	*19·76*	19·46	*19·83*	No ct
	H13N6	A/gull/Maryland/704/77	30·04	*39·67*	*31·85*	No ct
	H13N6	A/herring gull/Finland/Li9875/05	28·34	*36·33*	*29·83*	No ct
	H14N6	A/Mallard/Gurjev/244/82	33·54	*36·92*	*35·66*	No ct
	H15N6	A/shearwater/Western Australia/79	28·44	*37·09*	*29·91*	No ct
	H16N3	A/gull/Denmark/68110/02	29·53	*No ct*	*31·30*	No ct
	H16N3	A/gull/Sweden/03	32·40	*No ct*	*34·38*	No ct

For (i) the Ct values for the most sensitive M gene RRT PCR for a given isolate is shown in normal type, while Ct values for the two less sensitive M gene RRT PCR variants are shown in italic type.
*These samples produced bands when the amplified products were run on a 3% w/v agarose gel.

viral aetiology forms the primary submission selection criterion. All were tissue pools that included varying proportions of trachea and lung specimens. Virus isolation (VI) in EFEs at the time of submission divided these into 31 and 66 tissue specimens from pigs that were infected with SIV (Table 2) and uninfected, respectively. In addition, 104 nasal swabs from pigs in United Kingdom were collected by the VLA Regional Laboratories at Thirsk and Bury St Edmunds during the summer of 2009. These were obtained from swine that had been submitted for endemic disease investigations.

Thirty-nine clinical specimens were received between September and November 2009 from nine pig herds at nine different locations in six countries and included 24 respiratory swabs and 15 respiratory tissues (Table 3). Fifteen of these specimens (six swabs and nine tissues) were shown to be

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Table 2. List of archived tissue specimens collected in UK (n = 31) from pigs that were VI positive at the time of submission

Influenza A category	Influenza A subtype	Isolate name	Ct values: M gene RRT PCR: Original "avian"	Ct values: M gene RRT PCR: "Perfect match"	Ct values: M gene RRT PCR: "Combo"	Ct values: H1-118 RRT PCR	VI result (+/−) during current validation
UK pigs positive for avian-like swine H1N1	H1N1	A/swine/England/205200/92	*34·63*	34·21	*35·72*	No ct	−
	H1N1	A/swine/England/0204212/92	*30·95*	28·80	*30·61*	No ct	−
	H1N1	A/swine/England/0377301/93	*29·12*	27·96	*28·98*	No ct	−
	H1N1	A/swine/England/0281321/93	*38·04*	36·78	*No* ct	No ct	−
	H1N1	A/swine/England/0280326/93	No ct	No ct	No ct	No ct	−
	H1N1	A/swine/England/0378916/93	*25·60*	24·27	*25·27*	No ct	−
	H1N1	A/swine/England/020883/93	*27·42*	24·25	*25·96*	No ct	−
	H1N1	A/swine/England/0359774/93	*38·36*	34·65	*35·50*	No ct	−
	H1N1	A/swine/England/0281319/93	*36·13*	35·09	*35·90*	No ct	−
	H1N1	A/swine/England/079270/96	*32·42*	31·27	*32·57*	No ct	−
	H1N1	A/swine/England/076235/96	*22·20*	21·10	*21·76*	No ct	+
	H1N1	A/swine/England/108640/97	*32·82*	31·65	*33·01*	No ct	−
	H1N1	A/swine/England/095953/97	*28·33*	27·21	*28·29*	No ct	−
	H1N1	A/swine/England/101692/97	*19·39*	18·45	*19·56*	No ct	−
	H1N1	A/swine/England/113833/97	*27·97*	26·42	*27·63*	No ct	−
	H1N1	A/swine/England/379/08	*31·96*	29·63	*30·81*	No Ct	−
	H1N1	A/swine/England/471/08	*35·39*	32·43	*33·60*	No Ct	−
	H1N1	A/swine/England/57455/08	*No Ct*	35·43	*37·11*	No Ct	−
UK pigs positive for swine H1N2	H1N2	A/swine/England/410439/94	*24·42*	23·10	*24·09*	No Ct	−
	H1N2	A/swine/England/645913/96	*25·97*	24·18	*25·33*	No Ct	−
	H1N2	A/swine/England/661264/97	No Ct	No Ct	No Ct	No Ct	−
	H1N2	A/swine/England/997/08	*22·50*	20·12	*20·90*	No Ct	+
	H1N2	A/swine/England/3/09 (lung)	*38·11*	36·23	*37·47*	No Ct	−
	H1N2	A/swine/England/3/09 (trachea)	*No Ct*	37·39	*37·98*	No Ct	−
UK pigs positive for human-like swine H3N2	H3N2	A/swine/England/119404/91	33·65	*34·30*	*35·12*	No Ct	−
	H3N2	A/swine/England/241009/92	*30·11*	28·76	*29·69*	No Ct	−
	H3N2	A/swine/England/266546/93	No Ct	No Ct	No Ct	No Ct	−
	H3N2	A/swine/England/285044/93	*32·97*	30·64	*31·65*	No Ct	−
	H3N2	A/swine/England/399890/93	*29·22*	28·00	*28·65*	No Ct	+
	H3N2	A/swine/England/399892/93	*No Ct*	35·89	*36·03*	No Ct	−
	H3N2	A/swine/England/502321/94	*25·47*	22·44	*23·18*	No Ct	+

Archived tissues were pools that contained varying proportions of respiratory tissues, including trachea and/or lung, except for A/swine/England/3/09 where two distinct tissues were tested from the same pig. These samples were used to assess (i) M gene RRT PCRs (original avian protocol and two variants) and (ii) H1-118 RRT PCR. Ct values for the most sensitive M gene RRT PCR for a given tissue specimen is shown in normal type, while Ct values for the two less sensitive M gene RRT PCR variants are shown in italic type.

positive for H1N1v by non-RRT PCR approaches (Table 3), i.e. amplification of RNA extracted from the clinical specimen by conventional RT PCR using primers that had been designed specifically for the HA gene of current H1N1v isolates, available at: http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf

Amplicons were electrophoresed in 2% agarose and stained with RedSafe™ (iNtRON Biotechnology, Kyungki-Do, Korea) for visualisation, excised and purified from agarose using the QIAquick® Gel Extraction Kit (Qiagen, Crawley, UK). Nucleotide sequencing was performed using the Big Dye® Terminator v3·1 cycle sequencing kit (Applied Biosystems, Warrington, UK), and checked by BLAST analysis at: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome=blasthome, which confirmed these 15 specimens as H1N1v positive. In the case of four of these 15 H1N1v specimens, virus was grown in EFEs (Table 3) and RNA extracted from infective allantoic fluid, then similarly PCR amplified and sequenced to provide confirmation of H1N1v infection.

Table 3. Examination of clinical specimens derived from field cases of H1N1v-infected pigs using RRT PCR assays

Country of origin	Clinical Specimen (numbers)	Ct values of RRT PCR		Confirmatory criterion for H1N1v infection in herd
		''Perfect match'' M gene RRT PCR	H1-118 RRT PCR	
Singapore (Quarantine)	Nasal swab (1)	25·84	25·50	Sequencing and BLAST confirmation as H1N1v for both clinical specimens and egg-isolated virus
	Tracheal swab (1)	26·05	25·99	
N Ireland (a)	Lung (4)	21·48	22·34	Sequencing and BLAST confirmation as H1N1v for all four clinical specimens
		20·84	20·89	
		28·29	29·05	
		23·99	24·33	
N Ireland (b)	Lung (1)	30·26	30·20	Sequencing and BLAST confirmation as H1N1v for clinical specimen and egg-isolated virus
Norway	Nasal swabs (12)	23·04	23·14	Sequencing and BLAST confirmation as H1N1v for two swabs
		20·64	20·74	
		26·67	26·40	
		26·08	26·02	
		No Ct	No Ct	
		34·14	34·66	
		27·97	28·15	
		32·23	32·48	
		28·84	28·45	
		28·71	29·00	
		24·15	23·80	
		29·83	29·55	
Ireland	Lung (4)	23·71	24·24	Sequencing and BLAST confirmation as H1N1v for two lung specimens
		22·21	22·69	
		26·77	27·22	
		24·98	25·31	
N Ireland (c)	Lung (2)	37·50	37·93	Epidemiologically linked to other cases of H1N1v in swine in N Ireland & Ireland
		33·17	32·84	
Iceland	Nasal swabs (10)	30·64	29·92	Sequencing and BLAST confirmation as H1N1v for swab
		27·62	28·02	
		27·60	27·87	
		29·00	29·04	
		25·98	26·18	
		24·09	24·20	
		26·41	26·40	Sequencing and BLAST confirmation as H1N1v for swab
		28·17	28·25	
		23·87	24·45	
		23·23	23·38	
England (a)	Lung tonsil & trachea pools (3)	28·02	27·77	Sequencing and BLAST confirmation as H1N1v for this pooled tissue
		23·95	23·86	
		30·41	29·86	
England (b)	Lung tonsil & trachea pool (1)	21·95	22·30	Sequencing and BLAST confirmation as H1N1v for clinical specimen and egg-isolated virus

Ct values are shown for both generic ''Perfect match'' M gene RRT PCR and the specific ''H1-118'' RRT PCR assays.

Clinical specimens from pigs infected experimentally

Six Landrace hybrid pigs (age 4–5 weeks) were each infected experimentally by inoculation with 2 ml H1N1v (A/California/07/09), which contained $10^{5\cdot8}$ EID_{50} per animal via the intranasal route.[36] Twenty-three nasal swabs were obtained by swabbing the infected pigs daily from 1 to 7 days post-infection (dpi), and 10 pig tissues (lung $n = 5$, thoracic trachea $n = 5$) were obtained from animals killed humanely for post-mortem examination on 2,4 and 7 dpi (Table 4).

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Table 4. Results of testing clinical specimens from pigs infected experimentally with H1N1v (A/California/07/09)

Pig identifier	Days post-infection	Sample	Ct values: Perfect match M gene RRT PCR	Ct values: H1-118 RRT PCR	VI
1	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	28·57	29·67	+
	3	Nasal swab	26·68	28·86	+
	4	Nasal swab	29·53	32·36	+
	4	Lung	24·30	24·67	+
	4	Thoracic trachea	21·34	22·02	+
2	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	30·51	31·69	+
	2	Nasal swab	23·02	25·59	+
	3	Nasal swab	30·01	32·24	+
	4	Nasal swab	31·29	34·47	+
	5	Nasal swab	25·19	27·44	+
	6	Nasal swab	27·95	29·77	+
	7	Nasal swab	34·48	34·08	–
	7	Lung	29·72	30·25	+
	7	Thoracic trachea	No Ct	No Ct	–
3	1	Nasal swab	34·43	36·84	+
	2	Nasal swab	30·67	33·20	+
4	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·66	29·36	+
	2	Nasal swab	22·20	24·23	+
	2	Lung	No Ct	No Ct	–
	2	Thoracic trachea	No Ct	No Ct	–
5	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·51	29·35	+
	2	Nasal swab	22·24	24·25	+
	2	Lung	26·61	26·43	+
	2	Thoracic trachea	34·28	34·42	+
6	0	Nasal swab	No Ct	No Ct	ND
	1	Nasal swab	27·03	29·20	+
	2	Nasal swab	26·07	28·28	+
	3	Nasal swab	31·07	32·68	+
	4	Nasal swab	29·96	33·01	+
	5	Nasal swab	28·15	30·18	+
	6	Nasal swab	27·68	29·79	+
	7	Nasal swab	34·29	36·52	+
	7	Lung	20·33	21·17	+
	7	Thoracic trachea	30·82	31·51	+

Ct values are shown for ''perfect match'' M gene and ''H1-118'' RRT PCRs. ND indicates ''not done''.

Processing of clinical specimens, VI and RNA extraction

Swabs

One hundred and ten swabs from the field (104 from VLA Regional Laboratories during summer 2009; six from proven field cases of H1N1v infection, Table 3) and 23 swabs from pigs infected experimentally (Table 4) were stored at −70°C in 1 ml brain heart infusion broth containing antibiotics (BHIB). Swabs from the VLA Regional Laboratories and H1N1v experimentally infected pigs ($n = 127$) were tested by VI in 9- to 10-day-old EFEs by means of double inoculations via the allantoic and amniotic cavities, followed by a second EFE passage in the event of the first passage being VI negative. Influenza A virus growth was detected by a positive haemagglutination assay (HA) result

obtained by testing harvested amnio/allantoic fluid with chicken red blood cells.[27] In the case of six swabs from H1N1v-field-infected pigs, infection with this pandemic virus was independently proven by conventional PCR amplification, sequencing and BLAST searching as described earlier (Table 3).

Tissues

Organ homogenates (approximately 10% w/v in BHIB) were prepared from 97 archived UK tissue specimens (31 from SIV-positive pigs (Table 2) and 66 SIV-negative pigs by VI) and 15 tissues from H1N1v-field-infected pigs (September–November 2009; Table 3) by grinding with sterile sharp sand. For nine of 15 tissues from H1N1v-field-infected pigs, infection with this pandemic virus was independently proven by conventional PCR amplification, sequencing and BLAST searching as described earlier (Table 3). Ten tissue specimens from the experimentally infected pigs (Table 4) were disrupted in BHIB using a General Laboratory Homogenizer (Omni International, Marietta, GA, USA) to provide a similar 10% w/v suspension. All tissue homogenates were clarified by centrifugation for 1 minute prior to VI and RNA extraction. VI was carried out by inoculating clarified tissue homogenates into 9- to 10-day-old EFEs as described earlier for pig swabs.

The Mini Viral RNA kit (Qiagen) was used to extract RNA from allantoic fluids, BHIB swabs fluids and clarified tissue homogenates. This was performed either manually by the "spun column" method in accordance with the manufacturer's protocol, or by robotic RNA extraction by the same kit chemistry adapted to a Universal Biorobot (Qiagen).[34]

M gene RRT PCR assays

Comparison of the M gene primer and probe sequence were performed on the Influenza Research Database website http://www.fludb.org. Several programmes available on the site were used for the comparison of available swine influenza sequence information including the SNP Analysis, Blast and Alignment Viewer.[37] The primers and hydrolysis probe used initially were those from the M gene RRT PCR originally described by Spackman *et al.*[38] (Table 5) for global and generic detection of AIVs at final concentrations of 0·4 and 0·3 μm, respectively.[34] The reverse primer was modified in two variations of the M gene RRT PCR assay to investigate the detection of SIVs and H1N1v isolates. The first variant ("perfect match" M gene RRT PCR) included modification of the AIV reverse primer used by Spackman *et al.*[38] to provide a perfect match primer (reverse modified, i.e. "Rev-mod") with the corresponding region in the M gene of H1N1v isolates (Table 5),[39] i.e. accession number FJ966975 for the M gene sequence of A/California/07/09. This was Rev-mod: 5′-tgc aaa Gac aCT ttc Cag tct ctg-3′ [76–99] in which the four altered nucleotides in the reverse primer are indicated in upper case, while square brackets indicate nucleotide positions within FJ966975.

The second variant ("combo" M gene RRT PCR) included an equimolar mix of the original AIV reverse primer plus the above Rev-mod primer, with each included at 0·2 μm final concentration. Otherwise cycling conditions, temperatures and chemistry details for both variants were as outlined for the M gene RRT PCR,[34] in which the original M gene RRT PCR and the two variants produced the same 101- bp product. Two microlitres of extracted RNA were tested in each M gene RRT PCR in a final 25 μl volume using Mx 3000 RealTime PCR instruments (Stratagene, Amsterdam, The Netherlands).[34] Fluorescence thresholds for all RRT PCR experiments were determined using default settings in the supplied Stratagene MxPro software, and these were inspected visually after every experiment to ensure consistency.

H1-118 RRT PCR

Sequences of the H1 gene were acquired from a database listing pandemic H1N1v isolates at: http://www.ncbi.nlm.nih.gov/genomes/FLU/SwineFlu.html.[39] primerselect software from the Lasergene package (DNAstar, Madison, WI, USA) was used to guide RealTime PCR primer and probe design. Primers and a hydrolysis probe were designed to amplify a 118- bp product within the HA2 region ("H1-118" RRT PCR) of H1N1v isolates, these were

F118: 5′- AAT GCC GAA CTG TTG GTT CT -3′ [1315–1334] and

R118: 5′- CAA TTT CCT TGG CAT TGT TTT 3′ [1412–1432]

with nucleotide positions in square brackets corresponding to the H1 gene sequence in A/California/07/09 (accession number FJ981613).

The antisense hydrolysis probe (31 nucleotides long) was

118 pro-rev: FAM-5′- CTG GCT TCT TAC CTT TT*C ATA TAA GTT CTT C-3′ [1377-1407],

which was labelled with the FAM fluorophore at the 5′ end and with black hole quencher (BHQ1) at an internal T nucleotide (indicated as T*), while the 3′ terminal C nucleotide was modified to a dideoxy C to prevent probe extension by polymerase activity (Eurogentec, Liège (Luik), Belgium).

Primers and probe were included at final concentrations of 0·4 and 0·3 μm, respectively, and 2 μl of extracted RNA were tested in each H1-118 RRT PCR in a final 25 μl volume. Other chemistry details and cycling conditions were as outlined for the H7 HA2 RRT PCR[34] where Stratagene Mx 3000 RealTime PCR instruments were used. Occasionally, "H1-118" RRT PCR products were visually checked by electrophoresis in 3% (w/v) agarose gels according to standard procedures.[40]

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Table 5. Identification of nucleotide substitutions at the primer and probe binding sites for the matrix RRT-PCRs with 16 representative SIV and H1N1v isolates

Examples of influenza A isolates (subtype)	Accession number	M+24 F primer* AGA TGA GTC TTC TAA CCG AGG TCG	M+64 probe* TCA GGC CCC CTC AAA GCC GA	M−124 R primer* TGC AAA AAC ATC TTC AAG TCT CTG
A/California/07/09 (H1N1v)	FJ966975	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/England/195/09 (H1N1v)	GQ166660	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/swine/Italy/29021/09 (H1N1v)	CY053622	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -CT --- C-- --- ---
A/swine/England/WVL16/98 (H1N1 avian-like)	CY037955	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Geldern (Germany)/IDT2888/04 (H1N1 avian-like)	EU478821	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/swine/Stadtlohn (Germany)/IDT3853/05 (H1N2)	EU478843	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/swine/Sweden/1021/09 (H1N2)	GQ495135	--- --- --- --- --- --- --- ---	--C --- --- --- --- --- --	--- --- --- -C- --- C-- --- ---
A/swine/England/742104/95 (H3N2)	Unpublished	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Hungary/13509/07 (H3N2)	FJ798769	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- -C- --- C-- --- ---
A/wildboar/Germany/WS169/06 (H3N2)	AM746618	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- G-- --- --- C-- --- ---
A/swine/Quebec/5393/91 (H1N1)	AF188004	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- --- --- --- --- ---
A/swine/Kansas/77778/07 (H1N1)	GQ484361	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/Ontario/48235/04 (H1N2)	DQ280234	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- G-- --- --- --- --- --- ---
A/swine/Minnesota/SG-00239/07 (H1N2)	CY041906	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/North Carolina/R08-001877-D08-013371/08 (H3N2)	CY041850	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---
A/swine/Oklahoma/001142/09 (H3N2)	CY045554	--- --- --- --- --- --- --- ---	--- --- --- --- --- --- --	--- --- --- G-- --- G-- --- ---

*Primer and probe sequences at head of this table are as originally named and described by Spackman *et al* for the ''avian'' M gene RRT PCR,[38] written 5′ to 3′ from left to right. M gene sequences from H1N1v isolates guided modification of the original M–124 R primer to the ''Rev-mod'' primer used in the ''perfect match'' M gene RRT PCR described in this study.

Production of T7 *in vitro* RNA transcripts

In vitro RNA transcripts were made corresponding to M gene and "H1-118" amplified sequences of A/California/07/09 (H1N1v) and to the M gene amplified sequence of A/swine/England/742104/95 (H3N2). First, both H1N1v and H3N2 were amplified using the M gene and "H1-118" RRT PCR primer pairs as outlined earlier, but with the M gene reverse primer modified to identically match the target sequence for the two viruses (Table 5), and where the 5′ end of all reverse primers included the T7 promoter.[41] Conventional RT PCR conditions were as for the corresponding M gene and "H1-118 RRT" PCRs described in this study, except that the final magnesium chloride concentration was 2·5 mm, with ROX and probes excluded.[34] Amplicons were purified after gel electrophoresis in 3% agarose, and 10–20 ng/ul of each was added to a 100 μl volume T7 *in vitro* RNA transcription reaction (T7 RiboMAX Large Scale RNA Production System; Promega, Southampton, UK) that included 40 units of RNAsin (Promega). The reaction was incubated for four hours at 37°C. The volume was then adjusted to 120 μl by the addition of buffer and nine Kunitz units of DNAse I (RNase-Free DNase Set; Qiagen) where digestion proceeded for 30 minutes at 37°C, followed by T7 RNA transcript purification by spun columns using the outlined protocol for small transcript products <200 nucleotides (RNeasy Mini Kit; Qiagen).[34] The recovered 20 μl was again digested with fresh DNase I (7·5 Kunitz units) in an adjusted volume of 100 μl for 30 minutes at 37°C, followed by a second purification by spun columns. The concentration of the T7 *in vitro* transcripts in the 20 μl final eluate was determined using the RiboGreen RNA Quantitation kit (Invitrogen, Paisley, Scotland). The supplied ribosomal RNA standards were used to construct a calibration curve for RiboGreen fluorescence using the Mx3000 RealTime PCR instrument (Stratagene) in the quantitative plate read function and was used to calculate the concentration of the purified T7 *in vitro* transcribed RNA preparations. Successful DNase I digestion of template was established as previously.[34]

Conventional RT PCRs to identify H1N2 SIV infection

These were designed to investigate any UK clinical specimens that may be positive by M gene RRT PCR but negative by "H1-118" RRT PCR and negative by VI. Three HA gene alignments that included European SIV isolates of the H1N1 (avian-like swine), H1N2 and human-like swine H3N2 subtypes were constructed using the Megalign software from the Lasergene package. The Primerselect programme was then used to design appropriate primers to generate small (i.e. <230 bp) amplicons that were of sufficient size for sequencing and identification by BLAST searching. Five clinical specimens (one UK archived swine tissue submitted in January 2009 and four nasal swabs from UK Regional Labs, summer 2009) were positive by "perfect match" M gene RRT PCR but "H1-118" RRT PCR and VI negative. These were identified as being H1N2 SIV positive using the following five HA primer pairs for conventional RT PCR with forward (F) and reverse (R) primers indicated by suffixes: 218: 218F: AAA CGG GTG TTT TGA ATT CTA CCA CAA G, 218R: TGA TTG CCC CCA GGG AGA CTA AA; 226: 226F: GTT CAC CCC AGA AAT TGC AAA AAG AC, 226R: CCC CCG GGG TGT TTG ACA CT; 210: 210Fa: CAT GGC CCA AAC ACA GCG TAA AC, 210Fb: CAT GGC CCA AAC ACA ACG TAA CC (use as an equimolar mixture of two forward (F suffix) primers in 210 RT PCR), 210R: AGA TGG CCC TTT GAT CCT CTA TGT TAG A; 234: 234F: CAC TAC AAT TGG GGA AAT GCA GCA T, 234R: TGT GTT TGG GCC ATG AAC TTT CYT TAG; 187: 187F: ATG CCC AAA GTA CGT CAG GAG TAM AAA, 187R: TCC GCA GCA TAG CCA GAT CCC. These conventional RT PCR products were electrophoresed in 2% w/v agarose, purified and sequenced. Conventional RT PCR primers were similarly designed to amplify portions of the HA gene of European H1N1 (avian-like swine) and human-like H3N2 SIVs, and these sequences are available from the corresponding author on request.

Results

Bioinformatics evaluation of primer/probe-binding sequences for M gene RRT PCR

Analysis of the M gene primers and probe to available swine influenza sequences shows several different patterns based on geographical and temporal origins of the viruses (Table 5). M gene sequences are available from over 500 SIV isolates from North America, Europe and Asia, with different sequence patterns in each region (data not shown). The sequence for the forward M gene RRT PCR primer and the probe remains highly conserved for most isolates (Table 5). However, the reverse primer has extensive variability at primarily five different positions for H1N1v and SIV isolates compared to the "avian" M gene RRT PCR primers and probes originally designed by Spackman *et al.*[38] Current SIVs and H1N1v isolates have from zero to four nucleotide mismatches in the reverse primer (Table 5) that could potentially contribute to a decrease in sensitivity for some viruses. This influenced the design of the "perfect match" M gene RRT PCR "Rev-mod" primer for H1N1v detection, which is located at the same corresponding position as the "M−124 R" primer for the "avian" M gene RRT PCR (Table 5).

Analytical sensitivity and efficiency of M gene and H1-118 RRT PCRs

H1N1v isolate A/California/07/09 and SIV isolate A/swine/England/742104/95 (H3N2) were grown in EFEs

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and EID_{50} titres determined. RNA was extracted from these influenza A preparations and used to construct 10-fold dilution series, and both were tested by the three M gene RRT PCR assays, namely the "avian",[38] "combo" and "perfect match". The 10-fold dilution series from A/California/07/09 (H1N1v) was also tested by the "H1-118" RRT PCR. The detection limit corresponded to a viral titre (pre-RNA extraction) of 1×10^1 EID_{50}/ml for the "perfect match" M gene and "H1-118" RRT PCRs, which typically registered at Ct values 34–36. When a dilution series of quantified T7 *in vitro* RNA transcripts were tested, the detection limit of the "perfect match" M gene RRT PCR at these Ct values was 200 RNA copies and 2000 RNA copies for the H1N1v and H3N2 targets, respectively. This discrepancy in sensitivity may relate to differences in the M gene reverse primer binding sequence for the two influenza A viruses (Table 5), where A/swine/England/742104/95 (H3N2) has two mismatches with the "perfect match" M gene RRT PCR "Rev-mod" primer. The Stratagene Mx 3000 instrumentation software plotted a standard curve of Ct values against the logarithmic dilutions. An R^2 value of >0·98 indicated a straight line, in which the slope corresponded to an efficiency in the range 90–110% for the "combo" and "perfect match" M gene RRT PCRs for both the influenza A viruses, as well as for testing A/California/07/09 by the "H1-118" RRT PCR. These observations indicated optimised RRT PCR protocols.[42] However, the efficiency of the "avian" M gene RRT PCR fell outside these limits when testing both these viruses. This was unsurprising in view of M gene sequence mismatches for both A/California/07/09 and A/swine/England/742104/95 (H3N2) in the reverse primer (Table 5).

Influenza A virus laboratory isolates: M gene RRT PCRs

RNA extracted from 99 influenza A viruses were tested by the "avian",[38] "combo" and "perfect match" variations of the M gene RRT PCR assays. These RNA samples included those from influenza A virus laboratory isolates of veterinary significance, i.e. 51 SIV and 43 AIV isolates plus five H1N1v isolates (Table 1). For the five H1N1v isolates, the "perfect match" M gene RRT PCR was most sensitive as this test yielded the lowest threshold cycle (Ct) value, followed by "combo" RRT PCR, but the "avian" M gene RRT PCR was clearly the least sensitive with the highest Ct values (Table 1). In the case of A/swine/N Ireland/1012/09, the "avian" M gene RRT PCR failed to detect this RNA sample, presumably owing to it being of low titre (Table 1). It would appear that the modification of the reverse M gene RRT PCR primer in the "perfect match" variant assay contributed to this being the most sensitive approach for detecting the RNA of H1N1v isolates.

Fifty-one laboratory isolates of established SIVs were divided into five categories based on combinations of subtype and temporal and geographical origin (Table 1). For the two classical H1N1 SIVs isolated in United Kingdom in 1986 and 1996, the "avian" and "combo" M gene variant RRT PCR assays appeared to be the most sensitive. However, when 35 contemporary UK and European SIVs were tested, the "perfect match" M gene RRT PCR was the most sensitive for detecting 34 of the isolates in the avian-like swine H1N1, H1N2 and human-like swine H3N2 categories (Table 1). These three categories are representative of the SIVs that have been circulating in Europe for up to 30 years.[11] It should be noted that the "combo" M gene RRT PCR assay was slightly less sensitive, detecting the 35 contemporary European SIVs at Ct values that were higher, but by no more than 2 for the majority of these isolates (Table 1). The one exception was A/swine/Eire/89/96 (H1N1 avian-like), which was most sensitively detected by the original "avian" M gene RRT PCR (Table 1).

In contrast, the 14 North American SIVs were most sensitively detected by the "combo" M gene RRT PCR assay in which the lowest Ct values were obtained, followed by the "avian" M gene RRT PCR. The "perfect match" M gene RRT PCR assay was the least sensitive for detection of the 14 North American SIVs as the Ct values obtained were consistently higher than in the other two assays (Table 1). It can be inferred from these findings that the "perfect match" M gene RRT PCR assay would be the most sensitive to use for generic testing of contemporary European SIVs as well as any pig infections caused by H1N1v. However, for North American pigs, the "combo" M gene RRT PCR provided the most sensitive approach to generic SIV testing for 13 of 14 North American isolates, the one exception being A/swine/Nebraska/02013/08 (H1N1) where the "avian" M gene RRT PCR registered a slightly lower Ct value compared to the "combo" M gene RRT PCR (Table 1).

Representative isolates of all sixteen H-types were included in the 43 AIVs tested (Table 1). Forty-two of 43 were detected most sensitively by the "avian" M gene RRT PCR as this test consistently gave the lowest Ct values, the one exception being A/duck/Belgium/10157/07 (H12N5) (Table 1). This was followed by the "combo" M gene RRT PCR, while the "perfect match" M gene RRT PCR assay proved to be the least sensitive for the detection of the AIV isolates. The low sensitivity of the "perfect match" M gene RRT PCR assay resulted in "No Ct" being recorded for six AIV isolates, possibly because their RNA had been extracted from highly diluted virus preparations. One AIV RNA (A/mallard/Sweden/08, H7N7) was so highly diluted that a high Ct value (36·86) was obtained with the "avian" M gene RRT PCR, but "No Ct" registered by both the "perfect match" and "combo" M gene RRT PCR assays (Table 1).

Influenza A virus laboratory isolates: "H1-118" RRT PCR

The "H1-118" RRT PCR assay was used to test 94 SIV and AIV isolates plus five H1N1v isolates (Table 1). In the tests, comparable Ct values were obtained for the five H1N1v isolates to those observed in the "perfect match" M gene RRT PCR assay (Table 1). None of the 37 European SIV laboratory isolates, including the 22 H1N1 (20 avian-like swine and two classical) and eight H1N2 SIVs, were detected by the "H1-118" RRT PCR assay. The "H1-118" RRT PCR assay was designed specifically for differential detection of H1N1v influenza A viruses. Non-detection of contemporary, established European H1 SIVs by the "H1-118" RRT PCR as well as H1 AIVs indicated that it fulfils this condition (Table 1).

However, positive fluorescence signals were obtained in "H1-118" RRT PCR tests with two of the North American H1N1 SIVs, namely A/swine/Wisconsin/H04YS2/04 and A/swine/Nebraska/02013/08 (Table 1). Tests with the five other North American H1N1 SIVs produced negative fluorescence signals, but gel electrophoresis in 3% w/v agarose showed that all seven of the North American H1N1 isolates tested produced a band of the predicted 118 -bp size (Table 1). H1 gene sequences are available in the public databases for two of these North American H1N1 isolates, namely A/swine/Ontario/11112/04 (accession no. DQ280250) and A/swine/Indiana/1726/88 (accession no. M81707 or CY039925). A degree of conservation was observed in the primer binding sequences for the "H1-118" RRT PCR, while mismatches in the probe-binding region appeared to account for the failure to generate fluorescence signals (data not shown).

"H1-118" RRT PCR testing of both North American H1N2 isolates also produced the 118- bp band of predicted size. A/swine/Indiana/9K035/99 (H1N2) (AF250124) produced fluorescence in this test, but A/swine/Ontario/48235/04 (H1N2) (DQ280236) was fluorescence negative (Table 1). The A/swine/Indiana/9K035/99 H1 gene sequence has a perfect match with the primer and probe-binding regions for the "H1-118" RRT PCR, while the corresponding H1 gene sequence in A/swine/Ontario/48235/04 included significant mismatches in its probe-binding region (data not shown).

Non-influenza A viruses

The 14 non-influenza A viruses were tested by the three M gene RRT PCR assays and by the "H1-118" RRT PCR assay and only negative results were obtained.

Archived clinical specimens from pigs in UK

Ninety-seven frozen archived respiratory tissues that had been obtained from pigs in the United Kingdom since 1991 were tested. These were divided into two groups, those that had been obtained from pigs that were (i) SIV positive (n = 31) and (ii) SIV negative (n = 66) based on VI at the time of sample submission. All archived tissue specimens from positive pigs were retested by VI in the course of this study, as well as by the three M gene RRT PCR approaches and "H1-118" RRT PCR (Table 2). The 31 tissues from SIV-positive pigs included 18 tissues from pigs infected with avian-like swine H1N1 viruses between 1992 and 2008, six from pigs with H1N2 virus infections from 1994 to 2009 and seven from pigs infected with human-like swine H3N2 virus during 1991–1994 (Table 2). Three of these archived tissue samples from VI-positive pigs gave no Ct value with any of the M gene RRT PCR assays. No Ct value was obtained by the "avian" M gene RRT PCR with three further samples, although these were positive by the other two M gene assays (Table 2). One tissue specimen was negative by the "combo" M gene RRT PCR but gave high Ct's by the other two M gene RRT PCRs (Table 2). As in the case of the more contemporary SIV laboratory isolates from United Kingdom and European origin (Table 1), the "perfect match" M gene RRT PCR was shown to be the most sensitive of the M gene RRT PCR variants for the majority of the archived SIV-positive tissue specimens, as for 27 of 28 tissues the M gene "perfect match" RRT PCR registered the lowest Ct compared to the two other M gene RRT PCRs (Table 2). The one exception was the oldest tissue specimen, from which A/swine/England/119404/91 (H3N2) had been isolated; in this case, the original avian M gene RRT PCR was most sensitive (Table 2). These test results with archived tissue specimens obtained from the field reinforced the observation that the "perfect match" M gene RRT PCR is the most sensitive for detecting contemporary European and UK SIVs. All 31 archived UK tissue samples from SIV-positive pigs were negative by the "H1-118" RRT PCR assay (Table 2). Only four of these archived tissues were VI positive during the current validation study, this may reflect the age and/or storage history of the tissues, plus the use of only two egg passages for VI.

Sixty-six archived tissue specimens from SIV-negative pigs from United Kingdom (organ pools of lung, trachea and tonsil, or lung only) were tested by the "perfect match" M gene RRT PCR assay to assess its specificity. All gave "No Ct" results except one, which was submitted in January 2009 and produced a Ct value of 25·45 by the "perfect match" M gene RRT PCR. Repeat VI testing during the current validation study failed to recover viable influenza A virus from this specimen. However, investigation of this specimen by conventional RT PCR and amplicon sequencing identified an H1 gene that was highly suggestive of an H1N2 SIV. All 66 of these archived tissues from SIV-negative pigs were "No Ct" by the "H1-118" RRT PCR.

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Nasal swabs collected in the field by VLA Regional Laboratories

One hundred and four nasal swabs obtained from pigs in United Kingdom during the summer of 2009 were tested by VI in EFEs, the "perfect match" M gene RRT PCR assay and the "H1-118" RRT PCR assay. One hundred of these swabs were negative by all three tests. However, four swabs that originated from two UK pig herds gave negative results by VI and "H1-118 RRT" PCR but resulted in positive amplifications by "perfect match" M gene RRT PCR, with Ct values of 27·56 and 30·77 for one herd and 34·54 and 38·01 for the second herd. Investigation by conventional RT PCR and sequencing revealed an H1 gene that was suggestive of H1N2 SIV.

Respiratory swabs and tissues from H1N1v-field-infected pigs

Of the 39 specimens (24 respiratory swabs and 15 respiratory tissues) received between September and November 2009 from nine geographically diverse pig herds (Table 3), fifteen (six swabs and nine tissues) originating from eight pig herds had been shown to be H1N1v positive by non-RRT PCR approaches (Table 3). These and a further 24 specimens (18 swabs and six tissues) that included two lung specimens from an epidemiologically related ninth herd in Northern Ireland (NI "c"; Table 3) were tested by both the "perfect match" M gene and "H1-118" RRT PCRs. Twenty-two of the 24 swabs were positive by both RRT PCRs, with Ct value ranges of 20·64–34·14 and 20·74–34·66 for the "perfect match" M gene and "H1-118" RRT PCR assays, respectively (Table 3). The submission derived from herd "c" in NI that included two lung tissues gave positive results by both RRT PCRs for one specimen, but the second gave high Ct values (37·50 and 37·93 by "perfect match" M gene and "H1-118" RRT PCRs, respectively) that were just above the positive cut-off for the two tests (Table 3). However, the clear positive result for the first lung specimen had identified H1N1v infection in this pig herd. One swab from the Norwegian herd gave "No Ct" by both RRT PCR assays, but the other 11 nasal swabs from the same herd gave similar positive Ct values by both methods. The 15 tissues were all positive by both tests, with Ct value ranges of 20·84–33·17 and 20·89–32·84 for the "perfect match" M gene and "H1-118 RRT" PCR assays, respectively (Table 3).

Nasal swabs and tissues from pigs infected experimentally with H1N1v

Thirty-three clinical specimens collected from pigs infected experimentally with H1N1v [36] were tested by VI, the "perfect match" M gene RRT PCR assay and the "H1-118" RRT PCR assay (Table 4). These samples included 23 nasal swabs taken between 1 and 7 dpi, and post-mortem tissues (lung, $n = 5$; thoracic trachea, $n = 5$). Twenty-two of the 23 swabs gave positive results by VI and both RRT PCR assays, while one swab was weakly positive by the two RRT PCRs and VI negative (7 dpi, pig 2; Table 4). The Ct ranges for these swabs were 22·20–34·48 and 24·23–36·52 for "perfect match" M gene and "H1-118" RRT PCR assays, respectively. The 10 post-mortem tissues from the experimentally infected pigs gave concordant results by all three tests, i.e. seven were positive and three were negative (Table 4). The Ct ranges for these positive tissues were 20·33–34·28 and 21·17–34·42 for the "perfect match" M gene and "H1-118" RRT PCR assays, respectively.

Diagnostic sensitivity and specificity

The sensitivity and specificity were calculated for the "perfect match" M gene and "H1-118" RRT PCR assays from the clinical specimens (respiratory swabs and tissues) used in this validation study. For 249 clinical specimens, confirmatory results were available either as VI, or in the case of 15 H1N1v field specimens, through independent confirmation by amplicon sequencing (Table 3).

For 133 swabs, a diagnostic sensitivity of 100% and a specificity 95·2% were calculated for the "perfect match" M gene RRT PCR. With this assay, there were five results that were VI negative, but "perfect match" M gene RRT PCR positive (Table 6a). Evidence presented elsewhere in the Results strongly suggests that these five were genuine positives. When the swabs were tested by "H1-118 RRT" PCR, a diagnostic sensitivity of 100% and specificity of 99·1% was determined (Table 6b). There was only one swab, obtained from an experimentally infected pig at 7 dpi, that was VI negative but positive by "H1-118 RRT" PCR (Table 6b), this swab from pig 2 was also positive by "perfect match" M gene RRT PCR (Table 4).

In the case of the 97 archived pig tissues collected in United Kingdom between 1991 and 2009, the specimens were divided into those from SIV-positive ($n = 31$; Table 2) and SIV-negative ($n = 66$) pigs that had been identified by VI at the time of the original submission. By inclusion of 19 additional tissues from H1N1v-field-infected (Table 3) and experimentally infected (Table 4) pigs, a diagnostic sensitivity of 93·6% and a specificity of 98·6% were determined for the "perfect match" M gene RRT PCR (Table 7a). Three archived tissues from pigs that were VI positive gave negative results by "perfect match" M gene RRT PCR (Table 2), and one archived tissue was positive by "perfect match" M gene RRT PCR but VI negative (Table 7a). When the same tissues were tested by "H1-118" RRT PCR assay, the diagnostic sensitivity and specificity were both 100%, respectively (Table 7b).

Table 6: Diagnostic sensitivity and specificity for nasal swab samples (n=133) tested by (a) ''perfect match'' M gene RRT PCR assay and (b) ''H1-118'' RRT PCR assay.

n=133		VI or independent H1N1v identification +	–	Total
(a) * ''Perfect match'' M gene RRT PCR	+	28	5	33
	–	0	100	100
Total		28	105	133
(b) † ''H1-118'' RRT PCR	+	28	1	29
	–	0	104	104
Total		28	105	133

Sensitivity for both assays: 28/28+0 = 100%
* Specificity: 100/5+100 = 95·2%
† Specificity: 104/1+104 = 99·1%

Table 7: Diagnostic sensitivity and specificity for tissue samples (n=116) tested by (a) ''perfect match'' M gene RRT PCR assay and (b) ''H1-118'' RRT PCR assay.

n=116		VI or independent H1N1v identification +	–	Total
(a) * ''Perfect match'' M gene RRT PCR	+	44	1	45
	–	3	68	71
Total		47	69	116
(b) † ''H1-118'' RRT PCR	+	16	0	16
	–	0	100	100
Total		16	100	116

* Sensitivity: 44/44+3 = 93.6%; Specificity: 68/1+68 = 98.6%
† Sensitivity: 16/16+0 = 100%; Specificity: 100/0+100 = 100%

Discussion

In recent years, the M gene RRT PCR assay [38] has gained widespread acceptance as a validated method for the global detection of all AIV subtypes, and this method is referred to as a suitable screening assay in the OIE and EU Diagnostic Manuals for AI.[33,43] Although no international recommendations have been made concerning RRT PCR testing for SIVs, the highly conserved nature of the M gene in all influenza A viruses led to the M gene RRT PCR being considered as a candidate for an initial screening assay for detecting pigs infected with all SIVs, including any infections because of H1N1v. In this study, we have investigated the application of the ''avian'' M gene RRT PCR,[38] but sequence differences observed in the M gene of H1N1v isolates also prompted investigation of two new variations of the original M gene RRT PCR. The original ''avian'' M gene RRT PCR reverse primer (M−124 R),[38] with four nucleotide differences to H1N1v viruses (Table 5), clearly resulted in a significantly lower sensitivity when testing H1N1v laboratory isolates (Table 1). Consequently, the original avian M gene RRT PCR[38] should not be used for detection of H1N1v, and this deficiency of the diagnostic test needed to be addressed. Among the three variants of the M gene RRT PCR that were evaluated, the ''perfect match'' with the revised reverse primer (''Rev-mod'') provided the most sensitive results for many of the European SIVs (Tables 1 and 2). M gene sequence analysis showed that the ''Rev-mod'' primer compensated for two or three nucleotide mismatches in the corresponding region of the M gene from contemporary European SIVs (Table 5). However, experiments with T7 *in vitro* RNA transcripts corresponding to the amplified M gene region of A/swine/England/742104/95 (H3N2) suggested that the ''perfect match'' M gene RRT PCR may not be maximally sensitive for detection of all contemporary European SIVs, which again reflected sequence mismatches with the ''Rev-mod'' primer. Nevertheless, the ''perfect match'' M gene RRT PCR demonstrated successful detection of contemporary European SIVs in archived tissues from infected pigs (Table 2), plus five specimens (four nasal swabs from UK herds and one archived tissue, collected in 2009, highly likely to be H1N2), that were negative by VI (Tables 6 and 7). Importantly, the ''perfect match'' RRT PCR successfully detected both H1N1v laboratory isolates (Table 1) and clinical specimens from pigs that had been infected with H1N1v in the field (Table 3) and experimentally (Table 4).[36] The ''combo'' M gene RRT PCR assay was slightly less sensitive for detection of contemporary European SIVs and H1N1v (Tables 1 and 2).

However, for the testing of most North American SIVs that were included in this study, the ''perfect match'' M gene RRT PCR suffered from reduced or even poor sensitivity (Table 1). For these SIVs, improved sensitivity was restored by employing the ''combo'' M gene RRT PCR, where inclusion of the original ''avian'' M−124 R reverse primer reduced the number of nucleotide mismatches within the corresponding sequence of the North American SIVs' M gene (Tables 1 and 5). It must also be emphasised that the ''avian'' M gene RRT PCR remains the most sensitive of the three M gene RRT PCRs for the detection of AIVs (Table 1).

The diagnostic sensitivity and specificity of the ''perfect match'' M gene RRT PCR assay were 100% and 95·2%, respectively, from the testing of 133 swabs (Table 6a). The

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five discrepant results included a VI-negative nasal swab obtained from a H1N1v-experimentally infected pig at 7 dpi when viral shedding appeared to be waning (Ct 34·48), and this low titre was mirrored by the corresponding H1-118 RRT PCR (Ct 34·08; Table 4). The other four discrepant results were VI-negative swabs collected by UK Regional Laboratories during summer 2009 that were shown to be likely H1N2 SIV positives, so affirming these ''perfect match'' M gene RRT PCR results. This inferred a greater sensitivity for the RRT PCRs compared to VI. As regards ''perfect match'' M gene RRT PCR testing of the 116 swine tissues, however, there were four discrepant results, where a diagnostic sensitivity and specificity of 93·6% and 98·6%, respectively, were observed (Table 7a). The 96 UK archived swine tissues were classified as SIV positive or negative at the time of original submission based on VI. Three of the 31 tissues from SIV-positive pigs were negative (No Ct) by the ''perfect match'' M gene RRT PCR (Table 2). It was speculated that at the time of the original submission these pigs may have been classified as SIV positive based on a VI result from another localised tissue portion or clinical specimen that was no longer available for the current validation. It was also observed that only 4 of 31 tissues obtained from SIV-positive pigs were positive when VI was repeated through two egg passages during the current validation (Table 2). This may be a consequence of the long-term storage history of these archived tissues, where infectivity may have been compromised by repeated freeze-thawing, or even because of limiting VI to two egg passages. One archived tissue from an SIV-negative pig collected in 2009 was positive by ''perfect match'' M gene RRT PCR, negative by ''H1-118'' RRT PCR and VI (Table 7), but additional investigations suggested that this was a non-viable H1N2 specimen.

The H1-118 RRT PCR assay was designed for the differential detection of H1N1v infections of pigs. This method successfully detected five H1N1v laboratory isolates (Table 1) and the presence of H1N1v in 48 clinical specimens (29 swabs and 19 post-mortem tissues) in field-infected and experimentally infected pigs (Tables 3 and 4). It was also shown that the H1 genes from nine H1 AIVs, 30 European H1 SIVs (i.e. classical H1N1, contemporary avian-like swine H1N1 and H1N2) were not detected (Table 1) by this assay. In addition, no positive signal was obtained when 24 archived tissue samples obtained from pigs infected with European H1N1 and H1N2 SIVs were tested with the H1-118 RRT PCR assay (Table 2). This affirmed the ability of the H1-118 RRT PCR assay to differentiate H1N1v infections from those caused by the SIVs endemic in Europe. However, this test did show some cross-reactivity with North American H1 SIVs (Table 1). This was not unexpected in view of the evolutionary relationship between the H1 gene of North American triple reassortment SIVs and H1N1v.[5] Consequently, it would not be an appropriate test for differentiating H1N1v in North American pig infections.

The diagnostic sensitivity and specificity of the ''H1-118'' RRT PCR assay for 133 swabs were 100% and 99·1%, respectively (Table 6b); the one discrepant result was the swab obtained at 7 dpi from pig 2 infected experimentally with H1N1v when the titre of viral shedding was declining (Table 5). For the ''H1-118'' RRT PCR using tissue samples, both the diagnostic sensitivity and specificity of this assay were 100% (Table 7b).

Fifteen clinical specimens from H1N1v-field-infected pigs (Table 3) were independently proven as H1N1v positive by conventional RT PCR and amplicon sequencing because VI was not consistently successful for the identification of H1N1v. This is because H1N1v does not consistently haemagglutinate chicken red blood cells, which may not be observed until the third passage in EFEs (data not shown). A further 24 clinical specimens (18 swabs and six tissues) from H1N1v-field-infected herds were not included in the diagnostic sensitivity and specificity calculations, but testing by both RRT PCRs gave highly concordant results as judged by a comparison of Ct values (Table 3).

Public health laboratories have already described RRT PCR protocols for the detection of H1N1v in humans,[29–32] and in this study, we have validated RRT PCR testing for detection of H1N1v for veterinary purposes, specifically for detecting H1N1v in pigs. This included evaluation of a generic approach to detect known SIVs as well as H1N1v. This was achieved for European SIVs by the ''perfect mach'' M gene RRT PCR assay, which sensitively detected all known types of European SIVs in addition to H1N1v. However, it was noted that contemporary Irish SIVs may be the exception to this observation in Europe (Table 1). Testing of recent Irish SIVs by the various M gene RRT PCR assays suggests that the original ''avian'' M gene RRT PCR may be the more sensitive approach (Raleigh and Flynn, Personal Communication). In the case of North American SIVs, the ''combo'' M gene RRT PCR assay was most sensitive for generic detection, and this method was slightly less sensitive for the detection of H1N1v than the ''perfect match'' M gene RRT PCR assay. The ''H1-118'' RRT PCR assay was shown to be able to differentiate H1N1v from SIV infections in clinical samples derived from field-infected and experimentally infected pigs relevant to a European setting. Validation of the generic and differential RRT PCR assays involved not only testing of a range of laboratory-grown influenza A virus isolates, but also crucially included testing of the same set of clinical specimens from pigs infected with SIVs or H1N1v. Primers and probe for the differential ''H1-118'' RRT PCR were deliberately designed within the relatively conserved HA2 region of the HA gene of H1N1v. However, continuing molecular evolution the HA gene may

yet result in nucleotide changes within these primer and probe-binding sequences. Vigilance for relevant nucleotide changes by sequencing the HA genes of new H1N1v swine isolates may identify potential future modifications to the "H1-118" RRT PCR.

Validation of the RRT PCR assays was conducted from the perspective of veterinary contingency planning for the eventuality of H1N1v outbreaks in the European pig population. To date, H1N1v infections have been already documented in pigs in the Americas, Eurasia and Australasia [15,16,44] and examples of field cases have been included in this study (Table 3). As the human pandemic caused by H1N1v continues to spread globally, it is anticipated that further pig infections with H1N1v will be reported, and precedent suggests that these will be followed by sustained transmission within pig populations.[36] Analogous contingency planning was conducted in 2005, when the westward spread of H5N1 highly pathogenic AIV prompted the validation of a H5 RRT PCR for the detection of both this H5 virus and other Eurasian H5 AIVs.[45] In conclusion, it is possible to present these approaches as sensitive and specific methods for use in surveillance for SIV and current H1N1v isolates in European pig populations.

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Chapter 3
Avian Influenza Virus RNA Extraction from Tissue and Swab Material

Erica Spackman and David L. Suarez

Summary The efficient extraction and purification of viral RNA are critical for downstream molecular applications, whether it is the sensitive and specific detection of virus in clinical samples, virus gene cloning and expression, or quantification of the avian influenza (AI) virus by molecular methods from experimentally infected birds. Samples can generally be divided into two types: enriched (e.g., virus stocks) and clinical. Clinical type samples, which may be tissues or swab material, are the most difficult to process due to the complex sample composition and possibly low virus titers. In this chapter, two well-established procedures for the isolation of AI virus RNA from common clinical specimen types and enriched virus stocks for further molecular applications will be presented.

Keywords RNA; RNA extraction; molecular; nucleic acid; specimen processing; tissue; swab material.

1. Introduction

AI virus RNA can be isolated from numerous *in vitro* and *in vivo* virus sources; however, cell lysates, cell supernatants, or chicken embryo chorioallantoic fluid are the easiest to work with because the virus load is normally high and the sample composition is defined. When the RNA will be used for gene sequencing, cloning for characterization, or expression, the use of RNA from enriched stocks is recommended. Most general RNA extraction methods or commercial kits for total RNA or viral RNA extraction may be used with enriched samples.

In contrast, clinical diagnostic samples or samples from experimentally infected animals are complex mixtures of host cells, bacteria, and other material that can interfere with the detection of the AI virus with molecular diagnostic tests. The efficient extraction and purification of the viral RNA from this material are critical for sensitive and specific detection of the virus. Specific processing methods will depend on the sample type. For animal testing, the optimal sample type varies by species and virus strain (see Table 1), as, for example, the virus tissue tropism can differ based on the host and the virus may therefore be present in different amounts in different tissue

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types. Specimen species of origin can also affect the processing procedure for cloacal swabs or fecal samples since the host's diet may introduce RT-PCR inhibitors, which are not removed during RNA extraction. The diet of domestic poultry is generally uniform, allowing for more consistent processing, but samples from wild birds can vary greatly because of the variety of foods they can consume.

Clinical specimens and animal-origin specimens (swab material and tissue) may also contain substances that can degrade RNA once it is released from the virion. Specimens containing fecal material, such as cloacal swabs, are some of the most difficult to work with and require processing methods that inactivate RNases thoroughly and that are efficient at removing PCR inhibitors or other contaminants. Some general recommendations for the selection of RNA extraction method by sample type are provided in Table 1. See Chapter 2 for additional guidance on sample collection and handling.

Numerous commercial kits for total RNA extraction are available, and standard organic extraction methods may also be optimized for AI virus RNA. Here the procedures for two methods, a silicon binding column-based method that has been widely used with AI virus [1, 2] and a method using organic solvents for nucleic

Table 1 Processing Methods and Notes by Specimen Type

Specimen Type	Processing Notes
Oropharyngeal or tracheal swabs	Any method may be used. LPAI virus tends to replicate to higher titers in the respiratory tract of gallinaceous birds.
Cloacal swabs	Organic extraction preferred. Use of an internal positive control is highly recommended for RT-PCR and real-time RT-PCR (see Chapter 4). Low pathogenic AI virus tends to replicate to the highest titers in the intestinal tracts of waterfowl.
Tissue (i.e., lung, heart, etc.)	Prepare a 10% W/V homogenate and follow with an organic extraction. Highly pathogenic AI viruses can replicate to high levels in numerous tissues; low pathogenic AI viruses are optimally detected in the respiratory tracts of gallinaceous birds and the enteric tracts of water fowl.
Environmental samples (i.e., drag swabs)	Organic extraction preferred. Use of an internal positive control is highly recommended for RT-PCR. RT-PCR–based methods are not recommended if differentiation of viable from nonviable virus is necessary, in which case virus isolation is preferred [5].
Cell culture fluid	Any.
Amnioallantoic fluid (AAF)(egg fluid)	Any. Avoid using egg fluid with excessive yolk. AAF should appear clear to yellow and may be cloudy. Brown to red or solid yellow AAF, which may indicate yolk, bacterial, or blood contamination, should be avoided.

acid isolation, will be discussed. Although specific kits are mentioned, equivalent kits and suppliers may be used with optimization.

2. Materials

Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement.

2.1. Method 1. Silicon Nucleic Acid Binding Column

1. Nuclease-free water.
2. Nuclease-free plastic ware (i.e., microcentrifuge tubes) (*see* **Note 1**).
3. Qiagen RNeasy Mini Kit (Qiagen, Inc.) or equivalent (*see* **Note 2**).
4. 100% Ethanol.
5. 70% Ethanol (prepared with nuclease-free water).
6. 2-Mercaptoethanol (βME).
7. Sample material (*see* **Note 3**): oropharygeal or tracheal swab material, amnioallantoic fluid (AAF), cell culture material.

2.2. Method 2. Organic Extraction

1. Nuclease-free water.
2. Nuclease-free plastic ware (i.e., microcentrifuge tubes) (*see* **Note 1**).
3. Trizol LS reagent (Invitrogen, Inc.).
4. Chloroform.
5. Isopropanol.
6. Glycogen 5 mg/mL.
7. 70% Ethanol (prepared with nuclease-free water).
8. Sample material (*see* **Note 3**): oropharygeal, tracheal, or cloacal swab material, AAF, cell culture material, tissue.

3. Methods

3.1. Method 1. Silicon Nucleic Acid Binding Column

This method is best for AAF, cell culture material, tracheal swabs, or oropharyngeal swabs. This method is not recommended for tissue, cloacal swabs, or samples that may contain fecal material.

1. Prepare all buffers and reagents in accordance with the RNA extraction kit protocol (Qiagen RNeasy Mini Kit).
2. Vortex the sample (tracheal swabs in BHI or other media) for 3–5 s, and then centrifuge the sample for 5 min at ~5 Kg to pellet any large debris. Withdraw 500 µL of supernatant and place in a 1.5 mL microcentrifuge tube. Amnioallantoic fluid (AAF) and cell culture material may be added directly to the RLT buffer.
3. Add 500 µL of kit-supplied RLT buffer. Close the tube and vortex the sample for 5 s.
4. Add 500 µL of RNA-grade 70% ethanol to the tube and mix. Pulse-centrifuge to minimize aerosol generation.
5. Add 750 µL of the supernatant from step 3 to the RNeasy column and centrifuge for 15 s at ~12 Kg, empty the flow-through from the collection tube, and repeat (all of the sample/RLT/70% ethanol mix should be applied to the column). This step may be done with a vacuum manifold instead of a centrifuge.
6. Add 700 µL of RW1 buffer to the RNeasy column, centrifuge for 15 s at ~12Kg, and place the column in a clean collection tube (the collection tube with RW1 flow-through may be discarded and replaced with a fresh collection tube). This step may be done with a vacuum manifold instead of a centrifuge.
7. Add 500 µL of RPE buffer to the RNeasy column, centrifuge for 15 s at ~12 Kg, and empty the flow-through from the collection tube. This step may be done with a vacuum manifold instead of a centrifuge.
8. Repeat step 6 for a total of two washes with RPE buffer.
9. Centrifuge the empty RNeasy column an extra 2 min at ~14 Kg and discard the collection tube.
10. Place the RNeasy column in a 1.5 mL microfuge tube and add 50 µL of nuclease-free water to the column. Incubate at room temperature for 1 min. Elute RNA by centrifuging for 1 min at ~14 Kg. Discard the RNeasy column (*see* **Notes 4** and **5**).
11. The RNA sample may be stored at 4 °C for <1 week; storage for longer than 1 week should be at −70 °C or colder. Repeated freeze-thaw cycles should be avoided.

3.2. Method 2. Organic Extraction with Trizol LS Reagent

This method can be used with AAF, oral swabs, tracheal swabs, cloacal swabs, cell culture material, and tissues.

3.2.1. Sample Preparation

1. Cloacal swabs: Vortex vigorously for 7–10 s. Centrifuge for 5 min at 12,000 g. Extract RNA from the supernatant (see Section 3.2.2).
2. Tissues: Make a 10% homogenate of tissue in PBS. Centrifuge for 10 min at 12,000 g. Extract RNA from the supernatant (see Section 3.2.2).

3. AAF and cell culture material: Use 250 µL of the AAF or cell culture supernatant or lysate directly with 750 µL of Trizol LS (see Section 3.2.2).

3.2.2. RNA Extraction

1. Add 250 µL of the supernatant from the sample prepared as described in step 1 of Section 3.2.1, to 750 µL of Trizol LS reagent or equivalent. Vortex. Pulse-spin to remove liquid from the tube lid.
2. Add 200 µL of 100% chloroform to the sample/Trizol homogenate. Vortex for 15 s. Incubate at room temperature for 7 min.
3. Centrifuge at 12,000 g for 15 min at room temperature.
4. Transfer 400–450 µL of the upper aqueous layer to a separate microcentrifuge tube marked with the sample number. Because the transfer of organic phase material with the aqueous layer will reduce RNA yield and inhibit the RT-PCR reaction, take care to avoid pipetting any of the organic phase or interface material if present.
5. Add 500 µL of 100% isopropanol. Add 1 µL of 5 mg/mL stock glycogen to the isopropanol to aid RNA precipitation. Invert tube several times to mix, and incubate at room temperature for 10 min.
6. Centrifuge at 10,000 g for 10 min at 4 °C.
7. Decant liquid. Care should be taken to ensure that the RNA pellet is not disturbed. Add 1.0 mL of 70% or 80% ethanol. Mix gently.
8. Centrifuge at 10,000 g for 5 min at 4 °C.
9. Decant ethanol without dislodging the pellet. Invert the tube on a clean tissue wipe and allow to air-dry for 10 min or until all visible signs of moisture are gone. It is important not to let the RNA pellet overdry, as this will decrease its solubility.
10. Reconstitute the pellet in 50 µL of RNase-free water and incubate at 4 °C for 1 hr to overnight before using in downstream applications (*see* **Notes 4** and **5**).
11. The RNA sample may be stored at 4 °C for <1 week; storage for longer than 1 week should be at −70 °C or colder. Repeated freeze-thaw cycles should be avoided.

4. Notes

1. Use only RNA-grade (RNase-free) reagents and supplies for all reagents, buffers, and anything that may come in contact with the sample (e.g., mix any buffers to be used with RNA in RNase-free vessels). When possible, it is recommended that disposable RNAse-free labware is used since complete decontamination of glassware is difficult and time-consuming. Change gloves often to avoid cross-contamination and to minimize RNase contamination.

2. All kit-supplied buffers and reagents should be prepared in accordance with the kit instructions.
3. Samples collected for AI virus detection are often processed for more than one test. Therefore, when collecting samples for molecular methods, the samples are often handled to maintain viable virus. Since the AI virus is enveloped, maintenance of the cold chain while preventing freeze-thaw cycles and protein-rich pH-balanced buffers are critical. Brain-heart infusion (BHI) broth has been used extensively as the AI virus transport media based on work that compared numerous media and showed that BHI broth was superior in preserving AI virus [3].
4. Failure of the RNA extraction procedure may result in false negatives and can be difficult to identify as the cause. Use of an internal positive control for real-time RT-PCR is one way to ensure the quality of RNA extraction [4]. If RNA extraction fails:

   a. Confirm that all reagents have been properly made.
   b. Confirm that virus is present in the sample at adequate titers.
   c. Confirm that all materials were RNase-free.
   d. Repeat the extraction using the same or an alternative method.

5. For critical applications, some quality assurance may be helpful and can be done prior to the downstream application. Although UV spectroscopy may be used, it will give data for any and all RNA in a sample.

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VIROLOGY JOURNAL

SHORT REPORT Open Access

Susceptibility of turkeys to pandemic-H1N1 virus by reproductive tract insemination

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Abstract

The current pandemic influenza A H1N1 2009 (pH1N1) was first recognized in humans with acute respiratory diseases in April 2009 in Mexico, in swine in Canada in June, 2009 with respiratory disease, and in turkeys in Chile in June 2009 with a severe drop in egg production. Several experimental studies attempted to reproduce the disease in turkeys, but failed to produce respiratory infection in turkeys using standard inoculation routes. We demonstrated that pH1N1 virus can infect the reproductive tract of turkey hens after experimental intrauterine inoculation, causing decreased egg production. This route of exposure is realistic in modern turkey production because turkey hens are handled once a week for intrauterine insemination in order to produce fertile eggs. This understanding of virus exposure provides an improved understanding of the pathogenesis of the disease and can improve poultry husbandry to prevent disease outbreaks.

Findings

Because of the known susceptibility of turkeys to type A influenza viruses and the history of infection with triple reassortant viruses [1-6], when the pandemic influenza A H1N1 2009 (pH1N1) emerged, the possibility of turkeys becoming infected with the novel virus was investigated. However, experimental challenge with pH1N1 virus by the respiratory route showed that both turkey poults and adult turkey hens were resistant to infection [7-9], but infection was produced in young turkeys by the novel intracloacal route of inoculation (J. Pasick, personal communication). In August 2009, pH1N1 virus was detected in two turkey breeder farms in Chile presenting drops in egg production [10]. Epidemiological investigations on the possible source of infection identified workers with respiratory problems, and hen insemination as a risk factor for virus transmission to the birds. A second and third outbreak in turkey hens occurred in Canada, in September 2009, and in the USA, in November 2009, with a marked drop in egg production as the primary clinical sign of disease [11,12]. These three outbreaks of pH1N1 influenza in turkeys raised the question of how the turkey hens became infected when experimental evidence suggested that turkeys were refractory to respiratory infection.

In our previous study, 73-week-old turkey hens and 3-week-old turkey poults were intranasally inoculated with A/Mexico/4108/09 (H1N1) [8]. None of the turkeys developed clinical signs or died, no virus was detected in tissues, and all turkeys were negative for antibodies to the virus, indicating that they did not become infected. In another study, 21- and 70-day-old meat turkeys were oro-nasally inoculated with A/Italy/2810/2009 (H1N1) influenza virus. Virus was not recovered by molecular or conventional methods from blood, tracheal and cloacal swabs, lungs, intestine or muscle tissue, and only some birds seroconverted [9]. In a third study, inoculation of 3-week-old turkeys with A/CA/07/09 (H1N1) through the intranasal and intraocular route also failed to initiate infection [7].

In order to understand how the pH1N1 virus potentially had infected turkey breeders, we conducted a study in which we inoculated 53-week-old laying turkey hens with $10^{5.3}$ 50% cell culture infective doses of A/Chile/3536/2009 (H1N1) virus by three different routes. Eight hens were inoculated intranasally (IN), four hens were inoculated intracloacally (IC), and four hens were inoculated through the intrauterine (IU) route. Oropharyngeal and cloacal swabs were taken from all hens at days 2, 4, 7, 10, and 14 days post-inoculation (dpi), and lung, spleen, heart, kidney and oviduct were taken from one hen per group at 3 and 7 dpi for virus detection by quantitative real-time reverse transcriptase polymerase

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Table 1 Results of qRRT-PCR testing for pH1N1 virus in oropharyngeal and cloacal swabs of experimental turkey hens inoculated intranasally, intracloacally, or intrauterine with A/Chile/3536/2009 (H1N1) virus.

Groups	Sampling day (days post inoculation) for swabs									
	2		4		7		10		14	
	OP[a]	C[b]	OP	C	OP	C	OP	C	OP	C
IN[c]	0/8[d]	0/8	0/7	0/7	0/7	0/7	0/6	0/6	0/6	0/6
IC[e]	0/4	1/4($10^{4.7}$)	0/4	0/4	0/3	0/3	0/2	0/2	0/2	0/2
IU[f]	1/4 ($10^{4.7}$)[g]	1/4($10^{6.7}$)	3/3($10^{4.7}$)	3/3($10^{5.8}$)	0/3	3/3($10^{5.7}$)	0/2	1/2($10^{5.1}$)	1/2($10^{4.8}$)	0/2

[a] OP, oropharyngeal. [b] C, cloacal. [c] IN, intranasal. [d] number of virus positive/total sampled. [e] IC, intracloacal. [f] IU, intrauterine. [g] average titer of RNA positive samples. Previous studies have shown correlation between qRRT-PCR results and infectious titer of influenza A virus for oropharyngeal and cloacal swabs [15]. We report our qRRT-PCR data in relative equivalent units (REU) based on a standard curve for A/Chile/3536/2009 (H1N1) in mean chicken embryo infectious doses (EID_{50})

chain reaction (qRRT-PCR) assay targeted to the influenza virus matrix gene with the described modified reverse primer 3'-cagagactggaaagtgtctttgca-5' [8,13]. Tissues were also taken for histology and viral antigen detection by immunohistochemistry (IHC). For IHC, mouse monoclonal antibody P13C11, specific for influenza A nucleoprotein, was used. Sections were stained as previously described [14]. Serum was collected from the remaining turkeys at the end of the 14-day study for antibody testing by hemagglutination inhibition (HI).

None of the turkeys inoculated IN with the pH1N1 virus developed clinical signs. Turkeys inoculated IC or IU presented with mild diarrhea from 1 to 4 dpi. Turkeys inoculated by the IU route stopped laying eggs at 5 dpi, while turkeys IC inoculated laid eggs daily through 9 dpi. Turkeys IN-inoculated continued laying eggs until the end of the study. Turkeys inoculated IN or IC, necropsied at 3 and 7 dpi, presented no gross lesions and had active oviducts. The oviducts of the turkeys inoculated IU were congested or undergoing involution at 3 and 7 dpi, respectively. All IN-inoculated turkeys were negative for antibodies to the virus on 14 dpi. One of two IC-inoculated turkeys had a hemagglutination inhibition (HI) geometric mean antibody titer of 256, and both hens inoculated through the IU route had high HI titers (4096 and 8192) at 14 dpi. The two hens inoculated either IC or IU and necropsied at 7 dpi also seroconverted (64 and 256 HI titers, respectively). Virus

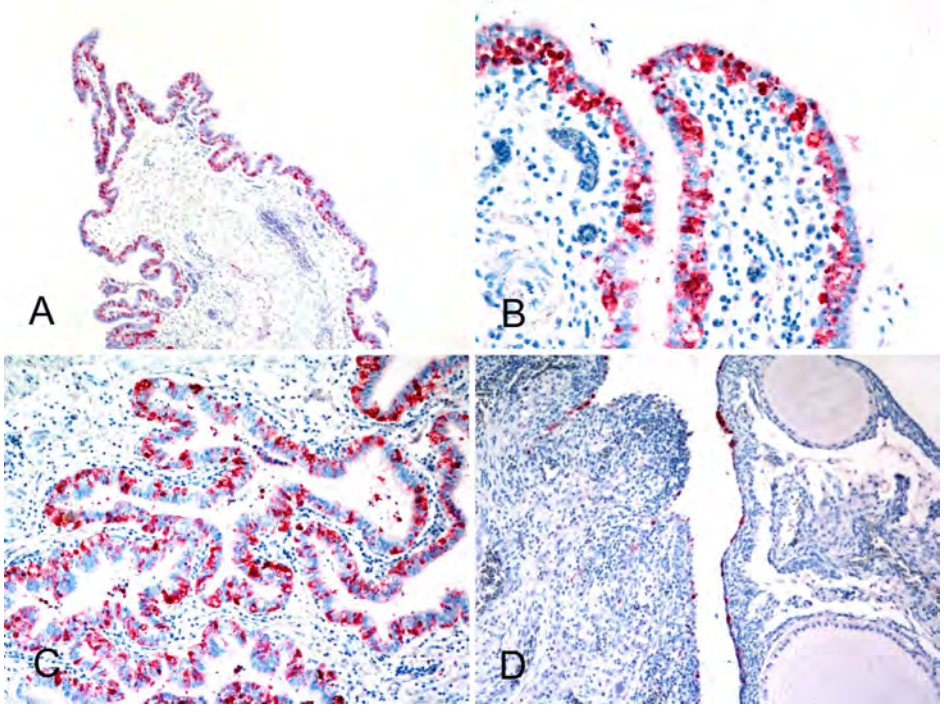

Figure 1 Photomicrographs of immunohistochemically strained reproductive tracts of turkey breeder hens IU-inoculated with pH1N1 virus. (A to C) Oviducts with influenza viral antigen in luminal lining epithelium, (D) Ovary with influenza viral antigen in surface germinal epithelium.

was detected in oropharyngeal and cloacal swabs from IU-inoculated turkeys from 2 to 14 dpi, and at 4 dpi from the cloacal swab of one IC-inoculated turkey (Table 1). Virus was detected in the oviduct of the turkeys IC- or IU-inoculated, and virus antigen was visualized by immunohistochemical staining in the surface germinal epithelium of the ovary and luminal epithelium lining the oviduct (Figure 1). No lesions or viral staining was present in any of the other tissues examined. No virus was detected in swabs or tissues from IN-inoculated turkeys.

In this study, and consistent with previous studies, turkeys IN-inoculated with the pH1N1 influenza virus did not become infected with the virus, although the respiratory route is considered the natural route of exposure for influenza A viruses in many animal species. However, IC or IU-inoculation with the virus resulted in pH1N1 virus infection. Such routes of exposure are realistic in modern turkey production because turkey hens are handled once a week for insemination, which deposits semen into the uterus, in order to produce fertile eggs, because modern tom turkeys are physically unable to efficiently breed naturally because of their large breast muscles. During this process, workers handle individual hens, manually everting the cloaca to locate the vagina for insertion of the insemination straw. Because of the close contact with infected humans, this routine insemination activity provided opportunity for initiating the infection process by either large droplet exposure during human sneezing activities or direct inoculation from infectious fomites on contaminated hands, and bird-to-bird transmission through mechanical fomite inoculation to the cloaca or reproductive tract by the inseminators. This is the first study to show infection by intrauterine exposure to influenza A virus in turkeys and such transmission is consistent with the proposed risk of infected insemination crews in cases of pH1N1 in Chilean turkey hens [10]. However, replication and shedding from the respiratory tract following IU-inoculation is perplexing considering IN-inoculation failed to produce infection. Possibly, the IU-inoculation and infection resulted in changes in the virus that allowed subsequent respiratory infection. Future studies will examine such viruses recovered from respiratory tract for changes in viral tissue tropism.

Abbreviations

dpi: days post-inoculation; HI: hemagglutination inhibition; IC: intracloacal; IHC: immunohistochemistry; IN: intranasal; IU: intrauterine; pH1N1: influenza A H1N1 2009; qRRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction

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Authors' contributions

MPJ participated in the design of the study, performed the animal study, read the histopathology and immunohistochemistry slides, and drafted the manuscript. JLW conducted virus isolation and serological assays. ES carried out the qRRT-PCR studies. DLS participated in the study design. DES conceived of the study, and participated in its design and coordination, and completed 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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Variability in Pathobiology of South Korean H5N1 High-Pathogenicity Avian Influenza Virus Infection for 5 Species of Migratory Waterfowl

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Abstract

The pathobiology of H5N1 high-pathogenicity avian influenza (HPAI) virus infection in wild waterfowl is poorly understood. This study examined the pathobiology of A/chicken/Korea/IS/06 (H5N1) HPAI in 5 migratory waterfowl species—mute swans (*Cygnus olor*), greylag geese (*Anser anser*), ruddy shelducks (*Tadorna ferruginea*), mandarin ducks (*Aix galericulata*), and mallard ducks (*Anas platyrhynchos*)—following intranasal inoculation or contact exposure, from which all birds became infected. In mute swans, this virus had strong vascular endothelial cell tropism, producing acute severe disease and 100% mortality; the virus was detected in various parenchymal cells; and necrotic and inflammatory changes were noted in a range of organs, including pancreas, brain, spleen, heart, oral cavity, adrenal gland, lung, and liver. The ruddy shelducks had 100% mortality, but time to death was delayed, and the lesions were primarily restricted to the brain, heart, pancreas, and spleen. The mandarin ducks had only a single mortality, with lesions similar to those in ruddy shelducks. The greylag geese became infected, developed neurological signs, and had residual meningoencephalitis when examined at termination but lacked mortality. The mallards had asymptomatic infection. These results indicate variation in the pathobiology of H5N1 virus infections in different species of wild waterfowl, ranging from severe, acute systemic disease with 100% mortality to asymptomatic infection of respiratory and gastrointestinal systems.

Keywords

avian influenza, birds, H5N1, immunohistochemistry, infectious diseases

Avian influenza (AI) is caused by infection with a type A influenz virus of the family Orthomyxoviridae.[41] Low-pathogenicity AI viruses have been isolated from numerous wild and domestic avian species,[37,41] and wild waterfowl are regarded as the primordial reservoir hosts of these viruses.[46] However, high-pathogenicity (HPAI) viruses arise from mutation of low-pathogenicity AI viruses as they circulate in poultry.[34,43] These HPAI viruses produce a severe systemic disease with near 100% mortality in chickens, turkeys, and other gallinaceous birds[31,41] but usually do not cause infection, clinical disease, or death in domestic waterfowl or wild birds, especially aquatic birds of the order Anseriformes (ducks, geese, swans).[1,6,25]

Since the isolation of H5N1 HPAI virus in 1996 from a domestic goose in Guangdong Province, China, descendants of this virus have developed variable abilities to infect and cause disease in domestic ducks and wild aquatic birds under natural and experimental settings.[3-5,7,11,14,38] In experimental infections, H5N1 HPAI viruses that were isolated between 1997 and 2001 in Hong Kong produced acute lesions and high mortality in chickens and other gallinaceous birds but only asymptomatic infections in domestic ducks or captive-raised aquatic birds.[27,31] However, the diversity of avian species for which H5N1 HPAI viruses are potentially infective, pathogenic, and lethal changed dramatically in late 2002, as shown by virus isolation from a variety of dead captive waterfowl in Penfold Park and Kowloon Park in Hong Kong, as well as from various waterfowl (ducks, geese, and swans), American flamingos (*Phoenicopterus ruber*), and more recently, tree sparrows (*Passer montanus*) in China.[9,11,20] In 2005, H5N1 HPAI virus was isolated from migratory birds found dead in Qinghai Lake of western China, a major breeding site for migratory birds, including bar-headed geese (*Anser indicus*).[10,23] In quick succession, mortality was found among domestic poultry and aquatic birds in western Siberia, Mongolia, Europe, Africa, and the Middle East and, in late 2006, reappeared in domestic ducks and

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chickens in South Korea and Japan.[35] The infection of a variety of wild birds under natural conditions raises concerns about the ecology, epidemiology, and pathobiology of these H5N1 HPAI viruses.[8,12]

This study was initiated (1) to determine the susceptibility of mute swans (*Cygnus olor*), greylag geese (*Anser anser*), ruddy shelducks (*Tadorna ferruginea*), mandarin ducks (*Aix galericulata*), and mallard ducks (*Anas platyrhynchos*) to intranasal inoculation with or contact exposure to the A/chicken/Korea/IS/06 (H5N1) HPAI virus and (2) to describe the lesions and the distribution of virus in each species. These birds were chosen because they are naturally occurring species in South Korea and migrate to and from mainland Asia where H5N1 HPAI virus has become endemic.

Materials and Methods

Virus

A/chicken/South Korea/IS/06 (Ck/Kr/06) (H5N1) AI virus was isolated from tissues collected from affected chickens during an outbreak in November 2006 (Avian Disease Division, National Veterinary Research and Quarantine Service, South Korea). Genetically, the virus is closely related to the clade 2.2 A/Bar-headed Gs/Qinghai/5/2005 and A/whooper swan/Monogolia/244/2005 (H5N1) HPAI viruses.[3,22] Virus-infected chorioallantoic fluid of second passage in 10-day-old embryonating chicken eggs was diluted 1:300 in brain–heart infusion medium (BHI) and used as the inoculum.

Animals

Mute swans, greylag geese, ruddy shelducks, mandarin ducks, and mallards were obtained from commercial captive-breeding sources and were sham or virus inoculated or contact exposed at 7, 7, 12, 8, and 12 weeks of age, respectively. Each species was housed in self-contained isolation units (Mark 4; Controlled Isolation Systems, San Diego, CA), ventilated under negative pressure with HEPA-filtered (ie, high-efficiency particulate air filtered) inlet and exhaust air. Feed and water were provided ad libitum. All birds used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee. All experiments were performed in a biosafety level 3–enhanced facility certified by the US Department of Agriculture.

Experimental Design

For each species, birds were separated into sham and virus-exposed groups. The sham groups contained 1 to 2 birds that were intranasally inoculated with 0.1 ml of noninfected chorioallantoic fluid diluted 1:300 in BHI medium. The sham birds were euthanized on the same day when the last virus-inoculated or contact-exposed bird died or were euthanized.

The virus-inoculated group contained 2 birds that were each inoculated intranasally with $10^{6.0}$ EID_{50} of the Ck/Kr/06 (H5N1) AI virus in a 0.1-ml dose. Twenty-four hours after intranasal inoculation, a naive bird from each species was placed in direct contact with the virus-inoculated birds (ie, contact exposed). The birds were monitored daily for illness or death.

Oropharyngeal and cloacal swabs were collected in BHI medium containing antimicrobial compounds (100 μg/ml gentamicin, 100 units/ml penicillin, and 5 μg/ml amphotericin B) from the virus-inoculated birds at 1, 2, 4, 7, and 14 days postinfection (DPI) and from the contact-exposed birds at 1, 3, 6, and 13 days postexposure (DPE). At 14 DPI (or 13 DPE), serum was collected from all surviving birds for serologic testing, and the birds were euthanized with intravenous sodium pentobarbital (100 mg/kg body weight). Necropsies were performed on all birds that died or were euthanized, and routine tissues were collected for histopathological and immunohistochemical evaluation.

Histopathology and Immunohistochemistry

Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μm and were routinely processed and stained with hematoxylin and eosin (HE). A duplicate 5-μm section was immunohistochemistry stained with a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen (SEPRL, Athens, GA) as the primary antibody. Procedures for immunohistochemistry followed those previously described.[29] Fast red was used as substrate chromogen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromogen deposition in the nucleus, which was often accompanied by chromogen deposition within the cytoplasm.

Virus Detection by Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

RNA was extracted from oropharyngeal and cloacal swab material as follows: 250 μl of swab material was added to 750 μl of TRIzol LS (Invitrogen Inc, Carlsbad, CA). The samples were mixed by vortexing and incubated at room temperature for 10 minutes; then, 200 μl of chloroform was added. The samples were vortexed again, incubated at room temperature for 10 minutes, and then centrifuged for 15 minutes at approximately 12,000 × *g*. The aqueous phase was collected, and RNA isolation was completed by extracting the RNA from the aqueous phase with the MagMAX AI/ND viral RNA isolation kit (Ambion, Inc, Austin TX) in accordance with the kit instructions using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRRT-PCR) for the influenza matrix gene was performed as previously described.[36] Samples with cycle threshold (CT) values over 38 were considered suspect and were confirmed as positive or negative by conventional reverse transcriptase polymerase chain reaction for the NS1 gene

Table 1. Clinical Features in Waterfowl Following Intranasal Inoculation or Contact Exposure With H5N1 High-Pathogenicity Avian Influenza Virus[a]

Species: Exposure Group	Morbidity	Clinical Signs	Mortality
Mute swan			
Virus inoculated	2 of 2[b] (3, 4)[c]	Incoordination (4, 5)	2 of 2[a] (3, 5)
Contact	1 of 1 (4–6)	Listlessness, incoordination (4, 5)	1 of 1 (6)
Ruddy shelduck			
Virus inoculated	2 of 2 (5–9)	Listlessness, poor physical condition (5–9)	2 of 2 (5, 10)
Contact	1 of 1 (5, 6)	Incoordination (5, 6)	1 of 1 (6)
Greylag goose			
Virus inoculated	2 of 2 (5–14)	Listlessness (5–14), incoordination (5–10)	0 of 2
Contact	1 of 1 (6–13)	Listlessness (5–13), incoordination (6–11)	0 of 2
Mandarin duck			
Virus inoculated	0 of 2	None detected	0 of 2
Contact	1 of 1 (4, 5)	Listlessness, poor physical condition (4, 5)	1 of 1 (5)
Mallard duck			
Virus inoculated	0 of 2	None detected	0 of 2
Contact	0 of 1	None detected	0 of 1

[a] Intranasally sham-inoculated control birds for each species lacked clinical and pathological evidence of virus infection.
[b] Ill/dead birds of total birds.
[c] Numbers in parentheses indicate "(days postinfection or days postexposure)" or the range therein.

followed by gel electrophoresis.[39] Because the NS1 test is not quantitative, CT values for the NS1-positive samples were calculated based on the matrix gene qRRT-PCR test.

Serologic Assays

Agar gel precipitin and hemagglutination inhibition (HI) tests were performed on the serum (preinoculation and postinoculation) by using standard procedures.[42] The HI tests were performed by using a 0.5% suspension of chicken erythrocytes in phosphate-buffered saline.

Results

Sham-Inoculated Controls

There was no morbidity or mortality observed in sham birds of the 5 species. These birds lacked evidence of infection, including absence of antibodies against AI virus at the beginning of the study and at 14 DPI; furthermore, no AI viral genome was detected by qRRT-PCR from oropharyngeal or cloacal swabs collected during the study. Histologically, random lymphoid nodules were occasionally observed in the liver and kidney. Nonspecific immunohistochemical staining, which was restricted to cytoplasmic granules of a few individual cells, was infrequently observed in the lymphoid tissues, including the spleen, tonsils, thymus, cloacal bursa, and liver. Immunohistochemical staining of this nature has been interpreted as nonspecific staining of mast cell granules.[29] In ruddy shelducks, the spleen had random foci of mild to moderate hemosiderosis.

Infectivity and Clinical Features

Morbidity and mortality following virus exposure to the Ck/Kr/06 H5N1 HPAI virus varied among the 5 species (Table 1). Virus inoculation and contact exposure in all mute swans and ruddy shelducks produced clinical disease—typically, listlessness, ruffled feathers, and neurological signs such as incoordination and head tremors, which terminated in death (Table 1). The contact-exposed mandarin duck died after exhibiting listlessness for a few days, but the virus-inoculated mandarin ducks lacked clinical signs. Virus inoculation or contact exposure produced mild neurological signs of incoordination in greylag geese with recovery by 10 DPI or 11 DPE. By contrast, virus inoculation and contact exposure in mallard ducks did not produce clinical signs or death.

The Ck/Kr/06 virus replicated in all virus-inoculated birds of all 5 species, and the virus spread to all contact-exposed birds (Table 2). Virus detection was more frequent from the oropharynx than cloaca, and the peak quantity was highest in oropharyngeal swab samples (ie, lowest CT values) for all species except for mallards, where the highest quantity was from a cloacal swab sample. Peak oropharyngeal swab quantity ranged from a low genome detection with 44.6 CT value in mallard ducks to a high genome detection with 25.5 CT value in mute swans. The duration of oropharyngeal virus shedding was relatively short for mallard ducks (4 DPI or 3 DPE), but virus was detected up to 7 DPI or 6 DPE in mandarin ducks and greylag geese and up to 10 DPI in virus-inoculated ruddy shelducks. Antinucleoprotein and anti-H5 hemagglutination inhibition antibodies were detected in all surviving greylag geese, mandarin ducks, and mallard ducks (Table 2), indicating that all birds became infected.

Gross Lesions

All mute swans had multiple white necrotic foci of 0.1 to 10.0 mm in diameter, with moderately coalescent hemorrhage in the pancreas and petechial hemorrhages in the epicardium

Table 2. Immunohistochemical Demonstration of Viral Antigen, Real-time Reverse Transcriptase Polymerase Chain Reaction for Avian Influenza Viral Genome, and Serological Data in Waterfowl Following Intranasal Inoculation or Contact Exposure With H5N1 Virus[a]

	IHC Tissues	qRRT-PCR[b]					
		Oropharyngeal Swab		Cloacal Swab			
Species: Exposure Group	Virus Detection	Virus Detection	CT Values	Virus Detection	CT Values	AGP	Serology HI Titer, Log_2
Mute swan							
Virus inoculated	2 of 2[c] (3, 5)[d]	2 of 2 (1, 2, 4)	38.8–27.4[e]	2 of 2 (2, 4)	41.6–37.0	NT	NT
Contact	1 of 1 (6)	1 of 1 (1, 3, 6)	42.6–25.5	1 of 1 (3)	38.8	NT	NT
Ruddy shelduck							
Virus inoculated	2 of 2 (5, 10)	2 of 2 (4, 10)	42.8–36.6	0 of 2	—	NT	NT
Contact	1 of 1 (6)	0 of 1	—	0 of 1	—	NT	NT
Greylag goose							
Virus inoculated	2 of 2 (14)	2 of 2 (1, 2, 4, 7)	44.0–31.2	1 of 2 (4)	39.9	2 of 2[†]	64, 1,024
Contact	0 of 1 (14)	1 of 1 (3, 6)	34.3–35.5	0 of 1	—	1 of 1	128
Mandarin duck							
Virus inoculated	0 of 2 (14)	2 of 2 (1, 2, 4, 7)	41.4–30.5	2 of 2 (4)	44.6–43.6	2 of 2	64, 128
Contact	1 of 1 (6)	1 of 1 (3, 6)	38.6–29.7	0 of 1	—	NT	NT
Mallard duck							
Virus inoculated	0 of 2 (14)	2 of 2 (1, 2, 4)	44.6–37.8	1 of 2 (4)	31.6	2 of 2	32, 64
Contact	0 of 1 (14)	1 of 1 (3)	40.6	0 of 1	—	1 of 1	16

[a] IHC, immunohistochemistry; qRRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; AGP, agar gel precipitation; HI, hemagglutination inhibition; CT, cycle threshold; NT, not tested because of lack of survivors.
[b] For qRRT-PCR, the quantity of viral genome is inversely proportional to CT value (ie, the lower the value, the greater the quantity of genome detected).
[c] Positive birds of total tested birds.
[d] Numbers in parentheses indicate "(days postinfection or days postexposure)" for IHC or "(days detected, days postinfection or days postexposure)" for qRRT-PCR.
[e] Virus titer range from positive swab samples based on qRRT-PCR.

and coronary fat of the heart. The lungs were moderately congested with edema in a single bird that died at 3 DPI. All 3 mute swans had hepatomegaly with friable parenchyma and congestion, a few petechial hemorrhages in the mucosa of the proventriculus, and moderately enlarged and congested in the spleen and kidney.

All 3 ruddy shelducks were severely dehydrated with mild to moderate dilation of the small intestine (especially the duodenum); they also had empty gastrointestinal tracts and green discolored feathers around the cloaca. The nasal sinuses and pharynx had excessive mucus accumulation. Two of 3 birds had multiple, pale, white 0.1- to 0.5-mm-diameter necrotic foci in the pancreas. The 2 virus-inoculated ruddy shelducks had congested and enlarged livers, spleens, and kidneys.

The contact-exposed mandarin duck that died 5 DPE had severe dehydration and green discolored feathers around the cloaca associated with diarrhea. In the intestine, especially the duodenum and jejunum, there was moderate congestion, severe dilation, and lack of luminal contents. The lungs were congested with ecchymotic hemorrhages. The spleen and kidney were moderately congested and increased in size. The liver was severely friable.

The 2 virus-inoculated mandarin ducks lacked lesions when examined at 14 DPI. The contact-exposed greylag goose euthanized 13 DPE had a 1.5-cm necrotic area extending from meninges into one cerebral hemisphere but not in the cerebellum. The 2 virus-inoculated geese lacked lesions when examined at 14 DPI. The virus-inoculated and contact-exposed mallards lacked lesions when examined at 14 DPI and 13 DPE, respectively.

Histology and Immunohistochemistry

Histological lesions and the corresponding viral antigen were distributed among multiple tissues in the mute swans, ruddy shelducks, greylag geese, and mandarin ducks but were lacking in all mallard ducks. Tables 3 and 4 summarize the tissue distribution and severity of histological lesions and AI viral staining.

Mute swans. Of the 5 species investigated, the most widespread distribution of histological lesions and viral antigen was observed in the mute swans. In this species, the most significant lesions were found in the oral cavity, pancreas, brain, lung, liver, spleen, heart, and adrenal gland. The oral mucosa had severe vacuolar degeneration and/or coagulative necrosis of squamous epithelium with associated intraepithelial viral antigen (Figs. 1A, 1B). The pancreata had moderate to severe, multifocal to confluent acinar necrosis with associated viral antigen in necrotic cells. The brain had focally extensive neuronal degeneration and necrosis, including vacuolation of the neuropil in the cerebrum and medulla. Mild to moderate lymphocytic perivascular cuffs, mild perivascular edema, and randomly scattered gliosis were also observed in the cerebrum and cerebellum. Viral antigen was detected in neurons, glial cells, neuropil, ependymal cells, epithelium of choroid plexi, and

Table 3. Average Severity of Histological Lesions in Waterfowl Exposed to H5N1 Virus by Contact With Infected Birds or Intranasal Inoculation[a]

Hosts	Nasal Cavity Epithelial Necrosis	Lung Interstitial Pneumonia	Heart Myofiber Necrosis	Brain Neuronal Necrosis	Meningitis	Pancreas Pancreatic Necrosis	Spleen Lymphoid Depletion	Liver Hepatocytic Necrosis	Adrenal Gland Epithelial Necrosis
Mute swans	+++ (3)	++ (3)	++ (3)	+++ (3)	—	+++ (3)	+++ (3)	++ (3)	+++ (3)
Ruddy sheldducks	++ (2)	—	++ (3)	++ (3)	—	++ (3)	+ (1)	± (2)	+++ (3)
Greylag geese	—	—	—	++ (3)	++ (3)	—	—	—	—
Mandarin duck[b]	++ (1)	—	++ (1)	++ (1)	++ (1)	+++ (1)	+++ (1)	+ (1)	—

[a] —, no lesion; ±, minimal; +, mild; ++, moderate; +++, severe. Number in parentheses indicates number of birds with histopathological changes. Mallard ducks lacked histological lesions and the corresponding viral antigen.
[b] Histological lesions of 1 bird that died at 5 days after contact exposure.

Table 4. Average Distribution of Avian Influenza Viral Antigen in the Tissues From Waterfowl Exposed to H5N1 Virus by Contact With Infected Birds or Intranasal Inoculation[a]

Hosts	Nasal Cavity Epithelia	Lung Histiocyte	Heart Myofiber	Blood Vessel Endothelia	Brain Neurons, Glial Cells, Ependyma	Peripheral Nerves Ganglion Cells	Spleen Phagocytes	Liver Hepatocytic Kupffer Cells	Pancreas Acinar Epithelia
Mute swans	+++ (3)	++ (2)	+++ (3)	++ (3)	+++ (3)	+ (1)	++ (3)	+++ (3)	+++ (3)
Ruddy Sheldducks	++ (2)	± (2)	++ (3)	—	+++ (3)	++ (3)	—	—	+++ (3)
Greylag geese	—	—	—	—	++ (2)	—	—	—	—
Mandarin duck[b]	++ (1)	—	—	—	+++ (1)	—	—	—	+++ (1)

[a] —, no staining; ±, minimal; +, mild; ++, moderate; +++, severe. Parentheses indicate number of birds with avian influenza viral antigen in the tissue. Mallard ducks lacked histological lesions and the corresponding viral antigen.
[b] Antigen distribution of 1 bird that died at 5 days after contact infection.

cerebellar Purkinje cells and neurons of granular layer. Moderate to severe multifocal coagulative necrosis of hepatocytes with minimal heterophilic to lymphocytic infiltration was identified, along with mild proliferation of Kupffer cells and necrosis of lymphocytes around portal areas.

Mild to severe histiocytic interstitial pneumonia was found with vascular-oriented pulmonary damage, including edema, congestion, hemorrhage, and, rarely, microthrombosis of capillaries (Fig 2A). The interstitial pneumonia was most severe, and the greatest quantity of viral antigen was detected in histiocytes and endothelium of blood vessels (Fig. 2B) of the mute swan that died at 3 DPE. In the nasal cavity, 2 virus-inoculated swans had moderate degeneration to necrosis of mucosal epithelium with congestion and/or hemorrhage. Viral antigen was found in the epithelium, submucosal histiocytes, and adherent cellular debris.

The heart had random, multifocal myocardial degeneration to necrosis with mild hemorrhage and moderate congestion (Fig. 3A). The spleen had severe lymphoid depletion from apoptosis and necrosis with edema and severe congestion. The corticotrophic cells and, less frequently, the chromaffin cells of the adrenal gland had moderate to severe multifocal to confluent areas of cytoplasmic vacuolar degeneration to coagulative necrosis. In addition, the cloacal bursa had severe lymphocyte depletion from lymphocytic necrosis and apoptosis not related to physiological regression (Fig. 4A). Viral antigen was associated with the histological lesions, commonly in cardiac myofibers (Fig. 3B), hepatocytes and Kupffer cells, splenic histiocytes, corticotrophic and chromaffin cells in the adrenals, and histiocytes and necrotic cellular debris in the cloacal bursa (Fig. 4B).

In the absence of histological lesions, viral antigen was infrequently identified in the parasympathetic ganglia within the submucosal and myenteric plexus of the small and large intestines; tubular epithelium of the kidney; vascular endothelium in intestine, heart, nasal cavity, and brain; skeletal muscle; epidermis; and pulp of feather follicles.

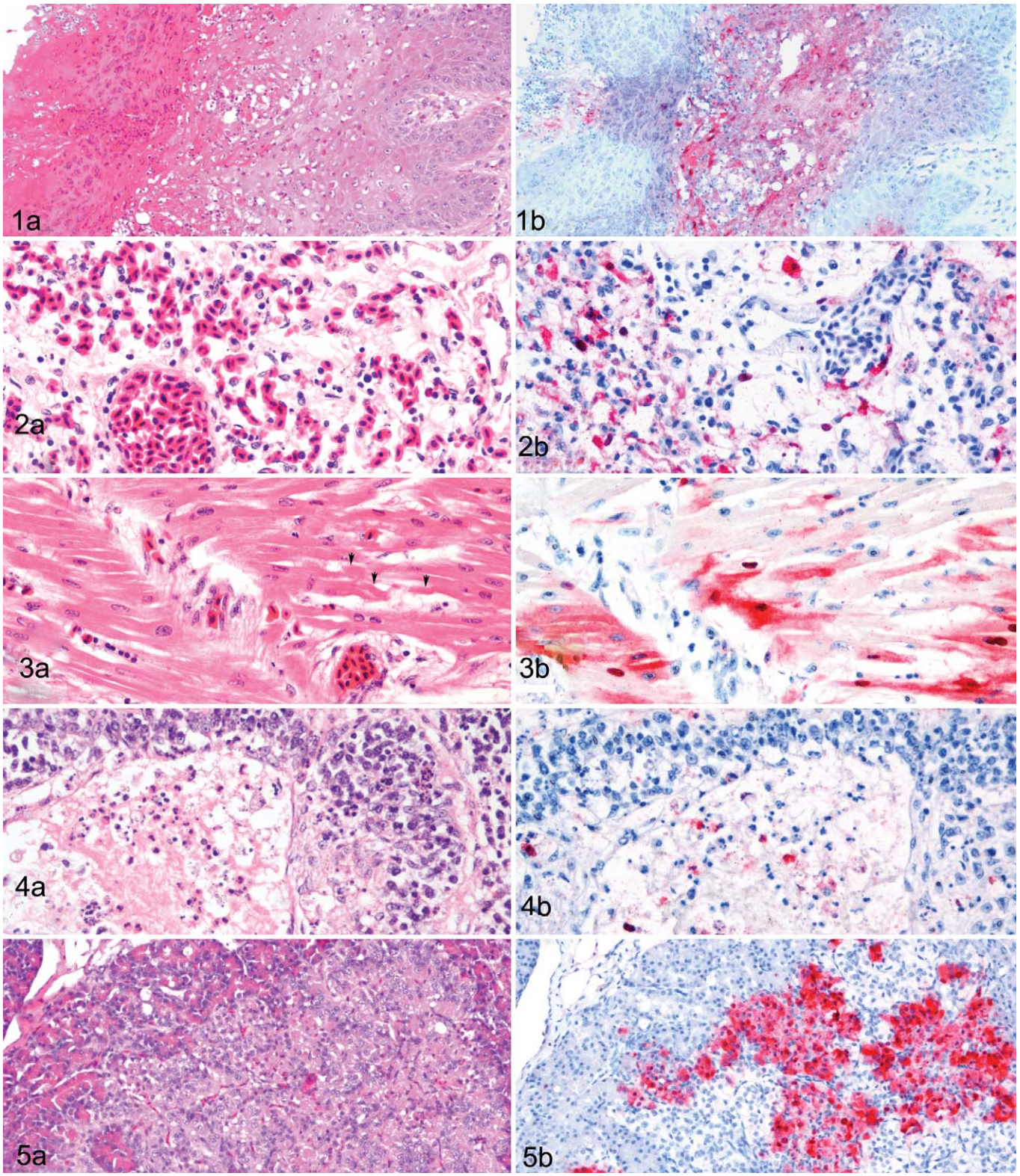

Figure 1. Oral cavity; mute swan, 5 days postinfection. A, severe vacuolar degeneration to coagulative necrosis of squamous epithelium of mucous membrane. HE. B, avian influenza viral antigen detected in intranuclear and intracytoplasmic locations within epithelium of mucosa. Biotin–streptavidin–peroxidase complex, hematoxylin counterstain. **Figure 2.** Lung; mute swan, 3 days postinfection. A, severe histiocytic interstitial pneumonia with moderate congestion and edema. HE. B, avian influenza viral antigen detected in the histiocytes and endothelial cells **Figure (continued).** (arrow) of small blood vessels. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 3.**

Ruddy shelducks. The distribution of lesions and antigenic staining paralleled those observed in the swans. However, the histological lesions, especially in the lungs, were generally less prominent and antigenic staining, less frequent. The pancreas, brain, heart, adrenal gland, and oral cavity were the tissues most affected in this species. The pancreata commonly had multifocal to confluent necrosis of exocrine parenchyma (Fig. 5A) and mild vacuolar degeneration of islets, with viral antigen being most consistent in acinar epithelium (Fig. 5B) and less frequently in islets cells. The cerebrum of the brain had moderate neuronal degeneration and necrosis, mild to moderate lymphocytic perivascular cuffs, mild perivascular edema and lymphocytic meningitis, and randomly scattered gliosis (Fig. 6A). The cerebellum had moderate necrosis of Purkinje cells, focal necrotic foci in the molecular layer, and some lymphocytic perivascular cuffs. Viral antigen was demonstrated in neuropil, neurons, glial cells (Fig. 6B), Purkinje cells and dendritic cells, granule cells, and ependymal cells. The heart had randomly scattered foci of myocyte degeneration with associated viral antigen. The adrenals commonly had degeneration and necrosis of corticotrophic cells and, less frequently, chromaffin cells, with associated viral antigen in the lesions (Figs. 7A, 7B). Mucosal epithelium of the oral cavity had focal degeneration and/or necrosis with abundant viral antigen. The spleen exhibited slight lymphocyte depletion, moderate sinusoidal congestion, and mild to moderate hemosiderosis. The liver had lysis of individual hepatocytes and mild proliferation of Kupffer cells. However, no viral antigen was detected in the liver or spleen. Viral antigen was demonstrated rarely to infrequently in scattered myofibers within the muscular layers of ventriculus (gizzard) and small and large intestines, ganglion cells in the myenteric plexi of the digestive tract, and histiocytes in the lungs. Lesions and viral antigen were absent in the trachea and skeletal muscle. An incidental finding was mild intestinal coccidiasis.

Mandarin ducks. One contact-exposed bird died at 5 DPE and had histological lesions similar to those in ruddy shelducks, but the distribution and intensity of viral antigen were more restricted in the mandarin duck, mainly affecting the nasal cavity, brain, and pancreas. Moderate to severe necrotizing rhinitis and sinusitis were present, and viral antigen was demonstrated in the necrotic cellular debris (Figs. 8A, 8B). The brain had multifocal moderate lymphocytic meningitis (Fig. 9A), diffuse moderate mononuclear perivascular cuffs, mild perivascular edema, mild focal gliosis, mild focal neuronal degeneration and necrosis, and minimal lymphocytic inflammation in the choroid plexus. Viral antigen was randomly distributed in the brain within ependymal cells, neurons, glial cells, and the epithelium of the choroid plexus (Fig. 9B). Moderate to marked multifocal necrotizing pancreatitis with mild lymphocytic inflammation was found, and viral staining was consistently demonstrated in pancreatic acinar epithelium. Additional histological changes observed in other organs included mild multifocal necrosis of cardiac myofibers, mild hepatocyte necrosis with minimal Kupffer cell hyperplasia, moderate lymphocytic depletion owing to apoptosis and necrosis in the spleen, and mild perivascular cuffs in the muscular layers of small intestine. Viral antigen was not detected in any other tissues.

The 2 intranasally infected mandarin ducks that were euthanized at 14 DPI lacked lesions and viral antigen, except for mild lymphocytic cuffs around blood vessels and peripheral nerves in the tunica muscularis of the small intestine (duodenum and jejunum).

Greylag geese. All greylag geese were euthanized at 14 DPI or 13 DPE and had lesions restricted to the brain, primarily in the cerebrum and midbrain. The brain lesions consisted of diffuse, severe malacia characterized by severe destruction of the neuropil, variable degrees of neuronal loss, moderate dystrophic calcification of necrotic neurons and neuropil, mild to moderate gitter cell and mononuclear cell infiltrates, severe lymphocytic perivascular cuffs, focal hemorrhage, and moderate spongiform degeneration (Fig. 10A). Surrounding the malacia was moderate neuronal satellitosis, marked mononuclear perivascular cuffs, and astrogliosis. The meninges had severe mononuclear cell infiltration with moderate edema. The cerebellum of the contact-exposed bird had focal neuronal necrosis. Viral antigen was infrequently detected in necrotic neurons and neuropil and rarely in glial cells and gitter cells within the malacia areas (Fig. 10B). Minor lesions that were identified included occasional urate deposition within kidney tubules and mononuclear cell infiltration around blood vessels and peripheral nerves. Viral antigen was lacking in all tissues other than the brain.

Mallard ducks. Specific lesions were lacking in all tissues, and viral antigen was not demonstrated.

Discussion

The clade 2.2 H5N1 HPAI viruses, represented by the prototype virus A/Bar-headed goose/Qinghai/1A/05, have spread from Asia to Europe and Africa, and they have been associated with mortality in swans, geese, and some wild ducks. However, mortality in the most common duck species, the mallard, has been infrequent.[8] In this study, we examined the infectivity and pathogenicity of a clade 2.2 virus, Ck/Kr/06, for 5 waterfowl

Heart; mute swan, 5 days postinfection. A, segmental necrosis of myofibers (arrowheads) with moderately swollen nuclei. HE. B, avian influenza viral antigen detected in the myofibers and endothelium of blood vessels. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 4.** Cloacal bursa; mute swan, 5 days postinfection. A, severe lymphocytic depletion with necrotic cellular debris. HE. B, avian influenza viral antigen detected in the cellular debris and histiocytes. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 5.** Pancreas; ruddy shelduck, 5 days postinfection. A, severe diffuse necrotizing pancreatitis. HE. B, avian influenza viral antigen detected in degenerating and necrotic acinar epithelial cells. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain.

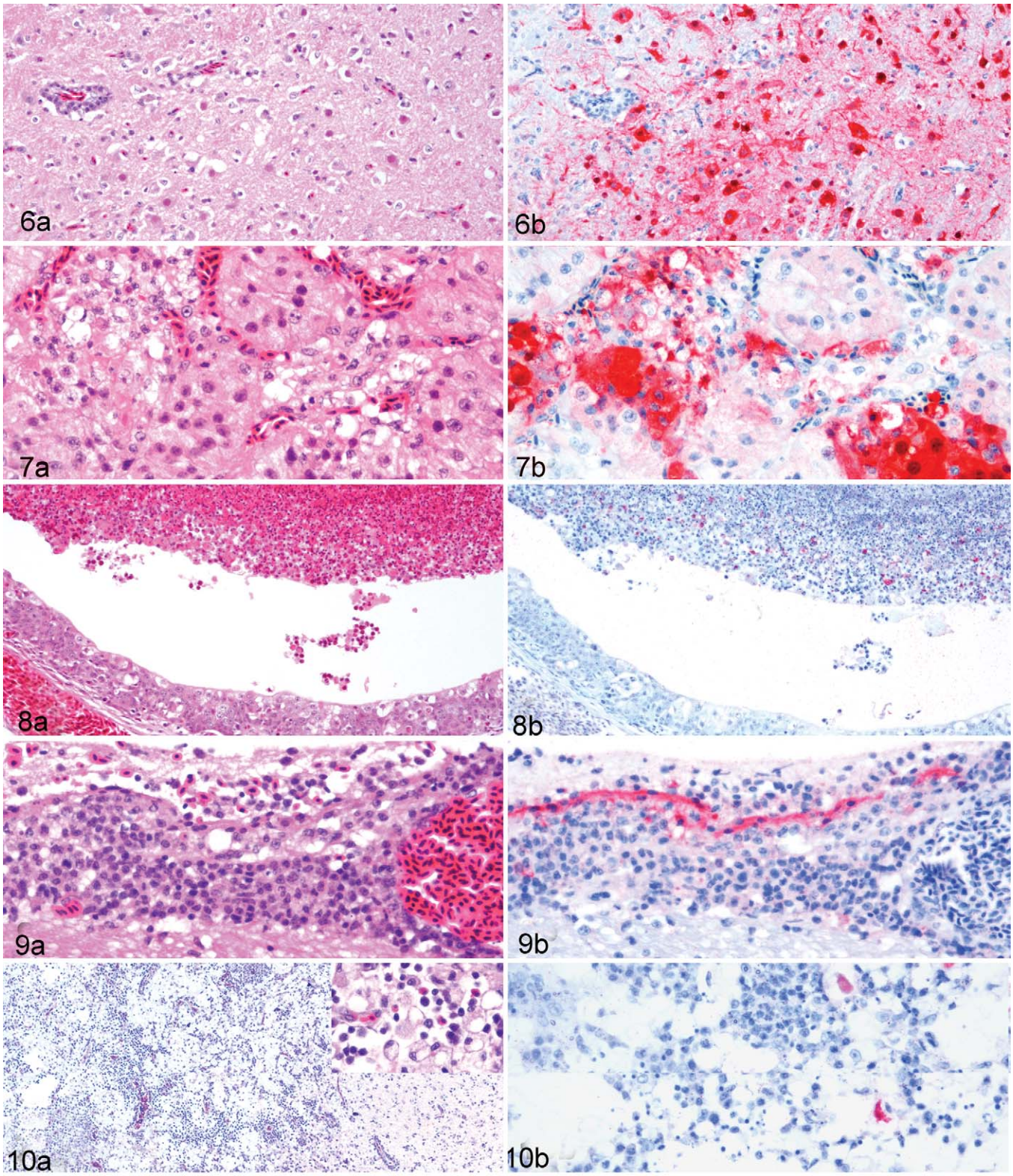

Figure 6. Brain; ruddy shelduck, 5 days postinfection. A, moderate degeneration and necrosis of neurons and glial cells with lymphocytic perivascular cuffings. HE. B, avian influenza viral antigen in neurons and glial cells. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 7.** Adrenal gland; ruddy shelduck, 5 days postinfection. A, moderate vacuolar degeneration to necrosis of adrenal corticotrophic cells. HE. B, avian influenza viral antigen detected in corticotrophic and chromaffin cells. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 8.** Nasal cavity; mandarin duck, 6 days postinfection. A, severe mucosal epithelial degeneration and **Figure 8 (continued).** necrosis with aggregated cellular debris in the airway. HE. B, avian influenza viral antigen detected in necrotic cellular

species that migrate between mainland Asia, Korean Peninsula, and Japan. All 5 waterfowl species became infected following virus inoculation or contact exposure and shed virus, mainly from the oropharynx and to lower frequency and quantity than from the digestive tract. However, there were species-related variability in the infections, as evident by differences in virus genome quantity detected in oropharynx and cloaca, severity of disease, distribution of viral antigen and lesions in tissues, and mortality patterns. In field cases associated with clade 2.2 H5N1 HPAI viruses in the European Union during the winter of 2006, most cases of mortality were identified in swans, and fewer cases were seen in ducks and geese, respectively.[24,33] However, within wild ducks, the mortality varied from a few to many, depending on the individual species.[19] Experimentally or in natural cases, the most severe infections in wild ducks were reported in wood ducks (*Aix sponsa*), tufted ducks (*Aythya fuligula*), and Eurasian pochards (*Aythya ferina*), whereas mallards were asymptomatically infected or had low mortality rates.[3,17,19]

Intranasal inoculation or contact exposure to the Ck/Kr/06 virus produced fatal infections in all mute swans, all ruddy shelducks, and 1 mandarin duck within 10 DPI or DPE. The vascular endothelial tropism (mute swans) and broad distribution of necrotic and inflammatory lesions in multiple visceral organs (mute swans, ruddy shelducks, and mandarin duck) with associated AI viral antigen staining suggest similar pathogenetic mechanisms for disseminated infections, as reported in gallinaceous birds and some other waterfowl infected with various HPAI viruses.[18,21,29,30,32] The current data are also consistent with field observations and other experimental studies that have shown (1) that swans and ruddy shelducks are highly susceptible to infection and the pathological effects of H5N1 HPAI viruses and (2) that such infections can be lethal, resulting from multiorgan virus replication and associated lesions.[3,4,8,16,17,19,24,26,44,45]

In chickens and other gallinaceous species, vascular damage (eg, severe pulmonary edema, congestion, microthrombosis) and viral antigen in the vascular endothelium have been commonly reported and may be responsible for fatal outcome.[40] However, such widespread vascular damage and viral antigen in endothelium have been less commonly reported in waterfowl, most recently in black swans and wood ducks but rarely in domestic Pekin ducks or mallards infected with HPAI viruses.[2-4,30] However, of 5 species examined in this study, only the mute swans had widespread vascular damage with antigen detected in the endothelium of capillaries, small arteries, and veins in various visceral organs, including lungs, intestine, heart, nasal cavity, and brain. These lesions could explain the early mortality possibly attributed to ischemia from vascular thrombosis and multiple organ failure or widespread dissemination of virus with multiorgan replication and damage.[40] Previous experimental studies in swans have identified endothelial tropism, but such tropism was less prominent in natural infections, suggesting multiple pathological mechanisms involved in disease and death.[4,16,26,44] Such endothelial tropism was most prominent in black swans that died at 2 and 3 DPI and less prominent in trumpeter (*Cygnus buccinator*), whooper (*Cygnus cygnus*), and mute swans that died at 4 to 9 DPI.[4]

In contrast to endothelial tropism, mute swans, ruddy shelducks, and mandarin ducks had multiple virus-associated necrotic lesions in many critical organs, suggesting that failure of one or more organs (eg, heart, adrenals, pancreas, brain, liver) was responsible for clinical signs and death. Moderate to severe necrotizing pancreatitis, myocarditis, and neuronal necrosis in brain or meningoencephalitis with associated viral antigen have been most commonly identified, and such tissue tropisms for H5N1 HPAI viruses have been demonstrated in multiple avian species, including migratory waterfowl.[3,4,16,17,26,28,44] However, some waterfowl species have less severe disease signs and outcomes, typically affecting only one or two systems (eg, nervous system). In our study, the greylag geese developed nonfatal clinical neurological disease, and at termination of the study, neurotropism was confirmed by severe cerebral malacia and meningoencephalitis and demonstration of viral antigen in areas of inflammation. Clinically and histopathologically, these neuropathological findings resemble those seen in domestic geese experimentally infected with clade 0 H5N1 HPAI virus isolated during 1997[30] and in Canada geese infected with clade 1 H5N1 HPAI virus from Vietnam.[28] This indicates that these recent clade 2.2 viruses have retained their tissue tropism for the central nervous system of geese but that they can have pathophysiological effects for other visceral organs. Despite no mortality in our experimental study, we assume that infected geese in their natural habitat succumb to starvation, dehydration, or predation because of the persistent neurological dysfunction.[13]

Despite considerable variation in quantity of viral genome detected and length of time for virus detection, mallards have had limited infections and no mortality with H5N1 HPAI viruses in experimental studies, including A/duck meat/Anyang/01 (clade 0), A/turkey/Turkey/1/2005 (clade 2.2), Ck/Kr/06 (clade 2.2), and A/Whooper swan/Mongolia/244/2005 (clade 2.2).[3,17] Similar asymptomatic infections have been produced in other duck species with H5N1 HPAI viruses, including northern pintail (*Anas acuta*), blue-winged teal (*Anas*

debris in the airway. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 9** Brain; mandarin duck, 6 days postinfection. A, extensively severe lymphocytic meningitis with necrosis of outer mesothelial cells. HE. B, avian influenza viral antigen detected in necrotic ependymal cells. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain. **Figure 10** Brain; greylag goose, 14 days postinfection. A, diffuse severe encephalomalacia with mononuclear cellular inflammatory reactions. HE. *Inset:* Severe gitter cells and lymphocyte infiltration in the neuropil. HE. B, avian influenza viral antigen detected in individual necrotic neurons. Biotin–streptavidin–peroxidase complex with hematoxylin counterstain.

discors), redhead duck (*Aythya americana*), common teal (*Anas crecca*), Eurasian wigeon (*Anas penelope*), and gadwall (*Anas strepera*).[3,17] By contrast, experimental infections in the domestic duck, which is the same genus and species as the mallard and thus has the mallard as the ancestor, has produced a range of outcomes, from asymptomatic infections to neurological disease to severe systemic infection with death.[14,15,27,40] Factors that determine severity of disease and fatal outcomes include virus strain and plaque morphology, dose of virus, age of experimental ducks, duck strain, and route of inoculation. Compared to the other 4 waterfowl species in our study, the mallards had the lowest quantity of viral genome detected in oropharyngeal and cloacal swabs and the shortest period of detection (4 DPI or 3 DPE) without clinical signs and pathological changes. Similarly, previous studies reported that when infected with other clade 2.2 H5N1 HPAI (ie, Qinghai-like) viruses,[3,17] domestic ducks lacked clinical or pathological evidence but could become infected and excrete different levels of virus, depending on the virus strain—that is, maximum titer of $10^{3.5}$ $TCID_{50}$/ml of A/turkey/Turkey/1/2005 (H5N1) and shed for 4 days or less, maximum titer of $10^{2.1}$ EID_{50}/ml of A/duck meat/Anyang/01 (H5N1) and shed for 2 days or less, and no virus shed with A/Whooper Swan/Mongolia/244/05 (H5N1). Interestingly, the authors of these studies drew opposite conclusions regarding the role that mallards play as potential long-distance vectors of H5N1 HPAI virus.

These results indicate a significant variation in the pathobiology of A/Chicken/Korea/IS/06 virus infections in different species of wild waterfowl. The mute swans had strong vascular tropism, 100% mortality, and the most diverse, acute, severe organ involvement. Ruddy shelducks and mandarin ducks had limited multiple-organ involvement with low to high mortality; greylag geese had pathobiology limited to the nervous system without mortality; and mallards had asymptomatic infection without clinical signs, thereby demonstrating the diverse clinical and biological outcomes following infection of waterfowl with H5N1 HPAI viruses.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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Single Assay for Simultaneous Detection and Differential Identification of Human and Avian Influenza Virus Types, Subtypes, and Emergent Variants

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Abstract

For more than four decades the cause of most type A influenza virus infections of humans has been attributed to only two viral subtypes, A/H1N1 or A/H3N2. In contrast, avian and other vertebrate species are a reservoir of type A influenza virus genome diversity, hosting strains representing at least 120 of 144 combinations of 16 viral hemagglutinin and 9 viral neuraminidase subtypes. Viral genome segment reassortments and mutations emerging within this reservoir may spawn new influenza virus strains as imminent epidemic or pandemic threats to human health and poultry production. Traditional methods to detect and differentiate influenza virus subtypes are either time-consuming and labor-intensive (culture-based) or remarkably insensitive (antibody-based). Molecular diagnostic assays based upon reverse transcriptase-polymerase chain reaction (RT-PCR) have short assay cycle time, and high analytical sensitivity and specificity. However, none of these diagnostic tests determine viral gene nucleotide sequences to distinguish strains and variants of a detected pathogen from one specimen to the next. Decision-quality, strain- and variant-specific pathogen gene sequence information may be critical for public health, infection control, surveillance, epidemiology, or medical/veterinary treatment planning. The Resequencing Pathogen Microarray (RPM-Flu) is a robust, highly multiplexed and target gene sequencing-based alternative to both traditional culture- or biomarker-based diagnostic tests. RPM-Flu is a single, simultaneous differential diagnostic assay for all subtype combinations of type A influenza viruses and for 30 other viral and bacterial pathogens that may cause influenza-like illness. These other pathogen targets of RPM-Flu may co-infect and compound the morbidity and/or mortality of patients with influenza. The informative specificity of a single RPM-Flu test represents specimen-specific viral gene sequences as determinants of virus type, A/HN subtype, virulence, host-range, and resistance to antiviral agents.

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Competing Interests: The authors from TessArae, LLC, (Tibbetts, Schafer, Lorence, Lichanska, Borsuk, Weslowski) are full-time employees of a for-profit limited liability corporation. The other authors have no financial conflicts of interest. TessArae, LLC holds the license to multiple United States Government Patents and Patents Pending, through the Department of the Navy, that relate to all commercial applications of Resequencing Pathogen Microarray (RPM) for screening, diagnosis and/or surveillance of pathogen-induced disease. Several contributing co-authors (including Stenger, Lin, Malanoski, Schnur and Tibbetts) are co-inventors of inventions covered by to the patents assigned to the U. S. Government, Department of the Navy.

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Introduction

There are sixteen recognized serological subtypes of type A influenza virus hemagglutinin (H1 through H16) and 9 type A neuraminidase subtypes (N1 through N9). Among the combinatorial diversity of 144 possible A/HN subtypes, relatively few subtypes have been identified as causes of human disease. Four pandemic outbreaks in the last century, one catastrophic, appear to have introduced the subsequently prevalent seasonal human influenza virus subtypes A/H1N1 (Spanish flu, 1918), A/H2N2

(Asian flu, 1957), A/H3N2 (Hong Kong flu, 1968), and A/H1N1 again (Swine flu, 1976; Russian flu, 1977). The current year 2009 has been marked by a late season pandemic-scale emergence of a novel A/H1N1 outbreak strain, raising immediate concerns for public health as well as for pork and poultry production industries worldwide.

As with the few common subtypes of human type A influenza viruses, there are similarly few subtypes of type A influenza viruses that are associated with most influenza infections of swine, horses or dogs. In distinct contrast, wildfowl species are natural hosts and a global reservoir for the majority of possible influenza A/HN subtypes. Many of these variant strains appear to be associated with endemic infections, often asymptomatic in avian hosts [1]. Incidental infections of humans by avian influenza viruses have been documented for avian influenza subtypes A/H5N1, A/H7N2, A/H7N3, A/H7N7, A/H9N2, A/H10N7 and A/H11N9. Recent outbreaks of "bird flu" may foreshadow an eventual pandemic outbreak, in the emergence of strains and variants with enhanced pathogenicity, virulence and transmissibility in human hosts. Examples of such outbreaks include A/H5N1 Hong Kong, 1997; H9N2 Hong Kong, 1999; A/H7N7 Netherlands, 2003; A/H5N1 Southeast Asia, 2004. Some avian A/H5 and A/H7 strains of influenza virus are recognized as highly pathogenic (HP) in domestic poultry and concerns arise that this phenotype may carry over to infections of humans. Since 1997, human infections associated with the Eurasian-African lineage of A/H5N1 HP avian influenza virus have been associated with 467 documented cases in 15 countries with high mortality (282 deaths) [2; updated 30 December 2009].

Fortunately, infectious transmission of such avian influenza virus strains between humans continues to be limited. However, history suggests that further evolution of these or other type A influenza strains could emerge as a next pandemic strain. Similarly, variant type A influenza virus strains have emerged from time to time, imposing serious costs and burdens upon poultry and livestock production.

Because the natural history and the molecular biology of influenza viruses reflect such viral genome diversity, there is a critical need for rapid, sensitive, specific, and informative assays to detect and characterize any subtype of influenza virus. Benchmark standard methods that employ propagation of virus in cell culture or in embryonating chicken eggs, with assays using panels of specific serological reagents, or reverse transcriptase polymerase chain reaction (RT-PCR)-based assays, using panels of short oligonucleotide primers and probes, are either slow and time consuming, or expensive. As prevailing strains of avian influenza continue to evolve and diverge, diagnostic assays that are based only on specific recognition of short signature sequences or peptide biomarker loci will increasingly fail, through false-positive and/or false-negative results. This will adversely impact critical decision-making.

This report describes a re-sequencing pathogen microarray (RPM)-based assay for simultaneous detection, identification and characterization of any subtype of type A human or avian influenza virus, based on rapid, sensitive and specimen-specific determination of nucleotide sequences from viral hemagglutinin, neuraminidase, and other genes.

Methods

Ethics Statement

All specimens described in this report that were originally obtained from human subjects were obtained with informed (verbal) consent of study participants allowing for further research use, following Institutional Review Board-approved research protocol NHRC.1999.0002, Triservice Population-Based Surveillance for Respiratory Pathogens Among High-Risk Military Personnel, CAPT Kevin Russell, MC, Principal Investigator. The NHRC IRB approved verbal consent for this protocol because of minimal risk to volunteers. Samples are collected for accredited diagnostic testing, and only used for further research in a de-identified manner with minimal demographic data (date and site of collection, age, sex), tested only for respiratory pathogens and analyzed in aggregate, and resulting data is not used for patient treatment or management. No part of this study was conducted as experimentation involving live vertebrate animals. Reference strains of avian influenza viruses were propagated in laboratory cultures for analysis of viral RNA.

Specimen Collection and Sample Processing

The Naval Health Research Center (NHRC) collects, analyzes, and archives throat-swab specimens from human subjects as part of respiratory infection surveillance at US basic military training facilities. Research use of donated specimens is permitted under the local Institutional Review Board's approved protocol-compliant informed consent (see Acknowledgments). Throat swabs are suspended in stabilizing transport media and archived frozen at −80°C. Viral culture and PCR-based testing protocols are used at the NHRC diagnostic laboratory to determine the presence or absence of influenza virus, and these assays are accredited by the College of American Pathologists as compliant with the Clinical Laboratory Improvement Amendments of 1988, and the Department of Defense Clinical Laboratory Improvement Program of 1994.

Cloacal swabs and/or tracheal swabs from migratory birds (mostly waterfowl) and commercial poultry; tracheal and lung tissue samples from dead birds; human throat swabs and one human lung sample from a deceased patient were collected by Naval Medical Research Unit No. 3 (NAMRU-3, Cairo, Egypt). Avian influenza viruses in these samples were cultured using chicken eggs and/or Madin-Darby canine kidney cell cultures (MDCK, American Type Culture Collection, CCL-34). Sample collection and viral culture techniques were as described [3]. Total RNA was extracted and purified from culture isolates, and 1:100 to 1:1000 dilutions were used for diagnostic evaluations. Aliquots were forwarded from NHRC to the Naval Research Laboratory (NRL, Washington, DC) for RPM-Flu assay (TessArray RPM-Flu 3.1 Kit (RPM-Flu), TessArae, LLC, Potomac Falls, VA).

The U.S. Department of Agriculture, Agriculture Research Service (USDA-ARS) Southeast Poultry Research Laboratory (SEPRL, Athens, GA) selected specimens from its reference strain archive for blinded analysis by RPM-Flu. These samples comprised 20 representative avian influenza A/HN strains and also included 2 avian paramyxoviruses as controls. Total nucleic acid extractions and purifications (MagNA Pure, Roche Applied Science, Indianapolis, IN) were performed following harvest from infected eggs at SEPRL. A 30 µl aliquot from the final preparation was frozen and forwarded for RPM-Flu analysis.

Reference Control Templates

The NRL group evaluated pathogen detection and identification capabilities of the RPM-Flu assay and kit components from TessArae, using control nucleic acid templates in assays *in lieu* of specimen total nucleic acid. Sources of control nucleic acid templates included type cultures of reference strain Eurasian-African A/H5N1 high pathogenicity influenza virus obtained from the Centers for Disease Control and Prevention (Atlanta, GA). Reference control templates as mixtures of the A/H1N1, A/

H3N2, and B strains of influenza virus were from live virus preparations (Influenza Virus Vaccine Live, Intranasal, FluMist, 2004–2005 Formula; MedImmune Inc., Gaithersburg, MD), or from inactivated virus preparations (Influenza Virus Vaccine [Fluvirin] Purified Surface Antigen Vaccine, 2006–2007 Formula, Package Insert; Chiron Corporation/Novartis Vaccines and Diagnostics, Inc., Emeryville, CA). Synthetic DNA preparations were purchased from Blue Heron Biotechnology (Bothell, WA) as templates to represent other type A influenza hemagglutinin (H2, and H4 through H16) and neuraminidase (N3 through N9) gene sequences, as well as other pathogen gene sequences that are used as detectors on the RPM-Flu array. In some cases, the NRL group outsourced (Macrogen USA, Gaithersburg, MD) corroborative *de novo* gene sequence determinations from amplified products from hemagglutinin (HA), matrix (M), neuraminidase (NA), non-structural (NS1) and RNA-dependent RNA polymerase subunit (PB2) genes.

RPM-Flu Assay

Figure 1 illustrates the pathogen gene re-sequencing capacity of the RPM-Flu assay as both complementary strands of 117,254 bp of gene sequences, distributed across 188 detector tiles, representing 30 different categories of viral and bacterial respiratory pathogens. The 30 other types of viruses and bacterial targeted by the RPM-Flu assay as agents of flu-like illness are identified in Supplemental Information Figure S1. A single total nucleic acid preparation from a single aliquot of a single specimen is simultaneously assayed for possible detection and illumination of specimen-specific gene sequences from each of the 188 independent target pathogen gene sequencing detector tiles on the array.

The largest segment of the RPM-Flu microarray (41,063 bp) is allocated to 40 detector tiles to represent the multiple subtypes of type A and type B HA and NA genes, selected type A M genes, and conserved NS and PB2 gene sequences from avian influenza virus A/H5N1.

Selected specimens were thawed to retrieve aliquots for extraction and purification of total nucleic acid for multiple assays, including RPM-Flu testing. Each RPM-Flu assay consumed about 30 µl (2%) of the originally archived specimen (about 1.5 ml). RPM-Flu analysis of specimen total nucleic acid followed the manufacturer's recommendations (TessArae, LLC; protocol details are provided with the RPM-Flu 3.1 Kit User Manual and are available online at www.tessarae.com).

The RPM-Flu assay protocol is executed as a series of specimen and sample processing steps, listed below and indicating approximate time required for each step:

1. Extraction of total nucleic acid; reverse transcription to convert target RNA sequences to cDNA – 1 hour
2. Multiplexed amplification of targeted gene sequences by thermal cycling – 2.5 hour
3. Pooling, purification and fragmentation of amplification products – 0.5 hour
4. End-labeling (biotinylation) of amplification products – 0.5 hour
5. Hybridization of labeled products to microarrays – 4 hour to 16 hour (overnight preferred)
6. Washing and staining arrays – 1.5 hour
7. Scanning arrays for data acquisition – 0.15 hour

The abbreviated 4-hour hybridization time (5. above) reduces assay cycle time to single day, which may be helpful if same-day test results are a critical consideration. The overnight (~16 hour) hybridization time is recommended and preferred to optimize

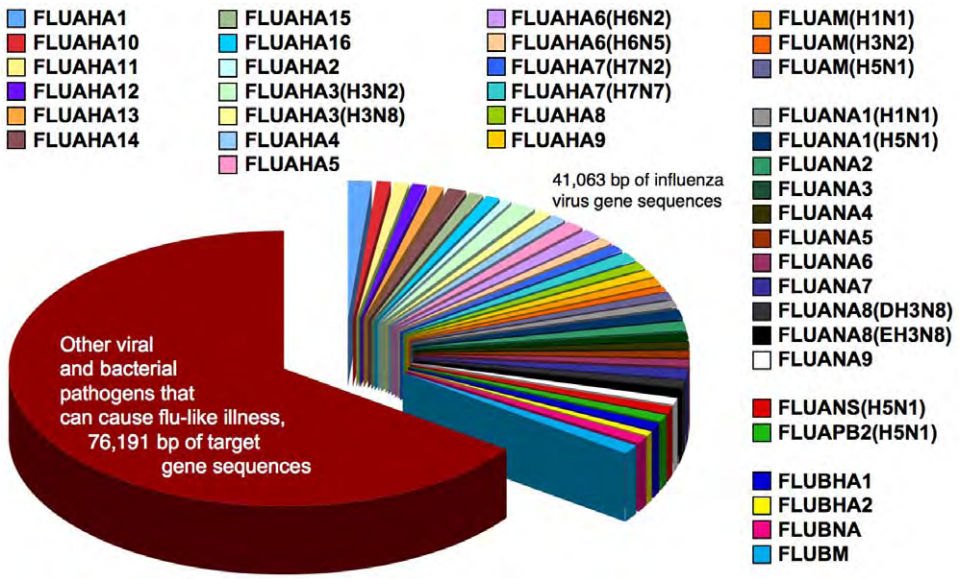

Figure 1. Influenza virus gene targets of the RPM-Flu assay. The TessArray RPM-Flu 3.1 allocates 40 detector tiles (41,063 bp) that represent influenza virus hemagglutinin genes (16 type A and 1 type B) and neuraminidase genes (9 type A and 1 type B), matrix genes (3 type A and 1 type B), and also the conserved NS and PB2 genes of avian influenza virus strain A/H5N1. The remaining 76,191 bp of re-sequencing capability on the microarray (as large red wedge) are distributed among 2 control detector tiles and 142 detector tiles that represent genes of other pathogens that may cause "flu-like" respiratory illness in humans. These other pathogens include viruses (adenovirus, enterovirus and rhinovirus, coronavirus, herpesvirus, measles virus, metapneumovirus, parainfluenzavirus, respiratory syncytial virus, rubella virus, and poxvirus [variola]) and bacteria (*Bacillus [anthracis]*, *Bordetella*, *Chlamydophila* and *Chlamydia*, *Corynebacterium*, *Francisella*, *Haemophilus*, *Klebsiella*, *Legionella*, *Moraxella*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Streptococcus*, *Staphylococcus* and *Yersinia*).
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both assay sensitivity and the quality and length(s) of assay-generated gene sequences from detected target pathogens.

RPM-Flu Assay Data Analysis

Analysis of RPM-Flu assay data now differs from earlier reported through the development of the prototype assay platform RPMv1 [4,5]. Each RPM-Flu assay of a specimen generates sequence data as base calls (A, G, C, or T) across each detector tile of Affymetrix CustomSeq-formatted microarrays [6] (see Affymetrix Technical Note, 2006, GeneChip CustomSeq Resequencing Array Base Calling Algorithm Version 2.0: Performance in Homozygous and Heterozygous SNP Detection). Image data acquired from GeneChip Instrument System scanning under GeneChip Operating Software control is translated by GSEQ sequence analysis software (Affymetrix Inc., Santa Clara, CA).

TessArray Sequence Analysis (TSEQ) software evaluates a "C3 Score" for each RPM-Flu detector tile, as a metric of detected DNA sequence quantity and quality. The C3 Score is the total number of GSEQ-identified nucleotides that appear in runs of three or more consecutive (non-N) base calls, expressed as percentage of the length (nucleotides) of each RPM-Flu detector tile sequence. This approach to definition of a detected sequence reduces background noise from spurious false-positive hybridizations of 25-base oligonucleotide probes that could lead to isolated and relatively uninformative single base calls.

Relaxed and stringent target pathogen detection and reporting thresholds for the RPM-Flu assay have been statistically and empirically established for all of the resequencing detector tiles at C3 Score >10 or C3 Score ≥ 20, respectively. RPM-Flu assay results from hundreds of clinical and field specimens as well as laboratory reference strains and synthetic DNA templates (aggregate data not shown) have demonstrated that these detection thresholds often represent C3 Scores that are more than 6 standard deviations ($>6\sigma$, $P<0.000003$) above the mean of C3 Scores from thousands of detector tiles from assays performed in the absence of positive control templates.

All sequences from an RPM-Flu assay that meet a detection and reporting threshold are automatically subjected to alignment-based search of the TessArray Validated Reference Sequence Database (VRSD) (most recently validated instance 10 August 2009), using a dedicated server-based implementation of the Basic Local Alignment Search Tool, BLAST, version 2.2.17 [7]. This enables identification of one or several most similar (and equivalently matching) sequence records for each target pathogen gene sequence that may be generated in the assay of every specimen. Adjustable parameters for the BLAST search are set as: word size 11 (or 7 if C3 Score <30), match/mismatch penalty $+1/-1$, gap costs as existence/extension $+2/+1$, low complexity region filter off, and mask options off.

In order to qualify for reporting of target pathogen detection using the relaxed threshold (C3 Score >10), a BLAST alignment and sequence similarity search using the assay-generated sequence of basecalls must return one or more most similar sequence records from the search database (as TessArae's VRSD) that are concordant with the identity of the tentatively identified target pathogen suggested by the identity of the particular resequencing detector tile.

Viral Stocks for Determinations of Assay Sensitivity

Aliquots from two throat swab specimens collected during the 2008–2009 influenza were used for propagation of stocks by infection of MDCK cell cultures.

A/California-NHRC/BRD10622N/2009(H1N1)
A/California-NHRC/BRD10601N/2009(H3N2)

Infected culture lysates were then diluted for infections of replicate shell vial cultures to determine infectious titers (performed by ViraPur, San Diego, CA). The resulting stocks of BRD10622N(H1N1) and BRD10601(H3N2) were determined to contain 3.16×10^7 $TCID_{50}$/ml and 1.78×10^7 $TCID_{50}$/ml, respectively.

Serial ten-fold dilutions of viral stocks for RPM-Flu assays were prepared using fresh cell culture media as diluent, from 10^0 (neat) to 10^{-3}, and then three-fold dilutions were prepared from 1:3,000 to 1:2,187,000. The routine RPM-Flu protocol for total nucleic extraction and purification was performed on each of the triplicate diluted samples of the viral stocks for identity-blinded triplicate RPM-Flu assays.

Results

RPM-Flu Specificity: Analysis of Trivalent Influenza Vaccine

The influenza virus subtype A/H1 gene detector tile 'HA1(H1N1)' on the RPM-Flu microarray is an array of 11,808 resequencing oligonucleotide probes (25-mers), representing the nucleotides 113 through 1,588 of the A/New Caledonia/20/99 (H1N1) strain hemagglutinin gene (GenBank Accession Number AY289929). This is the actual A/H1N1 strain of influenza virus representing the viral HA1 gene product in the 2004–2005 vaccine configuration.

An aliquot of FluMist vaccine (2004–2005) was subjected to RPM-Flu assay, from which 1,351 specific base calls were determined across the HA1(H1N1) detector tile, resulting in C3 Score = 91.5. BLAST analysis returned AY289929 A/New Caledonia/20/99 (H1N1) as the most similar sequence record from alignment-based search across the entire GenBank, with the limit (minimum) expectation value of 0.0 (or E-value = 1.0e-180).

Figure 2 shows the alignment of a 540-nucleotide segment of this RPM-Flu assay detected sequence, below corresponding intervals of the RPM-Flu detector tile sequence. This part of the RPM-Flu HA1(H1N1) detector tile spans the locus of the A/H1 hemagglutinin peptide cleavage site [8]. Each of the nine successive 60 bp RPM-Flu assay-detected HA1 sequence segments shown in the inset was subjected to BLAST alignment and similarity analysis. These short query sequences (60 bp) are identical matches to corresponding hemagglutinin sequence segments of many different A/H1N1 isolates, but in each case, the list of identical sequence record(s) returned by BLAST includes one or more A/New Caledonia/20/99 (H1N1) records. This includes those 60 bp lines with runs of up to 12 uncalled bases (N). With the exception of small gaps of uncalled bases, the detected sequence is identical to the prototype sequence of the RPM-Flu HA1(H1N1) detector tile.

This example of the integrity of RPM-Flu assay-generated target pathogen gene sequencing for detection and identification is reinforced by results for the other specific hemagglutinin, neuraminidase, and matrix gene sequences from the same RPM-Flu assay of the same aliquot of FluMist vaccine. Table 1 shows that the most similar sequence records returned from BLAST analysis of assay-generated HA1, NA1, HA3, NA2, M(H1N1), M(H3N2) gene sequences, as well as type B HA, NA and M gene sequences, all correctly correspond to the actual influenza virus strains used to represent these components in the 2004–2005 vaccine configuration. This result is achieved even for instances (HA3, NA2, B-HA, B-NA and both the type A and type B M

```
Upper sequence is detector tile HA1(H1N1):
  AY289929 HA1 Influenza A/New Caledonia/20/99(H1N1)
Lower sequence assay result from detector tile HA1(H1N1):
  RPM-Flu Assay of NRL_KB_FluVaccine_082206

  761                                                        820
    ATACAATAATATTTGAGGCAAATGGAAATCTAATAGCGCCATGGTATGCTTTTGCACTGA
    ATACAATAATATTTGAGGCAAATGGAAATCTAATAGCGCCATGGTATGCTTTTGCACTGA

  821                                                        880
    GTAGAGGCTTTGGATCAGGAATCATCACCTCAAATGCACCAATGGATGAATGTGATGCGA
    GTAGAGGCTTTGGATCAGGAATCATCACCTCAAATGCACCAATGGATGAATGTGATGCGA

  881                                                        940
    AGTGTCAAACACCTCAGGGAGCTATAAACAGCAGTCTTCCTTTCCAGAATGTACACCCAG
    AGTGTCAAACACCTCAGGGAGCTATAAACAGCAGTCTTCCTTTCCAGAATGTACACCCAG

  941                                                        1000
    TCACAATAGGAGAGTGTCCAAAGTATGTCAGGAGTGCAAAATTAAGGATGGTTACAGGAC
    TCACAATAGGAGAGTGTCCAAAGTATGTCAGGAGTGCAAAATnAAGGATGGnTACAGGAC

  1001                P  S  I  Q  S  R//G  L  F                1060
     TAAGGAACATCCCATCCATTCAATCCAGAGGTTTGTTTGGAGCCATTGCCGGTTTCATTG
     TAAGGAACATCCCnTCCATTCAATCCAGAGGTTTGTTTGGAGCCATTGCCGGTTTCATTG

  1061                                                       1120
     AAGGGGGGTGGACTGGAATGGTAGATGGGTGGTATGGTTATCATCATCAGAATGAGCAAG
     AAGnnnnGTGGACTGGAATGGTAGATGGGTGGTATGGTTATCATCATCAGAATGAGCAAG

  1121                                                       1180
     GATCTGGCTATGCTGCAGATCAAAAAAGTACACAAAATGCCATTAACGGGATTACAAACA
     GATCnggnnnnnnnnCAGATCAAAAAAGTnCACAAAATGCCATTAACGGGATTACAAACA

  1181                                                       1240
     AGGTGAATTCTGTAATTGAGAAAATGAACACTCAATTCACAGCTGTGGGCAAAGAATTCA
     AGGTGAnnnnnnnnnnnnnAGAAAATGAACACTCAATTCACngcnGTGGGCAAAGAATTCA

  1241                                                       1300
     ACAAATTGGAAAGAAGGATGGAAAACTTAAATAAAAAAGTTGATGATGGGTTTCTAGACA
     ACAAATTGGAAAGAAGGATGGAAAACTTAAATAAAAAAGTTGATGATGGGTTTCTAGACA
```

Figure 2. RPM-Flu assay-generated sequences from analysis of tri-valent influenza virus vaccine. A 540-nucleotide segment of the reported HA1 hemagglutinin sequence is shown aligned to the corresponding RPM-Flu HA1 gene detector tile sequence. This segment of the RPM-Flu HA1(H1N1) detector tile spans the locus of the A/H1 hemagglutinin peptide cleavage site (red box).
doi:10.1371/journal.pone.0008995.g002

genes) where the RPM-Flu detector tile sequences and actual vaccine gene sequences do not precisely match. This reflects the robust capability of the RPM-Flu resequencing microarray to determine accurately the match or alternative mismatch of each target gene nucleotide relative to corresponding nucleotide of the detector sequence. These results also demonstrate the non-interference of similar assay targets for RPM-Flu detection and identification of multiple target gene sequences from the same specimen.

Similarly specific typing, subtyping and strain identification results to those shown in Table 1 have been obtained from analyses of live and inactivated influenza vaccines configured for the 2005–2006, 2006–2007 and 2009–2010 seasons (results presented as SUPPLEMENTAL INFORMATION Tables S1, S2, S3 and S4). We note that results of assays using inactivated influenza virus vaccines identify detected type A matrix gene sequences are most closely (and correctly) matched by matrix gene sequence records from the A/Puerto Rico/8/34 (H1N1) master donor strain that is used for gene segment re-assortment to select for the inactivated vaccine strains.

The results in Table 1 are similar to those presented by Wang et al. [9, that report's Table 4]. That earlier work was performed using the RPMv1 prototype precursor platform of the TessArray RPM-Flu 3.1. The RPMv1 allocated only 7,691 base pairs (bp) to 9 different influenza-specific detector tiles, compared with the RPM-Flu assay that allocates 41,063 bp over 40 influenza virus-specific detector tiles (see Figure 1). The prototype RPMv1 assay also employed random primers in a reverse transcription and PCR-like protocol for total RNA amplification, and did not apply the C3 quantity/quality metric methodology described in this report for analysis of RPM-Flu assay-detected target pathogen gene sequences.

RPM-Flu Specificity: Mutually Exclusive Detection and Identification of Different Subtypes of Human and Avian Influenza Viruses

Table 2 presents HA, NA and M gene detector tile C3 Scores from RPM-Flu assays of samples representing prevalent human subtypes A/H1N1, A/H3N2, and type B influenza viruses, inactivated trivalent influenza virus vaccine, and selected A/HN subtypes of type A avian influenza viruses. C3 Scores that failed to meet or exceed the RPM-Flu assay detection and reporting thresholds are shown in parentheses and in smaller font size to

Table 1. Influenza type A and type B detector tile sequences and RPM-Flu assay-based identification of gene sequences by RPM-Flu assay of FluMist trivalent vaccine.

RPM-Flu detector title prototype sequences	C3 Score	E-value	SNPs[a]	Best matching sequence record (BLAST/GenBank)
Hemagglutinin genes				
A/New Caldedonia/20/1999 (H1N1)	64.0	1e-180	0/1039	A/New Caledonia/20/1999 (H1N1)
A/Canterbury/125/2005 (H3N2)	75.0	1e-180	6/1176	A/Wyoming/3/2003 (H3N2)
B/Malaysia/2506/2004	40.6	1e-155	10/451	B/Jilin/20/2003
B/Shanghai/361/2002	82.2	1e-180	6/750	B/Jilin/20/2003
Neuraminidase genes				
A/New Caldedonia/20/1999 (H1N1)	78.2	1e-180	0/971	A/New Caledonia/20/1999 (H1N1)
A/Canterbury/125/2005 (H3N2)	80.7	1e-180	7/999	A/Wyoming/3/2003 (H3N2)
B/Malaysia/2506/2004	61.7	1e-180	3/826	B/Jilin/20/2003[b]
Matrix genes				
A/Canterbury/100/2000 (H1N1)	69.5	1e-180	6/630	A/Ann Arbor/6/1960 (H2N2)
A/Canterbury/125/2005 (H3N2)	69.5	1e-180	14/626	A/Ann Arbor/6/1960 (H2N2)
B/Memphis/13/2003	81.6	1e-180	11/796	B/Ann Arbor/1/1966

[a]Number of observed single base polymorphisms (SNPs), as single base mismatches of detector and specimen gene sequences that are flanked by consensus base calls/number of specific base calls. The A/H1N1 detector tile and vaccine specimen hemagglutinin and neuraminidase gene sequences are concordant (bold font, underlined).

[b]The original RPM-Flu analysis on 21 Aug 2007 returned four best matching neuraminidase gene sequence records, as B/Yamagata/1246/2003, B/Taiwan/102/2005, B/Bangkok/460/2003, and B/Roma/4/2002. When subjected to BLAST analysis again on 09 Nov 2008, two more best matching neuraminidase gene sequence records were returned, as B/Taiwan/34/2004 and B/Jilin/20/2003. The actual influenza type B vaccine strain was B/Jilin/20/2003, but its neuraminidase gene sequence was not entered into the GenBank database until 21 Jul 2008.

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distinguish them from C3 Scores representing positive detection and identification of one or more of the RPM-Flu assay-targeted influenza viruses.

Except for the vaccine sample, assays of each sample reported sequences from only one hemagglutinin detector tile and one neuraminidase detector tile, those corresponding precisely to the specimen's A/HN subtype or B type.

The mean values of C3 Scores from the positive detector tiles in Table 2, and the background negative detector tiles, are respectively

Mean $C3_{pos} = 73.7 \pm 24.0$ (N = 22)

Mean $C3_{neg} = 3.2 \pm 2.3$ (N = 261)

[A six-sigma (6σ) detection threshold for this data set is C3 = 17.0]

The lowest positive control result from this data set was for the NA gene of the NHRC-type B influenza virus-positive specimen ($C3_{pos} = 13.7$). The same assay of this specimen generated higher C3 Scores for sequences from the B-HA (C3 = 31) and B-M (C3 = 34) detector tiles that exceeded the stringent detection threshold. The type B-NA sequence from the assay met the relaxed detection threshold (C3 Score >10), in part because BLAST analysis of the sequence returned nine most similar type B influenza virus NA gene sequence records. This corroborated a tentative detection report (relaxed threshold) that might have been made on the basis of the NA gene resequencing result alone. The specimen was collected in 2006 at U S Army Fort Leonard Wood, MO, and each of these most similar sequence records represented a NA gene from various circulating type B influenza virus strains collected during the same influenza season.

The RPM-Flu microarray includes four influenza virus matrix gene detector tiles, representing matrix gene sequences from selected reference strains of A/H1N1(human), A/H3N2(human), A/H5N1(avian) and B(human) types and subtypes. The results in Table 2 demonstrate specific discrimination and no apparent crosstalk between the three type A and the one type B matrix gene detectors. However for each of the human and avian subtypes of type A influenza viruses, it is generally the case that all three of the corresponding type A matrix gene detector tiles generate very similar and overlapping specimen-specific matrix gene sequences.

We have reviewed the lists of most similar sequence records returned by BLAST analysis of RPM-Flu assay-generated gene sequences from 12 different human (seasonal) A/H1N1 strains and 31 different human (seasonal) A/H3N2 strains.

Among RPM-Flu assay results from the 12 A/H1N1 samples there were 478, 800, 201, 348 and 312 most similar sequence records returned from BLAST analysis of sequences generated from the HA1(H1N1), NA1(H1N1), M(H1N1), M(H3N2), and NS(H5N1) detector tiles, respectively. Without exception, each of these most similar sequence records represents an A/H1N1 strain, including the 660 sequence records found to match the RPM-Flu sequences generated from the RPM-Flu M(H3N2) and NS(H5N1) detector tiles. Among the 31 A/H3N2 samples there were 754, 189, 1267, and 1678 most similar sequence records returned from the HA3(H3N2), NA2(H3N3), M(H1N1), and M(H3N2) detector tiles, respectively, and every one of these sequence records represents an A/H3N2 strain.

However, the NS (H5N1) detector tile generated 109 specimen-specific NS gene sequences from several of the A/H3N2 samples, and only 19 of the most NS gene sequence records were from A/H3N2 strains. The other NS gene sequence records that were found by BLAST to be most similar to the sequences generated from assays of A/H3N2 specimens represent subtypes A/H2N2 (12), A/H1N1 (73), A/H11N9 (2), A/H6N2 (2) and A/H3N6 (1). These results suggest that circulating strains of subtype A/H3N2 harbor diverse NS gene sequences, variations that presumably arise from frequent co-infections and genome segment reassortments in the field. On the other hand, the subtype A/H1N1 specimens led to RPM-Flu assay-generated NS gene sequences

Table 2. Detection of specific HA, NA and M gene sequences from individual type A influenza virus reference strains or trivalent vaccine mixture of reference strains.

Template →	NHRC A/H1N1	NHRC A/H3N2	NHRC type B	Fluvirin vaccine	USDA A/H4N6	USDA A/H7N1	USDA A/H8N4	NAMRU A/H10N7	USDA A/H14N5
HA Gene Detectors									
HA1(H1N1) human	**76.5**	(4.3)	(1.9)	**91.4**	(1.2)	(1.9)	(0.8)	(1.4)	(1.0)
HA2(H3N2) human	(4.0)	(4.6)	(2.1)	(1.8)	(1.3)	(2.3)	(1.2)	(0.4)	(2.1)
FLUAHA3 human	(2.6)	**89.4**	(3.0)	**94.3**	(3.9)	(6.9)	(3.3)	(0.0)	(4.1)
FLUBHA human	(5.9)	(9.6)	**31.0**	**95.9**	(4.2)	(3.1)	(3.5)	(1.9)	(2.4)
FLUAHA3 avian	(3.5)	(7.0)	(2.9)	(8.4)	(4.9)	(4.4)	(1.8)	(0.0)	(3.3)
FLUAHA4 avian	(3.3)	(6.3)	(1.8)	(2.0)	**65.0**	(0.7)	(0.6)	(1.1)	(5.6)
HA5(H5N1) avian	(5.1)	(7.5)	(3.6)	(2.5)	(2.3)	(2.3)	(2.4)	(0.4)	(1.9)
FLUAHA6 avian	(3.3)	(5.1)	(1.6)	(2.3)	(1.3)	(1.5)	(2.2)	(1.4)	(1.6)
FLUAHA7 avian	(2.4)	(6.6)	(3.7)	(5.6)	(2.3)	**88.5**	(3.0)	(2.9)	(4.3)
FLUAHA8 avian	(3.3)	(2.8)	(1.6)	(3.0)	(0.9)	(1.6)	**76.4**	(0.0)	(1.4)
FLUAHA9 avian	(2.5)	(5.8)	(3.9)	(3.4)	(2.5)	(1.5)	(2.7)	(1.5)	(2.9)
HA1(H1N1)0 avian	(2.9)	(5.7)	(3.0)	(0.3)	(1.2)	(4.9)	(0.3)	**61.8**	(0.7)
HA1(H1N1)1 avian	(4.5)	(5.1)	(4.0)	(1.7)	(4.0)	(3.4)	(3.4)	(2.3)	(2.5)
HA1(H1N1)2 avian	(2.1)	(4.5)	(0.6)	(0.7)	(0.3)	(0.5)	(2.4)	(0.3)	(0.0)
HA1(H1N1)3 avian	(2.1)	(6.1)	(1.6)	(1.5)	(0.4)	(0.3)	(0.5)	(0.0)	(0.3)
HA1(H1N1)4 avian	(4.2)	(8.6)	(3.3)	(3.3)	(5.7)	(4.9)	(3.4)	(2.4)	**86.3**
HA1(H1N1)5 avian	(5.3)	(5.8)	(3.4)	(1.8)	(1.2)	(4.0)	(1.7)	(0.0)	(1.8)
HA1(H1N1)6 avian	(3.9)	(4.8)	(2.6)	(1.2)	(0.4)	(0.4)	(0.8)	(0.4)	(0.3)
NA Gene Detectors									
NA1(H1N1) human	**84.9**	(6.9)	(2.5)	**87.8**	(2.7)	**14.0**	(2.1)	(1.4)	(3.0)
NA2(H3N2) human	(4.7)	**84.9**	(1.5)	**96.8**	(2.1)	(3.2)	(0.9)	(0.7)	(0.7)
FLUBNA human	(2.8)	(4.8)	**13.7**	**98.0**	(0.8)	(2.0)	(1.7)	(0.9)	(2.7)
NA1(H1N1) avian	(8.0)	(8.3)	(1.8)	(6.1)	(2.5)	**57.5**	(3.9)	(1.0)	(5.9)
FLUANA3 avian	(3.8)	(5.3)	(3.1)	(0.8)	(1.3)	(1.9)	(1.1)	(0.3)	(2.9)
FLUANA4 avian	(3.2)	(4.4)	(2.5)	(2.5)	(3.1)	(9.8)	**67.7**	(0.0)	(5.5)
FLUANA5 avian	(5.4)	(9.8)	(4.4)	(2.9)	(4.4)	(5.2)	(4.7)	(1.5)	**66.3**
FLUANA6 avian	(6.4)	(7.9)	(3.9)	(5.3)	**85.5**	(8.6)	(5.4)	(1.8)	(7.4)
FLUANA7 avian	(5.5)	(7.7)	(4.4)	(3.3)	(3.7)	(4.9)	(3.0)	**23.1**	(3.0)
FLUANA8 avian	(5.9)	(7.6)	(4.4)	(6.1)	(6.6)	(5.0)	(5.6)	(1.1)	(5.2)
FLUANA9 avian	(4.7)	(6.9)	(1.6)	(1.5)	(3.8)	(3.7)	(2.9)	(0.0)	(1.7)
M Gene Detectors									
M(H1N1)	**88.7**	**51.0**	(2.8)	**61.6**	**50.1**	**50.8**	**40.4**	**44.5**	**66.3**
M(H3N2)	**60.6**	**96.5**	(2.1)	**59.2**	**45.0**	**46.2**	**48.1**	**42.4**	**48.6**
M(H5N1)	**38.1**	**39.1**	(4.8)	**37.0**	**51.2**	**69.6**	**45.6**	**44.1**	**39.2**
FLUBM	(4.3)	(5.2)	**34.3**	**97.7**	(2.6)	(2.7)	(2.9)	(2.3)	(2.7)

C3 Scores from RPM-Flu gene sequence detector tiles demonstrate specific and differential detection of human and avian influenza virus A/HN subtypes and human type B influenza virus in assays with single control templates, field or clinical specimens, or the three-part inactivated virus vaccine mix (A/H1N1, A/H3N2, and B). C3 Scores in smaller font, within parentheses, represent sub-threshold negative detector tile reports.
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that are most similar to NS gene sequence records associated with subtype A/H1N1 strains.

Although it appears that the RPM-Flu assay-generated M gene sequences may have some utility to differentiate human A/H1N1 and human A/H3N2 subtypes, the NS gene sequences do not appear to be so reliable for the purpose of inferring A/HN subtype. This consideration will appear again later in RESULTS with the subject of indirect, inferred A/HN subtyping of type A avian influenza viruses.

Clinical Sensitivity and Clinical Specificity of the RPM-Flu Assay for Human Type A Influenza Viruses

The RPM-Flu assay was implemented in a blinded analysis of several hundred clinical specimens from the respiratory infectious disease archives at NHRC. Selected throat swab specimens had been collected over the period 2005–2007 from basic military trainees at several installations in the United States. The subjects were evaluated at the time of specimen collection and associated with one of three cohorts: apparently healthy and afebrile

individuals; those diagnosed with febrile respiratory illness (FRI); and those diagnosed with pneumonia (confirmed by chest x-ray).

A group of 298 specimens were analyzed using both the RPM-Flu assay and a validated benchmark RT-PCR assay for type A influenza virus described by Freed et al. [10]. Thirty specimens were identified as positive for type A influenza virus by both assays, and only 1 specimen was found to be negative by RPM-Flu and positive by RT-PCR. The remaining 267 specimens were found to be negative for influenza virus by both assays. All of the influenza-positive specimens were from participants that had been assigned to the FRI cohort.

Table 3 shows the calculation of high clinical sensitivity (97%) and even higher clinical specificity (100%) of RPM-Flu for detection and identification of type A human influenza virus, compared with results from testing the same specimens with the benchmark RT-PCR-based assay. Similarly high clinical sensitivity and high clinical specificity of RPM-Flu have been demonstrated with in the same ensemble of clinical specimens with respect to other simultaneously targeted pathogens of the assay, when similarly robust RT-PCR test panels were used for benchmark testing of matched specimens (e.g. human adenoviruses of subgroups B1, B2 and E; *Mycoplasma pneumoniae*; results not shown).

The benchmark RT-PCR type A influenza virus test used to assess clinical sensitivity and specificity of the RPM-Flu assay does not distinguish between the A/H1N1 and A/H3N2 subtypes. However the RPM-Flu assay is capable of simultaneous and mutually exclusive identification of a detected type A influenza virus as either seasonal subtype A/H1N1 or seasonal subtype A/H3N2. Results presented in Table 4 demonstrate this capability for the 30 specimens representing subtype A/H1N1 (5 specimens) or subtype A/H3N2 (25 specimens). Differentiation of the two prevalent subtypes is primarily based upon identification of assay-generated HA and NA sequences as matching HA1 or HA3 and/or NA1 or NA2, respectively. A secondary inference of A/HN subtype may be based on which of the M gene detectors M(H1N1) or M(H3N2) has the higher C3 Score. These indications of type A human influenza subtype are completely concordant with the corresponding subtypes associated with the most similar M gene sequence records from BLAST analysis of the sequences generated from both the M(H1N1) and the M(H3N2) detector tiles.

In the course of increasing prevalence of oseltamivir-resistant strains of A/H1N1 and amantadine-resistant strains of A/H3N2, the NHRC has implemented a new RT-PCR panel to determine the A/H1 or A/H3 subtype for specimens already shown to be type A influenza virus-positive by the earlier RT-PCR panel (David Metzgar, Chris Myers, personal communication). They have also validated the assay by demonstrating concordance of subtyping results from new RT-PCR panel with a cell culture/hemagglutinin immunofluorescence assay (HAI). Type B influenza virus-positive specimens (identified from the first tier RT-PCR typing panel) were negative controls for the second tier RT-PCR-based Type A subtyping panel.

Twelve isolates from the 2008–2009 influenza season, six each testing positive for A/H1 or A/H3 by the RT-PCR panel (and also by culture-HAI), were selected for RPM-Flu analysis. All of the specimens tested definitively positive in the initial (and only necessary) RPM-Flu assay as either A/H1N1 or A/H3N2, matching the results of both the RT-PCR and HAI benchmark

Table 3. Clinical sensitivity and clinical specificity of RPM-Flu for detection and identification of type A human influenza virus.

	RT-PCR Positive	RT-PCR Negative
RPM-Flu Positive	30	0
RPM-Flu Negative	1	267
Clinical Sensitivity = 96.8% ± 2.0% (95% confidence interval)		
Clinical Specificity = 100.0% ± 0.4% (95% confidence interval)		

RPM-Flu assay results for 298 clinical isolates are compared with results from benchmark RT-PCR test for type A influenza virus.
doi:10.1371/journal.pone.0008995.t003

Table 4. RPM-Flu assay results C3 scores of six detector tiles for detection and identification of A/H1N1 and A/H3N2 influenza virus subtypes.

Detector tile →	HA1	HA3	NA1	NA2	M (H1N1)	M (H3N2)
A/H1N1-positive specimens						
NHRC_117	76.5		84.9		88.7	50.6
NHRC_051	35.4		42.1		75.0	31.1
NHRC_022	27.1		38.8		81.2	34.4
NHRC_157	14.4		14.7		15.2	
NHRC_010			13.2		14.1	
A/H3N2-positive specimens						
NHRC_044		89.4		84.8	51.0	96.5
NHRC_053		89.0		87.5	49.0	93.6
NHRC_396		78.1		79.7	43.5	91.9
NHRC_075		62.9		69.0	39.3	91.6
NHRC_154		73.0		6.8	37.1	90.8
NHRC_388		72.4		64.3	36.4	88.4
NHRC_189		59.0		59.5	35.7	87.1
NHRC_080		56.1		56.1	36.8	86.2
NHRC_385		60.3		61.2	32.1	84.1
NHRC_016		48.7		51.8	33.0	84.1
NHRC_376		59.8		43.7	32.8	81.9
NHRC_176		59.9		58.0	30.4	80.4
NHRC_393		60.3		44.7	22.2	58.6
NHRC_055		39.0		37.5	22.6	62.1
NHRC_156		38.8		24.6	15.3	42.4
NHRC_040		31.8		30.1		30.5
NHRC_382		36.9		17.0		24.5
NHRC_057		23.6			13.6	42.9
NHRC_204		13.5				27.9
NHRC_049		24.6				21.8
NHRC_378		22.3				11.3
NHRC_366		19.0				10.1
NHRC_026		22.7				
NHRC_390		18.2				
NHRC_380		14.2				

Each specimen was independently corroborated as positive for type A influenza virus by benchmark RT-PCR analysis, noting that that assay does not resolve the subtype. The A/H1N1- and A/H3N2-positive specimens identified from RPM-Flu results are listed in descending order relative to the sum of positive detector tile C3 Scores (for H, N, and M). Empty cells indicate detector tiles that did not meet the relaxed detection and reporting threshold (C3 Score <10).
doi:10.1371/journal.pone.0008995.t004

Table 5. Concordance of RPM-Flu determination of A/H1N1 and A/H3N2 subtypes with benchmark RT-PCR HA1/HA3 subtyping panel and cell culture-HAI HA1/HA3 subtyping assays.

	C3 Scores from RPM-Flu Detector Tiles					Diagnostic Assay		
Specimen (_2008)	**HA1**	**NA1**	**M(H1N1):M(H3N2)**	**HA3**	**NA2**	**RPM-Flu**	**RT-PCR**	**Culture-HAI**
NHRC_SV_418	72	71	87>48[a]	4	6	A/H1N1	A & H1	A & H1
NHRC_SV_413	64	71	81>46[a]	3	7	A/H1N1	A & H1	A & H1
NHRC_SV_415	35	42	75>31[a]	7	13	A/H1N1	A & H1	A & H1
NHRC_SV_416	8	12	20>10[a]	2	6	A/H1N1	A & H1	A & H1
NHRC_SV_411	15	22	35>17[a]	4	10	A/H1N1	A & H1	A & H1
NHRC_AML_10622	73	83	84>45[a]	0	0	A/H1N1	A & H1	A & H1
NHRC_JB_46159	2	0	15<56[b]	43	34	A/H3N2	A & H3	A & H3
NHRC_JB_30553	1	0	18<62[b]	45	34	A/H3N2	A & H3	A & H3
NHRC_JB_35105	1	1	3<17[b]	19	7	A/H3N2	A & H3	A & H3
NHRC_JB_65320	1	0	3<23[b]	18	7	A/H3N2	A & H3	A & H3
NHRC_JB_46162	1	1	21<64[b]	52	42	A/H3N2	A & H3	A & H3
NHRC_AML_10601	1	1	43<96[b]	80	79	A/H3N2	A & H3	A & H3

[a]BLAST analysis of RPM-Flu assay-generated sequences from both of these M gene detector tiles returned most similar M gene sequence records that exclusively represent subtype A/H1N1 strains.
[b]BLAST analysis of RPM-Flu assay-generated sequences from both of these M gene detector tiles returned most similar M gene sequence records that exclusively represent subtype A/H3N2 strains.
doi:10.1371/journal.pone.0008995.t005

A/H1 and A/H3 assays. These results are summarized in Table 5, reinforcing the point of mutually exclusive detection and identification of specimen-specific gene sequences from the HA1 and NA1 detector tiles (as A/H1N1) or from the HA3 and NA2 detector tiles (as A/H3N2).

The results for M gene sequences from both the M(H1N1) and the M(H3N2) detector tiles for both sets of specimens shown in Table 5 again display significant crosstalk as overlapping and highly similar assay-generated sequences. In every example the two M gene sequences of an individual specimen, generated from the M(H1N1) and M(H3N2) gene detector tiles, are consistent with the A/HN subtype directly indicated the HA and NA gene sequences generated in the same RPM-Flu assay of the same specimen.

All 30 of the influenza-positive specimens described above were also reported by the individual RPM-Flu assays to be positive for at least one and up to six other viral and/or bacterial respiratory pathogens, including adenovirus, rhinovirus, coronavirus, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Pseudomonas spp.*, *Streptococcus mitis*, and *Streptococcus pneumoniae*. There was no obvious correlation of the distributions of other co-infecting pathogens with the particular subtype of infecting influenza virus, or with particular specimen collection venue(s). There was no data on clinical presentation or outcomes to correlate with particular states of co-infection(s).

We considered whether or not there may be interference with the detection and identification of specific influenza viruses in specimens that test positive in RPM-Flu assays for other viral and bacterial respiratory pathogens. The background distribution of C3 Scores for detection and identification of influenza viruses was estimated from RPM-Flu assays of 14 different blank samples (water or transport media negative controls). The average C3 Score of the 39 type A and type B influenza virus detector tiles to be 1.7 ± 1.1 ($N = 14$, results not shown).

Another set of 25 specimens were found to be negative for influenza virus by both RPM-Flu and benchmark RT-PCR tests, while the same RPM-Flu assays reported the specimens to be positive with apparently high load (C3 Score>75) of at least one other viral or bacterial respiratory pathogen (results not shown).

Simultaneous pathogen targets of the RPM-Flu assay that apparently do not interfere with detection and identification of influenza viruses include adenovirus (types Ad4, Ad7), coronavirus (strains OC43, 229E, NL63), rhinovirus (types A, B), metapneumovirus (types A, B), parainfluenzavirus (types 1, 2, 3), respiratory syncytial virus (RSV types A, B), *Chlamydophila pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (including MRSA strains), *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*.

Informational Specificity of RPM-Flu: Influenza Virus Genotypes and Resistance to Antiviral Agents

Subtype A/H3N2 influenza virus and Matrix (M) gene-based resistance to amantadine-like antiviral agents. The 25 A/H3N2-positive clinical specimens from the ensemble of 298 specimens described in the preceding section were collected between January 2007 and March 2007, at five different military basic training venues (Great Lakes Naval Training Station, IL; Marine Corps Recruit Depot, CA; Army Fort Leonard Wood, MO; Army Fort Jackson, SC; Army Fort Benning, GA). All but one of the RPM-Flu assay-generated HA3 gene sequences were most similar to BLAST-returned sequence records of A/H3N2 isolates that Nelson et al [11] associated with either major "clade a" from the 2006–2007 influenza outbreaks in the United States, or from the "N-lineage clade", a lineage originating from the 2005–2006 influenza season and continuing to circulate nationwide during the 2007–2008 season. The HA3 gene sequence from a single A/H3N2 specimen was found to be most similar to sequence records that Nelson et al [11] associated with the 2006–2007 "minor clade b". This minor clade b to represents a subpopulation of amantadine-sensitive A/H3N2 strains, in contrast to the resistant phenotype of the strains with the majority of circulating N- and a-clade strains.

At least five loci in the M2 trans-membrane peptide-encoding region of influenza virus M gene have been reported to confer some level of *in vitro* amantadine resistance [12,13]. This suggested examination of the RPM assay-generated M gene sequences from these A/H3N2-positive specimens, to evaluate possible correlations with resistance of the subject isolates to amantadine.

Figure 3 shows alignment of M gene sequences from 18 of the 25 A/H3N2-positive specimens, together representing all three of the clades described by Nelson et al [11].

The M gene sequences detected by RPM-Flu assay of the 24 amantadine-resistant N-lineage or clade-a specimens all reveal -AAT- as asparagine (N) codon 31 of the M2 peptide, a genotype consistent with amantadine resistance. The matrix gene sequence of the outlier clade-b specimen (NHRC_393) reveals -AGT- as serine (S) codon 31 of the M2 peptide, a genotype consistent with an amantadine-sensitive phenotype.

Subtype A/H1N1 influenza virus and neuraminidase NA1 gene-based resistance to oseltamivir-like antivirals. The five A/H1N1-positive clinical specimens from the ensemble of 298 specimens described in the preceding section were collected between January 2007 and March 2007, at two different military basic training venues (Army Fort Jackson, SC; Army Fort Benning, GA). BLAST analysis of the five RPM-Flu-detected HA1 gene sequences returned most similar reference sequence records including those that Nelson et al. [11] associated with A/H1N1 Major Clade A from the 2006–2007 outbreaks of influenza in the United States. Global surveillance during the two most recent influenza seasons (2007–2009) has revealed that most circulating A/H1N1 isolates are now resistant to oseltamivir, compared with oseltamivir sensitivity of most if not all A/H1N1 isolates from the 2006–2007 influenza season [14,15 (updated 13 June 2008)]. These more recent A/H1N1 strains appear to have a resistance phenotype due to mutation at the viral NA1 gene H275Y locus [16].

Neuraminidase gene sequences were detected and compared at this locus from RPM-Flu assays of specimens from the earlier 2006–2007 season and more recent 2007–2008 season specimens provided by NHRC for analysis. As shown in the Figure 4, the RPM-Flu assay-generated NA1 gene sequences from the older ensemble of clinical specimens (2006–2007) were concordant with those of the oseltamivir-sensitive, A/New Caldedonia/20/1999 (H1N1) RPM-Flu detector tile strain. BLAST analysis of these NA1 gene sequences returned most similar records including the oseltamivir-sensitive A/Ohio/UR06-0591/2007 and A/Oregon/UR06-0609/2007 strains. The '@' symbol in the inset indicates the C-T transition mutation corresponding to the H275Y mutation. On the other hand, the examples shown in the bottom half of the inset, representing 2007–2008 oseltamivir-resistant specimens from NHRC, had runs of 10 to 20 uncalled bases (N) spanning the NA1 gene's H275Y oseltamivir resistance locus. These results do not explicitly corroborate the H275Y resistance genotype, although they are consistent with diminished re-sequencing efficiency at sites of mismatching detector tile and specimen gene sequences. These runs of N-calls suggest two or more proximal nucleotide mismatches may be present at this NA1 gene locus of the selected NHRC 2008 specimens. There were 115

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2005-2006 N-lineage Clade:

NHRC_016    CCGCTTGNTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_055    CCGCTTGTNGTTGCCGCGAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_080    CCGCTTGTTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_176    CCGCTTGTTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATNTTGTGGATTCTT
NHRC_189    CCGCTNGNTGTTGCCGCGAATATCANTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_154    CCGCTNGTTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATNTTGTGGATTCTT

2006-2007 Major Clade a:

NHRC_040    CNGCTNGNTGTTGCCGCGAATATCATTGNGATCTNGCACTTGATATTGTGGATTCTT
NHRC_044    CCGCTNGNTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATNTTGTGGATTCTT
NHRC_053    CCGCTNGNTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATATTGTGGATTCTN
NHRC_156    CNGCTTGNTGTTGCCGCGAATATCATTGGGATCTNGCACTTGATATTGTGGATTCTT
NHRC_075    CCGCTNGNTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATNTTGTGGATTCTT
NHRC_385    CCGCTNGTTGTTGCCGCGAATATCNNTGNGATCTTGCACTTGATNNTGTGGNTTCNT
NHRC_382    CCGCTNGNTGTTGCCGCGAATATCANTGNGATCTTGCACTTGATNTTGTGGATTCTT
NHRC_388    CCGCTTGTTGTTGCCGCGAATATCNNTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_396    CCGCTNGTTGTTGCCGCGAATATCNNTGGGATCTTGCACTTGATATTGTGGATTCTT
NHRC_376    CCGCNNGNNGTTGCCGCGAATATCANTGGGATCTTGCACTTGATNTTGTGGATTCTT

Consensus:  CCGCTTGTTGTTGCCGCGAATATCATTGGGATCTTGCACTTGATNTTGTGGATTCTT
             P  L  V  V  A  A  N  I  I  G  I  L  H  L  I  L  W  I  L

2006-2007 Minor Clade b:

NHRC_393    CCGCNNNNNGTTGCCGCGAGTATCNTTGGGATCTTGCACTTGATATTGTGGATTCTT
             P  L  V  V  A  A  S  I  I  G  I  L  H  L  I  L  W  I  L
```

Figure 3. Alignment of M (matrix) gene sequences from 18 of 25 A/H3N2-positive specimens, representing three circulating 2006–2007 seasonal clades described by Nelson et al [11]. Twenty-four of the samples represent amantadine-resistant N-lineage or clade-a specimens, and each of these reveals -AAT- as asparagine (N) codon 31 of the M2 peptide (locus outlined in red box), a genotype consistent with the amantadine resistance phenotype. The matrix gene sequence of the single outlier clade-b sample (NHRC_393) reveals -AGT- as serine (S) codon 31 of the M2 peptide, a genotype consistent with the amantadine-sensitive phenotype.
doi:10.1371/journal.pone.0008995.g003

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2007 Tamiflu-sensitive                          H275Y
                                  A  P  N  F  Y  Y  E  E  C
                                                @
   A/Ohio/UR06-0591/2007          GCACCCAATTTTCATTATGAGGAATGT
   A/Oregon/UR06-0609/2007        GCACCCAATTTTCATTATGAGGAATGT
   NHRC_117_2007                  GCACCCAATTTTCATTATGAGGAATGT
   NHRC_051_2007                  GCACNNAATTTTCATTATGAGGAATGT
   NHRC_022_2007                  NCNNNNNNTTTTCANNATGAGGAATGT

RPM-Flu Detector:                 GCACCCAATTTTCATTATGAGGAATGT

   NHRC_413_2008                  GCACNNANTNNTNNNNNNNNNNNNNGC
   NHRC_415_2008                  GCACCNANNNNNNNNNNNNNNNNNNNNC
   NHRC_418_2008                  GCACCNANTNNNNATNNNNNGNNNNNC
   A/South Carolina/06/2008       GCACCCAATTTTTATTATGAGGAATGC
   A/Texas/16/2008                GCACCCAATTTTTATTATGAGGAATGC
                                              *@ *  *  *     *
                                  A  P  N  F  H  Y  E  E  C
2008 Tamiflu-resistant                          H275Y
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Figure 4. Comparison of RPM-Flu assay-generated NA1 (neuraminidase) gene sequences from two ensembles of clinical specimens, collected in 2006–2007 and in 2008, respectively. The older specimens have NA1 gene sequences that are concordant with those of the oseltamivir-sensitive, A/New Caldedonia/20/1999 (H1N1) RPM-Flu detector tile strain, including the determinant –CAT– histidine (H) codon at peptide position 275 (aligned locus in red box). BLAST analysis of these NA1 gene sequences returned most similar records including the oseltamivir-sensitive A/Ohio/UR06-0591/2007 and A/Oregon/UR06-0609/2007 strains. The '@' symbol in the inset indicates the C-T transition mutations encoding tyrosine (Y) at this locus of more recent strains such as A/South Carolina/06/2008 and A/Texas/16/2008. Three specimens (NHRC_413, _415, and _418) were also collected in 2008, and these failed to generate sequence through this locus from the oseltamivir-sensitive detector tile genotype. These results do not explicitly corroborate the H275Y resistance genotype, although they are consistent with diminished re-sequencing efficiency at sites of mismatching detector tile and specimen gene sequences. These runs of N-calls suggest two or more proximal nucleotide mismatches may be present at this NA1 gene locus of the selected NHRC 2008 specimens.
doi:10.1371/journal.pone.0008995.g004

most similar sequence records returned by BLAST for the three full-length RPM-Flu assay-generated NA1 gene sequences, and five of these sequence records indicated a second, H275Y-proximal mutation (at positions of asterisks on either side of the @ symbol). Any such pair of proximal mismatches in a specimen gene sequence (template) could disrupt hybridization of the sets of 25-nucleotide oligomer probes used to determine basecalls by the RPM-Flu re-sequencing method.

Future iterations of the RPM-Flu microarray design could provide one or more alternative NA1 gene sequence detector subtiles. Such assay design iterations would enable future RPM-Flu assays to deliver more explicit first-assay characterization of the oseltamivir genotype and phenotype.

RPM-Flu Sensitivity: Limits of Detection for A/H1N1 and A/H3N2 Isolates from the 2008–2009 Influenza Season

An isolate of subtype A/H1N1 and another of subtype A/H3N2 were selected by NHRC for single-passage propagation in MDCK cell cultures, followed by infectivity titrations in replicate shell vial cultures. The resulting neat stocks of BRD10622N(H1N1) and BRD10601(H3N2) were determined to contain 3.16×10^7 $TCID_{50}$/ml and 1.78×10^7 $TCID_{50}$/ml, respectively.

These two titered stocks were used to determine the analytical sensitivity of the RPM-Flu, by blinded assays of samples extracted from triplicate three-fold serial dilutions in the range of 1:1,000 to 1:2,187,000. Background C3 Scores were used as thresholds for reporting detection of either A/H1N1 or A/H3N2 in these limit dilution assays (as opposed to the relaxed and stringent thresholds used for reporting results from assays of clinical or field specimens). The background detector tiles represent 25 non-A/H1N1 and non-A/H3N2 detector tiles from each individual RPM-Flu assay, and a positive detection was reported if one of the target-specific detector tiles from the following lists had C3 score at least 3.29 standard deviations greater than the background mean C3 Score ($p<0.001$).

Figure 5 and Figure 6 illustrate the results of these endpoint dilution titrations of the titered A/H1N1 and titered A/H3N2 stocks, respectively, plotting the average highest C3 Score of A/H1N1 or A/H3N2 detector tiles for each of the triplicate assays at each dilution level. The percent positive assays (3/3, 2/3, 1/3 and/or 0/3) are also shown for the A/H1N1triplicate dilutions (Figure 5) and for the A/H3N2 triplicate dilutions (Figure 6). These unblinded dilution titration assay results were used to estimate 95% positive detection endpoints. Then one of the A/H1N1 and two of the A/H3N2 dilution tiers were expanded from three to twenty replicates, in order to demonstrate the high likelihood (≥95%) of positive detection and identification at the indicated dilution of the respective neat stocks.

From these results RPM-Flu assay, the 95% positive endpoint dilution limits of detection (LoD) be 3 $TCID_{50}$ for the A/H1N1 titered stock preparation and 6 $TCID_{50}$ for the A/ H3N2 titered stock preparation.

From the total of 41 independently extracted dilution specimens of the subtype-confirmed and titered A/H1N1 stock (Figure 5), 37 were reported to be influenza-positive by RPM-Flu assay, and all of these assays correctly reported by the RPM-Flu assays as subtype A/H1N1 (100% specificity). From a total of 58 independently extracted dilution specimens of the subtype-confirmed and titered A/H3N2 stock (Figure 6), 44 were reported to be influenza-positive by RPM-Flu assay, and all of these assays correctly reported by the RPM-Flu assays as subtype A/H3N2 (100% specificity). A total of 268 specimens from the NHRC clinical specimen ensemble were found to be negative for type A influenza virus by the RPM-Flu assay and 267 of these were also reported to be influenza virus-negative by benchmark RT-PCR assays for type A influenza virus, supporting the conclusion of 100% subtyping specificity for the RPM-Flu test.

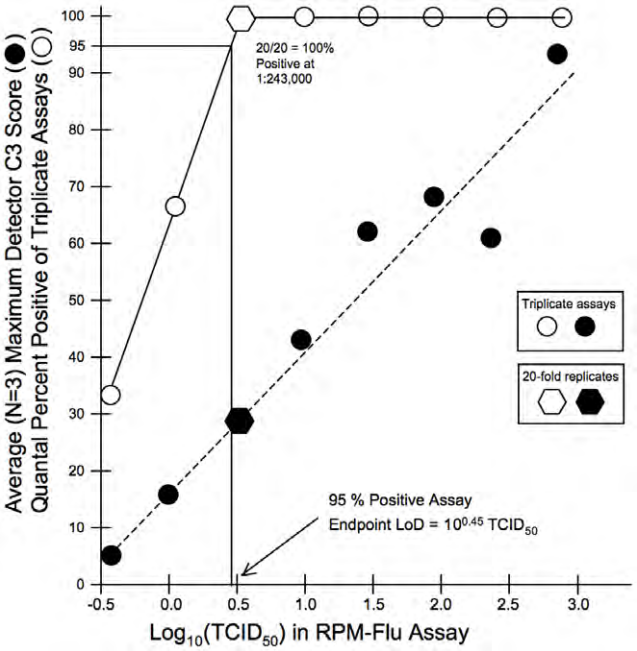

Figure 5. Limit of Detection (LoD) determination for seasonal A/H1N1 strain BRD10622. Neat stock of cell culture lysate (3.16× 10^7 $TCID_{50}$/ml) was diluted 1:1,000 in cell culture media. Triplicates at this dilution, and at serial three-fold dilutions to 1:2,187,000 were individually extracted and subjected to RPM-Flu assay. Black circles indicate average of the maximum C3 Score (from either HA1, NA1 or M(H1N1) detector tiles) at each dilution, plotted against log_{10} $TCID_{50}$ input per assay. Open circles represent the quantal percentage of positive assays (out of three) at each dilution tier. The open and black hexagons correspond to a dilution tier at which 20-fold replicates were assayed to demonstrate the 95% endpoint LoD, in this case corresponding to single assay input of about 3 $TCID_{50}$.
doi:10.1371/journal.pone.0008995.g005

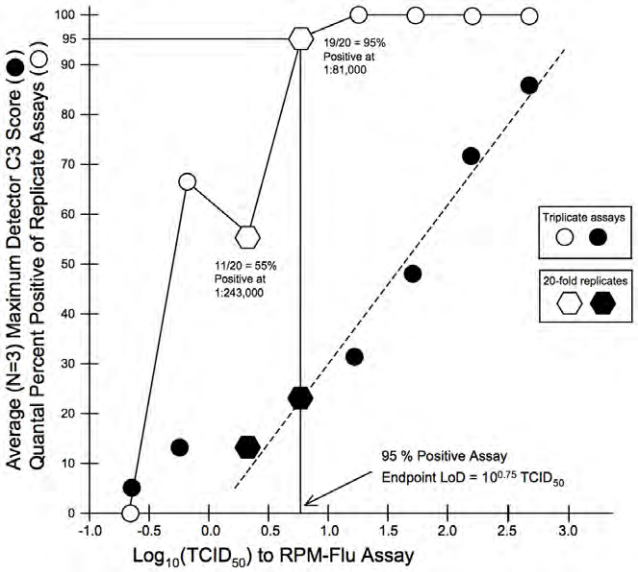

Figure 6. Limit of Detection (LoD) determination for seasonal A/H3N2 strain BRD10601. Neat stock of cell culture lysate (1.78× 10^7 $TCID_{50}$/ml) was diluted 1:1,000 in cell culture media. Triplicates at this dilution, and at serial three-fold dilutions to 1:2,187,000 were individually extracted and subjected to RPM-Flu assay. Black circles indicate average of the maximum C3 Score (from either HA3, NA2 or M(H3N2) detector tiles) at each dilution, plotted against log_{10} $TCID_{50}$ input per assay. Open circles represent the quantal percentage of positive assays (out of three) at each dilution tier. The open and black hexagons correspond to a dilution tier at which 20-fold replicates were assayed to demonstrate the 95% endpoint LoD, in this case corresponding to assay input of about 6 $TCID_{50}$.
doi:10.1371/journal.pone.0008995.g006

RPM-Flu Specificity: Direct Differentiation of Diverse Type A Avian Influenza Virus Subtypes Based upon RPM-Flu Assay-Generated HA and NA Gene Sequences

A collection of 23 reference strain specimens, representing diverse type A avian influenza virus subtypes, were propagated in embryonated eggs by SEPRL, then extracted for purification of total nucleic acid as input to RPM-Flu assays.

Table 6 presents the results for 19 of these 23 specimens reported by the RPM-Flu assays to be positive for avian influenza virus. In each case the RPM-Flu assay-generated HA and NA gene sequences identified the particular A/HN subtype that precisely matched the unblinded identifications of each reference strain. BLAST/GenBank analysis of these RPM-detected query sequences returned best matching sequence records with the same A/HN types as determined from the HA and NA detector tile C3 Scores. For 14 of these specimens, the single or multiple most similar HA and NA gene sequence records included the particular reference strain actually provided by SEPRL for analysis. These instances are highlighted as entries with flanking asterisks in the right column of Table 6).

The RPM-Flu assay of specimen USDA_13 was reported negative for detection of avian influenza virus, but the same assay also reported the specimen to be positive for avian metapneumovirus. This was revealed to be a correct result after unblinding of the specimen identification as a non-influenza control Type C avian metapneumovirus.

Three avian influenza virus-negative RPM-Flu assay results were also concordant with subsequently unblinded specimen information. USDA_5 was RPM-Flu negative for avian influenza and later confirmed to have been from an unsuccessful inoculation of the egg culture.

USDA_8 was reported by RPM-Flu assay to be A/H11N9, and while the initial identification from SEPRL was subtype A/H9N2, the original specimen apparently represented a mixed infection of H11N9 and H9N2. The HA component of the specimen was subjected to *de novo* gene sequencing and determined to be A/H11 strain. The RPM-detected neuraminidase sequence (868 bp, C3 = 88.7) unequivocally matched A/N9; the mean C3 Score of all other neuraminidase detector tiles in the assay was 3.1±1.6 (N = 10).

USDA_16 was correctly negative by RPM-Flu assay for avian influenza, but unblinding revealed this specimen to be a second negative control, containing instead avian paramyxovirus type I (Newcastle disease virus). After receiving this information, another aliquot of the same nucleic acid preparation from specimen USDA_16 was tested using an alternative RPM-TEI assay [17] for detecting tropical and emerging infections. This assay resulted in successful detection and identification of avian paramyxovirus type 1.

Specimen USDA_23 was received later as a substitute for the failed inoculum specimen USDA_5. The RPM-Flu assay of this specimen resulted in detection of sequence for neuraminidase type N4, with most similar sequence record representing an A/H8N4 strain. However, no hemagglutinin gene sequences were detected that met even the relaxed detection threshold. The highest C3 Scores among all RPM-Flu hemagglutinin detector tiles were

Table 6. RPM-Flu Assay detection and subtype identification of avian influenza in reference strain specimens provided by SEPRL.

Blinded sample ID	Highest HA C3 Score	Highest NA C3 Score	RPM-Flu Detection & Identification	Unblinded SEPRL Reference Strain
USDA_1	36.6	14.7	A/H1N1	* A/Turkey/Kansas/4880/80 (H1N1) *
USDA_2	30.0	85.3	A/H2N8	* A/Herring Gull/DE/677/88 (H2N8) *
USDA_3	65.6	78.0	A/H3N2	A/turkey/MN/366767/2005 (H3N2)
USDA_4	65.0	85.5	A/H4N6	* A/Blue Winged Teal/OH/240B/88 (H4N6) *
USDA_5	6.7	4.6	Negative for avian influenza virus	No avian influenza virus, Attributed to inoculum failure
USDA_6	12.1	18.6	A/H7N2	* A/quail/PA/20304/98 (H7N2) *
USDA_7	76.4	67.7	A/H8N4	A/turkey/CO/169118-13/02 (H8N4)
USDA_8	11.2	88.7	A/H11N9	Original inoculum as A/chicken/NJ/12220/97(H9N2) was found to be coinfected with A/H11N9 strain A/H11 sequence confirmed by DNA sequencing NA sequence pending
USDA_9	39.3	8.8	A/H10N7	A/quail/NJ/25254-22/95 (H10N7)
USDA_10	12.0	69.8	A/H11N3	A/chicken/NJ/4645/96(H11N3)
USDA_11	74.0	9.5	A/H12N5	* A/duck/LA/188D/87 (H12N5) *
USDA_12	16.8	18.0	A/H13N6	* A/Gull/Maryland/1824/1978(H13N6) *
USDA_13	9.8	4.9	Negative for avian influenza virus	No avian influenza virus Avian metapneumovirus (Colorado strain)
			Positive for avian metapneumovirus	
USDA_14	64.4	22.8	A/H5N3	* A/duck/Singapore/F119/97 (H5N3) *
USDA_15	10.8	30.3	A/H7N3	* A/chicken/Chile(F0)/176822/02 (H7N3) *
USDA_16	9.8	6.4	Negative for avian influenza virus	No avian influenza virus Avian paramyxovirus type 1 Newcastle Disease Virus California/02
			Positive for NDV using RPM-TEI	
USDA_17	10.5	19.2	A/H5N2	* A/chicken/Mex/26654-1374/94 (H5N2) *
USDA_18	88.5	57.5	A/H7N1	* A/turkey/Italy/4580/99 (H7N1) *
USDA_19	66.3	26.1	A/H7N3	* A/chicken/Pakistan/1369-CR2/95 (H7N3) *
USDA_20	27.6	24.1	A/H7N7	* A/chicken/Victoria/85 (H7N7) *
USDA_21	86.3	66.2	A/H14N5	* A/mallard/Gurjev/263/82 (H14N5) *
USDA_22	80.9	17.9	A/H15N9	* A/Shearwater/W. Australia/2576/79 (H15N9) *
USDA_23	10.4	57.0	A/H?/N4	No detection of HA sequence. Best matching record for NA4 sequence is A/H8N4.

For 14 of these specimens, the single or multiple most similar HA and NA gene sequence records matching sequences from the RPM-Flu assay included the particular reference strain actually provided by SEPRL for analysis. These instances are with asterisks flanking entries in the right column of Table 6). Other instances, without asterisks, represent assayed reference strains for which one or both HA and NA gene sequences had not yet been deposited in GenBank, but which were nevertheless concordant with sequences generated by the RPM-Flu assay.

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HA1(H1N1-1918), a very short detector tile, and FLUAHA7, but neither of these sub-threshold results triggered a positive detection report.

Lin et al (2009) recently reported another study of avian influenza viruses using RPM-Flu assays for 24 AH5N1-positive field and clinical specimens collected by the NAMRU-3 group from infected birds and humans [18]. Supplemental Table S5 summarizes results from our re-analysis of their original RPM-Flu assay data (GSEQ FASTA files), presenting C3 Scores as described in this report for assay-generated sequences from the subtype-specific HA and NA detector tiles, or from the RPM-Flu M, NS and PB2 detector tiles (that represent conserved gene sequences of a prototype A/H5N1 strain).

The RPM-Flu assay-generated HA5 gene sequences of all the A/H5N1-positive specimens from NAMRU-3 and also of the A/H5N2 reference strain specimen from SEPRL (Table 6, USDA_17) were consistent with an extended arginine-rich and lysine-rich motif spanning the HA5 peptide cleavage site, consistent with a viral high pathogenicity (HP) phenotype [19,20]. BLAST analysis of the RPM-Flu detected HA5 gene sequences from these strains returned most similar sequence records encoding a similar HP-like HA5 cleavage site motif.

The hemagglutinin RPM-Flu assay-generated HA5 gene sequence for the A/H5N3 reference strain (Table 6, USDA_14) had a significant gap of uncalled bases at the HA cleavage site locus, compared to the HP-like HA gene detector tile sequence A/

Viet Nam/1203/2004 (H5N1). The most similar hemagglutinin and neuraminidase gene sequence records returned by BLAST for HA and NA gene sequences of USDA_14 included strain A/duck/Singapore/3/1997 (H5N3), the actual reference strain submitted (blinded) for RPM-Flu analysis. The encoded hemagglutinin gene cleavage site for this reference A/H5N3 strain is consistent with a low pathogenicity (LP) phenotype.

A/duck/Singapore/3/1997(H5N3) -PQRE----TR/GLF- (LP)

A/Viet Nam/1203/2004 (H5N1) -PQRERRRKKR/GLF- (HP)

These results demonstrate efficacy of RPM-Flu for unequivocal diagnostic detection and identification of human and avian influenza virus subtypes, based upon direct determination of specimen-specific sequences of the hemagglutinin and neuraminidase genes. The two negative control specimens, USDA_13 and USDA_16, demonstrated the capability of the RPM-Flu assay to identify alternative pathogens to avian influenza virus as components of a more complete differential diagnosis assay for cases of avian influenza-like illness.

Prediction of Influenza Virus A/HN Subtype by Inference from Database Sequence Records That Are Most Similar to RPM-Flu Assay-Generated M, NS or PB Gene Sequences

The results presented in Table 6 demonstrated 19 of 19 reference strains of type A avian influenza viruses detected and correctly identified by corresponding A/HN subtype, based upon the specific HA and NA gene sequences revealed in the assays of each specimen. The same assays also generated specimen-specific M, NS and/or PB2 gene sequences. We used these results to poll the A/HN subtype(s) of the influenza virus strains corresponding to BLAST-returned sequence records found to be most similar to these relatively conserved non-HA and non-NA gene sequences (Supporting Information - Table S6, Table S7, Table S8).

BLAST analysis of the M gene sequences from the 19 avian influenza specimens returned 79 most similar sequence records, of which 40 (51%) were associated with different A/HN subgroups than determined from the corresponding specimens' HA and NA gene sequences. For five of the 19 specimens there were no most similar M gene sequences returned to match the specimen's actual A/HN subtype (Table S6).

BLAST analysis of the NS gene sequences from the 19 avian influenza specimens returned 63 most similar sequence records, of which 41 (65%) were associated with different A/HN subgroups than determined from the corresponding specimens' HA and NA gene sequences. For five of the 19 specimens there were no most similar NS gene sequences returned to match the specimen's actual A/HN subtype (Table S7).

BLAST analysis of the PB2 gene sequences from the 19 avian influenza specimens returned 58 most similar sequence records, of which 22 (38%) were associated with different A/HN subgroups than determined from the corresponding specimens' HA and NA gene sequences. Nine of the 19 specimens had no most similar PB2 gene sequences from the same A/HN subtype (Table S8).

These results suggest little predicate value of most type A influenza virus M, NS or PB2 sequences for determination of the A/HN subtype of the strain from which these sequences were identified.

We referred earlier to the complementary set of field and clinical samples (isolates, not original specimens) of type A avian influenza viruses provided by NAMRU-3 (Cairo, Egypt) and analyzed at NRL using RPM-Flu [18]. As noted earlier, Table S5 in SUPPLEMENTAL INFORMATION presents the C3 Scores from RPM-Flu assay-generated gene sequences data for these specimens.

Eight samples from this previously referenced set of clinical and field specimen isolates of avian influenza virus represented subtypes A/H10N7 (4), A/H7N7 (2), A/H11 (1) or A/H13 (1). In marked contrast to results from RPM-Flu assay-generated HA and NA gene sequences, there was poor correspondence of the actual specimen A/HN subtypes with the subtypes of sequence records returned from BLAST analysis of the assay-generated M, and NS gene sequences. Of 30 most similar M gene sequence records only 5 were from A/HN subtypes matching the subtypes of the corresponding specimens (Table S6). None of the 11 most similar NS gene sequence records matched the A/HN subtypes of the corresponding specimens (Table S7). There were no PB2 gene sequences reported from assays of these eight (non-A/H5N1) specimens (Table S5).

Twenty-one of the remaining specimens of the set reported by Lin et al [18] were of subtype A/H5N1, and the RPM-Flu assay results for these specimens are also included in Table S1, as C3 Scores for the sequences generated from the corresponding HA5, NA1, M, NS and PB2 gene detector tiles. For these 21 A/H5N1-positive specimens, the BLAST analyses of assay-generated M, NS and PB2 gene sequences exclusively represented subtype A/H5N1 avian influenza viruses, as 255 M gene, 146 NS gene and 310 PB2 gene sequence records.

This result for A/H5N1strains is reminiscent of the results presented earlier for subtypes A/H1N1 and A/H3N2 and type B human influenza virus sequence records that are found to be most similar to RPM-Flu assay-generated M, NS and PB2 gene sequences from the corresponding types and subtypes. It is interesting to consider that these results may reflect the highly biased over-representation of samples A/H5N1 sequences in the genome sequence database(s), reflecting much more of the natural diversity of non-HA and non-NA gene sequences than in more sparsely sampled (but naturally far more prevalent) avian influenza virus subtypes.

Discussion

Rapid and cost-effective microarray-based resequencing (RPM) of influenza virus hemagglutinin and neuraminidase genes provides sensitive detection and specific identification for types and subtypes of previously known as well as emergent strains and variants. These capabilities of the RPM platform stand out very favorably on comparisons with the most recently adopted diagnostic methodologies that are based on real-time reverse-transcription, polymerase chain reaction methods (RT-PCR). The FDA-cleared CDC rRT-PCR Flu Panel (510(k) 080570) enables detection and discrimination of influenza viruses having HA subtype components A/H1, A/H3 and A/H5, and also type B influenza virus. However the test provides no assurance that a positive test result correctly identifies one of the inferred target subtypes A/H1N1, A/H3N2 or A/H5N1; there is no neuraminidase subtype component evaluated as part of the test. Results presented in this report demonstrate that the TessArray RPM-Flu detect and identifies these three targeted subtypes of interest, but also directly distinguishes each of them among the 9 different A/HN subtype categories, or from the background of 144 combinatorial variations of influenza virus A/HN subtypes.

Sensitivity

The RPM-Flu assay leverages a multiplexed RT-PCR-like strategy to rapidly (~1 hour) amplify trace quantities of specific

target gene sequences so that they may be detected if actually present in a specimen. Results from this report demonstrated that the 95% endpoint limits of detection (LoD) for seasonal A/California-NHRC/BRD10622N/2009(H1N1) and seasonal A/California-NHRC/BRD10601N/2009(H3N2) influenza virus strains were 2.8 infectious doses ($TCID_{50}$) and 5.8 $TCID_{50}$, respectively. These strains have not been serially passaged at high multiplicity in either egg or MDCK cell cultures, so it is not likely that accumulations of defective viral particles would favorably bias determination of sensitivity with genome-based assays relative to infectious doses. Furthermore, these two strains were not selected *a priori* for favorable match of viral sequences with oligonucleotide primers used for target gene sequence amplification. The observed RPM-Flu LoD for each strain is of similar order of magnitude as comparable estimates of analytical sensitivity reported from RT-PCR based methods, Table 7.

Specificity

Each RPM assay of a single specimen is capable of generation from hundreds to thousands of nucleotides of multiple targeted pathogen gene sequence(s). Gene sequences generated directly from RPM-Flu assays may specify determinants of particular target pathogen phenotypes (virulence, drug resistance, host range, etc.), or support forensic queries of identity of detected strains in different specimens, or support genome diversity-based epidemiological tracking in the course of an infectious disease outbreak.

RPM-Flu assay-generated nucleotide sequences representing one or more target genes of each detected pathogen convey significantly more informative specificity than can be obtained from any singlet or multiples of RT-PCR tests. Some RT-PCR protocols are designed to glean additional detail regarding detected pathogen genotypes, through results from multiple (serially executed) RT-PCR panels, but this approach imposes additional testing costs and time delays and yield far less specific information than actual target gene sequences provided from a single RPM assay.

Some platforms claim to differentiate selected type A influenza virus subtypes based upon indirect detection or specification of non-HA and non-NA viral gene sequences [21,22]. Results in this report of RPM-Flu assay-generated viral gene sequences from a variety of reference strains indicate that matrix M gene sequences are correlated with the subtypes of only seasonal human A/H1N1 and seasonal A/H3N2 strains. In general, however, non-HA and non-NA viral gene sequences do not support reliable inference of type A influenza virus subtype.

Multiplicity

The RPM-Flu assay panel simultaneously addresses 30 different categories of viruses and bacteria that are more or less likely to be encountered in specimens related to respiratory infections (Figure 1, Figure S1). Each assay simultaneously enables detection and identification of targeted pathogens from one or more of 188 different target gene resequencing detector tiles.

For comparison, the recently FDA-cleared Luminex® Respiratory Viral Panel (RVP) Multiplex Nucleic Acid Detection Assay Respiratory Virus Panel (510(k) K063765) is a twelve-plex RT-PCR panel for detection and differentiation of a few strains and types of respiratory viruses including adenovirus, influenza A virus (as A/H1 or A/H3), respiratory syncytial virus, parainfluenza virus, metapneumovirus and rhinovirus (no bacterial respiratory pathogens tested). The RVP assay yields no specimen-specific viral gene sequences that could be used for more detailed identification and differentiation of detected virus strains and variants of detected virus(es).

For human and avian type A influenza viruses in particular, the RPM-Flu provides direct detection and identification capability for any of the combinatorial 144 A/HN subtypes that may be present in a specimen, using an ensemble of 39 different influenza virus target gene resequencing detectors. The Luminex RVP multiplex RT-PCR panel uses three of its twelve primer-probe sets to detect type A influenza virus (M gene-specific primer pair) and distinguish A/H1 from A/H3 (HA1- and HA3-specific primer

Table 7. Similar analytical sensitivity for seasonal influenza virus detection using RPM-Flu and rRT-PCR panel assays.

Platform	Tested Strains	Limit of Detection (LoD)
Resequencing Microarray:		
RPM-Flu	A/California-NHRC/BRD10622N/2009(H1N1)	3 $TCID_{50}$
RPM-Flu	A/California-NHRC/BRD10601N/2009(H3N2)	6 $TCID_{50}$
rRT-PCR Panel:		
JBAIDS[a]	A/Hawaii/15/2001 (H1N1)	2 $TCID_{50}$
JBAIDS[a]	A/Texas/71/2007 (H3N2)	15 $TCID_{50}$
CDC[a]	A/New Caledonia/20/1999(H1N1)	16 EID_{50}~1 $TCID_{50}$
CDC[a]	A/Hawaii/15/2001(H1N1)	32 EID_{50}~2 $TCID_{50}$
CDC[a]	A/New York/55/2004(H3N2)	160 EID_{50}~10 $TCID_{50}$
CDC[a]	A/Wisconsin/55/2004(H3N2)	32 EID_{50}~2 $TCID_{50}$

The Limit of Detection (LoD) for seasonal A/H1N1 and seasonal A/H3N2 influenza viruses are presented as the number of tissue culture ($TCID_{50}$) or egg culture (EID_{50}) infectious units used for each assay that are demonstrated to result in >95% positive assay outcomes. The relation of tissue culture and egg culture infectious units typically vary with respect to specific influenza virus strain and inoculum. For this table an estimated ratio of 16 EID_{50} : 1 $TCID_{50}$ is based upon results of testing the same A/Hawaii/15/2001 (H1N1) strain by both the JBAIDS and CDC rRT-PCR panels.

[a]The recently FDA-cleared 510(k)080570 the "CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel" is a test designed to specifically detect contemporary A/H1, A/H3 and A/H5 (Asian Lineage) influenza viruses in humans, as accessed 02 Oct 2009 at http://www.accessdata.fda.gov/cdrh_docs/pdf8/k080570.pdf.

In characterizations of analytical sensitivity of this test panel, the CDC has reported analytical LoD in terms of egg culture infectivity titers (EID_{50}/ml). The Department of Defense Joint Biological Agent Identification & Diagnostic System (JBAIDS) has implemented the same RT-PCR test and reported LoD in $TCID_{50}$ units of cell culture infectivity, as accessed 02 Oct 2009 at http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM180067.pdf.

We estimate CDC rRT-PCR LoD results shown in Table 7 using $TCID_{50}$ units based on the results of both CDC and JBAIDS for A/Hawaii/15/2001(H1N1).

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pairs). While this RT-PCR panel may infer influenza virus subtype, not even testing for the NA component, the assay result is only based on a pattern of signals from a small panel of RT-PCR primer-probes.

Some RT-PCR protocols require serial analysis with hierarchical panels of primer-probes in order to expand the practical multiplicity of pathogen targets or variants. This is the case with the benchmark PCR testing used in this report –a type A influenza virus-positive specimen may be identified in a first round RT-PCR panel, followed by testing with another panel to distinguish A/H1 from A/H3. Failure to establish subtype A/H1 or A/H3 in the follow-on test is a first tier screening results for possible identification of the 2009 Novel A/H1N1 influenza virus outbreak strain. According to the *FACT SHEET FOR HEALTHCARE PROVIDERS: INTERPRETING* CDC *HUMAN INFLUENZA VIRUS REAL-TIME RT-PCR DETECTION AND CHARACTERIZATION PANEL FOR RESPIRATORY SPECIMENS (NPS, NS, TS, NPS/TS, NA1) AND VIRAL CULTURE TEST RESULTS* (Authorized by FDA 02 May 2009),

> "The rRT-PCR Flu Panel (**NPS, NS, TS, NPS/TS, NA**) should be ordered to diagnose influenza A infections caused by influenza A or B and subtype determination of seasonal human influenza A virus (seasonal A/H1 or A/H3) to be used as a first tier test for the *in vitro* qualitative detection of novel influenza A (H1N1) virus in patients suspected of having novel influenza A (H1N1) infection. If the test result is positive for influenza A and negative for seasonal H1 and H3 subtypes, the laboratory should test the specimen with the Swine Influenza Real-time RT-PCR Detection Panel (rRT-PCR Swine Flu Panel) for the presumptive detection of novel influenza A (H1N1) infection."

It is not at all clear that such hierarchical RT-PCR protocols, attempting to increase analytical multiplicity through serial testing of the same specimen(s) at different laboratory venues over a period likely longer than a single day can be either efficient or cost-effective. Far more diagnostic intelligence about the patient and specimen would be available in first-day same-day results from a single RPM-Flu test of the original specimen.

Utility

Influenza infections do not necessarily preclude co-infection of the same individual by other viral and/or bacterial pathogens (including other strains of influenza virus). Such co-infections may confound a simple diagnosis of influenza, and may also compound morbidity and mortality for a co-infected patient. However it is not common practice that a positive result from a routine diagnostic influenza test would be followed by a call for one or more tests of possible secondary agents of infection. The single specimen single aliquot RPM-Flu assay simultaneously targets 30 different categories of viruses and bacteria, and these were each selected as a respiratory pathogens reported to cause "flu-like" symptoms at some stage of human infection.

Implementation of a highly multiplexed differential diagnostic assay as RPM-Flu would be of great advantage in anticipation of highly likely future influenza outbreaks, epidemics and pandemics. For many decades, leaders in the area of influenza epidemic and pandemic risk assessment and outbreak management have reviewed the pathology of influenza with respect to rapid deterioration and patient deaths attributable to secondary bacterial infections and pneumonia [23,24].

It has taken almost twenty-five years from the first research reports of the polymerase chain reaction [25] until recent regulatory clearances have enabled introduction specific PCR- and RT-PCR-based diagnostic assays and instrumentation into clinical practice. Such a traditional regulatory clearance timeline may assure safety and efficacy of diagnostic devices beyond imaginable limits of liability, but it also assures that best practice standards for clinical diagnostics are constrained to technologies that perform with nearly obsolete capabilities and specifications. Within the last decade resequencing microarrays have been introduced for research applications and clinical research [26,27,28]. It would be disappointing to anticipate that another decade or more may pass before benefits from diagnostic implementations of RPM or other highly multiplexed sequencing-based platforms can be realized in routine clinical diagnostics.

Supporting Information

Table S1 Analysis of 2009–2010 FluMist live virus trivalent vaccine as comparisons of Influenza type A and type B detector tile sequences to detection and identification of RPM-Flu assay-generated gene sequences. The strains configured in this vaccine are A/South Dakota/06/2007(H1N1), A/Uruguay/716/2007 (H3N2) and B/Brisbane/60/2008. The vaccine matrix genes and other non-HA, non-NA genes are from master donor strains A/Ann Arbor/6/1960(H2N2) and B/Ann Arbor/1/66.
Found at: doi:10.1371/journal.pone.0008995.s001 (0.06 MB DOC)

Table S2 Analysis of 2009–2010 Fluvirin inactivated virus trivalent vaccine as comparisons of Influenza type A and type B detector tile sequences to detection and identification of RPM-Flu assay-generated gene sequences. The strains configured in this vaccine are A/Brisbane/59/2007, IVR-148 (H1N1), A/Uruguay/716/2007(H3N2) and B/Brisbane/60/2008. The type A virus subtypes of the inactivated vaccines have matrix genes and other non-HA, non-NA genes derived from the master donor strain A/Puerto Rico/8/1934 (H1N1).
Found at: doi:10.1371/journal.pone.0008995.s002 (0.07 MB DOC)

Table S3 Analysis of 2005–2006 inactivated virus trivalent vaccine as comparisons of Influenza type A and type B detector tile sequences to detection and identification of RPM-Flu assay-generated gene sequences. The strains configured in this vaccine are A/New Caledonia/20/99 (H1N1), A/New York/55/2004(H3N2) and B/Jiangsu/10/2003. The type A virus subtypes of the inactivated vaccines have matrix genes and other non-HA, non-NA genes derived from the master donor strain A/Puerto Rico/8/1934 (H1N1). RPM-Flu detector title prototype sequences C3 Score BLAST E-value SNPsa Most similar sequence records from BLAST/GenBank include: Hemagglutinin genes A/New Caldedonia/20/1999 (H1N1) 62.3 1e-180 4/934 A/New Caldedonia/20/1999 A/Canterbury/125/2005 (H3N2) 84.6 1e-180 11/1269 A/New York/55/2004 B/Malaysia/2506/2004 25.2 2e-89 23/226 B/Jilin/20/2003 b B/Shanghai/361/2002 55.8 1e-180 20/502 B/Jiangsu/10/2003 b Neuraminidase genes A/New Caldedonia/20/1999 (H1N1) 51.6 1e-180 5/619 A/New Caldedonia/20/1999 A/Canterbury/125/2005 (H3N2) 89.8 1e-180 2/1077 A/New York/55/2004 B/Malaysia/2506/2004 55.2 1e-180 21/662 B/Jiangsu/10/2003 Matrix genes A/Canterbury/100/2000 (H1N1) 54.1 1e-180 24/459 A/Puerto Rico/8/1934(H1N1) A/Canterbury/125/2005 (H3N2) 52.4 1e-180 29/445 A/Puerto Rico/8/1934(H1N1) B/Memphis/13/2003 91.6 1e-180 14/870 B/Jiangsu/10/2003 a SNPs are single base call discrepancies between detector tile

sequence and assay generated sequence from labeled target DNA. The number of detected SNPs is shown relative to the number of bases called from the detector tile as contiguous runs of three or more base calls. b The B/Jilin/20/2003 and B/Jiangsu/10/2003 strains are equivalent B/Shanghai/361/2002-like strains as also used in 2004–2005 vaccine configurations (see Table 1).
Found at: doi:10.1371/journal.pone.0008995.s003 (0.06 MB DOC)

Table S4 Analysis of 2006–2007 inactivated virus trivalent vaccine as comparisons of Influenza type A and type B detector tile sequences to detection and identification of RPM-Flu assay-generated gene sequences. The strains configured in this vaccine are A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005(H3N2) and B/Malaysia/2506/2004. The type A virus subtypes of the inactivated vaccines have matrix genes and other non-HA, non-NA genes derived from the master donor strain A/Puerto Rico/8/1934 (H1N1).
Found at: doi:10.1371/journal.pone.0008995.s004 (0.06 MB DOC)

Table S5 RPM-Flu assay detection and identification of avian influenza viruses in field and clinical specimens obtained from human and avian hosts.
Found at: doi:10.1371/journal.pone.0008995.s005 (0.11 MB DOC)

Table S6 The A/HN subtypes associated with most similar sequence records for RPM-Flu assay-generated M gene sequences from 19 type A avian influenza viruses are not reliable indicators of the actual A/HN subtype. Forty of 79 (51%) most similar M gene sequence records are associated with different A/HN subtypes than independently determined from each specimen's specific HA and NA gene sequences (mismatches for 8 of 19 specimens).
Found at: doi:10.1371/journal.pone.0008995.s006 (0.07 MB DOC)

Table S7 The A/HN subtypes associated with most similar sequence records for RPM-Flu assay-generated NS gene sequences from 19 type A avian influenza viruses are not reliable indicators of the actual A/HN subtype. Forty-one of 63 (65%) most similar NS gene sequence records are associated with different A/HN subtypes than independently determined from each specimen's specific HA and NA gene sequences (mismatches for 11 of 19 specimens).
Found at: doi:10.1371/journal.pone.0008995.s007 (0.07 MB DOC)

Table S8 The A/HN subtypes associated with most similar sequence records for RPM-Flu assay-generated PB2 gene sequences from 19 type A avian influenza viruses are not reliable indicators of the actual A/HN subtype. Twenty-two of 58 (38%) most similar PB2 gene sequence records are associated with different A/HN subtypes than independently determined from each specimen's specific HA and NA gene sequences (mismatches for 12 of 19 specimens).
Found at: doi:10.1371/journal.pone.0008995.s008 (0.07 MB DOC)

Figure S1 All combinatorial subtypes of type A influenza virus strains and 30 different viral and bacterial pathogens causing influenza-like illness as represented on the RPM-Flu 3.1 resequencing pathogen microarray.
Found at: doi:10.1371/journal.pone.0008995.s009 (0.49 MB TIF)

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TessArae, LLC, performed RPM-Flu analysis at NHRC using NHRC-selected clinical specimens as part of the respiratory disease surveillance initiative of the Department of Defense Global Emerging Infections Surveillance and Response System (GEIS), a Division of the Armed Forces Health Surveillance Center, Silver Spring, MD.

RNA preparations from archived reference strains of avian influenza were provided through collaboration between TessArae, LLC, and the U.S. Department of Agriculture, Agricultural Research Service, Southeast Poultry Research Laboratory. Transfers of noninfectious RNA preparations from inoculated egg cultures were conformant to U.S. Veterinary Permit Number 105229, issued to TessArae, LLC, from the U.S. Department of Agriculture Animal and Plant Health Inspection Service.

The opinions and assertions contained herein are those of the authors and none are to be construed as those of the U.S. Department of Agriculture, U.S. Department of Defense, U.S. Department of the Navy, nor any other military service or government agency at large.

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.

Author Contributions

Conceived and designed the experiments: DM CAM KLR DJF DS DAS BL APM ZW JMS MCL KOS CT. Performed the experiments: JB SV CT KMB NCL AML. Analyzed the data: DM CAM KLR DJF PJB JB SV DS DAS BL APM ZW KMB NCL JMS LAB AML MCL BW KOS CT. Contributed reagents/materials/analysis tools: DM CAM KLR DJF PJB DS DAS BL ZW MDS. Wrote the paper: DM MCL CT.

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VIROLOGY JOURNAL

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Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995-2004

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Abstract

Background: Avian influenza virus (AIV) infections have caused heavy economic losses to the poultry industry in Pakistan as well as numerous other regions worldwide. The first introduction of H7N3 AIV to Pakistan occurred during 1995, since then H7N3, H9N2 and H5N1 AIVs have each been sporadically isolated. This report evaluates the genetic origin of the H7N3 viruses from Pakistan collected 1995-2004 and how they disseminated within the country. To accomplish this we produced whole genome sequences for 6 H7N3 viruses and data for the HA and NA genes of an additional 7 isolates. All available sequence from H7N3 AIV from Pakistan was included in the analysis.

Results: Phylogenetic analysis revealed that there were two introductions of H7 into Pakistan and one N3 introduction. Only one of the H7 introductions appears to have become established in poultry in Pakistan, while the other was isolated from two separate outbreaks 6 years apart. The data also shows that reassortment has occurred between H7N3 and H9N2 viruses in the field, likely during co-infection of poultry. Also, with the exception of these few reassortant isolates, all 8 genes in the predominant H7N3 virus lineage have evolved to be phylogenetically distinct.

Conclusions: Although rigorous control measures have been implemented in commercial poultry in Pakistan, AIV is sporadically transmitted to poultry and among the different poultry industry compartments (broilers, broiler breeders, table egg layers). Since there is one primary H7 lineage which persists and that has reassorted with the H9N2 AIV in poultry, it suggests that there is a reservoir with some link commercial poultry. On a general level, this offers insight into the molecular ecology of AIV in poultry where the virus has persisted despite vaccination and biosecurity. This data also illustrates the importance of sustained surveillance for AIVs in poultry.

Background

Avian Influenza Viruses (AIV) are among the most prominent viruses affecting animal and public health. AIV infections have caused heavy economic losses to the poultry industry world-wide. Several, sporadic outbreaks of AIV of different subtypes have occurred in Pakistan since the mid-1990's [1-3]. The first highly pathogenic (HP) AIV outbreak was observed in Pakistan in December 1994 at Salgran, near the capital city of Islamabad. The disease was controlled within 4-5 months by mass vaccination with a vaccine prepared from a field isolate [4]. Then in November 1998 an outbreak that was later identified as H9N2 low pathogenicity (LP) AIV occurred in North West Frontier Province in otherwise healthy flocks that had not been vaccinated for AIV [1,3]. In 2000-2001, another outbreak was observed in Central Pakistan (Punjab), caused by H7N3 LP AIV which was controlled by ring vaccination with an aqueous-based vaccine produced with a local AIV strain followed by administration of an oil-based vaccine. During early 2003, another outbreak of H7N3 LPAIV occurred in the Southern coastal region of the country, where more than 70% of the total commercial layer flocks in Pakistan are reared. Within months this LP AIV strain mutated to the HP form, producing a sudden increase in mortality and in November 2003 the H7N3 virus had an intravenous

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pathogenicity index of 2.8. A LP AIV of the H9N2 subtype, was isolated from some of the same flocks infected with the H7N3 HP AIV. This outbreak spread during the next 4 weeks, and primarily affected commercial table-egg layer flocks throughout the poultry estates in that area. The outbreak was controlled by adopting strict biosecurity measures, voluntary depopulation, strategic vaccination, and the implementation of a surveillance program in poultry populated areas throughout the country [4].

This study was conducted to characterize the genomes of the H7N3 type Influenza Viruses, circulating in Pakistan from 1995 through 2004, and to elucidate the pattern of AIV spread and reassortment within the country.

Results

H7 Hemagglutinin gene

There were 2 phylogenetic groups of H7 HA genes from AIV isolates from Pakistan (abbreviations defined in Table 1). There was a major group with 18 isolates (Figure 1) with 98.3-99.9% nucleotide (nt) identity (additional file 1). A second smaller group was comprised of 2 isolates: 35/Chakwal-01 and chicken/Pakistan/34668/95 (34668/Pak-95) which had between 88.6 and 89.5% nt identity with the HA genes of the other isolates from Pakistan. These two isolates, 35/Chakwal-01 and 34668/Pak-95, were most closely related to the HA gene of A/Parrot/NorthIreland/YF73-67/1973 (H7N1) (approximately 99.7% identity). The most closely related lineages were recent viruses from poultry in China (e.g. A/Duck/Nanchang/1904/1992) and the Eurasian lineage isolated in Italy during 1997-2003 both of which had 91.0-93.0% identity with the viruses from Pakistan (Figure 1).

Three deduced amino acid sequences for cleavage site of the HA genes from a total of 17 H7 viruses from Pakistan were observed. Thirteen of the viruses had the sequence PETPKRRK/R, two isolates (34669/Pak-95 and 447/Pak-95) had the sequence PETPKRKRK/R, and two

Table 1: Abbreviations used and GenBank accession numbers for H7N3 Influenza virus isolates included in this study as new sequence

Isolate	Abbreviation	HA	NA	NS	M	NP	PA	PB2	PB1
A/Chicken/Murree/NARC-01/1995	01/Murree-95	FJ577510	FJ577512	FJ577514	FJ577511	FJ577513	FJ577515	FJ577517	FJ577516
A/Chicken/Chakwal/NARC-35/2001	35/Chakwal-01	FJ577518	FJ577520	FJ577522	FJ577519	FJ577521	FJ577523	FJ577525	FJ577524
A/Chickem/Karachi/NARC-23/2003	23/Karachi-03	FJ577526	FJ577528	FJ577530	FJ577527	FJ577529	FJ577531	FJ577533	FJ577532
A/Chicken/Chakwal/NARC-46/2003	46/Chakwal-03	FJ577534	FJ577536	FJ577538	FJ577535	FJ577537	FJ577539	FJ577541	FJ577540
A/Chicken/Karachi/NARC-100/2004	100/Karachi-04	FJ577542	FJ577544	FJ577546	FJ577543	FJ577545	FJ577547	FJ577549	FJ577548
A/Chicken/Chakwal/NARC-148/2004	148/Chakwal-04	FJ577550	FJ577552	FJ577554	FJ577551	FJ577553	FJ577555	FJ577557	FJ577556
A/Chicken/Karachi/SPVC-1/2004	1/Karachi-04	FJ638319	FJ638320	-[a]	-	-	-	-	-
A/Chicken/Karachi/SPVC-2/2004	2/Karachi-04	FJ638321	FJ638322	-	-	-	-	-	-
A/Chicken/Karachi/SPVC-3/2004	3/Karachi-04	FJ638323	FJ638324	-	-	-	-	-	-
A/Chicken/Karachi/SPVC-4/2004	4/Karachi-04	FJ638325	FJ638326	-	-	-	-	-	-
A/Chicken/Karachi/SPVC-5/2004	5/Karachi-04	FJ638327	FJ638328	-	-	-	-	-	-
A/Chicken/Karachi/SPVC-6/2004	6/Karachi-04	FJ638329	FJ638330	-	-	-	-	-	-
A/Chicken/Karachi/SPVC-7/2004	7/Karachi-04	FJ638331	FJ638332	-	-	-	-	-	-

a. - No sequence data available.

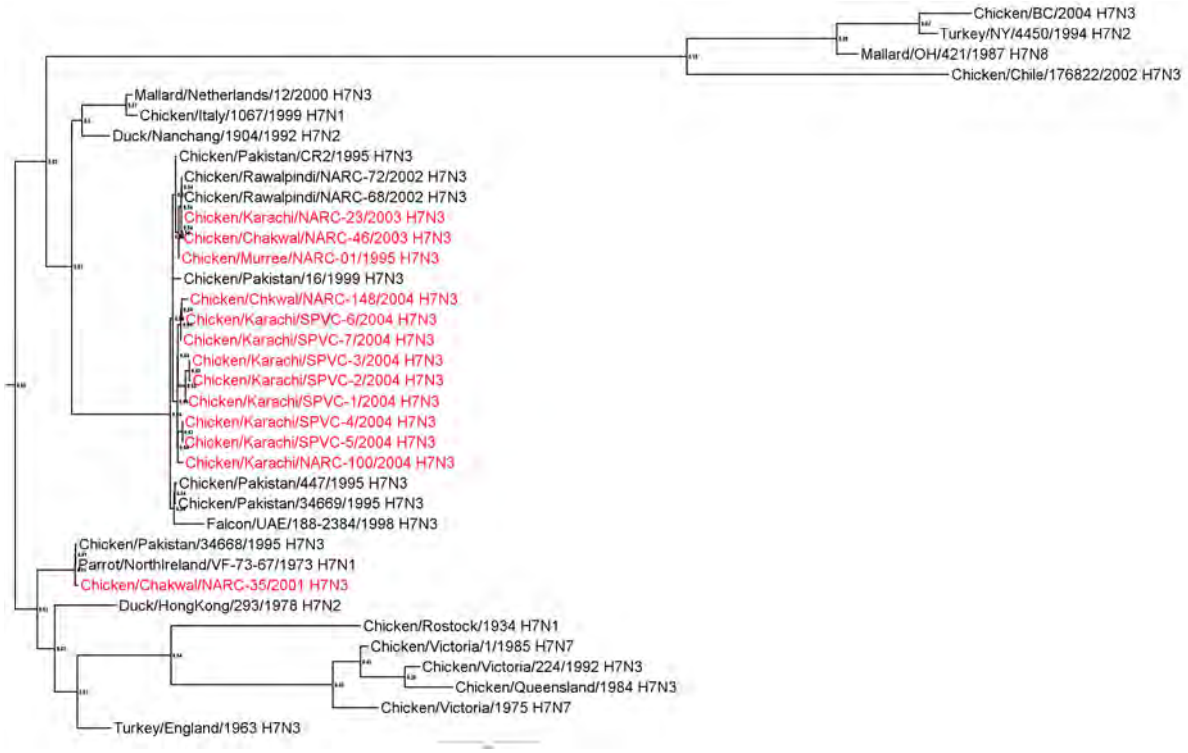

Figure 1 Phylogenetic tree of the Pakistani AIV H7 HA genes and other selected AIV isolates. The tree was constructed with merged duplicate runs of BEAST v. 1.4.8 using HKY substitution, empirical base frequency, gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule process tree prior with default operators with unweighted pair group mean with arithmetic average starting tree and a Markov Chain Monte Carlo length of 10^7.

isolates (35/Chakwal-01 and 34668/Pak-95) had the sequence PEIPKG/R. All were classified as HPAIV except 35/Chakwal-01 and 34668/Pak-95 which are LPAI viruses.

N3 Neuraminidase gene

All the NA genes from the Pakistani isolates were closely related to each other with 98.9-100% nt identity (additional file 2) and formed a single phylogenetic group (Figure 2). The most closely related N3 lineage with 93.9-95.0% identity were the poultry and wild bird viruses from Europe (Figure 2). All of the isolates from Pakistan had a full length NA stalk.

Non-structural gene

Most of the Pakistani H7N3 isolates had closely related NS genes (around 98.7-100% nt identity) (additional file 3) of which all were subtype A (Figure 3). Twenty-four encoded a truncated NS1 protein of 217 amino acids (aa). The isolates with the truncated NS1 all clustered together phylogenetically. Two isolates from the 2004 outbreak (100/Karachi-04 from Southern Pakistan, and A/Chicken/Mansehra/NARC-1282/2004 from Mansehra, Abbottabad in Northern Pakistan) encode the full-length (230 aa) NS1 gene. The NS genes from these two isolates are most closely related to the Pakistan H9N2 viruses from 2006 with around 97.0% nt identity. Another exception was 01/Murree-95, which had a 216 aa NS1 and a 120 aa NS2 protein which grouped with the other isolates with truncated NS1 proteins. The NS2 proteins of all other H7N3 isolates were a full length of 121 aa.

Matrix gene

The matrix genes of all the Pakistani H7N3 isolates had nt identity ranging from 99.2-100% (additional file 4) and phylogenetically assort into a single clade with the exception of 100/Karachi-04 which had about 90% identity with the other Pakistani Isolates (Figure 4). The most closely related matrix genes to 100/Karachi-04 are viruses from the Pakistan 2005-2008 H9N2 lineage which have around 97.5-98.5% nt identity with it.

Nucleoprotein gene

Nucleoprotein genes from the H7N3 Pakistani isolates were very closely related with nt identity above 99.7% with the exception of 100/Karachi-04 (additional file 5). The most closely related lineages to the main clade were wild bird and poultry isolates from Europe and Asia col-

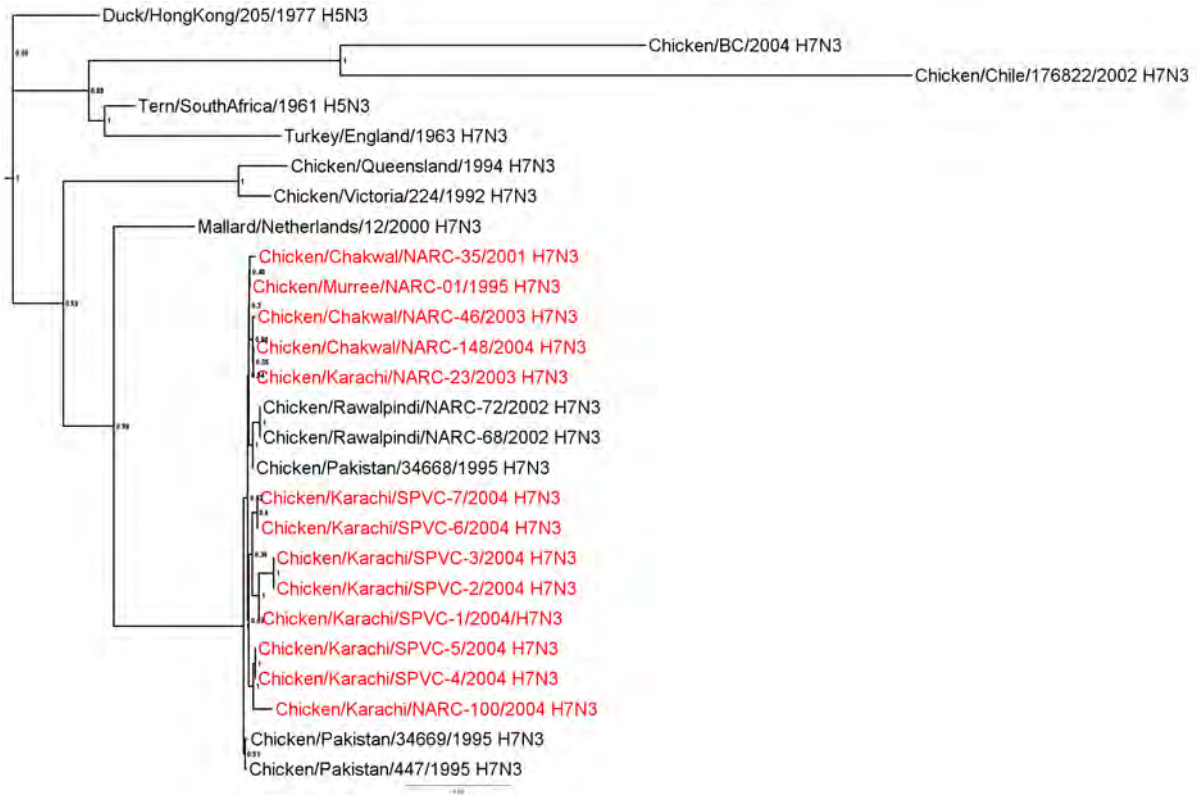

Figure 2 Phylogenetic tree of the Pakistani AIV N3 NA genes and other selected AIV isolates. The tree was constructed as described for figure 1.

lected in the 1990's with around 95% identity (Figure 5). The isolate 100/Karachi-04 had around 91% identity with the NP genes from the other H7N3 viruses and the most closely related lineage was the 1999 and 2005-2008 H9N2 viruses from Pakistan.

PA gene

Among the PA genes from the Pakistani H7N3 isolates the nt sequence identity was above 99.5% with the exception of 100/Karachi-04 which had around 92.0% nt identity with the other H7N3 isolates (additional file 6). Phylogenetically, the NP genes of all the Pakistani H7N3 viruses except 100/Karachi-04, assorted into a single cluster (Figure 6). The most closely related lineages to all the Pakistani viruses were European duck viruses and A/Quail/Dubai/303/2000 (H9N2).

PB1 gene

Nucleotide identity among the PB1 genes of Pakistani H7N3 isolates ranged from 99.6 to 100% (additional file 7), except 100/Karachi-04 which had nt identity of around of 92.7% to the other H7 isolates. Phylogenetic analysis showed that the PB1 of all the Pakistani H7N3 viruses grouped together as a distinct lineage and 100/Karachi-04 grouped with the 2005-2008 Pakistani H9N2 lineage of viruses (Figure 7).

PB2 gene

There was 99.1-100% nucleotide sequence identity among all the Pakistani isolates of the H7N3 subtype except chicken/Pakistan/447/1995 which had around 84% identity (additional file 8). Whereas, 92% and 90% nucleotide sequence similarity was observed with the isolates A/Quail/Dubai/303/2000 (H9N2) and A/turkey/England/50-92/1991 (H5N1), respectively which were the most related isolates from other lineages (Figure 8).

Discussion

The first H7N3 introduction of AIV into Pakistan in 1995 was LP then after circulating for a period of 6-8 months in the poultry population a HP virus emerged [1,5]. The details of AIV in Pakistan between 1995 and 2002 are limited due to the lack of an AIV surveillance program, which was established in 2002-2003. Prior to that there were some reports of seroconversion to AIV and some sporadic isolations of LPAIV from the poultry population [6] and from domestic birds that had been exported from Pakistan [7]. Importantly, although there was no standard vaccination program, a 1995 isolate was used by various poultry producers as an inactivated vaccine until 2003. Then in 2003-2004 a more extensive outbreak of H7N3 started in Karachi, in the Southern region of Pakistan, then spread in poultry throughout the country and was

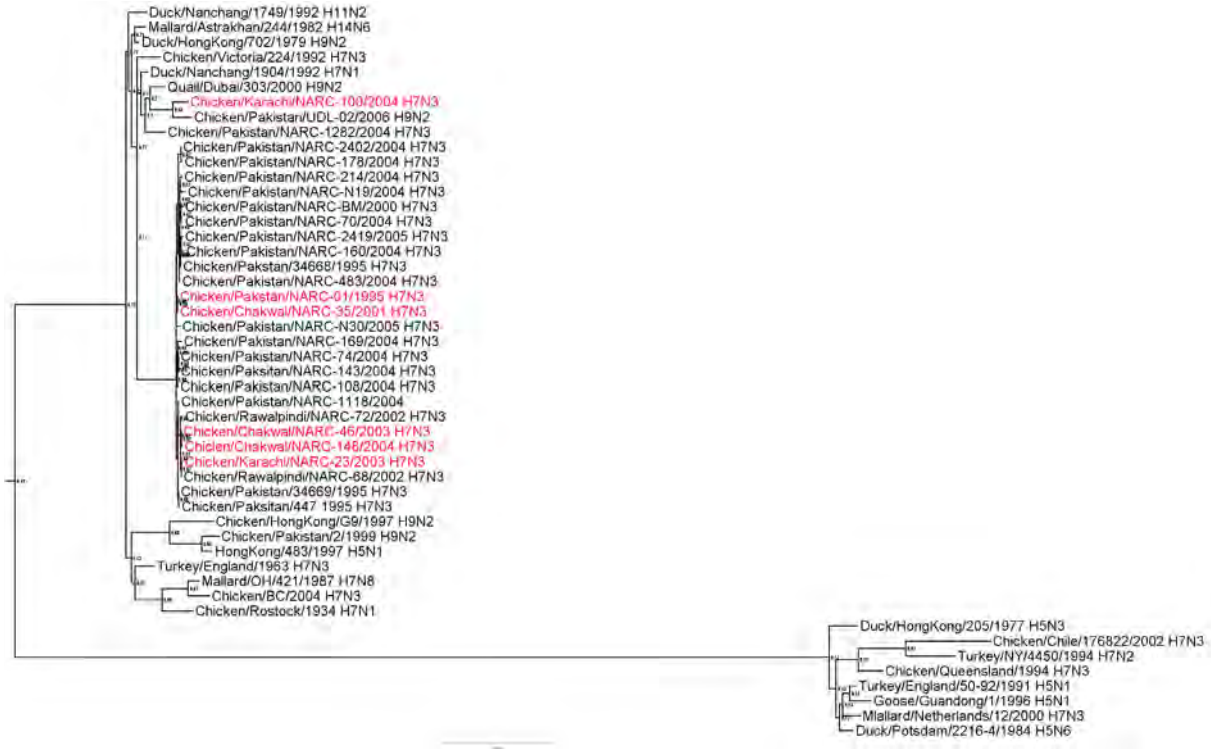

Figure 3 Phylogenetic tree of the Pakistani AIV NS genes and other selected AIV isolates. The tree was constructed as described for figure 1.

controlled with a combination of vaccination and biosecurity [4].

Not surprisingly, these viruses are most closely related to AIVs from Europe, Asia and Australia, which allows for speculation about the related lineages from which the initial viruses were introduced into Pakistan based on possible common ancestors, but not the specific origin of the viruses, i.e. how and when they were introduced. All 8 genes of the H7N3 viruses from Pakistan form distinct clades (at least 4% difference in nt identity with the next most closely related isolated lineage) with these exceptions: 1) the NS, M, NP, PA and PB1 of 100/Karachi-04 which groups with the H9N2 AIVs from Pakistan, and 2) the HA genes of 34668/Pak-95 and 35/Chakwal-01 which group with A/Parrot/NorthIreland/VF-73-67/73 (H7N1); 3) the PB2 of chicken/Pakistan/447/1995 which groups with a 1985 duck virus from Canada.

This suggests that the primary lineage of H7N3 AIVs in Pakistan are the result of a single initial introduction, probably from wild birds and have circulated long enough to evolve into separate clades from other lineages of AIV, including the H9N2 lineages described previously [2,8]. In addition and typical of AIV, the virus has undergone reassortment; 100/Karachi-04 appears to have emerged by reassortment with the H9N2 poultry viruses, therefore was likely generated by concomitant infection of poultry with both lineages, rather than through a novel introduction from wild birds. This agrees with a previous report on the H9N2 AIV lineages in Pakistan by Iqbal et al. [8]. Correlating with this, during this outbreak there were several cases where viruses of both lineages were isolated from the same flock. The HA genes of 34668/Pak-95 and 35/Chakwal-01, which are most closely related to A/Parrot/NorthernIreland/VF-73-67/1973 H7N1 and other viruses from European poultry from the 1970's, are an interesting case as a possible epidemiological link is not obvious. Lastly there is the PB2 gene of chicken/Pakistan/447/1995 which is most closely related to the PB2 of duck/Alberta/228/1985, which is suggestive of a reassortment event with wild bird origin AIVs.

Also, the majority of the H7N3 viruses have some genetic features which are consistent with adaptation to poultry, an HA proteolytic cleavage site (PCS) sequence with multiple basic amino acids [2,9,10] and a truncated NS1 protein [11-13]. However, the isolates do not have a neuraminidase stalk deletion, which is not uncommon in poultry adapted isolates [14].

Deduced amino acid sequences of the PCS of the 20 HA genes revealed three sequences, two of which are consistent with HP AIV, (PETPKRRK/R and PETPKRKRK/R), which is a mutation primarily observed in AIVs which have been circulating in chickens or turkeys. Field and clinical pathogenesis data are also consistent with these isolates being HP [1,2]. The remaining isolates,

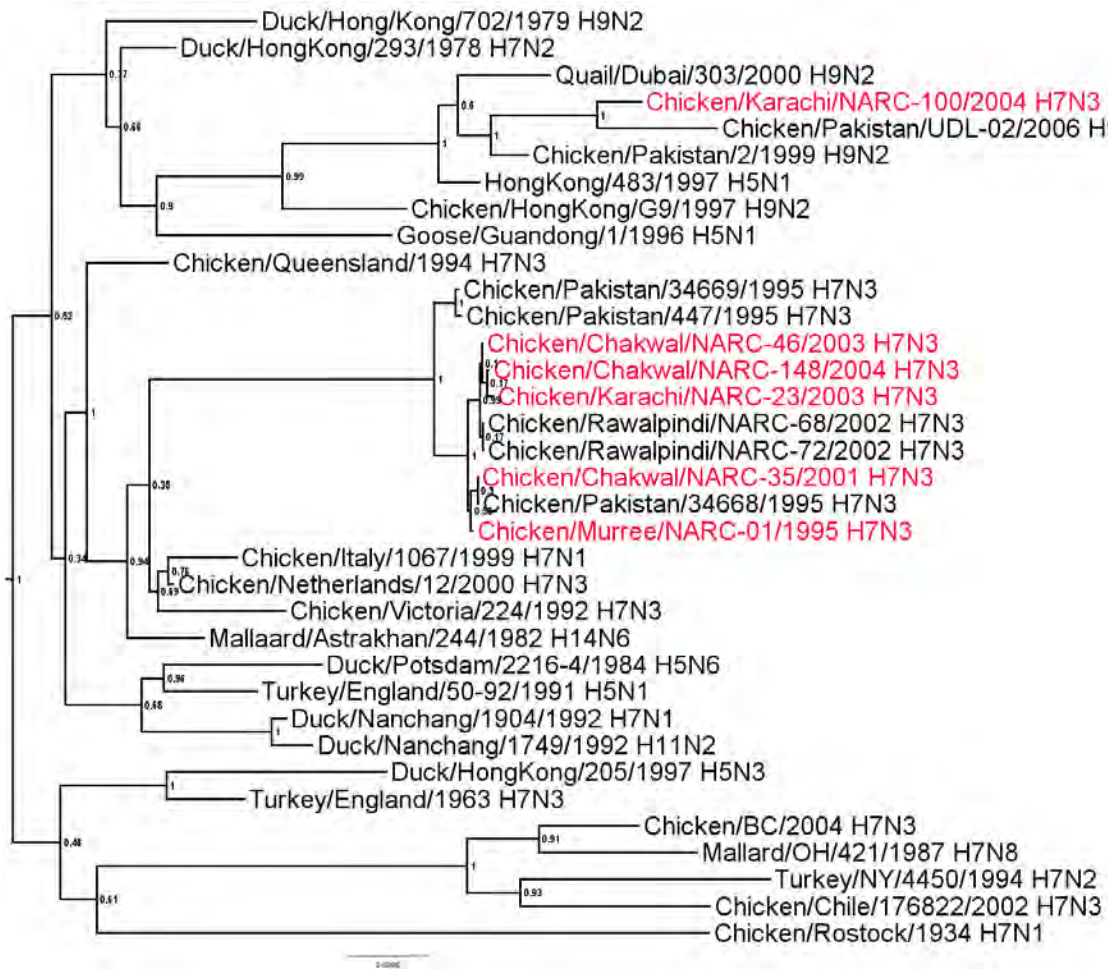

Figure 4 Phylogenetic tree of the Pakistani AIV M genes and other selected AIV isolates. The tree was constructed as described for figure 1.

35/Chakwal-01 and 34668/Pak-95, had a deduced PCS which is considered LP based on the OIE definition [15].

Although the functional reason is not known an apparent genetic adaptation of AIV to poultry appears to be the truncation of the NS1 protein [11,12,16]. The full length protein is 230 aa, while the NS1 of the H7N3 viruses isolated 1995 to 2003 are 217aa indicating that these viruses have adapted to poultry. In contrast, two viruses from 2004 (100/Karachi-04-H7N3 from April 2004 and one from Mansehra, Chicken/Pakistan/NARC-1282/2004, isolated in December 2004) have a full-length NS1 protein of 230aa. The two viruses with the full length NS1 proteins do not group in the main clade of the NS genes from H7N3 viruses from Pakistan, indicating they came from a separate introduction.

Conclusions

This report describes the genetic relationships among the H7N3 AIVs in Pakistan and their reassortment with wild bird viruses and the H9N2 AIVs in poultry in Pakistan. Although the specific sources of these viruses can not be known, sporadic isolations since their introduction suggest that despite vigorous control measures in commercial poultry there have been unidentified reservoirs within the country that have an epidemiological link with commercial poultry from different compartments (broilers, broiler breeders, table egg layers). The behavior of H7 viruses in Pakistan varied among the different regions of Pakistan during these outbreaks as some were controlled faster than others, this is likely to be due in at least part to local differences in vaccine use and control programs. The persistence of a single H7 lineage which causes sporadic outbreaks in geographically different regions of Pakistan suggests that there is a reservoir for which transmission is not completely controlled by vaccination and biosecurity, possibly backyard poultry. A reservoir in Pakistan is further supported by the genetic distance between the isolates in Pakistan and other recent isolates from the same region as the viruses in Pakistan have been able to evolve into a "Pakistani" lineage. Because out-

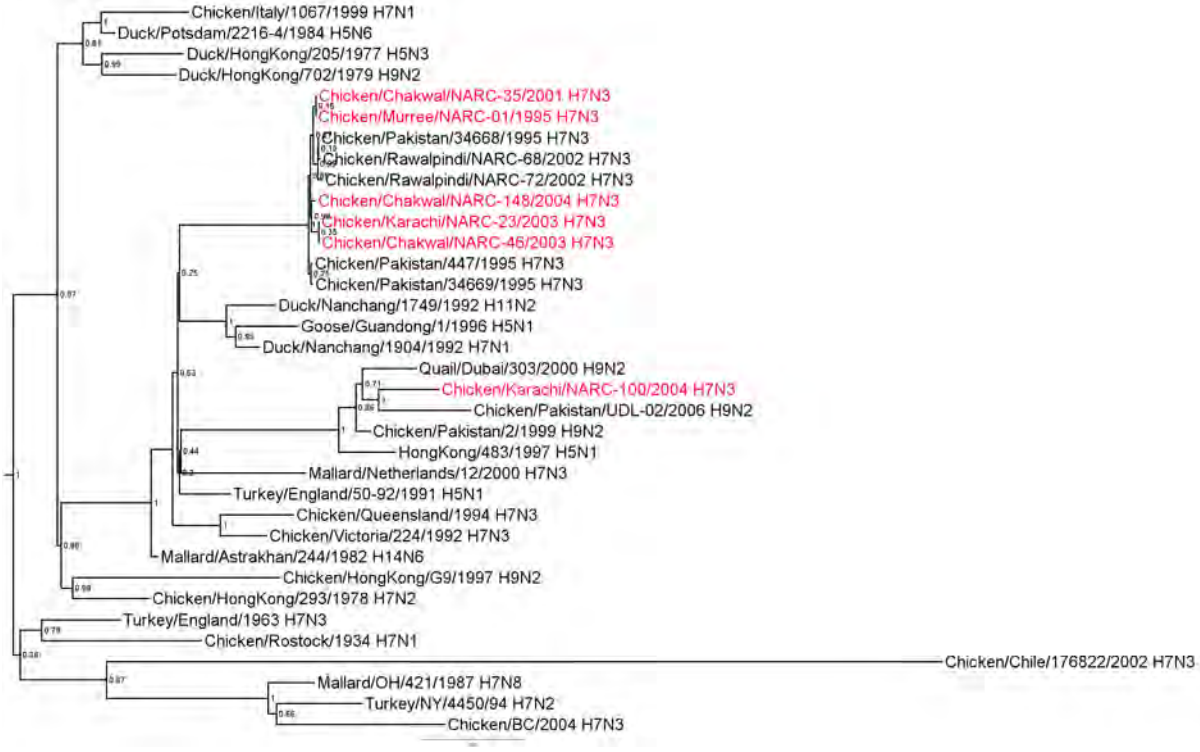

Figure 5 Phylogenetic tree of the Pakistani AIV NP genes and other selected AIV isolates. The tree was constructed as described for figure 1.

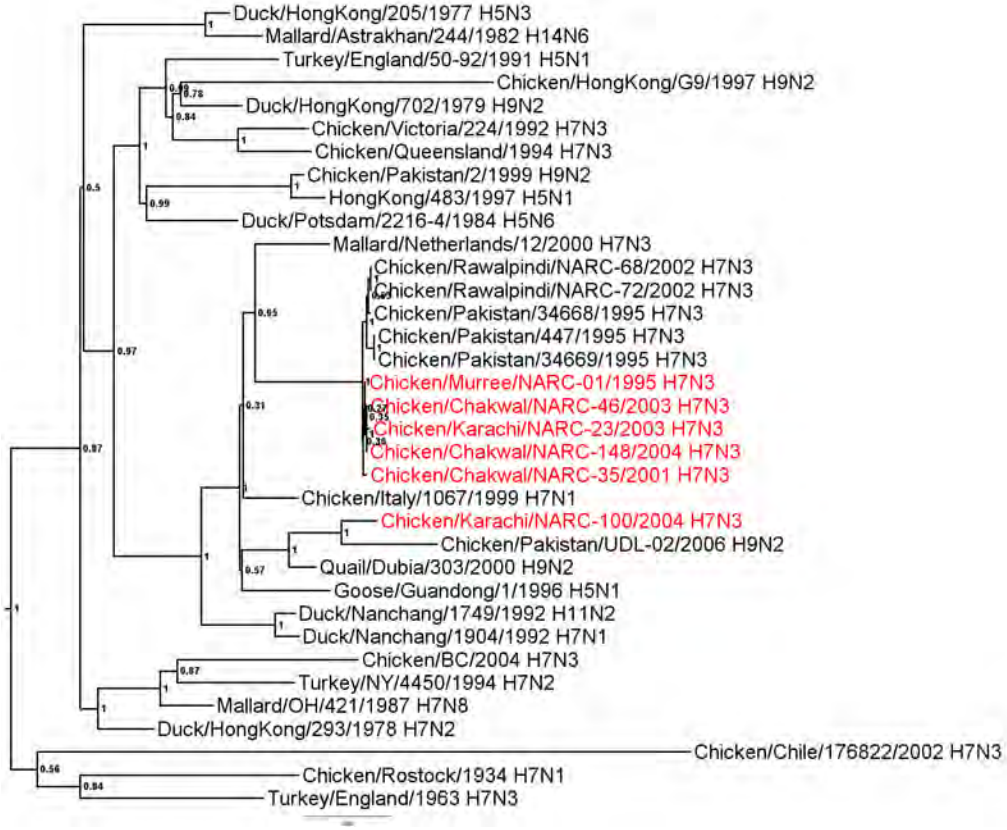

Figure 6 Phylogenetic tree of the Pakistani AIV PA genes and other selected AIV isolates. The tree was constructed as described for figure 1.

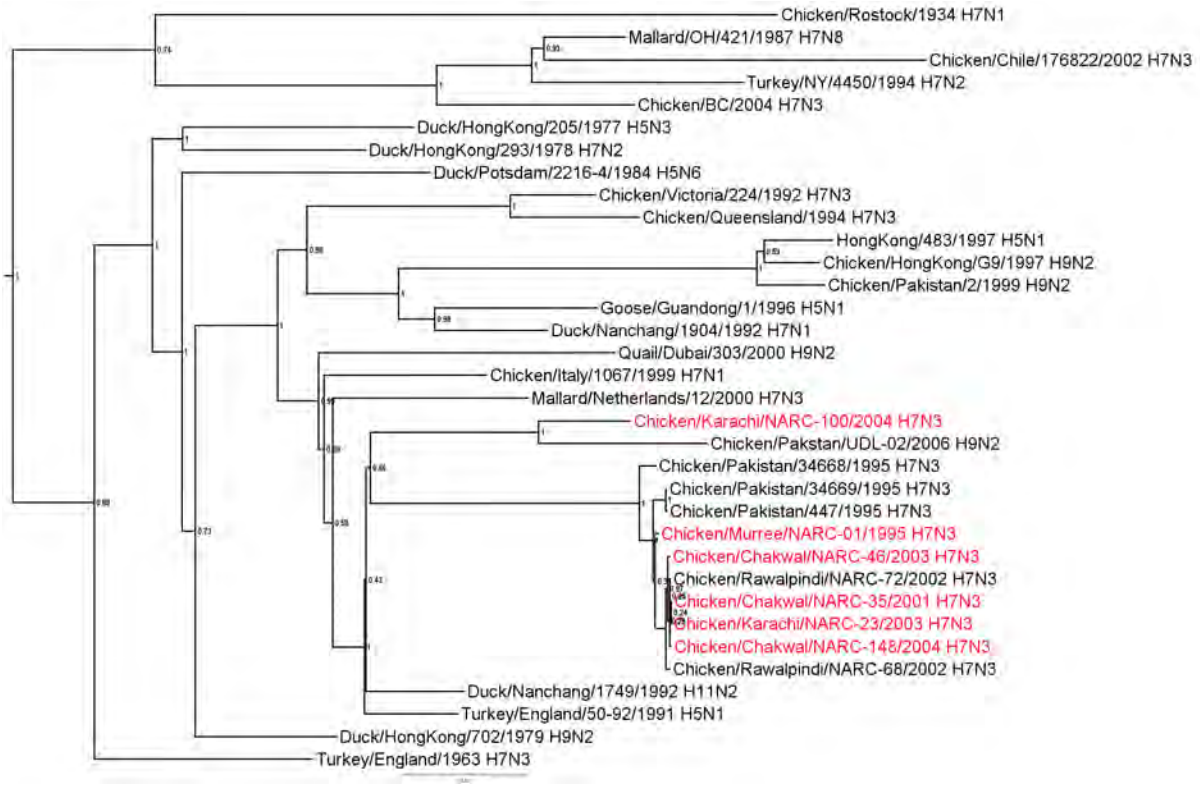

Figure 7 Phylogenetic tree of the Pakistani AIV PB1 genes and other selected AIV isolates. The tree was constructed as described for figure 1.

breaks with these and other AIVs have substantial economic impact in poultry, virus surveillance and uniform control activities need to be implemented on a sustainable basis in Pakistan. Additionally, recognition of any genetic changes in the circulating AIVs will help to implement better and more targeted control programs.

Methods

H7N3 AIV Isolates

Six AIV isolates (Table 1) were selected from the repository of National Reference Laboratory for Poultry Diseases (NRLPD) at the National Agricultural Research Centre (NARC), Islamabad, Pakistan. The isolates were selected to represent different times of isolation, different sectors of poultry production and different geographical origins as described below. An additional 7 isolates from 2004 from the Sindh Poultry Vaccine Centre (SPVC), Karachi, Pakistan were included (Table 1). All isolates were propagated in specific pathogen free embryonating chicken eggs by standard procedures [17].

Isolate 01/Murree-95 was from Murree (Salgran) about 30 Km East of the federal capital, Islamabad, which is densely populated with broiler breeders. Isolates 35/Chakwal-01, 46/Chakwal-03 and 148/Chakwal-04 were from the Chakwal region (the Central Region of Pakistan), which is about 100 Km to the Southwest of the Islamabad. Almost all type of poultry populations (broiler, layer, breeder and backyard) are reared in this region. The isolates 35/Chakwal-01 and 148/Chakwal-04 were from commercial layer flocks while isolate 46/Chakwal-03 was from a broiler flock. The remaining nine isolates were from the Karachi area, which is the Southern Coastal Region of Pakistan, located about 1,050 Km from Chakwal and 1,170 Km from Murree. More than 70% of the country's total commercial layer population is located in this region.

The AIV vaccination status of the flocks from which the isolates in the NRLPD repository originated is known. Three isolates, 01/Murree/95, 23/Karachi-03 and 46/Chakwal/03, were from the poultry flocks not vaccinated against AIV, while 35/Chakwal-01, 148/Chakwal-04 and 100/Karachi-04 were from vaccinated flocks.

Sequencing

Sequencing and analysis was conducted at Southeast Poultry Research Laboratory, US Department of Agriculture, Agricultural Research Service. Full genome sequence was generated for the 6 isolates from the NRLPD repository, while only the HA and NA genes of the 7 Karachi 2004 isolates from the SPVC repository were sequenced.

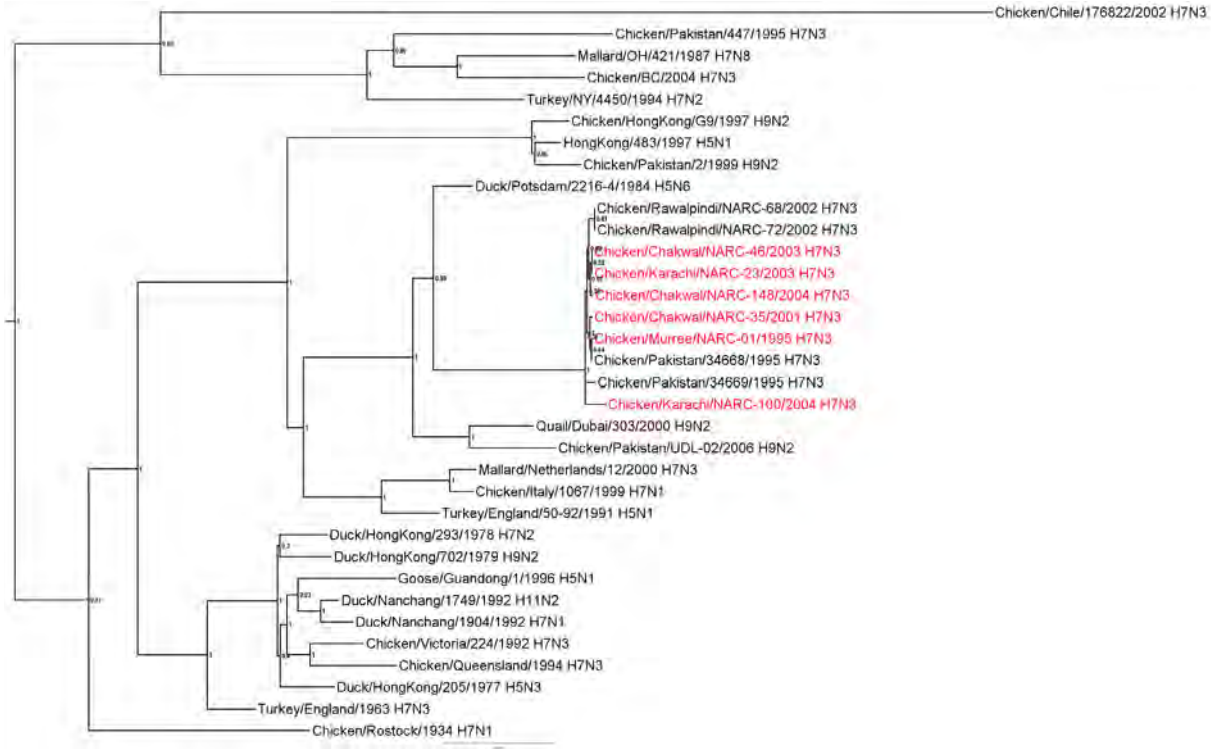

Figure 8 Phylogenetic tree of the Pakistani AIV PB2 genes and other selected AIV isolates. The tree was constructed as described for figure 1.

RNA was extracted from egg fluids with Trizol LS reagent (Invitrogen, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. Sequencing templates for individual influenza genes were produced by amplifying the full coding region of each gene by RT-PCR as previously described [18]. Templates were then purified by agarose gel extraction with the QIA-quick gel extraction kit (Qiagen, Inc., Valencia CA). The BigDye terminator kit (Applied Biosystems, Foster City, CA) was used for cycle sequencing and subsequently run on an AB 3730 (Applied Biosystems, Foster City, CA). GenBank accession numbers are given in Table 1.

Phylogenetic and Sequence Analysis

Phylogenetic analysis included any available sequence data from H7N3 isolates from Pakistan (therefore different numbers of each gene were analyzed), other closely related isolates based nucleotide sequence BLAST search, and numerous sequences from type A influenza isolates from numerous species, dates and regions. The trees shown here were constructed with selected isolates of different lineages to show the genes both in larger context of type A influenza and more closely related isolates. Sequences were aligned with Clustal V (Lasergene, V. 8.0.2 DNAStar, Madison WI). Trees were constructed with merged duplicate runs of BEAST v. 1.4.8 [19] using HKY substitution, empirical base frequency, gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule process tree prior with default operators with unweighted pair group mean with arithmetic average starting tree and a Markov Chain Monte Carlo length of 10^7.

Additional material

Additional file 1 Distance matrix of HA genes shown in figure 1. Similarity (upper triangle) and divergence (lower triangle) of influenza virus H7 HA genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 2 Distance matrix of NA genes shown in figure 2. Similarity (upper triangle) and divergence (lower triangle) of influenza virus N3 NA genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 3 Distance matrix of NS genes shown in figure 3. Similarity (upper triangle) and divergence (lower triangle) of influenza virus NS genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 4 Distance matrix of M genes shown in figure 4. Similarity (upper triangle) and divergence (lower triangle) of influenza virus M genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 5 Distance matrix of NP genes shown in figure 5. Similarity (upper triangle) and divergence (lower triangle) of influenza virus NP genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 6 Distance matrix of PA genes shown in figure 6. Similarity (upper triangle) and divergence (lower triangle) of influenza virus PA genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 7 Distance matrix of PB1 genes shown in figure 7. Similarity (upper triangle) and divergence (lower triangle) of influenza virus PB1 genes from Paksitani H7N3 isolates and other selected isolates.

Additional file 8 Distance matrix of PB2 genes shown in figure 8. Similarity (upper triangle) and divergence (lower triangle) of influenza virus PB2 genes from Paksitani H7N3 isolates and other selected isolates.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MAA, ZA, LS and NS produced and analyzed the sequence data. ES was involved in experimental design, performed the phylogenetic analysis and sequence analysis. DES, KN, AH and SR were involved in experimental design including providing the isolates and associated information. All authors have read an approved the final manuscript.

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Characterization and efficacy determination of commercially available Central American H5N2 avian influenza vaccines for poultry

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ABSTRACT

A poultry vaccination program was implemented in Central America beginning in January 1995 to control both H5N2 low (LPAI) and high pathogenicity avian influenza. This study was conducted to identify seed strain composition and the efficacy of 10 commercially available H5 vaccines against challenge with H5N2 LPAI viruses isolated from Latin America in 2003. The original 1994 vaccine seed virus in commercial inactivated vaccines did not significantly reduce challenge virus shed titers. However, two seed strains of inactivated vaccines, genetically more closely related to the challenge virus, did significantly reduce titers of challenge virus shed from respiratory tract. In addition, a live recombinant fowlpox virus vaccine containing a more distantly related Eurasian lineage H5 gene insert significantly reduced respiratory shedding as compared to sham vaccinates. These results demonstrate the feasibility of identifying vaccine seed strains in commercial finished products for regulatory verification and the need for periodic challenge testing against current field strains in order to select efficacious vaccine seed strains.

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1. Introduction

Antigenic drift has been observed in type A influenza viruses resulting from point mutations which ultimately change the hemagglutinin (HA) protein epitope structure [1]. These changes allow the virus to evade the host's acquired immunity regardless if the acquired immunity is from vaccination or natural infection, and such viruses can spread in a functionally naïve population. Therefore, every year human seasonal influenza vaccine formulation requires reevaluation by comparison with the major currently circulating strains and, periodic replacement of older strains with new variant strains to maintain efficacious human vaccines [2,3]. In contrast, broad immunity (i.e. immunity to variant strains of the same subtype as measured by reduction in viral titers and shed) to high pathogenicity avian influenza (HPAI) has been observed from avian influenza (AI) vaccination in chickens even when the HA protein sequences have as little as 86% nucleotide (or amino acid) identity [4,5]. This broad immunity might be attributed to the oil adjuvant systems used in inactivated vaccines to produce robust humoral response and the cell-mediated immunity from live recombinant virus vaccines. Prior to 1995, antigen drift of field viruses was of low concern because of the lack of endemicity of AI in most countries' commercial poultry and long term vaccination programs have historically been rare therefore, selective immune pressure on field viruses has been minimal [6,7].

H5N2 low pathogenicity avian influenza (LPAI) virus was first identified in Mexico in May 1994 and by November 1994 had mutated into HPAI virus. In January 1995, emergency eradication activities were initiated and included depopulation of affected farms, cleaning and disinfection of facilities, use of sentinel birds and vaccination [8,9]. The government of Mexico declared Mexico HPAI free in May 1995. However, the vaccination program was continued with the intent for vaccine use as a tool towards the eradication of H5N2 LPAI. More than 1 billion doses of inactivated emulsified vaccines were used between 1995 and 2001 and 459 million doses of recombinant fowlpox virus-vectored H5 AI vaccine were used between 1998 and 2001 [10]. However, after more than 14 years and 2 billion doses, H5N2 LPAI is still present in some parts of Mexico and genetically related viruses have periodically spread to Guatemala, El Salvador, Dominican Republic and Haiti, although no additional HPAI outbreaks have occurred [11]. One study phylogenetically analyzed the H5 HA gene from 52 LPAI virus isolates isolated 1993–2002 and concluded that most viruses isolated after the introduction of vaccine did not belong to the vaccine

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seed strain lineage and antigenic drift had occurred resulting in the lack of optimal protection observed in challenge studies when using an experimental, non-commercial inactivated oil-emulsion vaccine containing the original 1994 H5N2 vaccine seed strain [6]. Presently, there are concerns about viral antigenic drift and viral evolution due to selection pressure created by vaccinal immunity and how this could affect which seed strains of virus are used in commercial vaccines as well as their potency.

This study was conducted to identify the seed strain composition and evaluate the efficacy of commercial H5N2 vaccines used in some areas of Latin America following challenge with recent H5N2 LPAI field viruses. Our study demonstrates that 3 different inactivated seed strains were used in current commercially available inactivated vaccines as well as a live recombinant fowlpox virus vaccine with a Eurasian AI H5 gene insert (rFPV-AIV-H5). The inactivated vaccines using the original 1994 virus as the seed strain did not protect chickens against H5N2 LPAI virus challenge as evidenced by similar levels of respiratory viral replication and shedding as sham vaccinated chickens. By contrast, two inactivated vaccines using seed strains more closely related to the 2003 challenge virus and the live rFPV-AIV-H5 vaccine provided better protection based on significant reduction in respiratory virus shed.

2. Materials and methods

2.1. Viruses

Two closely related viruses from the same premises were selected as challenge viruses because they represent the most recent H5N2 avian influenza viruses available from Latin America to international reference laboratories. Virus stocks of A/chicken/Guatemala/270475-1/03 and A/chicken/Guatemala/270475-4/03 which share 98.9% identity in hemagglutinin protein were grown by passage in 9-day-old embryonating chicken eggs twice. Allantoic fluid was removed and diluted in brain-heart infusion (BHI) medium to a final titer of 10^6 50% embryo infectious doses (EID_{50}) per 0.1 ml as previously described [12].

2.2. Vaccines

Commercially available inactivated H5 whole virus vaccines, adjuvanted, in proprietary oil emulsions were used. A rFPV-AIV-H5 genetically engineered to contain the HA gene insert from A/turkey/Ireland/84 (H5H9) (rFPV-AI-H5) was also used

2.3. Vaccine seed strain identification

The most closely related isolate (MCRI) to the seed strain (and thus likely the seed strain) used to produce each vaccine was identified by sequencing. Total RNA was extracted from the inactivated, oil adjuvant vaccines by adding 100 μl vaccine to 900 μl TRIzol® LS reagent (Invitrogen Inc., Carlsbad, CA), mixing by vortexing, incubating at room temperature for 5 min, and then adding 200 μl of chloroform. The mixture was mixed by vortexing, incubated at room temperature a minimum of 10 min and centrifuged for 15 min at 12,000 × *g*. RNA extraction was completed with the top two phases using the MagMAX™ 96 AI/ND Viral RNA isolation kit (Ambion Inc., Austin, TX) in accordance with kit instructions using the KingFisher® magnetic particle processor (Thermo Scientific, Waltham, MA). DNA was extracted from 0.5 ml of a 1:10 dilution of the rFPV-AI-H5 vaccine (vaccine J) in PBS using the DNeasy® kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's instructions and eluted in 50 μl of nuclease-free water. The sequencing template was prepared by using 3 μl of DNA in a 50 μl PCR reaction using 2X PCR Master Mix (Promega Inc., Madison, WI) using the same primers and PCR conditions as the other vaccines

Sequencing templates were generated from the vaccine derived RNA by RT-PCR using primers directed to conserved sequences in the HA1 region of the H5 HA gene. The forward primer was H5 F645 5′-CTACCAGAACTCGAACACTTA-3′, and the reverse primer was R1082 5′-GCTCCAAAKAGKCCTCTTGT-3′. This primer pair failed to amplify the material in vaccine I, therefore a second set of primers using a conserved sequence at the 5′ end of the gene [13] with a reverse primer in the HA1 region of the gene; H5 R230 5′-AGC CAT CCA GCT ACA CTA CCA-3′ was used. Due to the poor yield and quality of RNA that can be obtained from inactivated, oil emulsion vaccines, we targeted relatively short segments and did not attempt to generate sequence for the full length HA genes.

A one-step RT-PCR kit (Qiagen Inc., Valencia, CA) was used in accordance with the kit instructions for a 25 μl reaction volume with 5 μl of total RNA template. Thermal cycling conditions were a reverse transcription step of one cycle of 50 °C for 30 min and 95 °C for 15 min then 35 cycles of 95 °C for 45 s, 53 °C for 1 min and 72 °C for 1 min. The RT-PCR products were visualized by agarose gel electrophoresis. Products of the expected size (485 bp) were excised, purified with the QIAquick® gel extraction kit (Qiagen Inc.) and directly sequenced with the primers used to produce the template. The sequencing template for the rFPV-AI-H5 vaccine was generated with the same primers and PCR cycling conditions listed above using the Promega 2X PCR MasterMix (Promega Inc.).

Cycle sequencing was performed with the BigDye® terminator kit (Applied Biosystems, Foster City, CA) and run on an ABI 3730 (Applied Biosystems). Contigs were assembled with SeqMan® 7.1 (DNAStar, Madison, WI) and were initially characterized by BLAST^SM search to find the most similar sequences in GenBank^SM. Phylogenetic analysis was performed by aligning the deduced amino acid sequences with Clustal V (Lasergene® 7.1, DNAStar, Madison, WI). Trees were constructed with merged duplicate runs with BEAST v. 1.4.8 [14] using Blosum62 substitution, Gamma + invariant site heterogeneity model, relaxed lognormal clock, Yule Process tree prior with default operators with UPGMA starting tree and MCMC length of 10^6.

2.4. Animals and housing

Animals were cared for and housed in accordance to an Institutional Animal Care and Use Committee approved animal use protocol at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, GA, USA. Experiments were performed in the USDA-certified Biosafety Level 3-enhanced facility [15]. Birds had *ad libitum* access to feed and water.

2.5. Experimental design: experiment 1, inactivated vaccines

Twelve-day-old specific pathogen free (SPF) White Plymouth Rock chickens from in-house flocks were bled for serum then vaccinated with the appropriate commercial vaccine subcutaneously in the nape of the neck as per manufacturer's instructions with 0.5 ml for vaccines A–H or 0.2 ml for vaccine I. At 3 weeks post-vaccination, birds were banded for identification, bled for serum and challenged intranasally (i.n.) with 10^6 EID_{50} per bird of A/chicken/Guatemala/270475-4/03 (H5N2). The sham vaccinated group received a vaccine prepared with non-infectious allantoic amniotic fluid mechanically emulsified in 4 parts of oil phase as previously described [16,17].

2.6. Experiment 2, recombinant fowlpox virus vaccine (rFPV-AI-H5)

Chickens were inoculated at 1 day of age, subcutaneously in nape of the neck with 0.2 ml per bird as per the manufac-

Table 1
Most closely related influenza isolate (MCRI) to each vaccine based on BLAST search of partial HA1 region of the HA gene and the percent amino acid (aa) identity. The percent aa identity for the entire HA gene of the MCRI with the challenge virus is also provided.

Vaccine	Most closely related isolate (MCRI)	%aa identity of vaccine with MCRI[a]	%aa identity of vaccine with the closest strain in SEPRL repository[b]	%aa identity of vaccine with the challenge strain[c]	%aa identity of vaccine with most recent H5N2 from Central America[d]
A	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	98.5	98.5[e]	89.6	86.9
B	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	100	100[e]	89.3	87.2
C	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	98.4	98.4[e]	88.4	87.5
D	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	98.5	98.5[e]	89.6	86.9
E	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	100	100[e]	88.5	84.5
F	A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)	98.3	98.3[e]	87.4	86.4
G	A/Chicken/Guatemala/45511-5/00 (H5N2)	96.9	97.7[f]	95.3	94.5
H	A/Chicken/Guatemala/45511-5/00 (H5N2)	97.0	98.4[f]	94.8	96.1
I	A/Chicken/Ibaraki(Japan)/1/05 (H5N2)	99.1	98[f]	94.8	94.9
J	A/Turkey/Ireland/1378/83	100	100[g]	90.4	80.9

[a] Based on a pair-wise alignment (Lipman–Pearson) of 95 amino acids (aa) from 232 to 327 for vaccines A through H, or aa positions 10–114 for vaccine I.
[b] Closest related H5N2 low pathogenicity virus of Central American lineage in SEPRL repository and was used as HI antigen in cross HI testing.
[c] Based on ClustalV multiple alignments. Challenge strains A/chicken/Guatemala/270475-4/03 (H5N2) for vaccine groups A–I and A/chicken/Guatemala/270475-1/03(H5N2) for vaccine group J.
[d] Hemagglutinin amino acid identity of vaccine seed strains and most recently released virus from Central America (A/chicken/Mexico/435/2005 (H5N2)).
[e] A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2).
[f] A/Chicken/Guatemala/194573/02 H5N2.
[g] A/Turkey/Ireland/1378/83.

turer's instructions. Three weeks post-vaccination, the chickens were challenged by the i.n. route with 10^6 EID_{50} per bird of A/Chicken/Guatemala/270475-1/03. The sham vaccinated received sterile diluent.

To evaluate virus shed for both experiments 1 and 2, oropharyngeal swabs were collected 3 days post-challenge (d.p.c.). At 14 d.p.c. birds were bled for serum and humanely euthanized.

2.7. Quantification of oropharyngeal virus shedding

Oropharyngeal swabs were collected at 3 d.p.c. and were stored at −70 °C until RNA isolations could be carried out. RNA was extracted by adding 750 μl TRIzol® LS (Invitrogen Inc., Carlsbad, CA) to 250 μl swab material. The swab material-TRIzol® LS material was mixed by vortexing and incubated at room temperature for 5 min, then 200 μl of chloroform was added. The material was mixed by vortexing, incubated at room temperature a minimum of 10 min and centrifuged for 15 min at 12,000 × *g*. Instead of precipitation with 2-propanol according to the manufacturer's instructions, the RNA extraction was completed by binding and eluting the RNA from the aqueous phase using the MagMAX™ 96 AI/ND Viral RNA isolation kit (Ambion Inc., Austin, TX) in accordance with kit instructions using the KingFisher® magnetic particle processor (Thermo Scientific, Waltham, MA).

Quantitative RRT-PCR (qRRT-PCR) which targets the influenza M gene [18] was performed using the SmartCycler® 2 (Cepheid Inc., Sunnyvale, CA) and the OneStep RT-PCR kit (Qiagen, Valencia, CA) in accordance with the U.S. National Veterinary Services Laboratories protocol AVSOP1521.01. The standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge virus run in duplicate and reported as relative equivalent units [REU] as EID_{50}/ml. qRRT-PCR detection limit was $10^{4.1}$ EID_{50}/ml or $10^{1.9}$ EID_{50} per reaction for A/chicken/Guatemala/270475-4/03 and $10^{3.7}$ EID_{50}/ml or $10^{1.5}$ EID_{50} per reaction for A/chicken/Guatemala/270475-1/03. If viral RNA was not detected from a sample then a number $10^{0.1}$ EID_{50}/ml lower than the respective lower detection limit was used for statistical analysis (i.e. $10^{4.0}$ EID_{50}/ml for A/chicken/Guatemala/270475-4/03 and $10^{3.6}$ EID_{50}/ml for A/chicken/Guatemala/270475-1/03).

2.8. Serology

Hemagglutination inhibition assays were performed to quantify antibody response to vaccine antigen and challenge virus antigen with serum collected pre- and post-challenge as previously described [12] using 0.5% suspension of chicken erythrocytes in phosphate buffered saline and most HI antigen from most closely related influenza isolate (MCRI) in our repository. For inactivated vaccine groups, cross HI tests were performed using antisera from a single chicken per group with 128-512 GMT to MCRI in our SEPRL avian influenza repository; i.e. either A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2) or A/Chicken/Guatemala/194573/02 (H5N2). The antisera were also tested against the challenge strains A/chicken/Guatemala/270475-1/03 (H5N2) and A/chicken/Guatemala/270475-4/03 (H5N2).

2.9. Statistical analysis

Statistical significance between groups was analyzed by ANOVA for both serological and virological testing. Pearson's correlation coefficient analysis was also carried out using pre-challenge vaccine strain antigen GMT or pre-challenge challenge strain antigen GMT and the respective vaccine group's titer for further statistical

Table 2
Antigenic relatedness of vaccine seed strains based on using antisera raised in SPF chickens against each vaccine and tested in hemagglutination inhibition test using four different antigens: two closest related viruses to vaccine seed strains in the repository and two challenge viruses.

HI test antigen	Antisera								
	Hid/94 (A)	Hid/94 (B)	Hid/94 (C)	Hid/94 (D)	Hid/94 (E)	Hid/94 (F)	Guat/194573/02 (G)	Guat/194573/02 (H)	Guat/194573/02 (I)
A/ck/Hidalgo/26654-1368/94 (H5N2)	256	128	512	256	256	512	32	16	<8
A/ck/Guatemala/194573/02 (H5N2)	32	32	64	128	8	64	128	128	256
A/ck/Guatemala/270475-1/03 (H5N2)	16	16	32	32	8	32	64	32	64
A/ck/Guatemala/270475-4/03 (H5N2)	32	32	64	64	16	64	128	256	256

analysis of serological data. All statistical analyses were carried out using Excel. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Vaccine seed strain identification

RNA was isolated from the commercial inactivated oil emulsified vaccines and DNA from the recombinant fowlpox virus-vectored AI vaccine, the HA1 region of the HA gene was sequenced and phylogenetically analyzed to evaluate protein sequence identity between vaccines and challenge virus and to identify the most closely related virus isolate. The AIV isolate with the most closely related HA gene to the sequence from each vaccine as determined by BLAST search and its identity to the challenge virus is provided in Table 1. Protein sequence comparison between the vaccine seed strains and the challenge virus A/chicken/Guatemala/270475-4/03 yielded percent identity ranging between 87.4 and 89.6 for vaccines A, B, C, D, E and F while analysis revealed vaccines G, H and I seed strains were more closely related to the challenge virus yielding 95.3%, 94.8% and 98.2% protein identity, respectively.

The most closely related virus isolate to the vaccines A, B, C, D, E, and F with 98.3–100% protein identity, was A/chicken/Hidalgo(Mexico)/232/94 (H5N2) which is the official H5N2 AI vaccine seed strain in Mexico. Vaccines G and H seed strains were most closely related to A/chicken/Guatemala/45511-5/00 (H5N2) with 96.9% and 97.0% protein identity, respectively. The most closely related strain to vaccine I was A/chicken/Ibaraki(Japan)/1/05 (H5N2) with 99.1% protein identity. Most closely related strain in our repository to vaccines G, H and I was A/Chicken/Guatemala/194573/02 (H5N2) with 97.7, 98.4 and 98.0% amino acid identity. The recombinant fowl pox-vectored AI vaccine (vaccine J) was verified to have the HA gene inserted from A/turkey/Ireland/1378/83 (H5N9) with 100% protein identity (Fig. 1).

Antigenic relatedness of vaccine seed strain to challenge virus were determine by HI tests. Sera from vaccines A–F had a 3–4 $\log_2$ reduction in HI titer when using challenge virus verses MCRI strain as test antigens (Table 2). However, vaccines G–I only had a 0–2 $\log_2$ reduction in titer when using challenge virus verses MCRI in the repository as test antigen (Table 2). This indicates vaccine seed strains A–F were antigenically more distantly related to the challenge virus while vaccine seed strains G–I were more closely related to the challenge virus.

3.2. Mortality and virus isolation from challenged birds

In experiment 1, only two birds died from the sham group on days 4 and 9. All chickens were tested for viral shedding on 3 d.p.c. from oropharyngeal swabs by qRRT-PCR. Chickens vaccinated with vaccines H or I showed both statistically significant decreases ($P \leq 0.05$) in the quantity of virus shed (vaccines H and I) and the number of birds per group shedding virus (vaccine H only) when compared to the sham group. In the sham group, 10 of 10 birds were positive by qRRT-PCR average REU $10^{7.4}$ EID_{50}/ml. In vaccine groups H and I, 0 of 10 birds and 6 of 10 ($10^{4.4}$ EID_{50}/ml average REU) birds were positive with qRRT-PCR, respectively. Chickens in vaccine groups A–G were positive (10 of 10 in each group) with qRRT-PCR average REU ranging between $10^{6.3}$ and $10^{7.5}$ EID_{50}/ml which were not significantly different when compared to the sham vaccinated group (Table 3).

In experiment 2, the efficacy of vaccine J was examined. The challenge virus used was Chicken/Guatemala/270475-1/03 which had 98.9% identity in hemagglutinin gene with Chicken/Guatemala/270475-4/03. No morbidity or mortality was observed for either vaccinated group or sham group after challenge. Viral shedding was detected and quantified by qRRT-PCR from RNA extracted from 3 d.p.c. oropharyngeal swabs. In the sham group, 8 of 10 birds were positive with a qRRT-PCR average REU $10^{5.2}$ EID_{50}/ml. Only 2 of 10 vaccinated birds were positive with an average REU of $10^{3.9}$ EID_{50}/ml which was significantly reduced in both number of

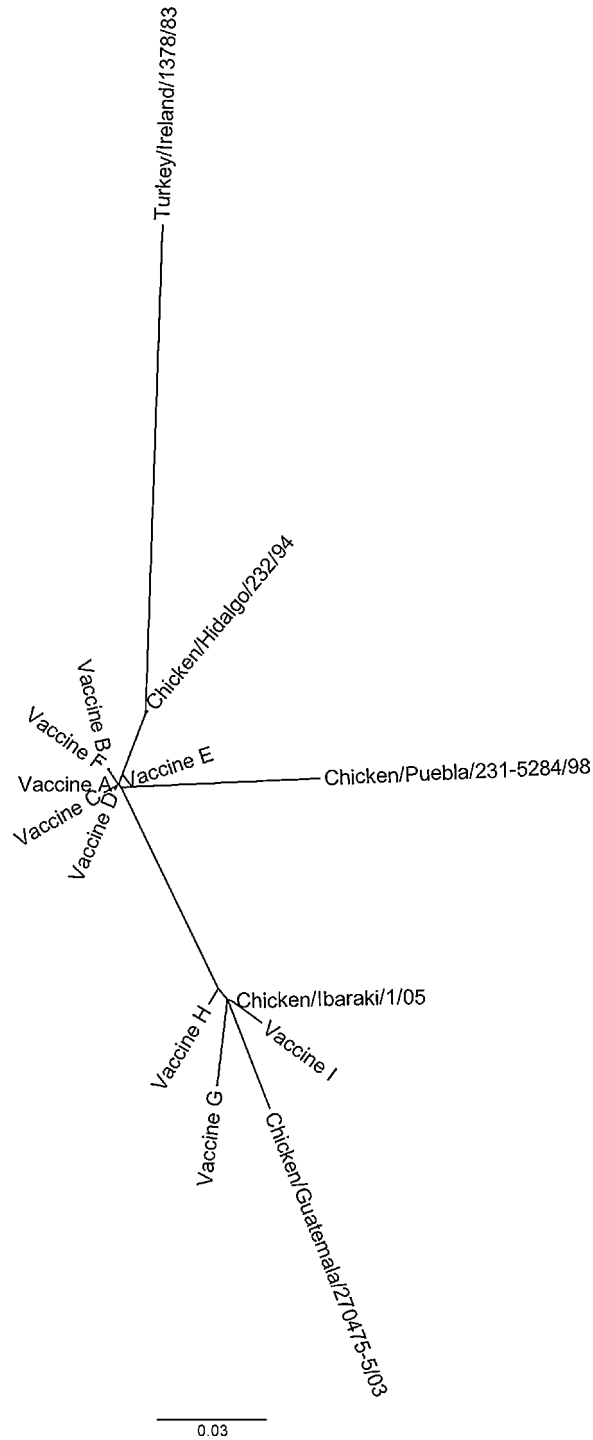

Fig. 1. Phylogenetic tree of partial H5 hemagglutinin sequences from the vaccines used in this study, challenge viruses, and vaccine seed strains. The tree was constructed with merged duplicate runs with BEAST v. 1.4.8 using Blosum62 substitution, Gamma + invariant site heterogeneity model, relaxed lognormal clock, Yule Process tree prior with default operators with UPGMA starting tree and MCMC length of 106.

Table 3
Number of chickens with challenge virus RNA isolated from oropharyngeal swabs (3 d.p.i.) and mean relative equivalent units [REU] ± S.D.

Vaccine group	# positive/total	REU ± S.D.
Sham	10/10	7.4 ± 0.58
A	10/10	7.4 ± 0.35
B	10/10	7.5 ± 0.19
C	10/10	7.4 ± 0.33
D	10/10	7.2 ± 0.33
E	10/10	7.0 ± 1.24
F	10/10	7.3 ± 0.41
G	10/10	6.3 ± 0.81
H	0/10	≤4.0
I	6/10	4.4 ± 1.96

birds shedding and the average titer of virus shed as compared to the sham group ($P \leq 0.05$).

3.3. Serology

Testing for H5 antibodies was conducted using hemagglutinin inhibition (HI) assays on serum. In experiment 1, all birds were negative for H5 antibodies pre-vaccination. Sham groups were negative for H5 antibodies both pre- and post-vaccination. Antibody response to each H5 vaccine is presented in Fig. 2. Respective vaccine specific antibodies were detected in all vaccinated groups post-vaccination and had geometric mean titers (GMT) ranging between 147 for vaccine groups A, B and F to 1351 in vaccine group I. Post-challenge vaccine specific antibody responses ranged between a GMT of 416 for vaccine group F and 4705 for vaccine group I (Fig. 2A). Post-vaccination antibody response using the challenge virus as HI antigen were ≤21 GMT for vaccine groups A–F, and were 39, 32 and 84 GMT for vaccine group G, H, and I, respectively. Post-challenge HI antibody response against the challenge virus was 64, 79, 137, 128, 49, 73, 294, 315, 512 and 39 GMT for vaccine groups A, B, C, D, E, F, G, H, I and the sham group, respectively (Fig. 2B).

In experiment 2, sham vaccinated animals were negative for H5 antibodies at both pre- and post-vaccination. Post-vaccination, vaccine group J induced an antibody response (49 GMT) to the vaccine strain, however there were no detectable antibodies specific for the challenge virus, chicken/Guatemala/270475-1/03. Post-challenge animals in vaccine group J had a GMT of 13 to the challenge strain.

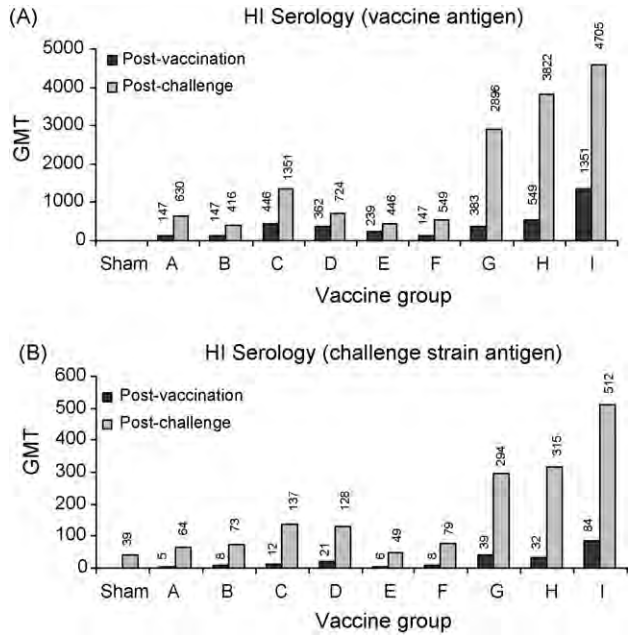

Fig. 2. Serological responses. (A) HI antibody response based on using vaccine strain as antigen on pre- and post-challenge sera. (B) HI antibody response based on challenge virus as antigen on pre- and post-challenge sera.

4. Discussion

Early studies with Latin American H5N2 LPAI viruses identified two sublineages of the HA gene: Puebla and Jalisco [19]. The original seed strain for inactivated vaccine used in Mexico was A/chicken/Mexico(Hidalgo)/232/94 (H5N2) and is from the Jalisco sublineage. A study conducted on viral isolates by Lee et al. [6] found both original sublineages had disappeared and been replaced with new sublineages, A and B. The study indicated sublineage A may have evolved from the Jalisco sublineage and another strain may have been the progenitor strain for sublineage B which includes viruses isolated from Guatemala and El Salvador [6]. Both genetic and antigenic drift of field viruses away from the original vaccine seed strain was demonstrated. In another study 18 AIV isolates collected between 2002 and 2006 from 9 regions in Mexico were analyzed and shown to be related to sublineage B and therefore more distantly related to the official vaccine strain [20]. These data support continued genetic and antigenic drift of the field viruses away from the historical vaccine seed strain from H5N2 poultry vaccines. One of the reasons for the observed antigenic drift could have been vaccine pressure, although the presence of immunity from infection of non-vaccinated flocks by H5N2 LPAI field viruses may also have contributed.

A critical question in ongoing evaluations of H5N2 LPAI vaccination programs is which seed strain or strains are used in commercial vaccines applied in the field. In our study, based on sequencing of short RNA fragments, we observed that inactivated H5N2 vaccines A, B, C, D, E, and F used the official 1994 Mexican seed strain. Vaccines G and H were produced with seed strains that were more recent and closely related to A/chicken/Guatemala/45511-5/00 (H5N2). Unexpectedly, the vaccine I seed strain was most closely related to an outbreak virus from Japan, A/chicken/Ibaraki(Japan)/1/05 (H5N2) (99.1% similarity in hemagglutinin), which is a North American lineage H5 virus and not an Asian lineage H5 virus. This later finding supports the possibility of the introduction of the North American lineage H5N2 virus into Asia via human activity since this virus lineage has not been identified in wild birds, but only in poultry of Latin America and the Caribbean Islands [21]. Vaccine J was a recombinant fowlpox vaccine and confirmed to contain an HA insert from A/turkey/Ireland/1378/83 (H5N9) as claimed by the manufacturer.

Potency is a measure of sufficient antigenic mass in the vaccine to produce a desired immune response, usually set by individual country licensing regulations to a minimal protective level. Potency can be directly evaluated with challenge studies which are both time consuming and expensive, requiring inoculation of birds with various doses of vaccine and looking at the prevention of mortality, reduction in challenge virus shed or serological response [7]. Because consistent induction of clinical signs and mortality are not usually observed in experimental infections with LPAI viruses, additional testing methods have been employed to discern if vaccines are potent. Commonly, HI assay was used to indirectly assess vaccine potency [7]. Previously for inactivated AI vaccines, protection of chickens from illness and/or death and reduction or elimination of challenge virus shed from the respiratory and alimentary tracts has been associated with an groups average HI titer at or above 139 to the challenge virus [22] or HI titer at or above 32 to protection from death [23]. All of the vaccines we tested produced a pre-challenge group average HI titer above 139 when using the vaccine seed strain as antigen indicating all the vaccines met minimum requirements for potency. However, based on the HI

titers using the challenge virus as antigen, none of the groups had a pre-challenge average HI titer above 84, raising questions concerning of inadequate protection, especially with groups A–F which had average HI titers below 32. Furthermore, analysis of individual antisera confirmed the lower antigenic relatedness of vaccines A–F seed strains as evident by 3–4 $\log_2$ drop in HI titer between MCRI strain and challenge virus when used as HI antigen, and higher antigenic relatedness of vaccines G–I as evident by 0–2 $\log_2$ drop in HI titer between MCRI and challenge strain when used as HI antigens.

In our study, the commercially available LPAI vaccines from Latin America were tested *in vivo* for efficacy by challenge with virus A/chicken/Guatemala/270475-4/03 (H5N2) or A/chicken/Guatemala/270475-1/03 (H5N2) which were originally isolated from the same farm and had 98.9% protein identity in the hemagglutinin proteins. Since LPAI virus do not produce consistent morbidity and mortality in experimental challenge, also confirmed in our study, we looked at alternative measures of protection. qRRT-PCR or virus isolation with titration have been used to measure reductions in LPAI and HPAI challenge virus replication and shedding from oropharyngeal and/or cloacal swabs for quantification of protection as previously described [24–26]. In our study, a significant decrease in challenge virus shedding based on viral RNA in oropharyngeal swabs at 3 d.p.c. was observed for vaccines H, I and J indicating less respiratory viral shedding. The other inactivated vaccines failed to significantly lower challenge virus shedding when compared to the sham group although the group G had a numeric 1 $\log_{10}$ reduction in viral shedding that was not statistically significant. This indicates for the inactivated virus vaccines (vaccines A–I) that only the seed strains most closely related to the challenge virus were protective, sharing >95% hemagglutinin similarity and producing no more than 2 $\log_2$ drop in HI titer between vaccine seed and challenge virus when used as antigen. However, the live recombinant fowl pox with AI hemagglutinin insert still provided protection as measured by significant reduction in challenge virus replication in respiratory tract, but this vaccine only had 83% hemagglutinin similarity with the challenge virus and HI testing demonstrated a 4 $\log_2$ drop in HI titer (data not shown). This suggests that use of HI serological testing can be helpful in predicting antigenic relatedness and protection from challenge LPAI virus replication and shedding, but such serology may not be predictive of protection for live vectored vaccines.

The situation in Latin America provides a unique opportunity in regards to viral drift, with vaccine pressure as one contributing factor. Mexico and Pakistan are the only two countries which have utilized an AI vaccine program for over a decade. However, since 2002, several countries in Asia have adopted vaccine use to control H5N1 HPAI, as it is considered more cost-effective and feasible than traditional stamping out/eradication policy in countries with endemic infections [27]. In association with such broad vaccine use there has been emergence of field strains that are resistant to immunity induced by classical vaccine seed strains in China, Egypt and Indonesia [28]. In experimental studies in Indonesia, the classic H5 inactivated vaccines were very potent, i.e. produced high HI titers in vaccinated birds, but failed to protect from mortality following Indonesian H5N1 HPAI virus challenge. In another recent experimental study, inactivated H5 reverse genetic AI vaccine seed strain using a North American HA produced significantly less reduction in oropharyngeal H5N1 HPAI challenge virus shedding as compared to a Eurasian HA gene insert vaccine [29]. In our study, vaccines H, I and J resulted in statistically significant reduction in virus shedding from the respiratory tract compared to the sham group. Vaccines H and J also resulted in a statistically significant decrease in the number of birds that shed virus. Only vaccine H reduced shedding to below detection limits. The challenge viruses used in our studies were from 2003 and were the most recent available to our laboratory which raises the question of how would newer H5N2 field viruses protect in relation to the 2003 challenge viruses. We assessed the genetic relatedness of hemagglutinin amino acid sequences from the vaccines seed strains and the most recently released sequence from Mexico, A/chicken/Mexico/435/2005 (H5N2) (Table 1). This more recent virus showed similar or lesser relatedness to the vaccine seed strains than the 2003 challenge viruses and would suggest similar poor protection from vaccines A–F. Predicted protection by vaccines G–J would be unknown against this field virus without some antigenic or challenge data. This findings have significant ramifications for the use of vaccine to control AI because seed strains should be evaluated and updated on a periodic basis to provide maximal reduction in replication and shedding of field viruses and thus reduce environmental contamination to prevent transmission to naïve flocks as well as reduce the risk of mutation into HPAI viruses. Potent and efficacious vaccines, applied in an appropriate manner are critical to prevent shedding of virus, reduce environmental contamination and transmission and thus become an effective tool in either control or eradication programs.

This study indicates the current inactivated commercial H5N2 vaccines in Latin America use more than one seed strain. Protection from inactivated vaccines is mainly from humoral immunity such that low HI titers may indicate poor cross protection from LPAI virus replication while moderate titers are suggestive of some level of protection [30]. Inactivated vaccines that use the older and genetically more distant 1994 H5N2 seed strain were ineffective in protecting against challenge with a more recent H5N2 LPAI virus while the inactivated vaccine with the seed strain most genetically related to the challenge virus (vaccine H) was protective as indicated by reduced replication and shedding of the challenge virus. Interestingly, live vectored vaccine J was protective against the newer H5N2 challenge virus despite the HA amino acid identity being only 83.0% to the challenge strain A/chicken/Guatemala/270475-1/03 (H5N2). This disparity in lack of protection by inactivated vaccine verses a recombinant fowlpox vaccine is unknown, but potentially is a result of broader cell-mediated immunity from the recombinant fowlpox vaccine. Indeed broad cross-protection has been demonstrated against HPAI viruses in previous studies [4,5]. Regardless, an effective vaccine must induce a robust response in the host to effectively neutralize the virus. Failure to do so may alternatively contribute to antigenic drift by selecting populations to which the vaccine offers little or no protection. This study highlights the need for continued monitoring of avian influenza field viruses and the need to evaluate and update poultry vaccine seed strains on a periodic basis.

Acknowledgements

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Validation of a Real-Time Reverse Transcriptase–PCR Assay for the Detection of H7 Avian Influenza Virus

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SUMMARY. This report describes the validation of an avian influenza virus (AIV) H7 subtype-specific real-time reverse transcriptase–PCR (rRT-PCR) assay developed at the Southeast Poultry Research Laboratory (SEPRL) for the detection of H7 AI in North and South American wild aquatic birds and poultry. The validation was a collaborative effort by the SEPRL and the National Veterinary Services Laboratories. The 2008 H7 rRT-PCR assay detects 10^1 50% embryo infectious doses per reaction, or 10^3–10^4 copies of transcribed H7 RNA. Diagnostic sensitivity and specificity were estimated to be 97.5% and 82.4%, respectively; the assay was shown to be specific for H7 AI when tested with >270 wild birds and poultry viruses. Following validation, the 2008 H7 rRT-PCR procedure was adopted as an official U.S. Department of Agriculture procedure for the detection of H7 AIV. The 2008 H7 assay replaced the previously used (2002) assay, which does not detect H7 viruses currently circulating in wild birds in North and South America.

RESUMEN. Validación de un método de transcripción reversa y reacción en cadena de la polimerasa en tiempo real para la detección del virus de la influenza aviar H7.

Este informe describe la validación de un método de transcripción reversa y reacción en cadena de la polimerasa en tiempo real específico para el subtipo H7 del virus de la influenza aviar (RRT-PCR) desarrollado en el Laboratorio de Investigación Avícola del Sureste (con las siglas en inglés SEPRL) para la detección de influenza aviar H7 en aves silvestres acuáticas y aves de corral de Norteamérica y de América del Sur. La validación fue un esfuerzo de colaboración entre el SEPRL y el Servicio Nacional de Laboratorios Veterinarios. El método rRT-PCR para H7 del año 2008 detecta 10^1 dosis infecciosas 50% por reacción, o 10^3–10^4 copias de ARN transcrito del gene H7. La sensibilidad y la especificidad diagnóstica se estimaron en 97.5% y 82.4%, respectivamente, el ensayo ha demostrado ser específico para el virus de la influenza aviar H7 cuando se probó con más de 270 virus de aves silvestres y aves de corral. Tras la validación, el método rRT-PCR para H7 del año 2008 fue adoptado como procedimiento oficial del Departamento de Agricultura de los Estados Unidos para la detección del virus de la influenza aviar H7. El ensayo del 2008 sustituyó al procedimiento para detectar al subtipo H7 utilizado anteriormente (2002), que no detecta los virus H7que circulan actualmente en aves silvestres en Norteamérica y en América del Sur.

Key words: avian influenza, poultry, real-time RT-PCR, surveillance, wild birds

Abbreviations: AI = avian influenza; AICAP = Prevention and Control of Avian Influenza in the United States; CL = cloacal; DPI = days postinoculation; DxSN = diagnostic sensitivity; DxSP = diagnostic specificity; EID_{50} = 50% embryo infectious dose; HPAI = highly pathogenic avian influenza; LPAI = low pathogenicity avian influenza; NVSL = National Veterinary Services Laboratories; OP = oropharyngeal; rRT-PCR = real-time reverse transcription–PCR; SPF = specific pathogen free; TN = true negative; TP = true positive; USDA = U.S. Department of Agriculture; VI = virus isolation

As part of the 2006 U.S. Department of Agriculture (USDA) and Department of the Interior interagency surveillance for H5N1 highly pathogenic avian influenza (HPAI) and the Prevention and Control of Avian Influenza in the United States (AICAP) surveillance programs, more than 164,000 swab specimens were screened by a matrix-based real-time reverse transcription–PCR (rRT-PCR) assay for detection of avian influenza (AI) virus in all 50 states. Specimens positive for AI by the matrix gene assay were further tested for H5 and H7 by subtype-specific rRT-PCR assays. The isolation of H7 AI virus from H7 rRT-PCR negative swabs and a 0% H7 rRT-PCR detection rate indicated that the 2002 USDA H7 rRT-PCR assay did not detect the lineage of H7 currently circulating in wild birds. The 2002 H7 assay developed by Spackman *et al.* (8) had been used by the USDA for detection of H7 AI in live bird market poultry as well as in commercial poultry in Virginia (2002), Delaware, and Connecticut (2004), but it failed to detect H7N9 in South Dakota (2008), HPAI H7N3 in Chile (2002), and low pathogenicity AI (LPAI) in wild aquatic birds (2006). A new (2008) assay for the detection of current North and South American lineages of H7 was validated to determine assay performance characteristics, including analytical and diagnostic sensitivity and specificity, limit of detection, and performance with alternative chemistry and real-time PCR platforms.

MATERIALS AND METHODS

Viruses. The avian and equine influenza virus strains used in the study as well as the avian respiratory pathogens obtained from the National Veterinary Services Laboratories (NVSL) reference collection were propagated in embryonated specific-pathogen-free (SPF) chicken eggs and subtyped with conventional hemagglutination inhibition and neuraminidase inhibition procedures (9). With the exception of A/Chile/176822-1/02 (H7N3) and A/British Columbia/CN-7-3/04

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Table 1. Limit of detection in terms of 50% embryo infectious dose (EID_{50}) for H7N1 ($10^{8.1}$ EID_{50}/ml) and H7N2 ($10^{7.4.5}$ EID_{50}/ml) with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries and AB7500Fast, AB7900HT, Cepheid SmartCycler®, and Roche LightCycler480™ real-time instruments. Testing was conducted in replicate.

	Limit of detection (in terms of EID_{50} per reaction for H7N1 and H7N2 avian influenza) with AgPath and Qiagen chemistries			
Instrument	H7N1 AgPath	H7N2 AgPath	H7N1 Qiagen	H7N2 Qiagen
AB7500Fast	10^{0-1} EID_{50}	10^{-1} EID_{50}	10^{1-2} EID_{50}	10^{0-1} EID_{50}
AB7900HT	10^{0} EID_{50}	10^{0} EID_{50}	10^{1} EID_{50}	10^{0} EID_{50}
Cepheid SC	10^{1} EID_{50}	10^{0} EID_{50}	10^{0} EID_{50}	10^{-1} EID_{50}
Roche LC480®	10^{-10} EID_{50}	10^{-10} EID_{50}	10^{0-1} EID_{50}	10^{-10} EID_{50}

(H7N3), all viruses were LPAI; the Chile and British Columbia H7N3 isolates were HPAI. In addition, 82 H7 AIVs (H7N1, H7N3, H7N4, H7N7, and H7N8 subtypes) and 157 non–H7 AI viruses isolated from wild birds were tested to evaluate analytical specificity. The H and N subtypes for all 239 wild bird isolates from the USDA/Department of the Interior and AICAP surveillance programs were determined by conventional hemagglutination inhibition/neuraminidase inhibition and/or molecular subtyping procedures. Tenfold serial dilutions of titered H7 reference viruses A/TY/Ont/18-2/00 (H7N1: $10^{8.1}$ 50% embryo infectious doses [EID_{50}]/ml) and A/CK/NY/273874/03 (H7N2: $10^{7.45}$ EID_{50}/ml) were tested in triplicate to determine the EID_{50} per reaction.

Diagnostic specimens and RNA extraction. Clinical specimens consisted of cloacal (CL) and oropharyngeal (OP) swabs collected from 4-to-5-wk-old SPF chickens experimentally inoculated with 10^6 EID_{50} A/TY/ONT/18-2/00(H7N1) as well as diagnostic poultry CL and OP swabs (n = 1578). From experimentally inoculated chickens, CL and OP swabs specimens were collected twice daily (morning and evening) between 1 and 8 days postinoculation (DPI) in 3.5 ml of brain heart infusion broth. Swabs were swirled in brain heart infusion broth to disperse contents, squeezed against the side of the tube to eliminate liquid, and then discarded. Swab supernatants were held at 4 C for up to 24 hr and were then processed for RNA extraction. Nucleic acid was extracted from 50 µl of clarified swab supernatant with MagMAX™ AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Nucleic acid extraction was conducted according to procedures described in the USDA/NVSL protocol "Real-Time RT-PCR for the Detection of Avian Influenza Virus and Identification of H5 and H7 subtypes in Clinical Samples" (AVPRO1510.03, available upon request) with a KingFisher (Thermo, Ontario, Canada) 96-well magnetic particle processor. The magnetic particle processor was programmed according to USDA/NVSL standard operating procedure AVSOP1522.01 (available upon request).

Virus isolation. Virus isolation was conducted in 9-to-11-day-old SPF chicken embryos with conventional procedures, as described previously (9). Briefly, 2.0 ml of swab supernatant was diluted in antibiotic suspension, incubated for 1 hr at room temperature, and inoculated (0.3 ml per embryo) into the allantoic sac of 9-to-11-day-old embryonating eggs (9). A second blind passage was made for all specimens that were negative for virus upon completion of the first passage and positive by rRT-PCR.

rRT-PCR. Nucleotide sequence for the primers and probe for the 2008 H7 assay were described by Spackman *et al.* (7). Validation testing conducted at the NVSL used 2.5 pmol/reaction of each primer and 1.5 pmol/reaction of FAM/black hole quencher probe. A 25-µl reaction volume, as described in USDA/NVSL AI rRT-PCR protocol (AVPRO1510.03, available upon request), was used for both the AgPath-ID™ (Ambion) and Qiagen One-Step RT-PCR® (Qiagen, Valencia, CA) kits with Applied Biosystems (AB)7500Fast (Applied Biosystems, Foster City, CA), AB7900HT, SmartCycler® (Cepheid, Sunnyvale, CA), and LightCycler480™ (Roche, Mannheim, Germany) real-time PCR instruments. Multiple PCR platforms and chemistries were compared with the serially diluted H7N1 and H7N2 reference viruses as part of the analytical sensitivity portion of the study. All diagnostic and analytical specificity validation testing was conducted with AgPath-ID™ chemistry and AB7500 instrumentation.

Phylogenetic analysis. Sequences of H7-subtype viruses isolated from wild birds in 2007 were generated at the NVSL as part of the bench validation for the 2008 H7 assay. The remaining sequences were obtained from the GenBank. An H7 phylogenetic tree was constructed using nucleotide sequences from the hemagglutinin gene and Lasergene software, version 8.0 (DNA Star, Madison, WI). Phylogenetic inference was conducted using the neighbor-joining method of inference with 1000 bootstraps.

RESULTS

The analytical sensitivity for the 2008 H7 rRT-PCR assay has been reported by Spackman *et al.* (7) to be 10^1 EID_{50} per reaction, or 10^3–10^4 copies of transcribed RNA. Limit of detection studies conducted with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries with AB7500Fast, AB7900HT, Roche LightCycler480®, and Cepheid SmartCycler® instrumentation supported the previously reported analytical sensitivity data (Table 1). The 2008 H7 assay is comparable to the USDA/NVSL matrix gene rRT-PCR assay. The AB7500Fast, AB7900HT, and Roche LightCycler480® platforms are approximately 10 times more sensitive than the Cepheid SmartCycler® instrument with AgPath-ID™ One-Step RT-PCR chemistry, and the Cepheid SmartCycler® instrument is approximately 10 times more sensitive than the alternative platforms with Qiagen One-Step RT-PCR® chemistry.

The 2008 H7 assay was found to be specific for North American and South American lineages of H7 AI and did not amplify AI of the H1–H6 and H8–H15 subtypes. The positive/negative detection limits for reference H1–15 AI viruses and poultry respiratory pathogens are shown in Table 2. One nonpoultry H7 reference virus, Eq/Prague/56 (H7N7), tested negative for H7 viral RNA by the 2008 H7 assay. In addition to the viruses listed in Table 2, 239 AI viruses isolated from North American wild birds were tested by the 2008 H7 assay. Of the 239 AI viruses tested, 100% (82 of 82) of the H7 subtype were detected by the 2008 H7 rRT-PCR assay: the remaining 157 non-H7 viruses were negative.

The diagnostic sensitivity (DxSN) and diagnostic specificity (DxSP) were determined by associating the 2008 H7 positive/negative categoric data with the known infection status of each animal using a two-way (2 × 2) table (Table 3). The known infection status of each animal was determined by virus isolation (VI), the gold standard for detection of AI virus. The true positive (TP) and true negative (TN) specimens were those specimens that tested positive for H7 AI by VI and the 2008 H7 test and those that tested negative for both tests, respectively. Specimens classified as false positive and false negative were those that were in disagreement with VI. Diagnostic sensitivity was calculated as TP/(TP + false negative), and DxSP was calculated as TN/(TN + false positive). The DxSN and DxSP of the 2008 H7 rRT-PCR assay as compared to VI

Table 2. Panel of reference H1–H15 avian influenza viruses and avian respiratory pathogens. Isolate name, subtype, and real-time RT-PCR positive/negative result for the 2008 H7 assay are listed.

Isolate	Subtype	+/−
DK/MN/764-1489/81	H1N2	−
A/NJ/8/76-Eq1	H1N7	−
Mallard/ALB/77/77	H2N3	−
Waterfowl/GA/03	H2N9	−
DK/MN/LQWR604/79	H3N4	−
Mallard/ALB/311/85	H3N6	−
DK/Czech/56	H4N6	−
DK/England/62	H4N8	−
MSwan/MI/451072-2/06	H5N1	−
DK/PA/454069/06	H5N1	−
CK/WA/13413/84	H5N2	−
CK/CA/101247/01	H6N2	−
Emu/TX/4259/93	H7N1	+
CK/NY/273874/03	H7N2	+
TY/UT/24721-10/95	H7N3	+
TY/OR/71	H7N3	+
CK/Chile/176822-1/02	H7N3	+
CK/BC/314514/04	H7N3	+
EQ/Prague/56	H7N7	−
Mallard/AK/495762-38/06	H7N8	+
TY/NE/505577/07	H7N9	+
TY/Ontario/6118/67	H8N4	−
TY/CA/6889/80	H9N2	−
CK/Germany/N/48	H10N7	−
DK/England/56-Bel	H11N1	−
Mallard/LALB/83	H12N5	−
Gull/MD/704/77	H13N6	−
Mallard/Gurjev/263/82	H14N5	−
Shearwater/Aust/1/73	H15N9	−
Avian paramyxovirus	APMV 1–4, 6–9	−
Infectious bronchitis	Mass	−
Avian metapneumovirus	Subtype C	−
Infectious laryngotrachitis virus		−

were estimated to be 97.5% and 82.4%, respectively. The positive/negative virus isolation and rRT-PCR data for the experimentally inoculated chickens included in the estimation of DxSN and DxSP were from 1–8 DPI CL and OP swabs. In the early phase of collection (2–3 DPI) many of the swabs, especially the OP swabs, were positive by H7 rRT-PCR and negative by VI. During the later phase of collection (7–8 DPI), a significant percentage of CL swab specimens were positive by H7 rRT-PCR and negative by VI. The greatest degree of correlation between the rRT-PCR and VI test occurred between 4 and 6 DPI.

The 2008 H7 assay was shown to detect North and South American lineages of H7 AI isolated from poultry and wild aquatic birds. The 2002 H7 assay was used for the detection of H7 AI from 2002 to 2007 in live bird markets in New York, New Jersey, and other northeastern states as well as for the detection of infections in commercial poultry in Delaware, Connecticut, and Virginia. The 2002 assay failed to detect a HP H7N3 in commercial poultry in Chile in 2002 and in British Columbia in 2004 as well as a LPAI H7N9 in commercial turkeys in South Dakota in 2007 (Fig. 1). In addition, the 2002 North American H7 assay failed to detect H7-subtype influenza from any of the AI matrix-positive specimens collected during the 2006 wild bird and AICAP surveillance periods. The 2008 H7 assay has increased the specificity of the USDA/NVSL procedure to include wild bird and poultry lineages circulating in North and South America (Fig. 1).

Table 3. Number of diagnostic swab specimens testing positive/negative for H7 AI by the 2008 H7 rRT-PCR assay and VI. Specimens included oropharyngeal and cloacal swabs collected from experimentally inoculated chickens and diagnostic poultry swab specimens (n = 1578).

	VI Positive	VI Negative		
rRT-PCR Positive	316 TP	221 FP	537	DxSN = 97.5%
rRT-PCR Negative	8 FN	1033 TN	1041	DxSP = 82.4%
Totals	324	1254		

DISCUSSION

rRT-PCR is shown to be an effective tool for the surveillance of AI in both commercial poultry and wild aquatic birds throughout the world (1,2,3,4,5,6). Surveillance and control programs normally use a matrix gene rRT-PCR assay for the detection of type A influenza and H5 and H7 subtype-specific assays for the detection H5 and H7 AI, which is critical for control of the disease and unhindered trade. As an effective surveillance tool, rRT-PCR tests require analytical and diagnostic validation as well as continued evaluation to assure that primers and probes will detect current lineages of AI. An essential component of continued evaluation of the assay is the isolation and subtype identification of AI viruses from all matrix-positive specimens. The 2008 H7 assay detects the current lineages of H7 circulating in North and South America, but it does not detect Eurasian lineages of H7 AI (7) or the H7 lineage of equine influenza. The 2008 H7 assay has been shown to be specific for H7 and does not detect other subtypes of AI or other poultry respiratory pathogens. In 2008 the NVSL received 281 presumptive H7 specimens for confirmation testing; 142 of the 281 presumptive H7 specimens were confirmed to be positive by the 2008 H7 assay.

A critical component of USDA preparation for an outbreak of HPAI is to increase the testing capacity of the National Animal Health Laboratory Network. Evaluation of alternative 96-well real-time platforms will increase testing capacity by allowing National Animal Health Laboratory Network labs to use alternative platforms that have been shown to be equivalent. The AB7500Fast, AB7900HT, Roche LightCycler480®, and Cepheid SmartCycler® instruments are similar in sensitivity, detecting approximately 10^{0-1} EID_{50} with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries. Robustness of the 2008 H7 assay was evaluated with an intra-assay and interassay variability study retrospective to analytical and diagnostic validation. Five within-run replicates demonstrated a <1.0 cycle threshold (Ct) difference, with tenfold serial dilutions of a reference virus over the linear range of the assay. Interassay variability studies demonstrated good between-run repeatability with the four previously identified platforms and two chemistries over the linear range of the assay.

The DxSN and DxSP of the assay were 97.5% and 82.4%, respectively, when compared to VI. The lower DxSP value is likely influenced by the inclusion of swabs from experimentally inoculated chickens in the validation study, in which viral RNA can be detected by RT-PCR but virus cannot be isolated, an insufficient number of negative specimens, and the use of a gold standard with a less

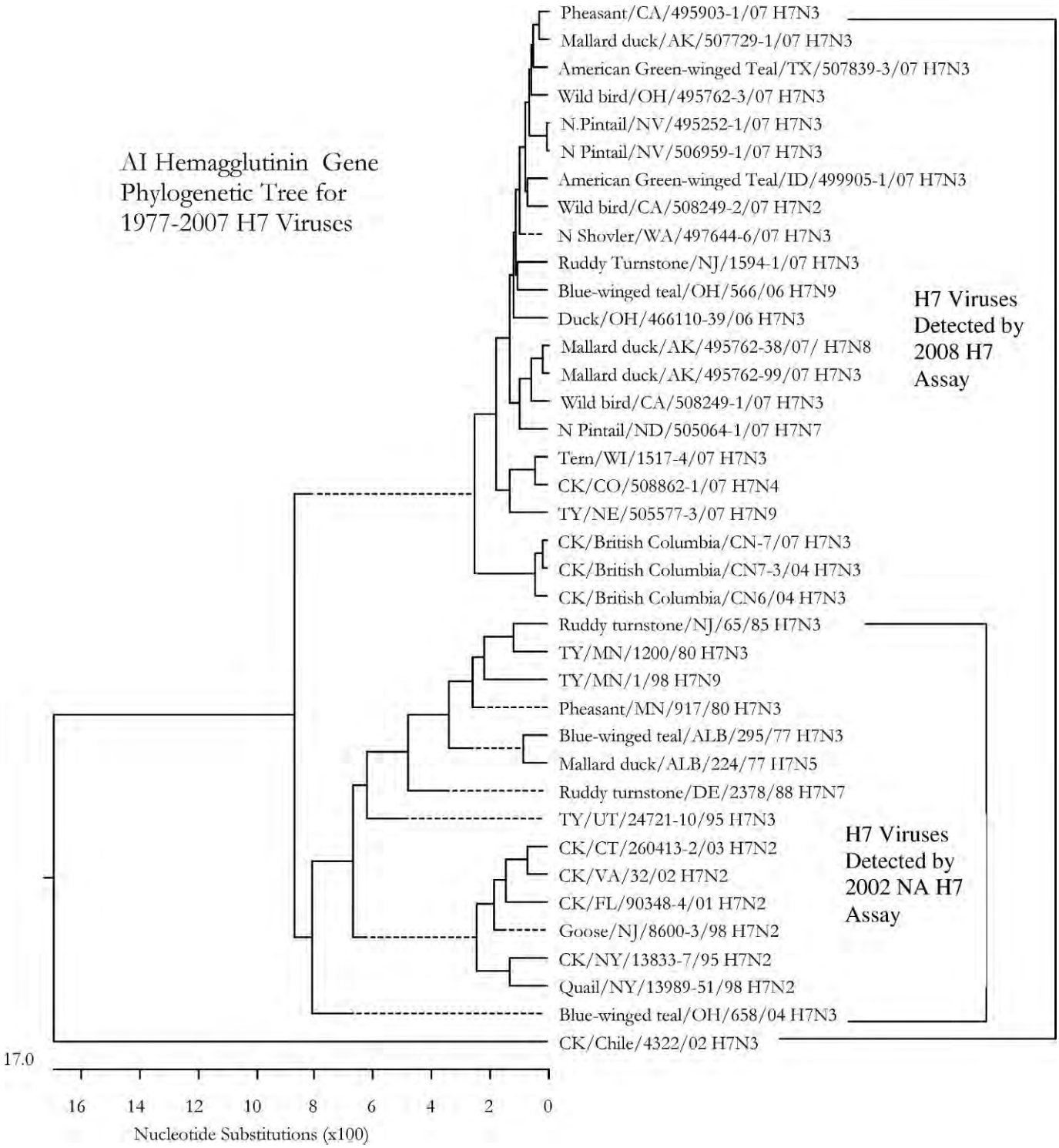

Fig. 1. Phylogenetic tree for the hemagglutinin gene of H7 viruses isolated between 1997 and 2007.

sensitive indicator system, the hemagglutination test. Conventional VI procedures use the hemagglutination test for identification of a hemagglutinating virus. The hemagglutination test detects approximately 4 logs of virus, as compared to the 10^1 EID_{50} detected by rRT-PCR. The 2008 H7 assay has replaced the 2002 H7 assay for the detection of H7 in commercial poultry and wild aquatic birds; as a critical component of the USDA AI surveillance program, the assay performance characteristics will continue to be evaluated.

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Pathogenesis of Pandemic Influenza A (H1N1) and Triple-Reassortant Swine Influenza A (H1) Viruses in Mice[▽]

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The pandemic H1N1 virus of 2009 (2009 H1N1) continues to cause illness worldwide, primarily in younger age groups. To better understand the pathogenesis of these viruses in mammals, we used a mouse model to evaluate the relative virulence of selected 2009 H1N1 viruses and compared them to a representative human triple-reassortant swine influenza virus that has circulated in pigs in the United States for over a decade preceding the current pandemic. Additional comparisons were made with the reconstructed 1918 virus, a 1976 H1N1 swine influenza virus, and a highly pathogenic H5N1 virus. Mice were inoculated intranasally with each virus and monitored for morbidity, mortality, viral replication, hemostatic parameters, cytokine production, and lung histology. All 2009 H1N1 viruses replicated efficiently in the lungs of mice and possessed a high degree of infectivity but did not cause lethal disease or exhibit extrapulmonary virus spread. Transient weight loss, lymphopenia, and proinflammatory cytokine and chemokine production were present following 2009 H1N1 virus infection, but these levels were generally muted compared with a triple-reassortant swine virus and the 1918 virus. 2009 H1N1 viruses isolated from fatal cases did not demonstrate enhanced virulence in this model compared with isolates from mild human cases. Histologically, infection with the 2009 viruses resulted in lesions in the lung varying from mild to moderate bronchiolitis with occasional necrosis of bronchiolar epithelium and mild to moderate peribronchiolar alveolitis. Taken together, these studies demonstrate that the 2009 H1N1 viruses exhibited mild to moderate virulence in mice compared with highly pathogenic viruses.

The 2009 (H1N1) influenza pandemic has resulted in laboratory-confirmed cases in over 200 countries with greater than 15,000 deaths worldwide (5). The majority of infected individuals have experienced uncomplicated, upper respiratory tract illness; cases have been distinguished by symptoms which include gastrointestinal distress and vomiting in approximately 40% of patients (7, 36). While the 2009 pandemic represents the greatest incidence of human infection with influenza viruses of swine origin to date, antigenically related swine lineage viruses have previously been associated with sporadic cases of human disease and death (11, 37). Prior to 2009, the largest cluster of H1N1 swine influenza cases occurred during an outbreak in 1976 which resulted in the infection of up to 230 soldiers at Fort Dix, NJ, with 13 severe cases and one fatality (12). The outbreak was limited to Fort Dix, possibly due to the poor transmissibility of this virus (21). Triple-reassortant swine H1N1 influenza viruses (containing avian, human, and swine genes) have additionally been associated with human infection since 2005 (11, 37). While gastrointestinal symptoms following seasonal influenza virus are uncommon, diarrhea was reported in 40% of patients infected with triple-reassortant swine H1N1 viruses, similar to cases early in the 2009 pandemic (37).

The hemagglutinin (HA) gene of 2009 H1N1 belongs to the classical swine lineage, which was first introduced into swine populations circa 1918 and shares antigenic similarity with the 1918 pandemic virus as well as the 1976 H1N1 virus and the more contemporary triple-reassortant swine influenza viruses (11, 16, 44). The 1918 HA gene has been previously shown to be essential for severe pulmonary lesion development and optimal virulence (19, 40). Thus, it is important to compare the relative virulence of the 2009 H1N1 viruses to that of other H1N1 viruses that have circulated over a span of more than 90 years. Mammalian models serve an invaluable role for the study of disease severity and outcome following influenza virus infection. Previous research evaluating classical swine influenza viruses has revealed that these viruses do not consistently exhibit high virulence in the mouse model. A/NJ/8/76 virus, isolated from the outbreak in Fort Dix, was lethal only for specific strains of mice or following mouse adaptation (9, 13). Two H1N1 viruses antigenically similar to the reconstructed 1918 virus, A/Swine/IA/15/30 and A/Swine/IA/31, replicated to high titers in the lungs of mice and caused substantial weight loss at the height of infection; however, only the 1931 virus isolate exhibited lethality in this model (26, 28). However, these studies largely occurred in the context of evaluating vaccine and antiviral efficacy and did not extensively study virus pathogenesis or the host response following infection.

Due to the rapid emergence of 2009 H1N1 viruses in humans, collaborative research has been undertaken to characterize viruses isolated from this pandemic in mammalian mod-

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TABLE 1. H1N1 and H5N1 viruses used in this study

Virus	Name in study	Subtype	Patient data
A/California/4/09	CA/4	H1N1	Pediatric uncomplicated, upper respiratory tract illness
A/Texas/15/09	TX/15	H1N1	Fatal pediatric respiratory illness
A/Mexico/4108/09	Mex/4108	H1N1	Hospitalized respiratory illness
A/Mexico/4482/09	Mex/4482	H1N1	Severe respiratory illness
A/Mexico/InDRE4487/09	Mex/4487	H1N1	Severe respiratory illness
A/New Jersey/8/76	NJ/8	H1N1	Severe respiratory illness
A/Ohio/2/07[a]	OH/2	H1N1	Pediatric uncomplicated, upper respiratory tract illness
rgA/South Carolina/1/18[a]	1918	H1N1	NA[b]
A/Vietnam/1203/04[a]	VN/1203	H5N1	Fatal pediatric respiratory illness

[a] As described in references 24, 37, and 40.
[b] NA, not applicable.

els, including the mouse, ferret, nonhuman primate, and pig (18, 23, 30). However, much of this work has been limited by a small number of novel isolates tested and a paucity of extensive side-by-side comparison with pertinent viruses outside seasonal H1N1 isolates. To better understand the capacity of viruses isolated from the 2009 pandemic to cause disease in the context of related viruses of swine origin or highly pathogenic viruses with pandemic potential, we expanded upon a mouse model for the study of 2009 H1N1 influenza viruses associated with human infection (23). Assessment of pathogenicity in the mouse model included histopathology analysis, hemostatic measurements, and cytokine production in the lung. This work revealed that a panel of swine origin H1N1 viruses exhibited lower pathogenicity relative to an H5N1 virus and the pandemic 1918 virus. Furthermore, a triple-reassortant H1N1 virus of swine origin isolated in 2007 possessed enhanced virulence in this model compared with swine origin 2009 pandemic H1N1 viruses or the swine origin virus that sickened humans in 1976.

MATERIALS AND METHODS

Viruses. Influenza A viruses of the H1N1 and H5N1 subtypes used in this study are shown in Table 1. Virus stocks were cultured consistent with the original isolate passage either in the allantoic cavity of 10-day-old embryonated hens' eggs (Mex/4108, Mex/4487, NJ/8, and VN/1203) or in Madin-Darby canine kidney (MDCK) cells (CA/4, TX/15, Mex/4482, OH/2, and 1918) at 37°C for 24 to 48 h as previously described (24, 40). Pooled allantoic fluid or cell supernatant was clarified by centrifugation and frozen in aliquots at −70°C. With the exception of NJ/8 (egg passage 14 [E14]), virus stocks were low-passage-number viruses (E1-E2 or cell passage 1 or 2 [C1-C2]), and the identities of virus genes were confirmed by sequence analysis to verify that no inadvertent mutations were present during the generation of virus stocks. The 50% egg infectious dose (EID_{50}) titer for egg-grown stocks was calculated by the method of Reed and Muench (35), following serial titration in eggs. Cell-grown stocks were titrated by standard plaque assay in MDCK cells as previously described for determination of PFU titer (46). All animal experiments with 2009 H1N1 viruses were conducted under biosafety level 3 enhanced (BSL3+) containment in accordance with guidelines of the World Health Organization (https://www.who.int/csr/resources/publications/swineflu/Laboratorybioriskmanagement.pdf).

Mouse *in vivo* experiments. Female BALB/c mice (Charles River Laboratories, Wilmington, MA), 6 to 8 weeks of age, were deeply anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin; Sigma-Aldrich, St. Louis, MO) and inoculated intranasally (i.n.) with 50 μl of infectious virus diluted in sterile phosphate-buffered saline (PBS). The 50% mouse infectious dose (MID_{50}) and 50% lethal dose (LD_{50}) were determined as previously described (22). Briefly, mice were inoculated with 10-fold dilutions (from 10^6 to 10^0 EID_{50} or PFU) of each virus. Three mice per group were euthanized on day 3 postinoculation (p.i.), and homogenized lungs were serially titrated in MDCK cells by standard plaque assay to determine the MID_{50} calculated by the method of Reed and Muench (35). Five mice per virus were monitored daily for 14 days p.i. for morbidity, as measured by weight loss, and mortality to determine the LD_{50}. Any mouse that lost >25% of its preinoculation body weight was euthanized. On days 3 and 6 p.i., three mice inoculated with 10^5 EID_{50} or PFU of each virus were euthanized for the collection of lungs, nose, spleen, intestine, thymus, and brain to determine replication and systemic spread of virus. Tissues were homogenized in 1 ml of cold PBS, and clarified homogenates were titrated in MDCK cells to determine virus infectivity, starting with undiluted sample (limit of detection, 10 PFU). Statistical significance for all experiments was determined using Student's *t* test.

Ocular inoculation of mice with H1N1 viruses was performed using 10^6 EID_{50} or PFU of indicated viruses in a volume of 5 μl following corneal scarification as previously described (2).

Pathology and immunohistochemistry. Lung sections were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (HE). A duplicate 5-μm section was immunohistochemically stained to demonstrate influenza A virus nucleoprotein by first microwaving the sections in Antigen Retrieval Citra solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2,000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein was applied and allowed to incubate for 12 h at 4°C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using the Mouse on Mouse system (M.O.M. kit; Vector Laboratories, Inc., Burlingame, CA) per the manufacturer's instructions. The 3-amino-9-ethylcarbazole (AEC) peroxidase substrate kit (Vector Laboratories Inc.) was used as the substrate chromogen, and hematoxylin was used as a counterstain. Histopathological lesions and immunohistochemical viral antigen detection were scored. For lesions, scoring was as follows: 1, no lesion; 2, mild inflammation and no or rare necrosis; 3, moderate inflammation with frequent necrotic cells; 4, severe inflammation with common necrosis and edema. For immunohistochemical viral antigen scoring, the following was used: 0, no antigen; 1, rare positive cells; 2, infrequent positive cells; 3, common positive cells; 4, widespread positive cells. For histopathological changes, morphological descriptions were provided.

Peripheral cell counts. On days 0, 3, and 6 p.i., blood was collected from the brachial artery of three to six mice inoculated with 10^5 EID_{50} or PFU of each virus. Blood was immediately placed in EDTA Vacutainer tubes (BD, Franklin Lakes, NJ), and complete blood counts were quantified using a Hemavet HV950FS instrument per the manufacturer's instructions (Drew Scientific, Inc., Oxford, CT). Absolute thymocyte counts were performed with single-cell suspensions of homogenized thymus diluted 1:10 with Turk's solution (2% acetic acid, 0.01% [vol/vol] crystal violet, double-distilled water), representative of three to six mice per group. Statistical significance of thymocyte counts was determined by analysis of variance (ANOVA) and Student's *t* test.

Cytokine quantification. Clarified day 3 and 6 p.i. lung homogenates from mice inoculated with 10^5 EID_{50} or PFU of each virus indicated ($n = 3$) were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BD Biosciences, San Diego, CA). Cytokines or chemokines analyzed were interleukin-6 (IL-6), IL-10, IL-12 (p40), and monocyte chemotactic protein 1 (MCP-1) (assay sensitivity, 15.6 pg/ml).

Sequence analysis. Genetic analyses of protein sequences were performed using the programs BioEdit and MUSCLE (8, 15).

RESULTS

Characterization of H1N1 viruses in mice. Human influenza A viruses of the H1 and H3 subtypes which cause seasonal

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TABLE 2. Mouse infectivity and tissue viral titers of H1N1 and H5N1 viruses

Virus	% wt loss (day p.i.)[a]	MID_{50}[b]	LD_{50}[b]	Viral titer[c]			
				Day 3 p.i.		Day 6 p.i.	
				Lung	Nose	Lung	Nose
CA/4	5.3 (8)	$10^{1.5}$	$>10^6$	5.9 ± 0.9	1.5 ± 0.2	6.2 ± 0.1	ND[d]
TX/15	1.5 (1)	$10^{0.5}$	$>10^6$	5.4 ± 1.0	ND	4.7 ± 0.8	ND
Mex/4108	3.5 (3)	$10^{1.5}$	$>10^6$	6.9 ± 0.3	1.6 ± 0.5	5.6 ± 0.3	1.3 ± 0.3
Mex/4482	19.0 (7)	$10^{0.25}$	$>10^6$	6.4 ± 0.2	1.2 ± 0.3	6.3 ± 0.4	ND
Mex/4487	9.7 (8)	$10^{2.75}$	$>10^6$	6.9 ± 0.4	1.7 ± 0.1	5.9 ± 0.2	2.6 ± 1.0
NJ/8	2.7 (1)	$10^{2.75}$	$>10^6$	6.0 ± 0.2	ND	5.9 ± 0.9	ND
OH/2	14.5 (7)	$10^{1.75}$	$10^{5.8}$	7.0 ± 0.3	1.4 ± 0.5	4.5 ± 0.1	ND
1918	22.4 (6)	$10^{0.75}$	$10^{3.5}$	6.7 ± 0.1	1.5 ± 0.4	6.2 ± 0.1	ND
VN/1203	23.4 (5)	$10^{1.5}$	$10^{1.3}$	7.3 ± 0.1	2.0 ± 0.5	6.1 ± 0.4	3.5 ± 1.5

[a] Mean maximum percent weight loss (5 mice per group) following inoculation with 10^5 PFU or EID_{50}.
[b] Fifty percent mouse infectious dose (MID_{50}) and 50% lethal dose (LD_{50}) are expressed as the PFU or EID_{50} required to give 1 MID_{50} or 1 LD_{50}, respectively.
[c] Virus endpoint titers are expressed as the mean $\log_{10}$ PFU/ml plus standard deviation of 3 to 6 mice per tissue.
[d] ND, not detected. The limit of virus detection was 10 PFU/ml.

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epidemics do not typically replicate efficiently in the mouse model, unlike avian influenza A viruses of the H5 and H7 subtypes, which replicate to high titers in the lungs of mice without prior adaptation and are capable of causing severe disease and death in this model (2, 10, 14, 22, 24). BALB/c mice were inoculated intranasally (i.n.) with multiple H1N1 viruses associated with human infection (Table 1). 2009 H1N1 viruses chosen for this study were isolated from either pediatric or adult patients with uncomplicated, severe, or fatal cases of respiratory illness.

All 2009 H1N1 viruses tested possessed low MID_{50} values ($10^{0.25}$ to $10^{2.75}$ EID_{50} or PFU), indicating a high degree of infectivity in this model without prior adaptation, similar to the 1918 and H5N1 viruses (Table 2). However, in contrast to the virulent 1918 and H5N1 viruses, the 2009 H1N1 viruses did not mount lethal infections in mice (LD_{50}, $>10^6$ EID_{50} or PFU), and with the exception of Mex/4482 virus, which caused 19% weight loss at the height of infection, morbidity was mild with <10% transient loss of initial body weight. All 2009 H1N1 viruses tested replicated to high titers ($10^{4.7}$ to $10^{6.9}$ EID_{50} or PFU) in the lungs of mice on days 3 and 6 postinoculation (p.i.). Virus was detected infrequently and at low titers in the nose at these times p.i. ($<10^2$ EID_{50} or PFU) (Table 2). Replication of all 2009 H1N1 viruses was restricted to respiratory tract tissues, indicating a lack of systemic spread of these viruses in this model (data not shown).

We next compared the virulence of 2009 H1N1 viruses to that of previous swine lineage influenza A viruses which have been associated with human disease. NJ/8 virus was isolated from throat swab material of a young military recruit during an outbreak at Fort Dix, NJ, in 1976. OH/2 virus, a triple-reassortant H1N1 virus containing avian, human, and swine genes, was isolated in 2007 from a 10-year-old female with uncomplicated, upper respiratory illness (37). Both viruses replicated in the respiratory tract of mice to titers comparable to those of the 2009 H1N1 isolates (Table 2). However, while NJ/8 virus caused insignificant morbidity, the triple-reassortant OH/2 virus resulted in greater weight loss, reached peak mean lung titers at day 3 p.i. of 10^7 PFU, and was lethal to mice at the highest dose of inoculum administered (LD_{50}, $10^{5.8}$ PFU) (Table 2).

In contrast to all 2009 H1N1 viruses tested, which were highly infectious but not lethal in the mouse, the reconstructed 1918 H1N1 virus and the highly pathogenic avian influenza (HPAI) H5N1 virus VN/1203 demonstrated enhanced morbidity and mortality in this model (Table 2) (24, 40). Mice inoculated with 1918 and VN/1203 viruses lost >20% of their initial body weight by day 5 p.i. and possessed LD_{50} values of $10^{3.5}$ PFU and $10^{1.3}$ EID_{50}, respectively. In contrast to all H1N1 subtype viruses tested, VN/1203 virus exhibited extrapulmonary spread following infection, with virus detected in the spleen, intestine, thymus, and brain (data not shown). Viral titers in the gastrointestinal tract were observed only with the H5N1 virus ($\log_{10}$ 2.7 ± 0.9 and 3.4 ± 1.4 PFU at days 3 and 6 p.i., respectively).

Reports of conjunctivitis following exposure to seasonal or avian influenza viruses indicate that influenza A viruses of multiple subtypes are capable of using the eye as a portal of entry (20). A recent study from our laboratory demonstrated the ability of selected H1N1 viruses to replicate in the lungs of mice following ocular inoculation (3). To assess the ability of 2009 H1N1 viruses to cause disease by this route, mice were inoculated by the ocular route following corneal scarification with Mex/4108, Mex/4482, NJ/8, or OH/2 virus. Mice inoculated by the ocular route did not exhibit substantial morbidity, and virus was not detected in the eye, nose, or lung of any mouse on day 3 or day 6 p.i. (data not shown). These results suggest that, unlike the case with some avian influenza viruses, the eye is not an efficient route of entry for swine origin H1N1 viruses in this model.

Histological pathology observed in H1N1 virus-infected mice. Histologically, all H1N1 virus infections produced lesions typical of influenza A virus infections: bronchiolitis and bronchitis with accompanying necrosis of respiratory epithelium and associated neutrophilic to histiocytic alveolitis (Table 3). However, the severity and character of necrosis and inflammation varied with individual virus strains. The mildest lesions were seen with TX/15, Mex/4108 and NJ/8 viruses (mean histological lesion score, 1.6 to 2.0), which produced mild bronchiolitis with minimal to no respiratory epithelial necrosis and only mild histiocytic alveolitis associated with terminal bronchioles (Fig. 1A and Table 3). Avian influenza viral antigen was rare to infrequent in alveolar epithelium and respiratory epithelium in

TABLE 3. Histopathological lesion and immunohistochemical viral antigen scores for mice intranasally inoculated with H1N1 influenza A viruses and sampled at 6 days p.i.

Virus strain	Alveolar		Bronchiolar and bronchial		Additional lesion features
	Histopathology score[a]	Viral antigen[b]	Histopathology score[a]	Viral antigen[b]	
CA/4	2.3	1.3	2	1.7	Histiocytic peribronchiolar alveolitis, some neutrophils; bronchiolitis with some necrosis
TX/15	1.6	0.3	1.3	0	Histiocytic alveolitis; bronchiolitis without necrosis
Mex/4108	1.6	1.7	2	2	Histiocytic alveolitis with uncommon neutrophils; bronchiolitis without necrosis
Mex/4482	2.6	2	2.6	1.5	Histiocytic peribronchiolar alveolitis, common neutrophils; bronchiolitis with some necrosis
Mex/4487	3	1.5	3	3	Histiocytic peribronchiolar alveolitis, common neutrophils; histiocytic to neutrophilic bronchiolitis with necrosis
NJ/8	2	1.5	2	2	Histiocytic alveolitis; lymphocytic bronchiolitis without necrosis
OH/2	2.6	1.6	2.6	1.7	Histiocytic peribronchiolar alveolitis; bronchiolitis with some necrosis
1918	3.3	3	2.5	0	Neutrophilic alveolitis with alveolar epithelial necrosis and edema and interlobular edema; neutrophilic bronchiolitis with severe necrosis
VN/1203	3.2	2	2.6	2.5	Neutrophilic peribronchiolar to diffuse alveolitis with edema and histiocytes; neutrophilic bronchiolitis with severe necrosis
Mock	1	0	1	0	No lesions

[a] Mean histopathological lesion scores for 2 or 3 mice. Histopathological lesion scores per mouse were determined as follows: 1, no lesion; 2, mild lesion; 3, moderate lesion; 4, severe lesion.

[b] Mean immunohistochemical viral antigen scores for 2 or 3 mice. Immunohistochemical viral antigen scores per mouse were determined as follows: 0, no antigen; 1, rare positive cells; 2, infrequent positive cells; 3, common positive cells; 4, widespread positive cells.

bronchioles (Fig. 1B). Infection with Mex/4487, Mex/4482, OH/2, and CA/4 viruses resulted in lesions (mean histological lesion score, 2.3 to 3.0) in the lung varying from mild to moderate bronchiolitis with occasional necrosis of bronchiolar epithelium and mild to moderate peribronchiolar alveolitis, mainly histiocytic but with some neutrophils (Fig. 1C). Avian influenza viral antigen was rare to common in alveolar epithelium and macrophages and rare to common in respiratory epithelium in bronchioles depending on individual animals and area of the lung (Fig. 1D). The most severe lesions observed (mean histological lesion score, 3.2 to 3.3) were with VN/1203 and 1918 viruses, which produced mild to severe necrosis of bronchiolar epithelium with neutrophilic inflammation and peribronchiolar to diffuse neutrophilic alveolitis with necrosis of alveolar epithelium and prominent alveolar to interlobular edema at the hilum (Fig. 1E to G). Avian influenza virus antigen was common to widespread in alveolar epithelium and macrophages and infrequent to common in respiratory epithelium of the bronchioles (Fig. 1H). In summary, 2009 H1N1 viruses were capable of causing histological lung pathology following infection in the mouse; however, the severity of necrosis and inflammation observed in alveoli was less pronounced compared with the highly pathogenic viruses VN/1203 and 1918. These highly pathogenic viruses had necrotic and neutrophilic inflammatory lesions focused on alveoli and with greater detection of viral antigen within the alveoli compared to 2009 H1N1 or seasonal H1N1 viruses (Table 3).

Analysis of lymphocyte populations in whole blood and thymus from 2009 H1N1 virus-infected mice. Infection with influenza viruses can lead to changes within the lymphohemopoietic system, and leukopenia and lymphopenia have been associated with both avian H5N1 human infection and human cases of triple-reassortant swine influenza (1, 37). This impairment is recapitulated in the mouse model, as previous work has noted severe depletion of lymphocytes in blood, lungs, and lymphoid tissue of mice infected with HPAI viruses (29, 41). To determine the extent of leukopenia resulting from infection with 2009 H1N1 viruses in mice, complete blood counts from mice inoculated with each virus were performed with a Hemavet multispecies hematology analyzer. Leukopenia was detected among all H1N1 viruses tested by day 6 p.i., with a statistically significant reduction in circulating white blood cells (WBCs) among TX/15- and Mex/4108-infected mice at this time p.i. (Table 4; Fig. 2). Differential blood counts of 2009 H1N1-infected mice revealed, on average, a 10% decrease of peripheral lymphocytes compared with uninfected mice on days 3 and 6 p.i. (Table 4; Fig. 2). The lethal OH/2 virus caused twice the reduction in the percentage of lymphocytes (approximately a 20% reduction from baseline levels) by day 6 p.i., which was significantly lower ($P < 0.01$) than that for all 2009 H1N1 viruses tested. Comparable levels of significant lymphopenia (>20% reduction from baseline levels) were also observed in mice infected with the highly virulent VN/1203 (H5N1) or 1918 virus. These levels were significantly lower than the lymphopenia induced by all 2009 H1N1 viruses tested ($P < 0.005$), demonstrating the enhanced virulence of these viruses in the mouse model. Because the pathogenesis of lethal influenza virus infections has been associated with depletion of thymocytes (41), we also determined the absolute thymocyte counts on days 3 and 6 p.i. (Table 4). All viruses which exhibited pronounced morbidity of >14% weight loss (Mex/4482, OH/2, VN/1203, and 1918) possessed significantly reduced (2.5- to 5-fold) total thymocyte counts by day 6 p.i. ($P < 0.01$). The greatest effect on the thymus followed 1918 virus infection: the number of leukocytes harvested from 1918-infected thymus tissue was 8-fold lower than the number of thymocytes from naïve mice. NJ/8 virus infection resulted in no depletion of leukocytes from the thymus, consistent with the insignificant morbidity and mortality observed.

Thrombocytopenia was documented following a human infection with triple-reassortant swine influenza virus in 2008 and is also a common observation among H5N1-infected patients

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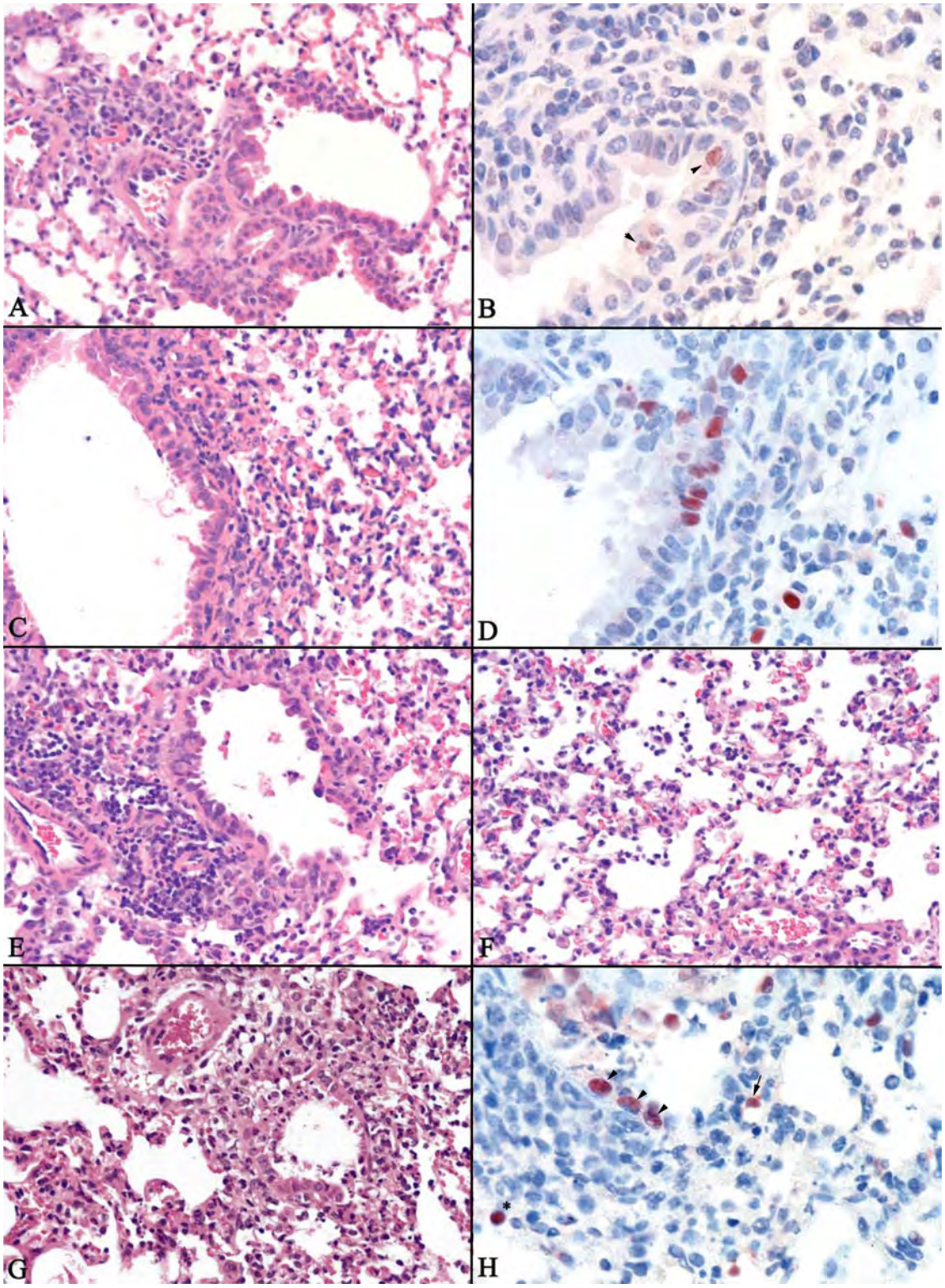

FIG. 1. Histopathologic changes and immunostaining of tissues from H1N1 virus-infected mice. Photomicrographs of hematoxylin-and-eosin-strained and immunohistochemically stained lung sections from mice at 6 days p.i. are shown. (A) Mild bronchiolitis with peribronchiolar lymphocytes and adjacent mild histiocytic alveolitis in an NJ/8 virus-infected mouse. (B) Weak influenza viral antigen staining in a few respiratory epithelial cells (arrowheads) in a bronchiole of an NJ/8 virus-infected mouse. (C) Mild epithelial necrosis in bronchiole and moderate histiocytic alveolitis with some neutrophils in a CA/4 virus-infected mouse. (D) Influenza viral antigen staining in respiratory epithelium bronchioles and in alveolar epithelium and histiocytes in alveoli of a CA/4 virus-infected mouse. (E) Moderate necrosis of bronchiole with alveoli containing edema and inflammatory cells in a 1918 virus-infected mouse. (F) Moderate neutrophilic alveolitis with edema, some histiocytes, and necrosis of alveolar epithelium in a 1918 virus-infected mouse. (G) Severe necrosis of bronchiole and neutrophilic alveolitis with edema and alveolar epithelial necrosis in a VN/1203 virus-infected mouse. (H) Influenza viral antigen commonly staining positive in respiratory epithelium of bronchioles (arrowheads) and in alveolar epithelium (arrow) and histiocytes (*) in alveoli in a 1918 virus-infected mouse.

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TABLE 4. Impact of viral infection on the mouse lymphohemopoietic system[d]

Virus	Day 3 p.i.					Day 6 p.i.				
	WBC[a]	% LY[b]	% NE[b]	% MO[b]	Leukocytes/ thymus[c]	WBC[a]	% LY[b]	% NE[b]	% MO[b]	Leukocytes/ thymus[c]
None (naïve mice)	2.0	76.2	16.7	6.2	7.7 ± 0.1	2.0	76.2	16.7	6.2	7.7 ± 0.1
CA/4	1.3	65.4*	27.9*	6.4	7.8 ± 0.2	1.5	68.2**	23.9*	7.4	7.7 ± 0.2
TX/15	2.2	68.3*	24.3	7.0	7.7 ± 0.1	1.0**	68.9*	21.0	8.9*	7.6 ± 0.2
Mex/4108	0.8**	62.2**	28.7**	8.8	7.7 ± 0.1	0.8**	65.3**	25.2**	8.5*	7.5 ± 0.2
Mex/4482	1.0**	67.9**	24.8**	6.4	7.3 ± 0.1**	1.5	73.2	19.5	6.6	7.3 ± 0.1**
Mex/4487	1.8	64.4**	29.4**	5.2	7.2 ± 0.1**	1.7	55.5**	34.9**	6.9	7.0 ± 0.1**
NJ/8	1.2**	74.5	17.3	6.9	7.7 ± 0.1	1.3*	68.3*	21.1	7.0	7.6 ± 0.1
OH/2	1.0**	59.7**	29.4**	7.8	7.5 ± 0.2	1.8	55.8**	34.7**	7.7	7.3 ± 0.2**
1918	1.2*	66.3*	23.1**	8.9	7.1 ± 0.1**	1.6	52.9**	38.1**	6.7	6.8 ± 0.2**
VN/1203	1.3*	50.3**	36.3**	9.9*	7.0 ± 0.1**	1.4*	48.2**	41.0**	6.8	7.1 ± 0.1**

[a] Number of white blood cells (WBC) in whole blood, expressed as thousands of WBCs per microliter of whole blood.
[b] Mean percentage of leukocytes that are lymphocytes (LY), neutrophils (NE), or monocytes (MO) from 3 to 7 mice per group.
[c] Numbers of leukocytes are expressed as the mean $\log_{10}$ ± standard deviation of 3 to 6 thymi.
[d] Statistical significance: *, $P < 0.05$, and **, $P < 0.005$, compared with naïve controls.

with severe illness (37, 39, 43). However, platelet counts did not drop below baseline levels during the height of infection with any virus tested in mice (data not shown). While reduced levels of hemoglobin have been detected among confirmed H5N1-infected patients, erythrocyte counts did not consistently fall below baseline levels in these experiments (data not shown) (39, 42). In summary, we found that lymphopenia in peripheral blood and a reduction in thymus cellularity were associated with swine, avian, and previous pandemic influenza viruses possessing increased pathogenicity in the mouse model.

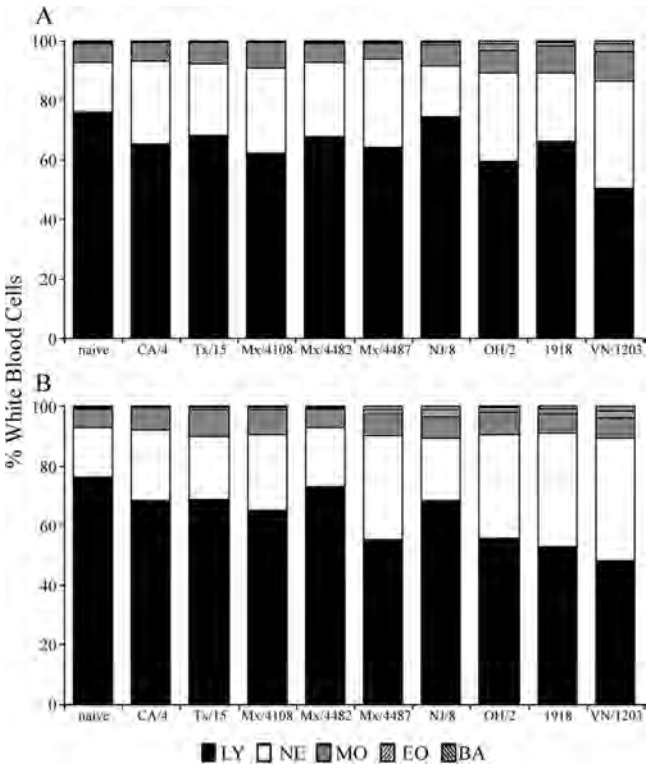

FIG. 2. Kinetic analysis of circulating lymphocytes following influenza virus infection. BALB/c mice (3 or 4 mice/group) were inoculated i.n. with 10^5 EID_{50} or PFU of each virus shown. Blood was collected on day 3 (A) or 6 (B) p.i. in EDTA Vacutainer tubes and analyzed with a hematology scanner. Blood collected immediately prior to inoculation was included as a baseline control (naïve). The average percentages of lymphocytes (LY), neutrophils (NE), monocytes (MO), eosinophils (EO), and basophils (BA) in whole blood are shown.

Cytokine production following primary infection with 2009 H1N1 viruses in mice. Despite efficient replication in mice of all pandemic 2009 H1N1 isolates tested, morbidity following infection varied between strains. To determine if there was a correlation between severe disease and proinflammatory cytokine production in the mouse lung with 2009 H1N1 viruses, tissue homogenates from mice inoculated with 10^5 EID_{50} or PFU were tested by ELISA for the presence of key cytokines previously shown to be differentially induced following seasonal and 2009 pandemic H1N1 virus infection (18). Interleukin 12 [IL-12 (p40)] is involved with T-cell proliferation and contributes toward the stimulation of gamma interferon (IFN-γ) and other cytokines (34). Production of IL-12 did not differ significantly between 2009 H1N1 viruses tested (Fig. 3). Compared with 2009 isolates, OH/2 virus-infected lungs possessed 3-fold-higher levels of IL-12 on day 3 p.i. ($P < 0.001$) and VN/1203 virus-infected lungs possessed 3-fold-higher levels on day 6 p.i. ($P < 0.001$). Influenza virus infection results in the production of several chemokines, including monocyte chemotactic protein 1 (MCP-1), which plays a role in the recruitment of leukocytes to sites of infection. Excessive production of this and other chemokines has been associated with the severe disease observed with H5N1 virus infection (6). MCP-1 production did not differ significantly between 2009 H1N1 viruses (Fig. 3). However, production of MCP-1 was on average 2-fold higher following infection with OH/2 virus on days 3 and 6 p.i. ($P < 0.001$), with 1918-infected lungs producing similarly elevated levels of MCP-1 by day 6 p.i. ($P < 0.001$) compared with 2009 H1N1 viruses. VN/1203-infected lungs possessed significantly higher levels of MCP-1, 10- to 14-fold above those of 2009 H1N1 viruses ($P < 0.001$, days 3 and 6 p.i.). Levels of both IL-12 and MCP-1 in NJ/8 virus-infected lungs were significantly lower (2- to 5-fold) than those in 2009 H1N1 viruses tested ($P < 0.005$, days 3 and 6 p.i.). Production of the cytokines IL-6 and IL-10 did not vary substantially between viruses tested (data not shown). In summary, while differences between 2009 H1N1 virus isolates were not observed, we found that OH/2 virus infection consistently produced ele-

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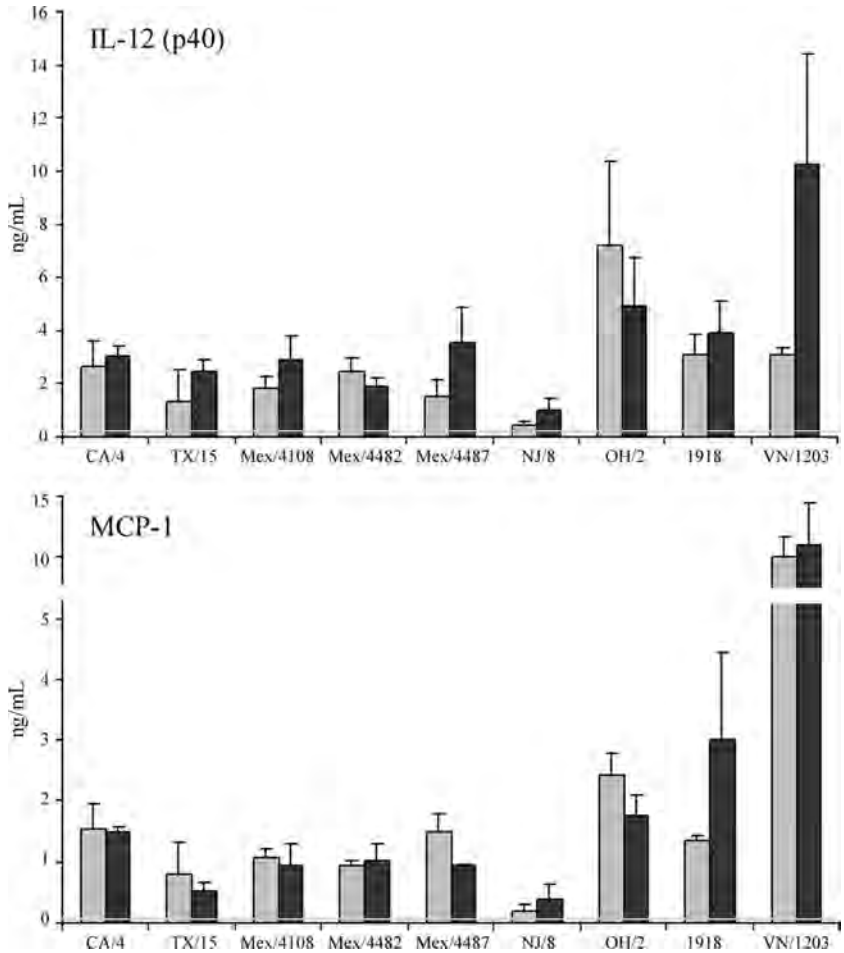

FIG. 3. Determination of IL-12 and MCP-1 levels in H1N1- and H5N1-infected mouse lungs. BALB/c mice (3 or 4 mice/group) were inoculated i.n. with 10^5 EID_{50} or PFU of each virus shown. Lungs were removed on day 3 (gray bars) or 6 (black bars) p.i. and frozen at −70°C until processed. Clarified cell lysates from lungs homogenized in 1 ml PBS were assayed by ELISA and are presented as the mean levels plus standard deviations. Constitutive levels of IL-12 (0.38 ng/ml) or MCP-1 (0.07 ng/ml) present in the lungs were determined by harvesting normal, uninfected BALB/c lungs and are denoted in the figure by a gray line.

vated levels of cytokines in the lungs compared with 2009 isolates, while NJ/8 virus infection consistently produced reduced levels.

Molecular analysis of 2009 H1N1 viruses. The molecular determinants of pathogenicity in mice infected with HPAI H5N1 and 1918 viruses have been characterized (32, 38). The absence of known molecular determinants that confer pathogenicity in 2009 H1N1 viruses was previously discussed in the work of Garten et al. (11). To identify a possible correlation between molecular factors and virus pathogenicity in mammalian species, we searched for molecular differences among 2009 H1N1 viruses and between these and other viruses used in this study. As shown in Table 5, the 2009 H1N1 viruses in this study

TABLE 5. Amino acid differences among 2009 H1N1 viruses used in this study

Virus	Amino acid[c]																				
	PB2; 82	PA			HA[a]							NP				NA[b]		M2; 82	NS1; 45	NS2; 67
		224	275	581	83 (91)	108 (115)	197 (200)	210 (213)	223 (226)	321 (323)	444	16	100	101	373	106	248 (247)			
CA/4	N	**P**	L	M	**P**	V	**T**	F	Q	**I**	V	G	V	D	T	V	N	S	G	E
TX/15	N	S	L	M	S	**M**	A	**L**	Q	V	V	**D**	V	D	T	V	N	S	**R**	**K**
Mex/4108	N	S	L	**L**	S	V	A	F	Q	V	**I**	G	V	**G**	**I**	V	N	**N**	G	E
Mex/4482	N	S	L	M	S	V	A	F	Q	V	V	G	**I**	D	T	**I**	**D**	S	G	E
Mex/4487	**S**	S	**I**	M	S	V	A	F	**R**	V	V	G	V	D	T	V	N	S	G	E

[a] H3 numbering is in parentheses.
[b] N2 numbering is in parentheses.
[c] Nonconserved amino acids are highlighted by boldface.

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possess amino acid differences in 8 out of 11 viral proteins. Overall, these amino acid changes do not map to known functional sites in viral proteins that readily explain variations in virus function, replication, or pathogenicity in mice. Two 2009 H1N1 viruses, Mex/4482 and Mex/4487, possessed amino acid changes in NA and HA proteins, respectively. Mex/4482 was one of the first viruses isolated from a fatal case that contained two amino acid changes in the NA (V106I and N247D). Mex/4487 virus contained an HA amino acid change located in the receptor binding site (Q226R).

The enhanced virulence of 1918 and VN/1203 viruses in this study prompted us to compare molecular features of these viruses to those of the H1N1 viruses examined here. The presence of a lysine at position 627 in PB2 is a known correlate of pathogenicity in the murine model (17, 38). In this study, only VN/1203, 1918, and NJ/8 viruses were found to possess a lysine at this position. A full-length PB1-F2 protein (90 amino acids [aa]) was present in VN/1203, 1918, and OH/2 viruses but not in NJ/8 virus or any of the 2009 H1N1 isolates. A full-length NS1 protein (230 aa) was present in the 1918 virus only; all 2009 H1N1 viruses tested possessed a 219-aa-long NS1 protein. In summary, molecular features which are frequently found among viruses of high pathogenicity in mammalian models were not detected in 2009 H1N1 viruses used in this study.

DISCUSSION

The rapid introduction of swine origin H1N1 viruses into human populations in 2009 necessitated the detailed study of these viruses to better understand their capacity to cause disease in mammalian species. Because of the similarity in the HA proteins of the 2009 H1N1 viruses and classical swine H1N1, including the 1918 pandemic virus, a side-by-side comparison was made among these viruses representing a span of approximately 91 years. This animal model has proven a useful tool for the study of influenza viruses with pandemic potential, due to its utility in measuring infectivity, pathogenesis, and subsequent application for the evaluation of vaccine and antiviral candidates. In this study, we used this model to assess the relative virulence of 2009 H1N1 viruses isolated from human patients compared with both swine lineage influenza viruses previously associated with human infection and highly pathogenic H1N1 and H5N1 viruses. Importantly, these studies revealed that 2009 H1N1 viruses exhibited mild to moderate virulence in mice compared with highly pathogenic avian H5N1 or 1918 viruses, as defined by morbidity, mortality, hemostatic assessments, lung histopathology, and lung cytokine production. Viruses isolated from severe or fatal human cases did not possess heightened virulence in this model. As the 2009 H1N1 pandemic continues to cause human infection and disease, this information will contribute toward a more comprehensive understanding of these viruses in the context of previous human infections with swine lineage influenza viruses and provide a well-characterized model for the study of antiviral and other therapeutic strategies against this emergent pandemic strain.

Viruses isolated from 2009 pandemic H1N1 patients replicated efficiently in the lungs of mice and sporadically and to lower titers in the noses and were restricted to the murine respiratory tract. This pattern of virus replication is similar to that observed with the 1918 reconstructed virus in mice (40). A recent study by Itoh et al., which assessed 2009 H1N1 virus replication in the mouse, confirmed this lack of systemic spread but detected virus at high titers in nasal turbinates (18). This study also reported that select 2009 H1N1 viruses were lethal to mice at high inoculum doses, a finding not recapitulated here. Differences between laboratories in anesthesia agents used, in addition to laboratory-specific conditions, may have contributed to the discordance in severity of disease observed between studies. In agreement with previous work, the 2009 H1N1 viruses tested here produced pulmonary pathology similar to those of other influenza A viruses and included mild to moderate bronchiolitis and alveolitis (18). Necrosis was not a common feature, and neutrophilic responses were typically mild. In contrast, the 1918 and VN/1203 viruses produced moderate to severe inflammation, and necrosis of bronchiolar and alveolar epithelium was frequent, as was alveolar and hilar interstitial edema.

Gastrointestinal symptoms were reported in approximately 40% of 2009 H1N1 virus-infected patients, and virus was recovered from the intestinal tract of ferrets following inoculation with 2009 H1N1 viruses; however, the 2009 H1N1 viruses tested here were not recovered from intestinal mouse tissue (7, 23). We also assessed the ability of 2009 H1N1 viruses to cause disease following ocular inoculation, as some influenza virus subtypes can use this portal of entry to initiate virus infection (3). The inability of 2009 H1N1 viruses to mount a productive infection in mice following ocular inoculation recapitulates the absence of conjunctivitis reported following 2009 H1N1 human infection and further suggests that the primary mode of 2009 H1N1 virus transmission is via the respiratory tract (7, 23, 30).

Collection of blood from mice during the acute phase of infection was performed to assess the influence of 2009 H1N1 virus infection on lymphocyte populations and other hemostatic parameters. Severe influenza virus infection in humans with H5N1 and 2009 H1N1 viruses has been associated with leukopenia, lymphopenia, and thrombocytopenia; these clinical characteristics have not been consistently observed among mild cases of 2009 H1N1 virus infection in humans (4, 37, 39). Highly virulent viruses, including the reconstructed 1918 virus and the H5N1 virus used in this study, have previously been shown to result in peripheral blood lymphopenia in the mouse model (24, 33). While transient lymphopenia was observed in mice following 2009 H1N1 virus infection, there was significantly less overall lymphocyte depletion compared with highly virulent viruses or the triple-reassortant OH/2 virus (Table 4; Fig. 2). Further clinical data from 2009 H1N1 virus-infected patients will contribute toward a comprehensive understanding of the change in hemostatic parameters following infection with this pandemic virus. There is currently a paucity of information pertaining to the consequence of influenza virus infection on complete blood counts in the mouse model as presented in this study in Table 4. By comparing viruses of different subtypes and degrees of virulence, we are better able to place in context the results obtained from 2009 H1N1 virus-infected mice.

The dysregulation of cytokines and chemokines during infection with H5N1 avian influenza viruses has been proposed to contribute to the severity of disease in human patients (25). Currently, there is little information on the proinflammatory

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response induced by 2009 H1N1 viruses and its relevance to disease severity in humans. A recent study described the increased production of several cytokines in mice infected with the 2009 H1N1 virus CA/4 compared with a seasonal H1N1 virus (18). As these experiments were performed with only one 2009 H1N1 isolate and did not offer a side-by-side comparison with viruses known to elicit hypercytokinemia following infection, selected analytes were chosen for further study here to more broadly assess the induction of cytokines and chemokines following infection with multiple human 2009 H1N1 isolates. We did not observe substantial differences in mouse cytokine production among 2009 H1N1 viruses isolated from patients in the early stages of the pandemic. Levels of IL-12 and MCP-1 following 2009 H1N1 virus infection were significantly higher compared with NJ/8 virus, similar to the results in the study by Itoh et al., which demonstrated elevated levels of cytokines from CA/4 virus compared with a less virulent seasonal H1N1 virus (18). Conversely, cytokine production following OH/2 virus infection was elevated compared with 2009 H1N1 viruses, which correlates with the heightened pathogenicity observed with the triple-reassortant virus in mice. Furthermore, production of IL-12 and MCP-1 by H1N1 viruses in this study was significantly reduced compared with the H5N1 virus VN/1203, similarly to a previous report demonstrating excessive release of cytokines by H5N1 viruses compared with H1N1 viruses, including the 1918 reconstructed virus (33). Our results agree with a recent study which found weak cytokine production following 2009 H1N1 virus infection in human macrophages and dendritic cells (31). These findings suggest that hypercytokinemia is not a general feature of 2009 H1N1 viruses, including those viruses isolated from fatal cases.

Similarly to previously published studies, we did not observe molecular features among 2009 H1N1 viruses which are known to correlate with virus pathogenicity in the mouse model. The E627K substitution in PB2 has been associated with enhanced virulence of HPAI viruses and is also present in the reconstructed 1918 virus (17, 40). The presence of this amino acid in NJ/8 virus indicates that not all H1N1 viruses which bear this substitution possess high virulence in the mouse model. The accessory protein PB1-F2 has known roles in virus pathogenicity and inflammation following influenza virus infection (27, 45). All viruses in this study which possessed a full-length PB1-F2 protein (VN/1203, 1918, and OH/2), and only these viruses, were capable of mounting a lethal infection in the mouse model. Future study addressing the potential for enhanced pathogenicity of 2009 H1N1 viruses following the introduction of a full-length PB1-F2 is warranted. The triple-reassortant H1N1 virus OH/2 demonstrated enhanced morbidity and mortality in mice compared with 2009 H1N1 viruses, along with elevated levels of proinflammatory cytokines and more pronounced dysregulation of hemostatic and lymphostatic parameters. The genetic composition of OH/2 virus, a member of the swH1-gamma lineage, generally aligns with 2009 H1N1 viruses, with the exception of Eurasian and not classical swine lineage NA and M genes present in 2009 isolates (11, 37). Triple-reassortant H1N1 viruses, including OH/2 virus, remain antigenically similar to the 2009 H1N1 isolates (11). Detailed study of the contribution of individual genes to the heightened pathogenicity of OH/2 virus found in this study compared with 2009 isolates will allow for a greater understanding of the potential for these viruses to cause severe disease.

Well-characterized mammalian models have afforded the opportunity for a more comprehensive understanding of previous pandemic viruses and those viruses considered to hold pandemic potential (24, 40). Establishing similar models for 2009 H1N1 viruses, such as the mouse model presented here, will accelerate the identification of those molecular correlates which contribute to the unique features of this newest pandemic strain and support efforts to limit the spread and severity of disease via the use of vaccines and antivirals. Direct comparison *in vivo* between 2009 H1N1 viruses and previous viruses of swine origin associated with human infection additionally presents the opportunity to more closely examine viruses within this lineage as they evolved from causing sporadic human infections to acquiring properties which resulted in the first pandemic of the 21st century.

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Lethal Dissemination of H5N1 Influenza Virus Is Associated with Dysregulation of Inflammation and Lipoxin Signaling in a Mouse Model of Infection▽†

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Periodic outbreaks of highly pathogenic avian H5N1 influenza viruses and the current H1N1 pandemic highlight the need for a more detailed understanding of influenza virus pathogenesis. To investigate the host transcriptional response induced by pathogenic influenza viruses, we used a functional-genomics approach to compare gene expression profiles in lungs from 129S6/SvEv mice infected with either the fully reconstructed H1N1 1918 pandemic virus (1918) or the highly pathogenic avian H5N1 virus Vietnam/1203/04 (VN/1203). Although the viruses reached similar titers in the lung and caused lethal infections, the mean time of death was 6 days for VN/1203-infected animals and 9 days for mice infected with the 1918 virus. VN/1203-infected animals also exhibited an earlier and more potent inflammatory response. This response included induction of genes encoding components of the inflammasome. VN/1203 was also able to disseminate to multiple organs, including the brain, which correlated with changes in the expression of genes associated with hematological functions and lipoxin biogenesis and signaling. Both viruses elicited expression of type I interferon (IFN)-regulated genes in wild-type mice and to a lesser extent in mice lacking the type I IFN receptor, suggesting alternative or redundant pathways for IFN signaling. Our findings suggest that VN/1203 is more pathogenic in mice as a consequence of several factors, including the early and sustained induction of the inflammatory response, the additive or synergistic effects of upregulated components of the immune response, and inhibition of lipoxin-mediated anti-inflammatory responses, which correlated with the ability of VN/1203 to disseminate to extrapulmonary organs.

The World Health Organization officially declared on 11 June 2009 that spread of a new swine origin H1N1 influenza virus had reached the level of a global pandemic (29). Although less virulent than the H1N1 virus responsible for the 1918 influenza pandemic, initial studies indicate that this virus is more pathogenic than seasonal H1N1 influenza viruses (10). The continued emergence of new influenza viruses highlights the need to better understand influenza virus-host interactions and mechanisms of pathogenicity. Such an understanding is necessary to facilitate the development of safe and effective therapeutics and vaccines, critical aspects of preparedness for the current and future pandemics.

A clear reminder of the lethal potential of influenza virus infection is the 1918 pandemic, which resulted in over 50 million deaths worldwide (14). In addition, highly pathogenic avian H5N1 influenza viruses continue to circulate in diverse parts of the world (31, 45), and with human infections resulting in a greater than 50% mortality rate, there is considerable concern over the potential for a deadly new pandemic. Here, we sought to compare the host transcriptional responses to the reconstructed 1918 virus and the avian H5N1 virus Vietnam/1203/04 (VN/1203), with the goal of gaining insights into the underlying mechanisms that make these viruses so lethal.

Recently, we reported a study comparing the host response to 1918 and VN/1203 influenza viruses in a macaque model of infection (8). In that study, all 1918-infected macaques died while all VN/1203-infected animals recovered. Interestingly, only the 1918 virus induced the expression of genes encoding components of the inflammasome early during infection, pointing out different transcriptional mechanisms during infection with highly pathogenic H5N1 and H1N1 influenza viruses. Results from an independent study using mouse infection models suggest there are also differences in lethality and disease progression between the avian H5N1 influenza strains A/Hong Kong/483/97 and A/Hong Kong/486/97 (40). In that study, alpha/beta interferon receptor (IFN-α/βR)-deficient mice succumbed faster than wild-type mice to either H5N1 virus. In addition, there was systemic infection of IFN-α/β receptor-deficient mice with both viruses.

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In the present study, we examined the host responses to the 1918 virus and VN/1203 in wild-type 129S6/SvEv mice; additional findings were obtained from IFN-α/β receptor-deficient mice and mouse embryonic fibroblasts derived from these animals. The VN/1203 virus was more pathogenic than the 1918 virus and was able to disseminate to extrapulmonary organs. Global gene expression profiling data revealed possible mechanisms underlying the greater pathogenicity of the VN/1203 virus.

MATERIALS AND METHODS

Viruses. The reconstructed 1918 H1N1 virus (42) (1918), possessing the A/South Carolina/1/18 hemagglutinin (HA), and VN/1203 were previously shown to be highly virulent for both mice and ferrets (23, 42–43). The 1918 virus was generated utilizing the 12-plasmid reverse-genetics system in a mixture of Madin-Darby canine kidney (MDCK) (ATCC, Manassas, VA) and 293T (ATCC) cells as previously described (42). The titers of virus stocks were determined by plaque assay on MDCK cells, and stocks were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT) and 1% penicillin/streptomycin (Gibco). The reconstructed 1918 and VN/1203 viruses were grown as previously described (33). All virus challenge experiments were performed under the guidance of the U.S. National Select Agent Program in negative-pressure HEPA-filtered biosafety level 3 enhanced (BSL-3+) laboratories and with the use of a battery-powered Racal HEPA filter respirator (Racal Health and Safety Inc., Frederick, MD) according to Biomedical Microbiological and Biomedical Laboratory procedures (35).

Mouse experiments. All animal research was conducted according to the guidance of CDC's Institutional Animal Care and Use Committee in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. IFN-α/βR-deficient (IFNR1$^{-/-}$) mice on the 129 background were obtained from Herbert Virgin at Washington University, St. Louis, MO. Wild-type SvEv129 (129S6/SvEv) mice (44) were used as age-matched controls in these experiments (Taconic, Bar Harbor, ME). Similar to the majority of inbred mouse strains, 129S6/SvEv animals carry defective alleles of the Mx1 gene (38). Eight-to-10-week-old female 129S6/SvEv and IFNR1$^{-/-}$ mice (on a 129 background) were anesthetized by intraperitoneal injection of 0.2 ml of 2,2,2-tribromoethanol in tert-amylalcohol (Avertin; Sigma-Aldrich, Milwaukee, WI). Ten times the 50% lethal dose (LD_{50}), 3.2×10^4 PFU (1918) or 7×10^3 PFU (VN/1203), in 50 μl of infectious virus diluted in phosphate-buffered saline (PBS) was inoculated intranasally (i.n.). Fifty percent mouse infectious dose (MID_{50}) and LD_{50} titers were determined by inoculating groups of seven mice i.n. with serial 10-fold dilutions of virus. For MID_{50} determination, three mice from each group were euthanized on day 4 postinoculation (p.i.), and lungs were collected and homogenized in 1 ml of cold PBS. The homogenates were frozen at −70°C and later thawed before centrifugation and titration for virus infectivity in eggs. The four remaining mice in each group were checked daily for disease signs and death for 14 days p.i. MID_{50} and LD_{50} titers were calculated by the method of Reed and Muench (34) and were expressed as the 50% egg infective dose (EID_{50}) value corresponding to 1 MID_{50} or LD_{50}.

Replication of 1918 and H5N1 viruses in mice (3 animals/group/time point) was examined by determining the virus titers in lung (days 1, 3, and 4), spleen, liver, kidney, and brain (days 3 and 5) following i.n. inoculation with 10^6 EID_{50} of virus. Clarified homogenates were titrated for virus infectivity in eggs from an initial dilution of 1:10 (lung), making the limit of virus detection $10^{1.2}$ EID_{50}/ml. Additional inoculated mice (6 animals/group/virus, making a total of 24 animals) were followed for morbidity and mortality. For RNA isolation, lungs (2 animals/group/time point) were frozen in individual tubes and stored in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) as previously described (7). In addition, groups of uninfected 129S6/SvEv (6 animals) and IFNR1$^{-/-}$ (6 animals) mice were intranasally treated with 10,000 units of recombinant human IFN-α A/D (R&D Systems, Minneapolis, MN) and euthanized 8 and 24 h posttreatment.

Infection of MEFs. Mouse embryonic fibroblasts (MEFs) derived from wild-type 129S6/SvEv mice or IFNR1$^{-/-}$ mice generated on the 129S6/SvEv background (27) were grown as monolayers in high-glucose Dulbecco's modified Eagle's medium (hgDMEM) supplemented to contain 10% heat-inactivated fetal calf serum (HyClone Laboratories), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 μM 2-mercaptoethanol, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml). MDCK cells were grown as monolayers in hgDMEM supplemented to contain 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml). Near-confluent monolayers of cells were mock infected with PBS alone or infected with influenza virus diluted in infection medium (hgDMEM supplemented to contain 2% heat-inactivated calf serum, 2 mM L-glutamine, penicillin G [50 units/ml], streptomycin sulfate [50 μg/ml], and 50 mM HEPES) to the indicated multiplicity of infection (MOI). After 45 min of adsorption at 4°C, virus and medium were removed. Fresh infection medium was added to the cells, and the infections were allowed to proceed at 37°C until the indicated time postinfection.

Histopathology and immunohistochemistry (IHC). Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were cut to 5 μm and stained with hematoxylin and eosin (HE). Methods for immunohistochemical detection of influenza A viral antigen have been previously described (32). A duplicate 5-μm section was immunohistochemically stained to demonstrate influenza A virus nucleoprotein by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2,000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein was applied and allowed to incubate for 12 h at 4°C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using the Mouse on Mouse System (M.O.M. kit; Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. The AEC-Peroxidase Substrate Kit (Vector Laboratories Inc.) was used as the substrate chromogen, and hematoxylin was used as a counterstain.

Microarray analysis and bioinformatics. For all gene expression analyses, separate microarrays were run for each experimental sample. This included 2 animals/time point for 1918 virus-infected mice (24 animals total) or 3 animals/time point for VN/1203-infected mice (36 animals total). Equal masses of total RNA isolated from lungs collected from infected mice were amplified with a Low RNA Input Linear Amplification Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Global gene expression in infected lungs was compared to genetic-match mock control RNA for each group prepared from a pool of equal masses of total RNA from lung tissue of three uninfected mice. Probe labeling and microarray slide hybridization were performed as described elsewhere (16). Briefly, probes were hybridized on Agilent mouse oligonucleotide microarray slides (approximately 20,000 unique mouse genes). The slides were scanned with an Agilent DNA microarray scanner, and image data were processed using Agilent Feature Extractor (AFE) version 8.1.1.1. The AFE software was used to perform image analysis, including significance of signal and spatial detrending, and to apply a universal error model (for these hybridizations, the most conservative error model was applied). To correct for dye bias, AFE software was also used to perform linear and lowess (intensity dependent) dye normalization. The raw data were then loaded into a custom-designed laboratory information management system (LIMS). The raw data and associated sample information were subsequently loaded into Rosetta Resolver 7.2 (Rosetta Biosoftware, Seattle, WA). Each single microarray experiment incorporated reverse dye labeling techniques and resulted in two measurements for each gene ($n = 2$), allowing the calculation of a mean ratio between expression levels, standard deviations, and P values within the Rosetta Resolver System (Rosetta Biosoftware, Seattle, WA).

The Resolver system performs a squeeze operation that creates ratio profiles by combining replicates while applying error weighting. The error weighting consists of adjusting for additive and multiplicative noise. A P value is generated that represents the probability that a gene is differentially expressed. In this study, a threshold P value of 0.01 was used to identify genes that were significantly differentially expressed. The Resolver system then combines ratio profiles to create ratio experiments using an error-weighted average as described previously (38a). Spotfire Decision Site 9.1.1 (Spotfire, Somerville, MA) and Ingenuity Pathways Analysis (IPA) 8.0 (Ingenuity Systems, Redwood city, CA) were also used for data analysis and mining. Primary gene expression data are available at http://viromics.washington.edu, in accordance with proposed minimum information about a microarray experiment (MIAME) standards.

Functional and network analyses of statistically significant gene expression changes were performed using IPA. Analysis considered all genes from the data set that met the 2-fold ($P < 0.01$) change cutoff and that were associated with biological functions in the Ingenuity Pathways Knowledge Base. For all analyses, Fisher's exact test was used to determine the probability that each biological function assigned to the genes within each data set was due to chance alone.

qRT-PCR. Reverse transcription (RT)-PCR was performed on samples from individual animals to validate specific cellular gene expression changes detected by microarray. The QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA) was used to generate cDNA. Quantitative real-time RT-PCR (qRT-PCR)

was run on an ABI 7500 PCR system, using TaqMan chemistry (Applied Biosystems, Foster City, CA). Gene expression assays specific to mouse cellular genes were purchased from Applied Biosystems. Differences in gene expression are represented as $\log_{10}$ relative quantification to a calibrator and normalized to a reference, using the $2^{-\Delta\Delta CT}$ method (20).

RESULTS

VN/1203 is more pathogenic than the 1918 virus in mice. Our previous studies of macaques infected with either VN/1203 or the 1918 virus indicated that the 1918 virus is more pathogenic in this animal model (8). However, of the 498 confirmed human cases of avian H5N1 influenza reported to the World Health Organization as of May 2010, 294 had resulted in death, representing a case fatality rate approaching 60%. Although there may be mild infections that go undiagnosed, H5N1 viruses appear to be significantly more pathogenic than the 1918 virus in humans (28). Although the genetic, anatomical, and physiological similarities between macaques and humans suggest that macaques provide a highly relevant animal model, the mouse infection model is widely used because it provides an approximation of human disease; larger numbers of animals can be used to improve statistical significance; and, importantly, a wide variety of gene knockout animals are available. Therefore, to gain additional insights into the host response to these viruses, we used a mouse infection model that included wild-type mice and mice lacking the type I IFN receptor, a critical component of the innate immune response. When inoculated into wild-type 129S6/SvEv mice, both VN/1203 and the 1918 virus caused lethal infections; however, the mean time of death was 6 days for VN/1203-infected animals and 9 days for mice infected with the 1918 virus (Fig. 1A). Animals were sacrificed according to weight loss euthanasia criteria (see Fig. S1 in the supplemental material). Moreover, VN/1203 disseminated to extrapulmonary organs, as demonstrated by detection of virus in brain and spleen (Fig. 1B), whereas the 1918 virus was not detected outside the respiratory tract. The observed differences in viral dissemination and time to death could not be attributed to differences in viral titers in the lungs, as the two viruses were present at similar levels at all time points examined (Fig. 1C).

Histopathology results indicated that two of six animals infected with the 1918 virus had mild epithelial necrosis in bronchioles and minimal peribronchiolar alveolitis at day 1 p.i. By day 3 p.i., three of the animals still lacked lesions and three presented mild to severe necrotizing bronchiolitis with associated neutrophilic casts in large bronchioles, peribronchiolar edema and inflammation, and moderate peribronchiolar neutrophilic-to-histiocytic alveolitis. By 4 days p.i., pneumonia and bronchiolitis were consistent in all animals and were slightly more severe than those observed at day 3 p.i. No lesions were observed in heart, spleen, brain, kidney, or liver in 1918-infected animals (see Fig. S2A and B in the supplemental material). Animals infected with VN/1203 presented at day 1 p.i. with moderate necrotizing bronchiolitis with neutrophilic inflammation and minimal peribronchiolar alveolitis. By days 3 and 4 p.i., the airway lesions were accompanied by minimal to mild peribronchiolar alveolitis. VN/1203-infected mice presented no lesions in extrapulmonary organs (see Fig. S2C and D in the supplemental material), but virus was detected in brain and spleen. Viral antigen was commonly present in bronchiolar epithelial cells and less commonly in alveolar epithelial cells and macrophages (see Fig. S2E and F in the supplemental material). Staining was more intense and more widely distributed in VN/1203-infected mice than in 1918 virus-infected mice. In summary, both viruses caused lethal infections, with death occurring approximately 2 days sooner among VN/1203-infected mice. VN/1203 disseminated to brain and spleen by 5

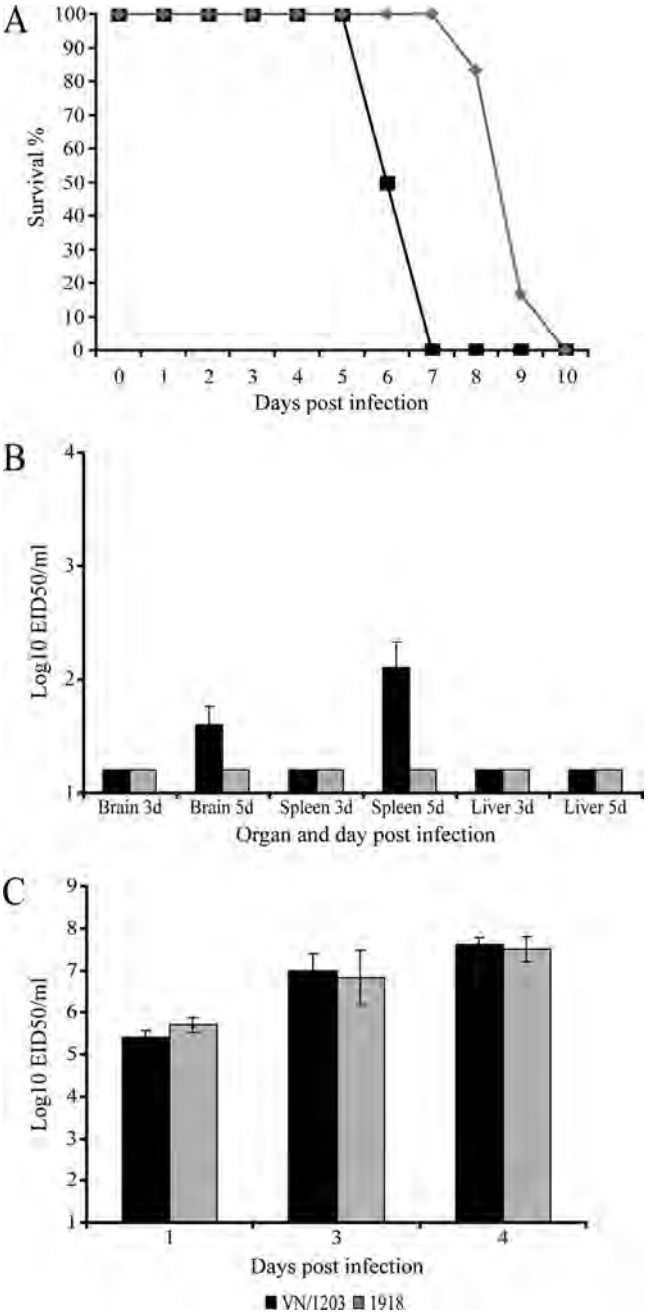

FIG. 1. VN/1203 infection is more pathogenic than 1918 infection in a mouse model of infection. (A) Mortality data from a total of 12 mice (6 mice/virus). (B) Viral dissemination data from a total of 12 mice (3 mice/virus/time point). d, day(s). (C) Lung virus titer data from a total of 18 wild-type mice (3 mice/virus/time point) infected with 1918 or VN/1203 virus. The limit of detection in this assay is indicated by black and grey bars at 1.2×10^1 EID_{50}/ml.

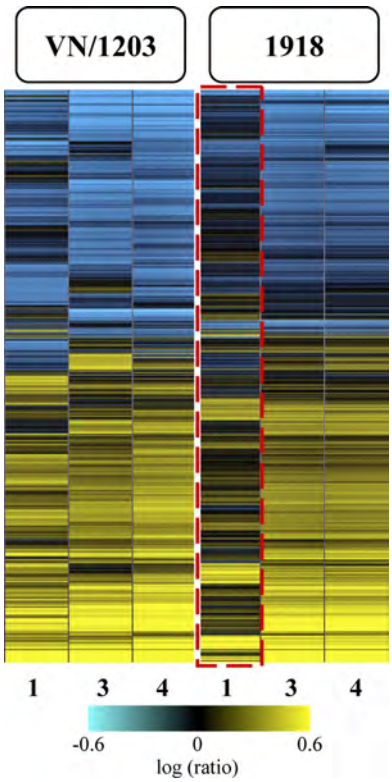

FIG. 2. Global transcriptional responses to highly pathogenic 1918 and VN/1203 viruses. Shown is microarray analysis of whole lung tissue from 1918 and VN/1203 virus-infected wild-type mice at 1, 3, and 4 days p.i. The heat map illustrates the global transcriptional profile of 3,470 genes activated within cutoff values of ≥2-fold change and $P \leq 0.01$. The genes shown in yellow were upregulated and those in blue were downregulated in infected relative to mock-infected animals. The box outlined in red indicates the attenuated transcriptional response in 1918 virus-infected animals.

days p.i., but without noticeable tissue damage to those organs. The absence of pathology in extrapulmonary organs may be due to detection of recent virus replication that had not yet resulted in observable lesions. Alternatively, lesions may have been random and focal and not present on the samples examined.

VN/1203 elicits an earlier and more robust host transcriptional response. In order to discover aspects of the host response that could explain differences in pathogenicity and dissemination, we used a functional-genomics approach to investigate the host transcriptional response elicited in the lungs of mice infected with VN/1203 or the 1918 virus. When analyzing the global transcriptional response, we found that VN/1203 infection induced the differential expression of numerous genes at all time points analyzed. Whereas the day 1 transcriptional response was attenuated in animals infected with the 1918 virus relative to the response induced by VN/1203, at later time points, the host responses to the two viruses were more similar (Fig. 2). This more robust transcriptional response to VN/1203 correlates with our observations that all VN/1203-infected animals showed moderate airway lesions at day 1 p.i., although we have not directly evaluated the extent to which specific gene expression changes are responsible for lesion development.

The VN/1203 and 1918 viruses differentially regulate key cellular response pathways. We next used Ingenuity Pathways Analysis to identify functional categories of differentially expressed genes. Our analyses showed that many of these genes were related to the inflammatory response (Fig. 3A) and included the upregulation of inflammasome genes in VN/1203-infected mice (but not in 1918-infected mice) at day 1 p.i. The upregulation of inflammasome genes in VN/1203-infected mice is also illustrated in the functional network shown in Fig. 3B. In this network, genes depicted in blue were upregulated by VN/1203 but not by the 1918 virus, whereas genes depicted in orange were upregulated by VN/1203 virus but downregulated by the 1918 virus. Of particular note, the key inflammasome components CASP1 (caspase 1), interleukin 1β (IL-1β), and NLRP3 (nucleotide-binding domain and leucine-rich-repeat-containing protein 3) were upregulated in response to VN/1203 infection.

In addition, VN/1203 also upregulated the expression of tumor necrosis factor alpha (TNF-α), a potent inflammatory molecule; IFN-γ; eukaryotic initiation factor 2 AK2 (eIF2AK2); protein kinase RNA activated (PKR); and additional chemokines and inflammation-related genes. Quantitative RT-PCR analyses were performed to verify the expression of specific transcripts, including IL-1β, NLRP3, CASP1, and TNF-α. The results from this method correlated well with the microarray results (see Fig. S3 in the supplemental material). Later during infection (days 3 and 4 p.i.), the inflammatory responses induced by the two viruses were still different but did not involve the inflammasome, and the numbers of genes differentially regulated were fewer than observed on day 1. This striking difference in the quality of the early inflammatory responses to two highly pathogenic influenza viruses correlates with the severity of disease.

We also observed that at day 1 p.i., VN/1203 infection induced the differential expression of genes associated with viral sensing, neutrophil activation, NF-κB signaling, and chemokine signaling (Fig. 4). Quantitative RT-PCR analyses were performed to verify the expression of specific transcripts, including IFN-β1 and IFN-γ. The results from this method correlated well with the microarray results (see Fig. S4A and B in the supplemental material). Because the products of many of these genes play important roles in regulating cellular gene expression, the activation of these genes may also be associated with the earlier and more robust host transcriptional and inflammatory response observed in VN/1203-infected animals.

VN/1203 dissemination correlates with sustained activation of the inflammatory response and altered hematological function and lipoxin signaling. To attempt to gain insight into the ability of VN/1203 to disseminate to the brain and spleen, we also devised an analysis strategy to identify gene expression changes that correlated with tissue dissemination. Briefly, gene expression data from all time points were stratified into four groups: wild-type mice infected by the 1918 virus, wild-type mice infected by VN/1203, IFNR1$^{-/-}$ mice infected by the 1918 virus, and IFNR1$^{-/-}$ mice infected by VN/1203. Among these groups, only wild-type mice infected by the 1918 virus showed no dissemination during infection. Differentially expressed genes in each group compared to mock infection were identified (≥2.0-fold change), and pathway analysis was car-

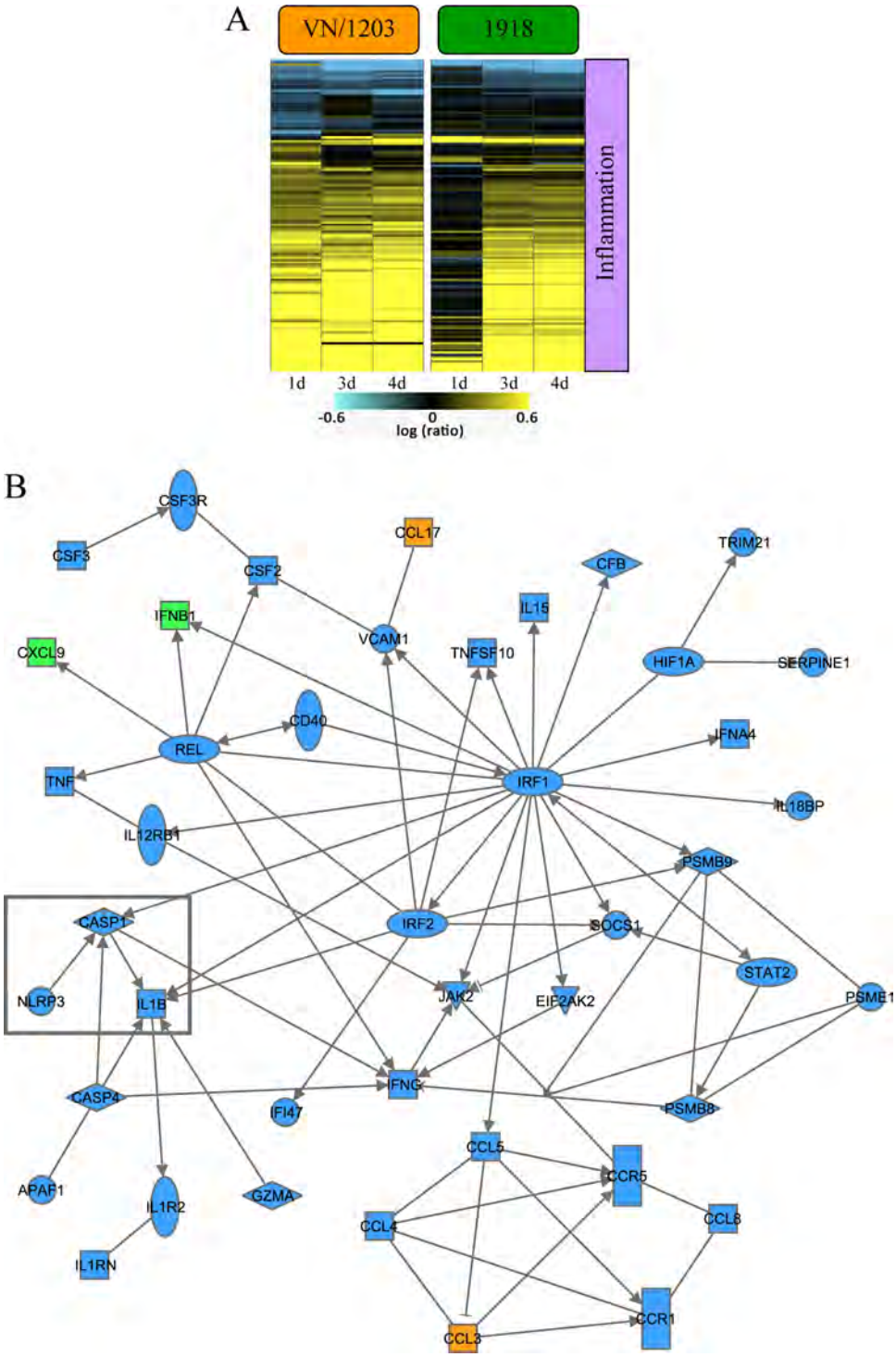

FIG. 3. VN/1203 virus differentially regulates the expression of inflammatory response genes. (A) Heat map illustrating ANOVA results for 142 inflammatory genes differentially transcribed within cutoff values of ≥2-fold change and ANOVA $P \leq 0.01$ in wild-type mouse lungs infected with 1918 or VN/1203 virus. (B) Biological-network analysis of the top functional category inflammatory response ($P = 4.22E-16$) determined by Ingenuity Pathway Analysis. This analysis highlights a subset of genes that were upregulated during VN/1203 infection but not during 1918 infection (blue shading). Another subset of genes was anti-coregulated, that is, upregulated during VN/1203 infection but downregulated during 1918 infection (orange shading). A third subset of genes was upregulated by both viruses (green shading). The boxed genes highlight the differential regulation of the inflammasome components CASP1, IL-1β, and NLRP3.

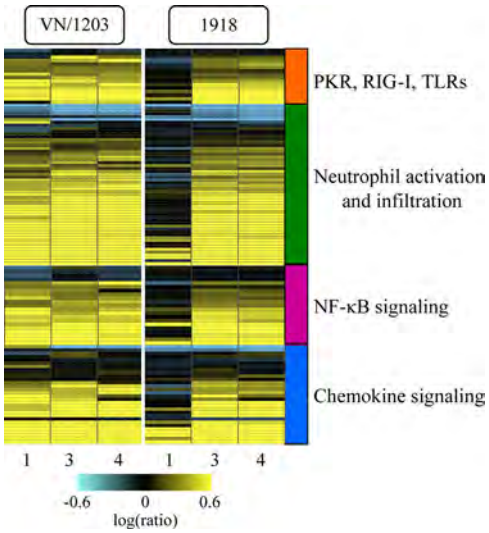

FIG. 4. The VN/1203 and 1918 viruses differentially regulate key cellular signaling pathways. Shown is ANOVA of microarray data from lung samples using cutoff values of ≥2-fold change and ANOVA $P \leq 0.01$ comparing the infections of wild-type animals with 1918 and VN/1203 viruses. The results were uploaded into IPA for functional analysis. Selected canonical pathways were analyzed, and the results are presented as heat maps illustrating the expression of genes associated with viral sensing, neutrophil activation, NF-κB signaling, and chemokine signaling to 1918 and VN/1203 viruses. Yellow indicates upregulated genes; blue indicates downregulated genes.

ried out using Ingenuity Pathway Analysis and GeneGo MetaCore. Two functional categories, which included genes associated with hematological development and function and genes associated with the inhibitory effects of lipoxin on inflammation, leukocyte trafficking, and neutrophil-mediated tissue injury, showed statistically significant changes in the groups associated with virus dissemination but not the group not associated (wild-type mice infected by 1918). Many of the genes in the hematological development and function category are associated with proinflammatory responses and were strongly upregulated during VN/1203 infection (Fig. 5A). Lipoxins, in contrast, are eicosanoids with potent anti-inflammatory properties (37). The gene responsible for lipoxin biogenesis, Alox5, was downregulated during VN/1203 infection (Fig. 5B), as was the gene encoding suppressor of cytokine signaling 2 (Socs2), the expression of which can be induced by lipoxins to control proinflammatory responses (22). These finding suggest that not only does VN/1203 infection result in the induction of numerous proinflammatory genes, but lipoxin-mediated anti-inflammatory responses are also impaired. No change in the expression of Alox5, Socs2, or Fprl1 (the primary receptor for lipoxins) was observed in response to infection with the 1918 virus.

We also investigated the observed dissemination of the 1918 virus to brain and spleen during infection of IFNR1$^{-/-}$ mice. Our results indicated that the host responses elicited by 1918 virus infection were distinctly different in wild-type and immune-deficient animals. While the activation of important host functions, such as immune cell trafficking, inflammatory response, and cell death, were almost at similar levels in both mouse strains by day 1 p.i., these functions changed dramatically in wild-type mice, which responded to 1918 infection by an steady increase in the expression of genes associated with these functions by days 3 and 4 p.i. However, far fewer genes in these categories were transcriptionally activated in IFNR1$^{-/-}$ mice by days 3 and 4 p.i. (see Fig. S5 in the supplemental material).

VN/1203 and 1918 viruses induce the activation of type I IFN-regulated genes even in the absence of the IFN-α/β receptor. Activation of IFN signaling is an important component of the innate antiviral response, and our previous studies of the 1918 virus in mice and VN/1203 in macaques suggested differences in IFN signaling in response to these viruses (6, 17). We therefore sought to directly compare the IFN response to these viruses in the mouse infection model. To identify IFN-regulated genes, we treated uninfected mice with IFN and identified the resulting differential expression of genes in the lungs. We also took advantage of mouse genetics and used IFNR1$^{-/-}$ mice to determine the importance of an intact IFN response to the host response to these viruses.

To identify IFN-regulated genes, we treated wild-type or IFNR1$^{-/-}$ mice with 10,000 U of recombinant human IFN-α A/D and harvested lung tissue at 8 and 24 h after treatment. These samples were then used to generate a set of 854 type I IFN-regulated genes, which was used as a common gene set for clustering and analysis of variance (ANOVA) of gene expression data from virus-infected animals. We found that activation of type I IFN-regulated genes was stronger in VN/1203-infected mice than in mice infected with the 1918 virus and that VN/1203 elicited steady and sustained activation of these genes even in animals lacking the IFN-α/β receptor (Fig. 6A). In animals infected with the 1918 virus, there was little activation of the IFN-regulated genes at day 1 p.i., but by days 3 and 4, IFN-regulated gene expression reached almost the same level as that induced by VN/1203. Since it was possible that IFNR1$^{-/-}$ mice compensated for the absence of IFNR1 with increased expression of IFN-λ3 (also known as IL28B) (4, 18), we used quantitative RT-PCR to detect IL28B in lung samples at 1, 3, and 4 days p.i. We found that IL28B was not overexpressed in the IFNR1$^{-/-}$ mice and that expression correlated with the temporal regulation of IFN-β1 and, to a lesser extent, IFN-γ (see Fig. S4C in the supplemental material).

IFNR1$^{-/-}$ mice infected with either virus died sooner than infected wild-type animals, and IFNR1$^{-/-}$ mice infected with VN/1203 died approximately 3 days sooner than those infected with the 1918 virus (see Fig. S6A in the supplemental material). The percentage of survival was determined by the weight loss parameter, according to the CDC's Institutional Animal Care and Use Committee (see Fig. S1B in the supplemental material). VN/1203 disseminated to brain, liver, and spleen, and in contrast to wild-type animals, the 1918 virus was also detected in the brains and spleens of IFNR1$^{-/-}$ mice at day 5 p.i. (see Fig. S6B in the supplemental material). As in the case of wild-type mice, viral dissemination in the IFNR1$^{-/-}$ mice did not correlate with the viral titer (see Fig. S6C in the supplemental material). Even though the 1918 virus was detected in the brains and spleens of IFNR1$^{-/-}$ mice at day 5 p.i., histopathology revealed no lesions in these organs (Fig. 7A and B). Lesions in spleens were observed in VN/1203-infected IFNR1$^{-/-}$ mice (Fig. 7C and D). Staining for influenza virus antigen in the bronchus was more uniformly distributed in

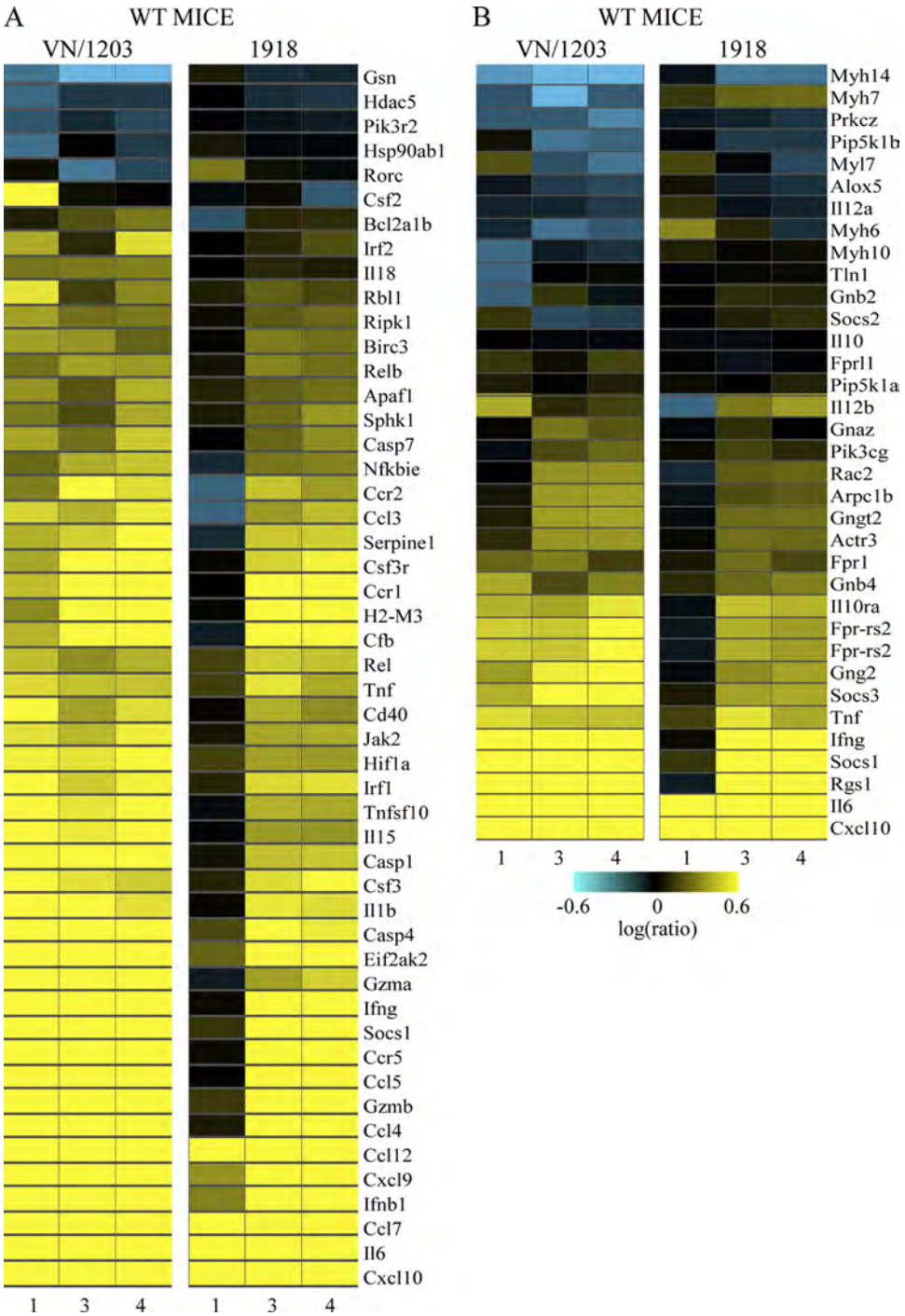

FIG. 5. Association of hematological function and lipoxin signaling with VN/1203 virus dissemination. (A) Differential regulation of hematological system development- and function-related genes. (B) Differential regulation of lipoxin signaling. Shown is ANOVA of lung gene expression data using cutoff values of ≥2-fold change and ANOVA $P \leq 0.01$ comparing the infection of wild-type animals with VN/1203 or the 1918 virus. The results were uploaded into IPA for functional analysis. Selected biological functions were analyzed, and the results are presented as heat maps. Yellow indicates upregulated genes; blue indicates downregulated genes. WT, wild type.

VN/1203-infected than 1918-infected mice, and staining was more uniform in bronchiolar epithelium than in alveolar cells (Fig. 7E and F).

To determine whether we could recapitulate our results using a simpler system, we performed *in vitro* 1918 and VN/1203 infection experiments using MEFs from wild-type and IFNR1$^{-/-}$ mice. Cells were harvested at 8 and 24 h p.i. for microarray analysis. Our analyses showed that wild-type and IFNR1$^{-/-}$ MEFs both exhibited a type I IFN response to VN/1203 infection, whereas the 1918 virus elicited weak acti-

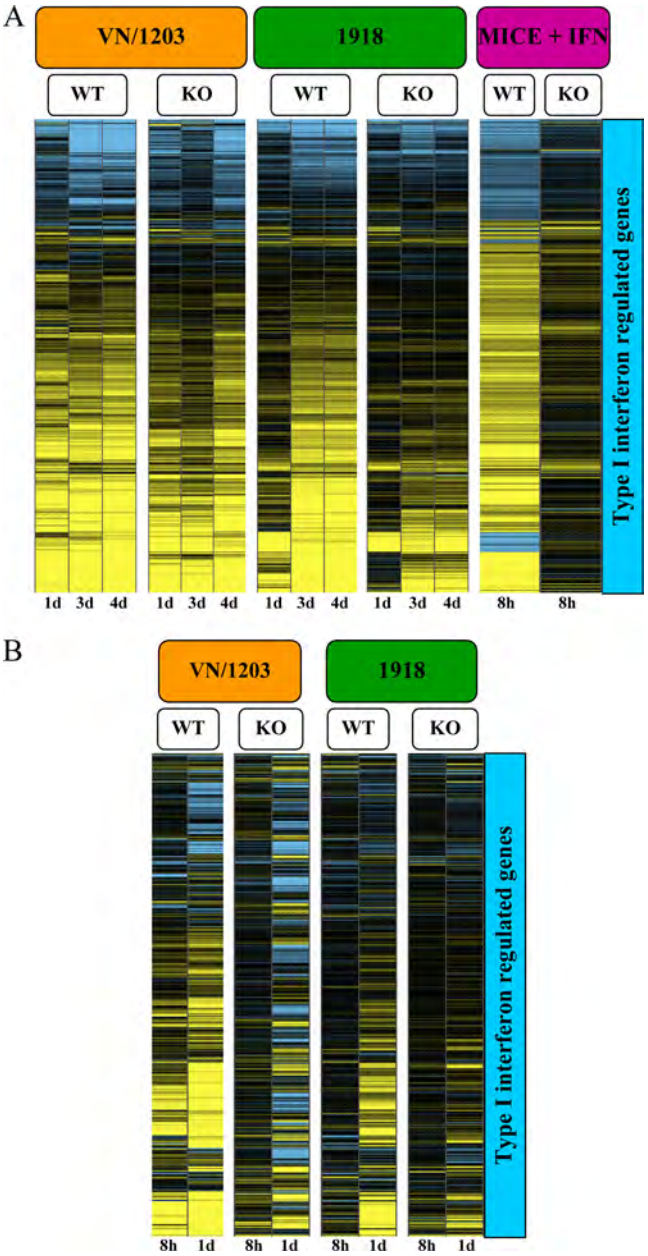

FIG. 6. Expression of interferon-regulated genes in response to interferon treatment or VN/1203 or 1918 infection. (A) Expression of type I interferon response genes in wild-type or IFNR1$^{-/-}$ mice infected with 1918 and VN/1203 viruses. Expression was determined by ANOVA of microarray data using cutoff values of ≥2-fold change and ANOVA $P \leq 0.01$, respectively, from mock-infected wild-type mouse lung samples treated with interferon or untreated. This analysis generated a subset of 854 genes (right) that were used to cluster the mouse data. KO, knockout. (B) Expression of type I interferon response genes in MEFs infected with 1918 and VN/1203 viruses. Expression was determined by ANOVA analysis using cutoff values of ≥2-fold change and ANOVA $P \leq 0.01$, respectively.

vation of IFN-regulated genes. Interestingly, 1918 infection of MEFs recapitulated the delayed activation of the type I IFN response that was observed in mouse lungs (Fig. 6B). These results clearly indicate a significant difference in the host responses to the H1N1 1918 virus and the avian H5N1 VN/1203 virus and correlate well with our mouse microarray data. The combination of stronger inflammatory and type I IFN responses induced by VN/1203 virus likely contributes to the increased pathogenesis of this virus in the mouse model.

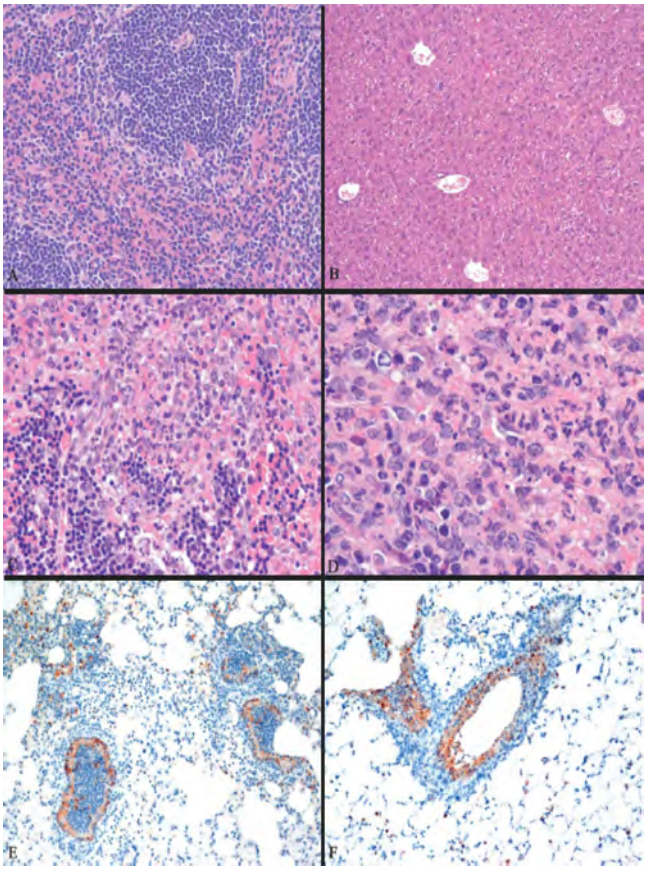

FIG. 7. Histopathology analysis of 1918 and VN/1203 infections in IFNR1$^{-/-}$ mice. (A) Normal spleen of an IFNR1$^{-/-}$ mouse infected by 1918 virus at day 1 p.i. (B) Normal liver of an IFNR1$^{-/-}$ mouse infected by 1918 virus at day 4 p.i. (C) Apoptotic spleen of an IFNR1$^{-/-}$ mouse infected by VN/1203 virus at day 1 p.i. (D) Lymphocyte depletion in the periarteriolar lymphatic sheath with heterophilic splenitis of an IFNR1$^{-/-}$ mouse infected by VN/1203 virus at day 1 p.i. (E) Common viral staining in bronchus of an IFNR1$^{-/-}$ mouse infected by 1918 virus at day 3 p.i. (F) Intense antigen staining in the bronchus of an IFNR1$^{-/-}$ mouse infected by VN/1203 virus at day 3 p.i.

DISCUSSION

In the present study, we observed that the highly pathogenic H5N1 VN/1203 virus was able to disseminate to extrapulmonary organs regardless of the expression of the type I IFN receptor. However, whereas the 1918 virus did not disseminate in wild-type mice (Fig. 1B), dissemination to brain and spleen was observed in the absence of the type I IFN receptor (see Fig. S6B in the supplemental material), although no damage to these tissues was apparent. These results clearly indicate a role for the IFN response in the control of influenza infection, as has been previously described (11, 40). Moreover, systemic disease and tissue damage, as suggested by necrosis of spleen and liver tissue, was observed only in IFNR1$^{-/-}$ mice infected with VN/1203 (Fig. 7). However, IFN signaling was not suffi-

cient to prevent a lethal outcome, nor could it control the spread of VN/1203 to extrapulmonary organs (Fig. 1A and B). The observed dissemination to extrapulmonary organs could not be explained by differences in viral loads, since the viruses exhibited similar titers in the lungs at the measured time points (Fig. 1C; see Fig. S6C in the supplemental material).

VN/1203 induced an earlier and stronger host response. An additional distinction between the viruses used in this study was the time to death following infection. In wild-type and IFNR1$^{-/-}$ mice, VN/1203-infected animals died sooner than 1918-infected mice. In addition, IFNR1$^{-/-}$ animals infected with either VN/1203 or the 1918 virus died sooner than infected immunocompetent wild-type mice. Similar results have recently been reported by Szretter et al. during infection of wild-type and IFNR1$^{-/-}$ mice with A/Hong Kong/483/97, a highly pathogenic H5N1 influenza virus (40).

We used global gene expression profiling to determine whether differences in the host transcriptional response to infection would provide clues to differences in disease outcome. Because the mouse LD_{50} for VN/1203 was lower than that of the 1918 virus, we normalized the experimental design by using the LD_{50} of each virus to determine the inoculum. Although this resulted in infection with different amounts of virus, this approach allowed a more accurate comparison of the host response to these viruses, and viral titers were nearly equivalent at all measured time points. Mice infected with VN/1203 elicited a strong transcriptional activation of the innate response at 1 day p.i. that persisted throughout infection (Fig. 2). In contrast, the host transcriptional response was delayed in 1918 virus-infected mice. Extensive functional-genomics analysis using Ingenuity Pathways Analysis showed that the inflammatory response was one of the most significant functional categories differentially regulated by the VN/1203 and 1918 viruses (Fig. 3A). In particular, we found that at day 1 p.i., the VN/1203 virus upregulated genes associated with the inflammatory response, including the inflammasome components NLRP3, IL-1β, and CASP1, whose products are important for the innate immune response to influenza virus infection (Fig. 3B) (3, 13, 41).

Our data indicate that as early as day 1 p.i., VN/1203 is able to exacerbate the inflammatory response and trigger the type I IFN response. These responses are sustained throughout infection, which likely favors a "cytokine storm" response that is detrimental to the host (30). In-depth analysis of the transcriptional profiles suggested that the delayed host response during 1918 infection (Fig. 2) was a consequence of the inability of the host immune response to activate the transcription of critical cell signaling pathways involved in the classical response to viral pathogens (Fig. 4). For example, the transcriptional activation of the pattern recognition receptors (PRRs) toll-like receptors (TLRs) and RIG-I helicase were almost nonexistent at 1 day p.i. Furthermore, the activation of genes encoding proteins that modulate the immune response, such as chemokines and NF-κB, was also delayed. The activation of the effector components PKR, inflammatory response, and neutrophil activation and infiltration were also unchanged. There was activation of these cell signaling categories by 3 and 4 days p.i., although to a lesser extent than during VN/1203 infection.

Dissemination of VN/1203 in wild-type mice correlates with sustained activation of the inflammatory response and altered hematological function and lipoxin signaling. Our results indicated that VN/1203 infection induced strong activation of the inflammatory response (Fig. 3A), including inflammasome genes, at day 1 p.i. (Fig. 3B). Virus dissemination and an intense inflammatory response have also been reported as features of H5N1 pathogenesis in humans (9). The physiological consequence of acute inflammation is the transvasation of immune cells from the blood to the infected tissues and the production of chemokines, cytokines, and vasoactive amines. Activated neutrophils release reactive oxygen and nitrogen species that act on pathogen and host targets in an indiscriminate manner (25).

We used a bioinformatics approach to attempt to gain insight into why VN/1203 was able to disseminate during infection of wild-type mice. This analysis revealed two biological categories associated with dissemination. The first category was the differential regulation of hematological system development and function (Fig. 5A), which was steadily activated at all time points analyzed. These same genes were activated only later and to a lesser extent during 1918 virus infection. The second identified category was the inhibitory effect of lipoxin signaling (Fig. 5B). These results depict a scenario where VN/1203 elicits a strong and steady upregulation of genes involved in the hematological function, a critical physiological response to acute inflammation, to trigger the resolution and healing processes at the sites of inflammation. Recently, it has been reported that people with severe cases of human swine H1N1 influenza infection are at risk to develop blood clots in the lungs (1), suggesting that dysregulation of the hematological system is likely important for the severity of disease.

Using an independent analytical tool for functional analysis (GeneGo), we also found a small set of genes related to hematological function whose expression was differentially regulated by the two viruses. These genes include fibrinogen α, β, and γ (FGA, FGB, and FGG); thrombomodulin (F2); and coagulation factor 3 (F3), which are components of the blood coagulation pathway (data not shown). Interestingly, it has been reported that disseminated intravascular coagulation generated by strong activation of the coagulation system that results in microvascular thrombosis (24) may be a contributing factor for multisystem organ failure during Ebola virus infection (12, 36). These results may suggest that the robust expression of hematological-function genes related to proinflammatory responses and blood coagulation system genes may contribute to VN/1203 virus dissemination.

In addition, we found that loss of the inhibitory action of lipoxin on neutrophil function was also associated with dissemination of VN/1203 in wild-type mice. Lipoxins play a role in the modulation of immune responses during microbial infection (21) and have potent anti-inflammatory properties in diseases such as tuberculosis, toxoplasmosis, arthritis, and cystic fibrosis (2, 5, 15, 21, 39). Studies of *Toxoplasma gondii* and *Mycobacterium tuberculosis* infection indicate that lipoxin-dependent inhibition of proinflammatory type 1 responses favor transmission and propagation of these pathogens (21). In particular, lipoxins inhibit important defense mechanisms, such as leukocyte migration, translocation of NF-κB, leukotriene function, TNF-induced chemokine production, natural killer cell function, and expression of chemokine receptor and adhesion molecules. The differential transcriptional regulation of lipoxin signaling and its inhibitory

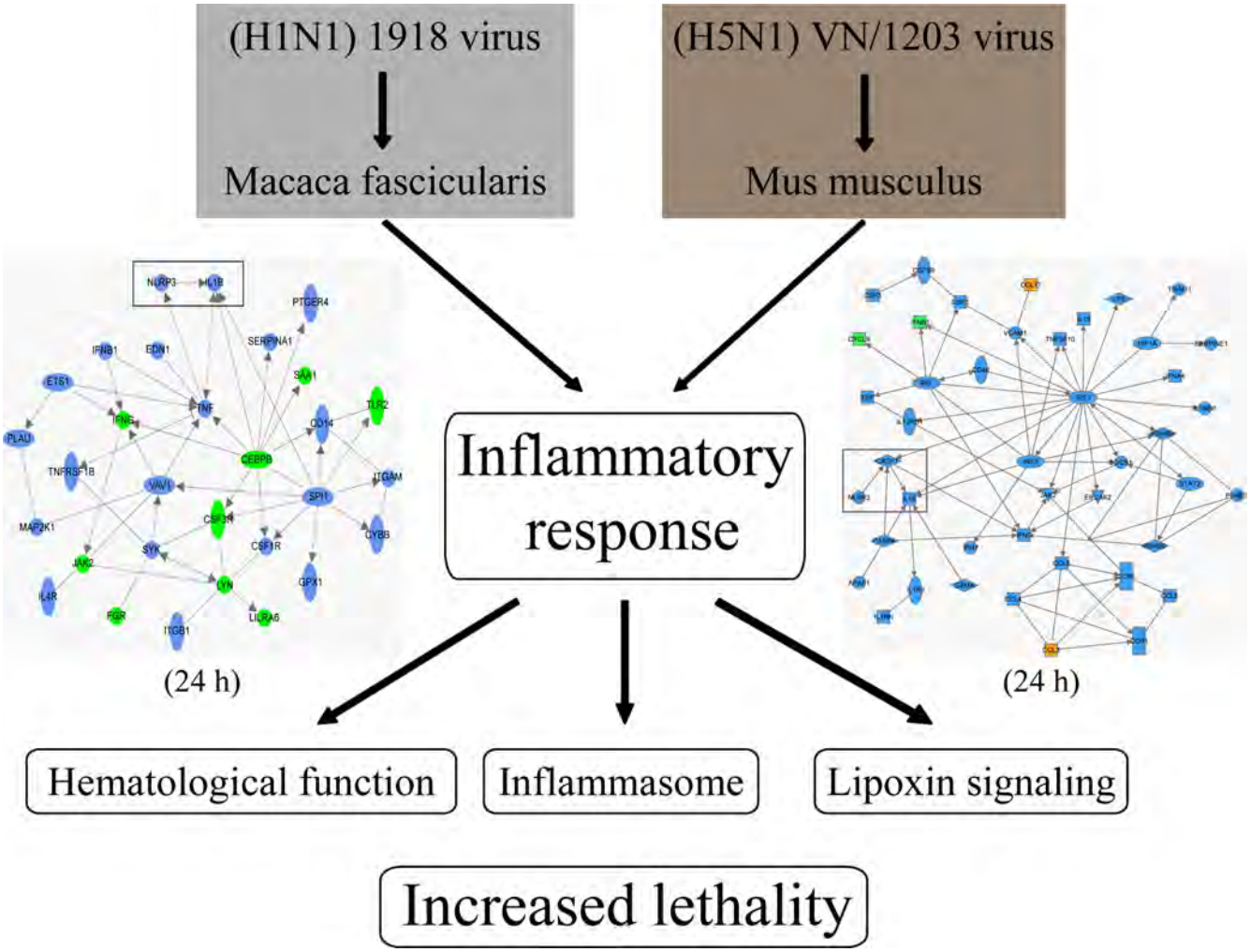

FIG. 8. Model illustrating the consolidation of data obtained from macaques and mice infected with highly pathogenic 1918 and VN/1203 viruses. Provided is an illustration depicting our current understanding of influenza virus infections in macaques and mice. The network diagrams for macaques (8) and mice (Fig. 3B) emphasize the differential regulation of the inflammatory response and activation of inflammasome components that was found during 1918 and VN/1203 virus infections, respectively.

action on neutrophil functions may be important factors in differences in VN/1203 and 1918 virus pathogenesis.

Another avenue that we investigated was the detected dissemination of the 1918 virus in IFNR1$^{-/-}$ animals. Our data indicate that infection of wild-type mice with the 1918 virus resulted in the activation of important host functions, such as the inflammatory response, immune cell trafficking, and cell death, which at day 1 p.i. were at levels equivalent to those of the response elicited in IFNR1$^{-/-}$ mice. However, while wild-type animals produced a robust and steady increase in the activation of genes associated with these functions at days 3 and 4 p.i., this response was weak in IFNR1$^{-/-}$ animals (see Fig. S5 in the supplemental material). These results suggest that an intact immune response is necessary to control dissemination of the 1918 virus. However, this response must be controlled, since the exacerbated inflammatory response elicited by VN/1203 was also correlated with virus dissemination.

VN/1203 and the 1918 virus induce the activation of type I IFN-regulated genes even in the absence of the IFN-α/β receptor. Gene expression data from 1918 and VN/1203 infections indicated that both viruses induced the activation of type I IFN-regulated genes in wild-type mice. Interestingly, the absence of IFNR1 did not completely abrogate the expression of type I IFN-related genes in response to either VN/1203 or the 1918 virus, and VN/1203 in particular elicited a strong IFN response (Fig. 6A). Our results suggest that the response to VN/1203 infection may be due in part to differences in the activation of TLRs and NF-κB signaling cascades. Recently, it has been shown that IFN-λ is important for controlling influenza replication in mice (4, 26). Therefore, a formal possibility in our experiments was that some of the observed transcriptional activation changes were due in part to the compensatory effects of IFN-λ expression. However, our quantitative RT-PCR results suggest that this was not the case (see Fig. S4C in the supplemental material).

The mouse model is a complex system in which cell infiltration is a landmark of the host response. Using MEFs, a system in which there is no cell infiltration, we were able to recapit-

ulate our *in vivo* results. MEFs also exhibited a delay in the transcriptional activation of the innate response to 1918 infection relative to that elicited by VN/1203 (Fig. 6B). These results suggest that in mice or in mouse-derived cells, VN/1203 elicits a stronger IFN response that correlates with disease outcome. These data illustrate the complex and delicate homeostatic balance of the host response against highly pathogenic influenza infections.

Taken together, our results suggest that VN/1203-infected mice likely died faster as a consequence of several factors: (i) the early and sustained induction of an inflammatory response that may be detrimental rather than protective to the host; (ii) the additive and/or synergistic effects of the virus-sensing (TLRs and RIG-I), modulator (chemokines, NF-κB, and IFN), and effector (PKR, neutrophil infiltration, and inflammation) components of the immune response, which were transcriptionally induced in a steady manner throughout infection; (iii) the inhibition of lipoxin-mediated anti-inflammatory responses; and (iv) the ability of VN/1203 to disseminate to extrapulmonary organs.

Interestingly, our findings using the mouse model show intriguing differences and similarities compared with our findings using a cynomolgus macaque model of influenza virus infection (8). We previously reported that all 1918 virus-infected macaques succumbed to infection, whereas, despite the fact that there were initial signs of tissue damage, all VN/1203-infected macaques recovered. Interestingly, in macaques, it was the 1918 virus that upregulated key inflammasome components, whereas these genes were downregulated during VN/1203 infection. Consolidation of our data from macaque (8) and mouse infection studies suggests that the early and sustained upregulation of the inflammatory response is detrimental to the host (Fig. 8). It is also worth noting that in both animal models upregulation of the inflammatory response took place at 24 h p.i. (in macaques by the 1918 virus and in mice by VN/1203). It is also possible that differences in cellular microRNA expression between mouse and macaque may affect disease outcome, as we have recently reported that cellular microRNAs influence the inflammatory response to influenza virus infection (19). Together, our findings highlight the importance of the inflammatory response and suggest new lines of experimentation, including the infection of inflammasome knockout mice and specific targeting of the Alox5 gene, responsible for lipoxin biogenesis. The inflammatory response is also an attractive target for drug intervention, and the development of new drugs that target the inflammatory response may prevent severe tissue damage.

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AVIAN DISEASES 54:522–526, 2010

Review Article—

The High Susceptibility of Turkeys to Influenza Viruses of Different Origins Implies Their Importance as Potential Intermediate Hosts

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SUMMARY. Several previous reports and our studies show that waterfowl-origin influenza viruses can be more easily transmitted to domestic turkeys than chickens. Similarly, studies indicate turkeys to be better hosts for low pathogenic avian influenza viruses isolated from commercial poultry operations and live bird markets in comparison to chickens. Low 50% infectious-dose titers of wild bird as well as poultry-adapted viruses for turkeys further suggest that turkeys can be easily infected following a low-dose exposure. Also, interspecies transmission of swine influenza viruses to turkeys occurs frequently. These findings suggest the role of turkeys as suitable intermediate hosts that can be easily infected with influenza viruses of different origins and that turkeys can act as source of infection for other land-based poultry or even mammals.

RESUMEN. *Estudio Recapitulativo*—La alta susceptibilidad de los pavos al virus de la influenza de diferentes orígenes implica su importancia como posibles huéspedes intermediarios.

Varios reportes previos y los estudios de nuestro laboratorio muestran que los virus de la influenza originados de aves acuáticas puede ser más fácilmente transmitidos a los pavos domésticos que a los pollos. De la misma manera, los estudios indican que los pavos son mejores hospederos que los pollos para virus de la influenza aviar de baja patogenicidad aislados de operaciones avícolas comerciales y de mercados de aves vivas. Títulos bajos de dosis infecciosas 50% de virus con origen en aves silvestres así como virus adaptados en pavos, sugieren que los pavos pueden ser fácilmente infectados después de una exposición con dosis virales bajas. Además, ocurre con frecuencia la transmisión entre especies por el virus de la influenza porcina a los pavos. Estos hallazgos sugieren el papel de los pavos como huéspedes intermediarios que pueden ser fácilmente infectados por virus de la influenza de diferentes orígenes y que los pavos pueden actuar como fuente de infección para otras especies aviares comerciales, o incluso mamíferos.

Key words: turkeys, influenza, interspecies transmission

Abbreviations: EID_{50} = egg infectious dose; HI = hemagglutination inhibition; Gal = galactose; LBM = live bird market; LPAI = low pathogenic avian influenza; MAA = *Maackia amurensis* agglutinin; RRT-PCR = reverse transcriptase–polymerase chain reaction; SA = sialic acid; SNA = *Sambucus nigra* agglutinin; TR = triple-reassortant

Wild waterfowl, gulls, and shorebirds are believed to be the natural hosts and reservoirs of influenza A viruses (53). Influenza viruses in all other species of mammals and birds are derived from the wild bird reservoirs, in which all the 16 hemagglutinin and 9 neuraminidase subtypes have been found (11,60). In these reservoirs, influenza viruses are considered to be in a state of evolutionary stasis and infections are usually asymptomatic. Though they are not natural hosts, different species of domestic poultry have been found to be infected with influenza viruses as a result of exposure to wild bird reservoirs or contaminated environment from these reservoirs. In most cases, low pathogenic influenza infections in domestic poultry result in mild respiratory disease, reduction in egg production, and occasionally increased mortality (9). Surveillance studies indicate that low pathogenic influenza infections are widespread among commercial poultry. Among domestic bird species, infections have been most commonly reported from Galliformes and Anseriformes, which include chickens, turkeys, quails, guineafowl, pheasants, patridges, ducks, and geese. Psittaciformes (parrots, cockatoos, parakeets), Casuariiformes (emu), Struthioniformes (ostrich), and Rheiformes (rhea) have been found to be susceptible under natural conditions. Infections of some free-living birds like starlings and sparrows have been found following their close association with infected birds in poultry farms (9,53). Experimental infections also indicate the susceptibility of different domestic bird species, including chickens, ducks, turkeys, quails, pigeons, and pheasants to influenza viral infections from different sources (23,27,32,37,38,45,46,48,49). In addition to lowered production, increased mortality, and accompanying economic losses, major concerns with infection of poultry with low pathogenic influenza viruses are the ability of these species to act as bridging hosts for influenza infections from wild bird reservoirs to other land-based poultry and mammals including humans. Also, there is threat of these viruses mutating to the highly pathogenic forms after many replication cycles in poultry (37,60). Among the different domestic birds that can be infected with influenza viruses, chickens and turkeys have gained importance in the United States, considering their economic and public health significance.

Turkeys are susceptible to many wild aquatic bird influenza viruses. It has been documented that multiple subtypes of influenza viruses can establish stable lineages in domestic poultry (29,50,59). Though the source of influenza infections for domestic bird species are believed to be wild bird reservoirs, studies indicate that domestic

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poultry can be infected with influenza viruses from different sources, for example, swine influenza infections of turkeys. Avian influenza infections have been recorded from turkeys worldwide (1,2,3,7,33,55). Similarly, multiple subtypes of influenza viruses have been isolated from healthy and diseased chickens; however, it is not known whether the influenza virus was transmitted directly to chickens from an aquatic bird, or if the virus acquired expanded host-range capabilities by replication in other avian species, prior to infection of chickens. Several observations show that waterfowl origin influenza virus can be more easily transmitted to domestic turkeys than chickens. In the United States, since the mid-1960s, influenza virus infections have been seen consistently in turkeys in California and Minnesota, where turkeys were range reared and farms were heavily concentrated and situated on migratory waterfowl flyways (20). Campitelli *et al.* identified for the first time that the H7N3 viruses from turkeys in Italy were derived directly from influenza strains circulating in wild waterfowl (7). Several previous studies and field data document that turkeys may be more susceptible to wild bird influenza viruses than chickens (19,48,54). Studies on replication and intraspecies transmission characteristics of eight low pathogenic H5 subtype wild bird isolates (one from mute swan, four from mallards, and four from ruddy turnstones) in chickens and turkeys in our lab also indicated the higher susceptibility of turkeys to mallard and ruddy turnstone viruses in comparison to chickens (unpublished data).

Studies on the minimum infectious dose of wild bird viruses for different domestic bird species are limited. Determination of 50% infectious dose titers of wild bird viruses of H5 subtype indicated >1000 times higher dose for infection of chickens in comparison to turkeys (48,54, unpubl. data). The mean 50% infectious dose titers for different wild bird isolates for chickens were in the range of $10^{6.3}$–$10^{8.3}$ 50% egg infectious dose (EID_{50}), whereas for turkeys, the range was from $10^{2.5}$ to $10^{4.2}$ EID_{50}. Although data from other subtypes are not available, the low minimum infectious dose suggests that turkeys can be easily infected following a low dose exposure and confirms the higher susceptibility of turkeys to influenza viruses of wild bird origin, especially of the H5 subtype. Their high susceptibility also indicates that turkeys could be good sentinel hosts in influenza surveillance studies and/or better model hosts for studies on adaptation of wild bird origin influenza viruses for domestic poultry. Once domestic birds are infected, influenza viruses in them undergo rapid evolution (17,66). It is possible that wild bird viruses circulating in turkeys could undergo favorable changes that permit their adaptation to chickens and other domestic poultry. Hence, the potential role of turkeys as bridging hosts for introduction of low pathogenic waterfowl-origin influenza viruses into other domestic poultry cannot be ruled out. Although confinement rearing has become the norm in the United States and other developed countries, turkeys could be significant players in supporting and transmitting influenza infections to other land-based poultry where wild birds have free access to domestic poultry.

Influenza viruses isolated from domestic birds can replicate and transmit among turkeys. Though detailed experimental studies on the replication and transmission characteristics of domestic bird-origin influenza viruses in turkeys are lacking, available evidence indicates susceptibility of turkeys to low pathogenic avian influenza (LPAI) viruses isolated from commercial poultry operations and live bird markets (26,54,56). Among commercial poultry operations, more LPAI outbreaks have been reported in turkeys in comparison to chickens (53). Experimental studies with a LPAI H7N2 isolate that caused an outbreak among commercial poultry primarily infecting turkeys indicated a lower 50% infectious dose titer for turkeys than chickens. Also, 20–158-fold higher infectious virus was recovered from turkeys in comparison to chickens (56). A similar outbreak with H7 subtype viruses in poultry in Italy in 1999 primarily affected turkey flocks (64). The high susceptibility of turkeys might be explained by the low minimum infectious dose (about 10-fold less viral load for infection of turkeys in comparison to chickens) required for infection. Our studies using a highly poultry-adapted strain of LPAI, A/parrot/California/04, showed a lower 50% infectious dose titer of $10^{1.4}$ 50% EID_{50} /0.2 ml for turkeys in comparison to a $10^{2.6}$ EID_{50}/0.2-ml dose for chickens. However, it should be noted that minimum infectious dose required may vary with virus strains as well as the genetic constitution of the birds used in the studies. Hence, caution should be undertaken when generalizing the results from studies using few isolates on a particular turkey breed. Also, under field conditions, where secondary infections or other factors can reduce immune responses, susceptibility and clinical disease may be exacerbated. The higher susceptibility in an outbreak area may also be an indication of the higher population of turkey flocks in the outbreak area. In spite of the limitations of experimental infection studies, they are important to understand the biological properties of viruses and host ecologies. In addition to wild bird isolates as previously described, 12 low pathogenic influenza virus isolates (which includes duck, chicken, pheasant, and emu isolates) from live bird markets (LBMs) as well as chicken and turkey isolates from commercial poultry operations also revealed turkeys to be highly susceptible hosts for influenza viruses of different degrees of adaptation for domestic poultry (unpubl. data). These viruses were inoculated at a dose of $10^{6.0}$ EID_{50}/0.2 ml through choanal route to chickens and turkeys. Influenza isolates from LBMs are believed to be highly poultry adapted, as they provide favorable environment for adaptation of influenza viruses for a variety of hosts, including chickens, turkeys, pheasants, quails, and ducks, and have been implicated as the source of influenza outbreaks in commercial poultry operations as well as in humans. LBMs in New York and New Jersey have been implicated as significant sources of influenza viruses for commercial chicken and turkey operations in the United States (36,42). The high susceptibility of turkeys to domestic bird isolates again underscores their importance as potential bridging hosts for influenza viruses from different origins and domestic poultry.

Interspecies transmission of swine influenza viruses to turkeys: a frequent event. Currently three subtypes of influenza viruses are commonly found in pigs worldwide, H1N1, H1N2, and H3N2 (5,40). These viruses were found to be derived either from mammalian or avian viruses or their reassortants (6,8). H1N1 influenza viruses were first isolated from pigs in the United States in 1930 (43), and until the late 1990s, classical H1N1 lineage was the predominant influenza subtype in pigs in the United States (34). The first reported isolation of swine influenza viruses in turkeys was in 1980–81 (22). Since then, transmission of swine H1N1 influenza viruses to turkeys has been documented several times (4,10,28,30,61) and in the majority of the cases, the turkey flocks were housed in close proximity to swine herds (51). In 1998, triple-reassortant (TR) influenza viruses containing gene segments derived from recent human (HA, NA, and PB1), swine (NS, NP, and M), and avian (PB2, PA) viruses were first isolated from pigs and since then, have spread over much of the U.S. swine population (58,65). Since 2003, the swine-origin TR H3N2 viruses have been isolated from the U.S. turkey population (8,55). The early reports of swine TR H3N2 viruses crossing the species barrier to infect turkeys were from farms in Minnesota and North Carolina in 2003 (8). Later, the TR H3N2 viruses have been isolated from turkey flocks in different

parts of the United States (55,63). In 2002, Suarez *et al.* reported the isolation of TR H1N2 influenza virus with gene segments derived from swine, human, and avian lineages from turkey breeder hens associated with sudden drops in egg production (51). This virus was also believed to have a swine source of infection and provides additional evidence of cross-species transmission of swine influenza viruses to turkeys. Among the different domestic bird species that are susceptible to swine influenza viruses, including chickens, more field cases have been reported with turkeys, indicating that turkeys may be naturally more susceptible than chickens and other domestic poultry (41,42). Experimental infections also supported the findings that turkeys are more susceptible to influenza infections than chickens. The ability of the turkey TRs to replicate in quail, but not the swine TRs, also indicate the potential role of turkeys as bridging hosts for influenza viruses to other land-based poultry, mammals, or even humans (8). The ability of these swine-origin viruses to infect turkeys is not limited to birds of the younger age group that are usually used for experimental infection studies. Several previous reports document drops in egg production in turkeys due to H1N1 (30,44), reassortant H1N2 (51), and H3N2 viruses (8,35,39,55). Hence, along with the concern of introducing and establishing new influenza lineages in turkeys, interspecies transmission of swine influenza viruses to turkeys can be economically significant. The strong staining for avian receptors and high viral titers in the oviduct and drastic declines in egg production following infection are indicators that influenza viral infections alone without any concurrent infections can result in economic losses under field conditions (39). A recent study reported that turkey embryos are susceptible to swine TR H3N2 influenza viruses, and continuous passage of the swine viruses have been found to result in mutations in HA similar to those found in turkey TR H3N2 viruses (47). However, although embryonated turkey eggs may serve as inexpensive tools to study the adaptation and interspecies transmission of swine influenza viruses, turkey embryos may not mimic the biology of a bird, especially in terms of immune responses and several issues including the reproducibility of the HA mutations with other swine viruses, and the changes in other gene segments should be considered in the study.

The receptor profile in turkeys supports their role as intermediate hosts. Different sialic acid types and linkages in different species of birds and mammals are believed to be barriers for efficient interspecies transmission of influenza viruses. Influenza virus receptors on host cells are believed to be terminal sialic acid (SA) residues that are bound to glycans through an α2,3 or α2,6 linkage, mediated by sialyltransferases that are expressed in a cell- and species-specific manner (12). It is believed that duck intestinal epithelial cells express α2,3SA-galactose (gal) receptors, whereas tracheal epithelial cells of humans mainly express α2,6SA-gal receptors. Avian viruses are believed to bind α2,3SA-gal linked receptors preferentially, and influenza viruses from humans preferentially recognize human-type receptors (31). Apart from the SA-gal linkages to penultimate sugars, the binding of viruses can be affected by structure of more distant parts of the oligosaccharide chain (13,21,52).

Plant lectins, *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA), that are specific to α2,3SA-gal and α2,6SA-gal terminated sugars are usually used to identify the type of receptors present in different tissue sections. Epithelial cells from trachea and intestines of chickens and quail have been found to have moieties that can bind SNA and MAA and to bind viruses showing human- and avian-type receptor specificity (15,18,57). Our studies using MAA and SNA on turkey tracheal epithelium indicated that turkeys also possess α2,3SA-gal– and α2,6SA-gal–terminated sialyloligosaccharide residues with 80%–90% and 70% of the tracheal epithelial cells, respectively, showing positive staining. The colon sections showed only the presence of α2,3SA-gal receptors (unpubl. data). Previous lectin studies have demonstrated that turkey ovaries contain both α2,3SA-gal and α2,6SA-gal receptors and support our results on the presence of both types of receptors on turkey tissues (25). The presence of avian- and human-type receptors in turkeys explains their higher susceptibility to wild and domestic bird origin and swine viruses. Although different viral and host factors may play roles in successful viral replication, adaptation, and transmission, these findings strengthen the argument that turkeys can be infected with influenza viruses containing mammalian hemagglutinin gene segments and can act as potential intermediate hosts for interspecies transmission and spread of reassortant viruses between birds and humans.

The actual viral binding to the receptors observed have been found to be affected by different factors other than receptor specificity. Avian influenza viruses have been found to bind more strongly to Neu5Ac receptors than to Neu5Gc-containing receptors (21,24,31). Also, duck viruses have been found to bind with greater affinity to Neu5Ac(α2-3)Gal(β1-3)GalNAc-containing receptors than Neu5Ac(α2-3)Gal(β1-4)GlcNAc-containing receptors, indicating that distant parts of the oligosaccharide chains affect binding of the duck viruses (14). Viruses from gulls and shore birds preferentially bind to Neu2Acα2-3gal receptors similar to ducks (16,62). However, H13 viruses were found to bind to 2-3 sialic acid linkages weakly and not to discriminate between β1-3 and β1-4 linkages (16). The length of the gangliosides was also found to have an effect on viral binding. Duck viruses bound to gangliosides with short sugar chains that were found to be abundant in duck intestines, whereas chicken viruses bound more strongly to gangliosides with long sugar chains that were abundant in chicken intestinal tissues (15). As expected, in accordance with the detection of SAα2,3-gal and SAα2,6-gal receptors, epithelial cells from quail and chicken intestines were found to bind both avian- and human-type viruses (18). Similar studies on epithelial cells from trachea and intestines of turkeys are necessary to confirm that they play important roles as intermediate hosts where influenza viruses of avian and human origin can be amplified and spread to susceptible hosts.

CONCLUDING REMARKS

The inherent susceptibility of turkeys to wild bird, domestic bird, and swine origin low-pathogenic influenza viruses signify the important role of turkeys as the domestic bird species that could act as the potential entry points and the bridging species for influenza viral entry into agricultural and commercial poultry systems. Also, the fact that turkeys are easily infected with aquatic bird and swine viruses and the ability of these viruses to reassort increases the probability that further reassortments and evolution of these viruses can take place in these turkey hosts.

Mixing of poultry and outdoor rearing could favor adaptation of low-pathogenic influenza viruses from different sources for domestic poultry and pose serious health risks for birds and other mammals. In the United States alone, turkey consumption has increased more than 100% since 1970, and turkey meat is one of most popular protein choices for consumers. Thus, it is important to control and prevent influenza infection in turkeys, not only to prevent transmission, but also to control the burden of disease in turkeys, to maintain wholesome poultry and poultry product markets.

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Cell Surface Display of Highly Pathogenic Avian Influenza Virus Hemagglutinin on the Surface of *Pichia pastoris* Cells Using α-Agglutinin for Production of Oral Vaccines

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Yeast is an ideal organism to express viral antigens because yeast glycosylate proteins more similarly to mammals than bacteria. Expression of proteins in yeast is relatively fast and inexpensive. In addition to the convenience of production, for purposes of vaccination, yeast has been shown to have natural adjuvant activity making the expressed proteins more immunogenic when administered along with yeast cell wall components. Development of genetic systems to display foreign proteins on the surface of yeast via fusion to glycosylphosphatidylinositol-anchored (GPI) proteins has further simplified the purification of recombinant proteins by not requiring harsh treatments for cellular lysis or protein purification. We have expressed the hemagglutinin protein from a highly pathogenic avian influenza (HPAI) virus [A/Egret/HK/757.2/02], subtype H5N1, on the surface of the yeast strain Pichia pastoris*, as an anchored C-terminal fusion with the* Saccharomyces cerevisiae *GPI-anchored cell wall protein, α-agglutinin. Surface expression of the hemagglutinin fusion protein was demonstrated by immunofluorescence microscopy. Functionally, the fusion protein retained hemagglutinin agglutinating activity, and oral vaccination with the yeast resulted in production of virus neutralizing antibodies. This study represents the first steps in the generation of a yeast-based vaccine for protection against highly pathogenic strains of avian influenza.* Published 2009 American Institute of Chemical Engineers† *Biotechnol. Prog.,* 26: 542–547, 2010
Keywords: Pichia pastoris, cell surface display, hemagglutinin, α-agglutinin, H5N1, avian influenza

Introduction

Highly pathogenic avian influenza (HPAI) infections result in devastating poultry loses around the world. Traditional vaccination methods for HPAI viruses require costly and time-consuming injection of individual birds, often multiple times, in order to provide adequate protection. Furthermore, methods to develop and manufacture these vaccines are time consuming and somewhat limited in the ability to generate vaccines quickly in response to the constant evolution of new influenza field strains. The use of the eukaryotic yeast as an expression system for influenza proteins can remedy these problems. As an eukaryotic organism yeast glycosylates proteins more similarly to that of mammalian proteins than those expressed in bacteria. This is an important criterion when developing vaccines because glycan moieties have been demonstrated to be important in the generation of neutralizing antibodies.[1-3] Expression of proteins in yeast is fast, inexpensive, and yeast have the added benefit of providing high-quality nutrition when given in the feed. Yeast are generally regarded as safe and benign organisms and would therefore allow long-term feeding of yeast expressing recombinant proteins in order to maintain immunity over time with limited side effects. In addition to the convenience of production, components of the yeast cell wall have been shown to contain natural adjuvant activity. Therefore, the expressed recombinant proteins are more immunogenic when administered with the yeast cell wall components.[4,5]

Previously, researchers developed several different yeast expression systems utilizing the covalently linked GPI-anchored proteins as the basis for displaying foreign proteins on the yeast cell surface.[6] One of the widely utilized GPI-anchored proteins is the *Saccharomyces cerevisiae* protein, α-agglutinin (*AG*α1). The C-terminus of α-agglutinin has been successfully used to display several foreign proteins such as α-amylase and red sea bream iridovirus 380R antigen on surface of *S. cerevisiae*.[7,8] More recently, the use of the methylotrophic yeast *Pichia pastoris* has been used to display enhanced green fluorescence protein (EGFP) and *K. lactis* yellow enzyme (KYE) using the α-agglutinin C-terminal anchoring method. Methanol-inducible *P. pastoris* expression vectors are also commercially available.[9,10]

This study describes the creation and characterization of a *P. pastoris* strain expressing the hemagglutinin antigen of a (H5N1) HPAI virus A/Egret/HK/757.2/02 as a potential oral vaccine for poultry. To accomplish this, the hemagglutinin antigen was fused to the C-terminus of the *S. cerevisiae* GPI-protein, α-agglutinin. Immunofluorescence microscopy confirmed the display of hemagglutinin fusion protein on the

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surface of *P. pastoris* cells and the hemagglutinin fusion protein retained its hemagglutination (HA) activity. Oral vaccination of chickens with the H5-fusion-expressing yeast resulted in production of virus neutralizing antibodies.

Materials and Methods

Plasmids, strains, and media

Competent DH5α *Escherichia coli* cells were purchased from Zymo Research Corp (Orange, CA) and used for all standard recombinant DNA manipulations and were grown at 37°C in LB medium containing 100 μg/mL of ampicillin for selection according to standards methods.[11] The wild-type *S. cerevisiae* strain was a kind gift from Dr. Judith Fridovich-Keil, Emory University, Atlanta, GA. *Pichia pastoris* strain GS115, and *P. pastoris* expression plasmid pPIC9K, for secreted expression, were purchased from Invitrogen (Carlsbad, CA). Yeast strains were propagated in yeast extract peptone dextrose (YEPD) medium (US Biologicals, Swampscott, MA). Minimal glycerol medium and minimal methanol medium were prepared as recommended by Invitrogen.

Genomic DNA isolation from yeast strains

All genomic DNA was isolated using YeaStar yeast genomic DNA kit (Zymo Research).

Construction of P. pastoris *cell surface display plasmids*

Nucleotides 970–1950 (including the stop codon) of the GPI-anchored protein *AG*α1 (α-agglutinin) were amplified using high-fidelity PCR polymerase (Invitrogen) from *S. cerevisiae* genomic DNA using primers: *AG*α1 EcoRI F 5′-gatgc gaattcGGTCGGAACCTCG-3′ and *AG*α1 AvrII R 5′-gcctcagtca cctaggTTAGAATAGCAGGTACGAC-3′. The 980 bp PCR product was digested with *Eco*RI and *Avr*II, gel purified using Gene Clean II kit (MP Biomedical LLC., Solon, OH), and cloned into the *Eco*RI and *Avr*II restriction endonuclease sites of pPIC9K expression vector (Invitrogen) generating a plasmid designated *AG*α1.pPIC9K.

The influenza hemagglutinin gene of the strain A/Egret/Hong Kong/757.2/02 (H5N1 subtype) was enzmatically amplified using high-fidelty PCR polymerase (Invitrogen) and primers H5 SnaBI F 5′-gactgc tacgtaGACCAAATTTG-CATTGGTTACC-3′ and H5 MfeI R 5′-cacgtca caattgTTG-GTAAGTTCCTATTGATTCCAA -3′ corresponding to nucleotides 49 and 1593, respectively. Recombinant plasmid rEgret HA.pHH21 plasmid containing the full H5 gene derived by reverse transcriptase PCR of viral RNA isolated from A/Egret/Hong Kong/757.2/02 virus was used as the template.[12] The 1.55 kb amplification product containing the H5 coding sequence minus the coding sequences for the cytoplasmic tail and transmembrane domain was digested with *Sna*BI and *Mfe*I restriction enzymes, gel purified using Gene Clean II kit (MP Biomedical LLC.), and ligated into the *AG*α1.pPIC9k vector previously digested with *Sna*BI and *Eco*RI restriction endonucleases. The resulting plasmid, designated H5.*AG*α1.pPIC9K, contained the H5, *AG*α1, and vector sequences all in frame (Figure 1). The nucleotide sequence, confirming the lack of mutations and continuity of the reading frame, was determined using an ABI Big Dye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) and a 3730 XL DNA Analyzer (Applied Biosystems) sequencer.

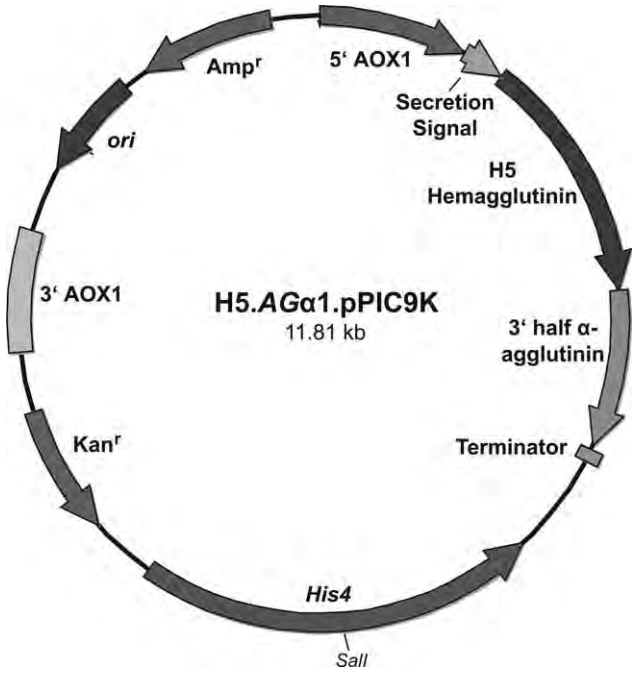

Figure 1. Recombinant *P. pastoris* expression vector for cell surface display of the influenza H5 antigen.

Plasmid H5.*AG*α1.pPIC9K contains influenza virus H5 antigen fused in frame with the 3′ terminus of α-agglutinin from *S. cerevisiae*, under the control of the AOX1 methanol-inducible promoter.

Yeast transformations and expression of fusion proteins

The recombinant plasmid, H5.*AG*α1.pPIC9K, and control plasmid, *AG*α1.pPIC9K, were digested with *Sal*I restriction endonuclease for 4 h at 37°C. The *P. pastoris* yeast strain GS115 (Invitrogen) was grown in YEPD medium at 30°C to an OD_{600} of 1.5. Cells were washed with sterile water, pretreated with 10 mM DTT, and electroporated as previously described using 40 ng of digested plasmid DNA.[13] The resulting auxotrophs (His+) were selected for on minimal dextrose medium lacking histidine. Further selection was obtained by plating His+ colonies onto YEPD medium containing up to 4 mg/mL G418 antibiotic (US Biologicals). This process was repeated to rule out false positive colonies. The location of the recombinant expression vector within the yeast genome was confirmed using PCR with genomic DNA isolated from His+, G418 resistant colonies and were tested for correct genomic DNA integration of the expression vectors by isolating the genomic DNA and performing PCR with the manufacturer's recommended primers (Invitrogen) confirming the correct size inserts were present.

H5.*AG*α1.pPIC9K and *AG*α1.pPIC9K integrated yeast were grown overnight in 100 mL minimal glycerol medium, resuspended in 400 mL buffered minimal methanol medium to an OD_{600} of 1, and grown at 27.5°C in a table top shaking incubator. Methanol was added every 24 h to maintain a 0.5% of methanol concentration. Cells were harvested after 46 h, and cell pellets were stored at −80°C until use.

Immunofluorescence labeling of recombinant yeast

Expression of the H5.*AG*α1 fusion protein on the cell wall of *P. pastoris* was investigated using immunofluorescence

microscopy. Cells were washed in water two times, applied to glass slides, and fixed with 100% acetone for 3 min. Fixed slides were a washed in 1× phosphate buffered saline (PBS) and then incubated with an in-house generated chicken anti-A/Turkey/Wisconsin/68 serum (H5N9) (Southeast Poultry Research Laboratory, Athens, GA) at a dilution of 1:300 for 1 h at room temperature. Slides were then washed three times with 1× PBS and incubated with mouse anti-chicken FITC-conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL) diluted 1:500 in 1× PBS for 45 min at room temperature before washing three times in 1× PBS. Immunofluorescence was detected using a Nikon E600 fluorescence microscope (Nikon Instruments Nipon, Japan).

Cell wall protein extractions

Proteins comprising the cell wall of *P. pastoris* were isolated as previously described[14] with the following modifications: cells were centrifuged and washed three times with water. Isolation buffer (10 mM Tris-HCl, pH 7.8, 1 mM PMSF) was added to the cells (10 mg wet cell weight) along with 100 μL of 0.5 mm glass beads (Research Products International, Mt. Prospect, IL) and vigorously shaken using a vortex mixer at high speed for 5 min at 4°C. The supernatant and two 1 mM NaCl-1mM PMSF washes were pooled and centrifuged at 1,000*g* for 5 min at 4°C. The pellets were then washed three times with 1 mM PMSF. Pellets were resuspended in 20 μL of 100 mM sodium acetate, pH 5. Laminarinase β-1,3-glucanase (Sigma–Aldrich, St. Louis, MO) was added (0.5 mU per 10 mg of cell walls) and incubated at 37°C for 2 h. An additional 0.5 mU of laminarinase was then added, and the incubation was continued for another 2 h at 37°C. After centrifugation at 10,000*g* for 5 min the supernatants containing cell wall proteins were harvested. Protein concentrations were determined using Bio-Rad DC protein assays (Bio-Rad, Hercules, CA).

Western blot analysis

Cell wall protein extracts (30 μg total protein) produced by laminarinase digestions were separated on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen). Proteins were transferred to polyvinylidene-difluoride membranes using Invitrogen's XCell II system. Membranes were blocked in TBS-T buffer (0.1% Tween-20, 150 mM NaCl, 10 mM Tris, pH 8.3) containing 5% milk for 1 h at room temperature. Membranes were incubated with a 1:500 dilution of primary antibody (chicken anti-A/TK/Wisconsin/68 serum) for 1 h at room temperature followed by three 5 min washes in TBS-T buffer. The membranes were incubated with a HRP-conjugated goat anti-chicken IgG-Fc antibody (Bethyl Laboratories, Montgomery, TX) diluted 1:2,000 for 30 min at room temperature. After three additional TBS-T washes, Sigma Fast DAB developing solution (Sigma–Aldrich) was used to visualize the bands.

*HA assay of H5.*AG*α1 displaying yeast cells*

Hemagglutinating activity of the H5.*AG*α1 fusion protein was determined by standard influenza HA assays measuring the ability of the hemagglutinin protein to attach to red blood cells (RBCs) and agglutinate them.[15] Yeast cells expressing the H5.*AG*α1 fusion protein and those just expressing *AG*α1 (negative control) were washed twice in 1× PBS and resuspended in 1× PBS to an OD_{600} of 10. Yeast cells (50 μL) were incubated with equal volumes of washed 0.5% chicken RBCs in 1× PBS for 4 h at room temperature in round bottom 96-well plates. HA activity was scored as previously described.[16] Briefly, HA positive wells had agglutination of the RBCs resulting in no settling of the RBCs. HA negative wells resulted in settling of the RBCs forming a RBC button on the bottom of the wells.

Preparation of the Yeast Vaccines and Vaccination of Chickens. All yeast used for vaccination were washed in sterile 1× PBS before being resuspended in 1× PBS as follows. Yeast expressing the H5 hemagglutinin were prepared with 6.7×10^9 cells/mL in 2.5 mL and administered using oral gavage (on weeks 1, 2, 3, 4, 5, and 7) to 10 white leghorn specific pathogen free chickens (*Gallus gallus*) from Southeast Poultry Research Laboratory flocks, starting at 2 weeks of age. Blood was drawn from 10 chickens 7 weeks post vaccination for collection of serum. Blood was also collected from a control group containing 10 chickens not administered with the yeast vaccine.

Virus Neutralization Assays. An equal amount of serum from each of the 10 yeast vaccinated chickens, or controls, was pooled. The pooled sera (600 μL) was incubated with A/Egret/HK/757.2/03 (H5N1) virus dilutions (600 μL) at $10^{1.38}$, $10^{0.38}$, $10^{0.038}$, and $10^{0.0038}$ egg infectious doses (EID_{50}) in a 50 μL volume at room temperature for 30 min. Six embryonating chicken eggs were inoculated with 100 μL of the pooled sera/virus mixes per virus dilution. After 4 days, the number of hemagglutinin positive eggs were determined by HA assays.[16] All work with HPAI viruses was conducted in a BSL-3 enhanced facility at the Southeast Poultry Research Laboratory, ARS, USDA, Athens, GA.

Results and Discussion

Creation of a P. pastoris *strain expressing H5 fused to the C-terminus of* AG*α1 on its cell surface*

Previously, the methylotrophic yeast *P. pastoris* had been used to express and display foreign proteins such as EGFP or KYE on the yeast cell surface, anchored using the C-terminus of α-agglutinin from *S. cerevisiae*.[9,10] Here, the *P. pastoris* system was also used to create an expression plasmid H5.*AG*α1.pPIC9K construct (Figure 1) that expressed the ectodomain of the H5 hemagglutinin protein from the H5N1 HPAI virus A/Egret/Hong Kong/757.2/02. This is the first step in hopes of creating of a yeast-based vaccine against HPAI. To accomplish this, the commercially available pPIC9K expression vector was used to create a H5- *AG*α1 fusion protein. This vector is unique as it contains the α-factor pre-pro leader sequence, which allows for secretion and export of the foreign protein across the membrane and places foreign gene expression under the control of the methanol inducible alcohol oxidase utilization gene 1 (*AOX*1) promoter.[17] The H5 gene had the original influenza 5′ signal sequence deleted in order to allow the yeast α-factor pre-pro leader sequence to efficiently secrete the fusion protein without interference from the endogenous signal sequence. The H5 transmembrane and cytoplasmic tail regions were also deleted to ensure that the fusion protein would not integrate into the plasma membrane and would be expressed on the surface of the cell wall. The expressed protein contained 515 amino acids of the H5 protein fused in

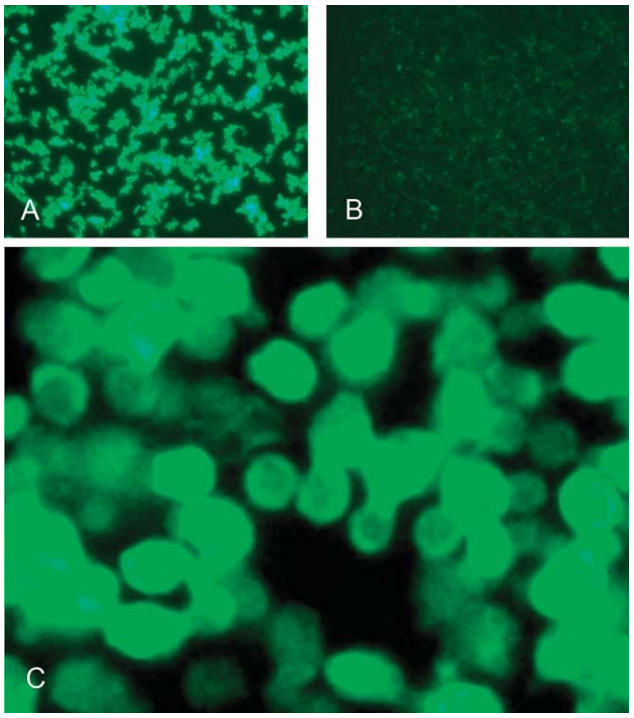

Figure 2. Immunofluorescence labeling.

Recombinant *P. pastoris* cells expressing either H5.*AG*α1 (Panels A and C) or the control *AG*α1 (Panel B) proteins on their cell surfaces were tested for H5 protein expression using anti-A/Turkey/Wisconsin/68 (H5N9 subtype) serum with anti-chicken FITC-conjugated antibody as the secondary antibody.

frame to the C-terminus (327 amino acids) of the *S. cerevisiae* α-agglutinin (*AG*α1) protein.

*H5.*AGα1 *fusion proteins are correctly targeted and immobilized on the surface of* P. pastoris *cells*

The production of recombinant protein in a foreign organism can lead to improper folding and localization. To confirm the proper folding, secretion, and targeting to the cell surface of the expressed H5.*AG*α1 fusion protein, immunofluorescence microscopy was used to visualize the location of the fusion protein (Figure 2). Only the H5.*AG*α1 integrated *P. pastoris* strain was recognized by the anti-H5 antibody on the cell surface (Panel A and C), whereas the negative control *AG*α1 strain (Panel B) showed minimal background fluorescence. This confirmed the specificity of the immunofluorescence labeling system and correct localization of the fusion protein on the cell wall.

To further verify that the H5 fusion protein was associated with the yeast cell walls, cell walls of both H5.*AGα1* and *AGα1* yeast were treated with laminarinase to extract proteins from the cell wall attached via *β*-1, 3-glucans (Figure 3). Extracts were then separated on SDS-PAGE gels and immunoblotted for H5 protein detection. It has been reported that the HA0 H5 protein of HPAI viruses is cleaved at the polybasic amino acid cleavage sequence by cellular proteases resulting in two cleavage products, designated HA1 and HA2,[18] and expression of H5 in *P. pastoris* also has been shown to result in two cleavage products without the addition of exogenous proteases.[19] The H5.*AGα1* fusion protein extracts showed bands at ~110 kDa and ~57 kDa after laminarinase enzyme extraction, corresponding to the cleaved and uncleaved H5.*AGα1* products (Figure 3, Lane 2). The

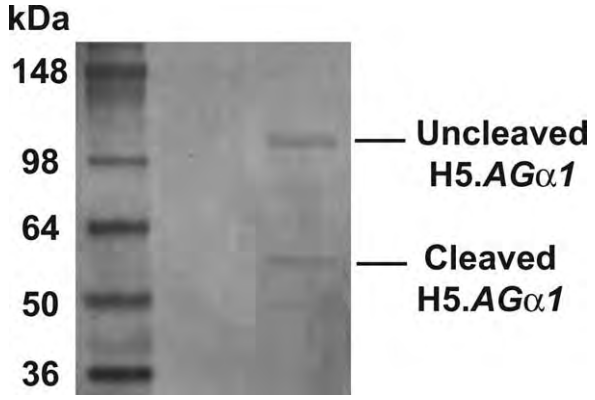

Figure 3. Western blot analysis of laminarinase extracted cell wall proteins.

Recombinant *P. pastoris* cells expressing either *AG*α1 (Lane 1) or the control H5.*AG*α1 (Lane 2) proteins were tested for H5 protein expression on their cell surfaces. Cell walls were isolated by laminarinase extraction and run on a SDS-PAGE gel (30 *μ*g total protein per lane).

molecular weights were slightly greater in the cell wall extracts than the calculated values of the cleavage products of 91.2 kDa (HA0.*AGα1*) and 53 kDa (HA2. *AGα1*), respectively, which is consistent with the fusion protein products being glycosylated by the *P. pastoris* cells. In addition, the third cleavage product (HA1) predicted to be 38 kDa was not observed on the Western blot and may result from lack of recognition of HA1 by the polyclonal antibody used. As expected, no H5 specific signal was detected (Figure 3, Lane 1) with laminarinase-treated cell wall proteins extracted from the *AGα1* expressing control yeast. These results confirmed that the H5. *AGα1* fusion proteins were correctly attached to the yeast cell walls through *β*-1, 3-glucans.

*H5.*AGα*1 fusion protein expression on the cell surface results in functional hemagglutinating activity*

One of the classical diagnostic assays for influenza viruses are their ability to bind RBCs and causing rosette formation—the hemagglutination process.[15] This process is due to binding of the hemagglutinin to terminal sialic acids of glycoproteins on the cell surface of permissive cells.[20] This ability of the HA protein to attach to RBCs was used to further confirm the expressed H5.*AGα1* fusion protein on the yeast cell surface was conformationally and functionally intact. Incubation of HA expressing cells with chicken RBCs provided a visual method to determine hemagglutination. When HA is present, it binds to the RBCs, agglutinates them, and prevents them from settling. In the absence of HA, the RBCs just settle to the bottom of the round well forming a button pattern. Hemagglutination using whole yeast cells was measured after yeast were incubated with chicken RBCs (Figure 4). The button pattern of settling RBCs can be seen in control well containing just PBS (no yeast). Wells containing yeast expressing *AG*α1 also resulted in settling of the RBCs; however, the cosettling of the yeast resulted in a slightly more diffuse settlement pattern of the RBCs than the negative control alone, which did not contain interfering yeast cells. Despite the interference of the yeast cells, it was still clear that the *AG*α1 yeast control wells were not able to keep the RBCs from settling. Therefore, hemagglutination only occurred when RBCs were incubated with yeast expressing the HA.*AG*α1-fusion protein indicating

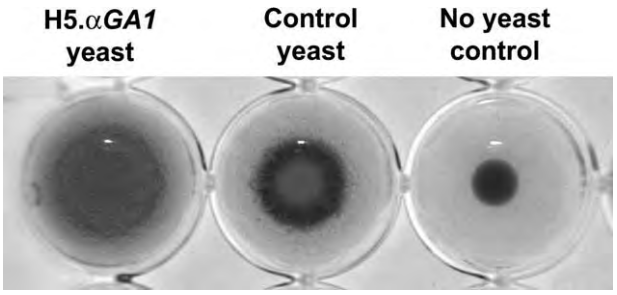

Figure 4. Hemagglutination activity of recombinant *P. pastoris* strains.

Hemagglutination activity by the H5.*AG*α1 fusion protein on the cell surface was measured in cells (OD_{600} of 10). Agglutination of chicken red blood cells was tested by incubating yeast cells with 0.5% chicken red blood cells at room temperature for 4 h and observing the RBC settlement pattern.

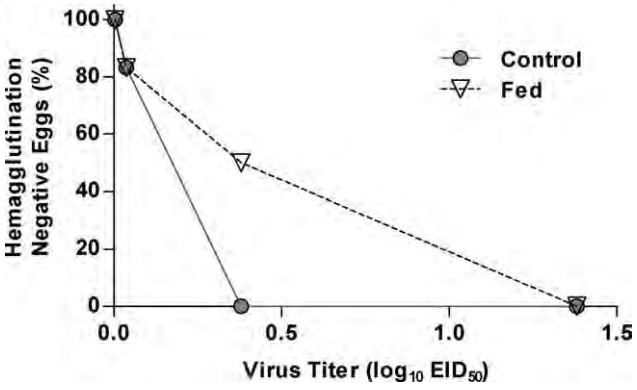

Figure 5. Virus neutralization assays.

Chickens were orally vaccinated with whole H5.*AG*α1 expressing yeast by oral feeding. Sera taken from 10 chickens per group, 7 weeks post vaccination, were pooled and mixed with an equal volume of A/Egret/Hong Kong/757.2/02 (H5N1) virus dilutions. After a 30 min incubation, embryonating chicken eggs were inoculated with the serum/virus mixtures, and hemagglutinating activity was assayed 4 days later.

the expressed protein retained its conformation and functionality when displayed on the cell wall.

*Oral vaccination of chickens with H5.*AGα*1-expressing yeast produces virus neutralizing antibodies*

The effectiveness of the H5.*AG*α1-expressing yeast as an oral vaccine against H5N1 HPAI viruses was evaluated. Chickens were orally vaccinated and, 7 weeks post vaccination, the serum was tested for production of virus neutralizing antibodies. The virus neutralization assay measures the ability of antibodies produced by the vaccinated chickens to bind and neutralize HPAI virus particles, thus, preventing further infection of embryonating chicken eggs after inoculation. Positive hemagglutination in the eggs indicates the virus was not completely neutralized, allowing the virus to replicate. Figure 5 shows the results of the virus neutralization assay using the H5N1 A/Egret/Hong Kong/757.2/02 virus. Although the results of hemagglutinin inhibition assays (data not shown) for detection of H5 antibodies were below the level of detection for the assay, the more sensitive virus neutralization assay showed the orally vaccinated group was able to produce virus neutralizing antibodies in the serum, resulting in 50% hemagglutination negative eggs at the 0.38 virus dilution, compared with the control group that showed 100% hemagglutination positive eggs at the same dilution. This indicates that the use of oral vaccination using this system has potential use as a vaccine. Although the higher titer virus dilution was not sufficiently neutralized by the control or vaccinated groups, further changes in yeast dosage and timing of vaccination of chickens will help to optimize this vaccine system. Inclusion of the H5.*AG*α1-expressing yeast in the chicken feed may help to maintain long-term antibody production in the chickens without any added labor for the process of vaccination, possibly allowing vaccination against multiple influenza subtypes simultaneously without detriment to the health of the vaccinated chickens. Other possible delivery methods such as aerosolized yeast suspension could also be examined to find the most efficacious route.

Conclusion

In this study, the hemagglutinin protein from a highly pathogenic strain of avian influenza was expressed on the cell surface of *P. pastoris*. Immunofluorescence demonstrated its cellular localization on the surface of yeast, and its ability to hemagglutinate RBCs demonstrated the expressed H5.*AG*α1 fusion protein retained its function and appeared to be in a native conformation. Ultimately, virus neutralizing antibodies were produced in response to oral administration of the H5.*AG*α1-expressing yeast to chickens, demonstrating the potential of this application to impact methods of vaccination against HPAI virus infections.

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Mucosal vaccination with a codon-optimized hemagglutinin gene expressed by attenuated *Salmonella* elicits a protective immune response in chickens against highly pathogenic avian influenza

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ABSTRACT

The purpose of this study was to evaluate clinical protection from challenge conferred by two attenuated *Salmonella enteria* serovar *typhimurium* vaccine strains expressing the hemagglutinin (HA1) gene from a highly pathogenic avian influenza (HPAI) H5N1 (A/whooper swan/Mongolia/3/2005), under control of the anaerobically inducible nir15 promoter. Two-week-old White Leghorn chickens were immunized by oral gavage with one milliliter doses of >109 *Salmonella* colony-forming units once weekly for 4 weeks prior to challenge. Expression of recombinant protein was confirmed via Western blot. Serum and mucosal gavage samples were collected prior to, and following immunization and antibodies against avian influenza HA were confirmed by Western blot and hemagglutination-inhibition (HI) assay. Chickens were challenged with homologous (A/whooper swan/Mongolia/3/2005), or heterologous (A/Chicken/Queretaro/14588-19/95) HPAI virus strains. Chickens immunized with attenuated *Salmonella* strains containing plasmid expression vector (pTETnir15HA) demonstrated a statistically significant increase in survival compared to control groups. Results provide evidence of effectiveness of attenuated *Salmonella* strains for delivery of recombinant avian influenza HA antigens and induction of mucosal and systemic immune responses protective against lethal challenge with HPAI.

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1. Introduction

The influenza hemagglutinin (HA) glycoprotein is responsible for attachment and fusion of the viral and endosomal membrane during virus entry [1]. Protective immunity against avian influenza depends largely on the development of immune response against epitopes of the HA glycoprotein [2–4]. Various experimental DNA vaccines and vectored vaccines based on influenza hemagglutinin antigens have shown efficacy in chickens against avian influenza challenge [3–6].

Attenuated *Salmonella typhimurium* strains have being evaluated for use as live vaccines for delivery of a variety of bacterial, viral, and parasitic antigens to mucosal lymphoid tissue, including the HA of avian influenza [7–10]. Vaccines administered via mucosal routes can induce mucosal, cell-mediated, as well as systemic immune responses, while parentally administered vaccines induce mostly humoral immunity [11]. *Salmonella* invades enterocytes, replicates and persists in the M-cells of Peyer's patches, and survives in macrophages, which allows bacterial antigens to be processed and presented to immune cells, and prevent the induction of oral tolerance [11]. In addition, the stimulation of local immune responses at the site of pathogen entry may aid in protection against clinical disease and reduce viral shedding and reduction of transmission to other susceptible hosts [3].

The goal of creating attenuated bacterial strains for use as vaccines is the inactivation of genes that attenuate proliferation and persistence of the strain, without significantly compromising its immunogenicity [7]. *Salmonella* strains with deletions in the aromatic amino acid (Δaro) biosynthetic pathway such as BRD509 are severely attenuated, but have been shown to retain their immunogenicity in mice [12–15]. These nutritional auxotrophs cannot synthesize chorismic acid, a key intermediate in the synthesis the aromatic amino acids, *p*-aminobenzoate (PABA), and dihydrozybennzoate (DHB). The attenuated *Salmonella* Δaro mutants require precursor compounds that are not synthesized by mammalian organisms and therefore, they should not proliferate and persist within even immuno-compromised host organisms [15]. The Δaro mutants of *S. typhimurium* have been shown to be safe and immunogenic when administered as live vaccines to chickens [16–18]. Attenuated *Salmonella* strains

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Table 1
DNA sequences of primers used for the construction of the pTET*nir15*HA expression plasmid.

Primer name	Direction	Nucleotide sequence
HAcontr689PCR	r	TAGCTACGTTTAATGGTCGGATAC
HABamHI	f	CATTCC*GGATCC***ATG**GAAAAAATTGTGCTGCTGCT
HASmaI	r	AATTGC*CCCGGG*TTAAATACAAATACGGCACT
optHA158	f	GCCTGAAACGCGAAGAAAT
optHA157	r	CAGTTTGCCGTTGTGGGTTTT
pTETApaIBglISDHA1.1	f	AAGGTAGGCGGTAGGGCCC*AGATCT*TAATCATCCACAGGAGACTTTCTG **ATG**GAAAAAATTGTGCTGCTGCTGGCGATT
pTETApaBglIISDHA1.3	f	AAGGTAGGCGGTAGGGCCCAGATCT
pTETHABamHI	r	AGGCGGGCTAGCGGATCCTTAAATACAAATACGGCACT
pTET3644	f	CCACCTGACGTCTAAGAAACCATT
pTET281	r	GGTGGATAGCTTTGCCGTTGATGC

f: forward; r: reverse.
Restriction sites are designated in italic, the start codon is in bold, the stop codon is underlined, and the ribosomal binding site Shine-Dalgardo (SD) sequence is indicated by smaller font.

in which deletions in the host specific of DNA (*hsd*) genes for restriction (r^-) and/or modification (m^+), are efficient recipients of unmodified DNA [19]. The auxotrophic *S. typhimurium* r^-m^+ strains LB5000 and LB5010, have also been used as vaccine strains delivering a variety of antigens, including an AI H3 epitope which conferred partial protection against lethal challenge in mice [19].

The problem of toxicity of over expressed foreign protein or loss of the expression plasmid may be overcome by use of *in vivo* inducible promoters, allowing stable expression the cloned antigen. The naturally occurring *Escherichia coli* promoters *nir*B and *nir*15 are induced by the reduction in oxygen tension which occurs upon phagocytosis of *Salmonella* by macrophages or M-cells in intestinal Peyer's patches [12,13]. These *in vivo* inducible promoters have been shown to be effective for controlled delivery of recombinant proteins in attenuated *Salmonella* strains [7,12,13]. Foreign antigens expressed under control of *nir15* promoter using the pTET expression plasmid and auxotrophic *S. typhimurium* strains have been shown to successfully induce immune responses in chickens [7,13,20–22].

We expressed the immunogenic HA protein derived from HPAI H5N1 in bacterial vectors under the control of an *in vivo* inducible promoter and evaluated the protection against avian influenza virus challenge in chickens. A pTET, plasmid-based expression vector was constructed using the A/whooper swan/Mongolia/3/2006 (H5N1) HA gene sequence, optimized for codon usage of *Salmonella enterica*, under the control of the anaerobically induced *nir*15 promoter (pTET*nir*15HA). We demonstrated the avian influenza virus HA was successfully expressed *in vitro* by Western blot analysis, and *in vivo* by detection of HI antibodies in vaccinated birds. Following lethal challenge, the attenuated recombinant bacterial vaccine induced partially protective immune responses against lethal challenge by both the homologous HPAI virus of Asian lineage, and a heterologous HPAI virus of North American lineage.

2. Materials and methods

2.1. Viral strains and culture

Two HPAI strains were used for challenge: A/Whooper Swan/Mongolia/3/2005 (H5N1) (WM05) and A/chicken/Queretaro/14855-19/95 (H5N2) (CQ95). The LPAI A/Ck/PA/1370/83 (H5N2), or the HPAI A/Chicken/Queretaro/14588-19/95 (H5N2) virus was used as antigen in HA and HI assays. Viruses were propagated and titrated in 9–11 days of embryonation, specific pathogen free (SPF) chicken eggs according to standard procedure [23].

2.2. Bacterial strains and culture

The restriction-deficient, modification-proficient *S. typhimurium* LT2 LB5010 (r^-m^+) strain was purchased from the *Salmonella* genetic stock center (University Calgary, Calgary, Canada) [19], and the auxotrophic *S. typhimurium* BRD509 (Δ*aroA*, Δ*aroD*) strain was provided by D. Pickard (Wellcome Trust Sanger Institute, Cambridge, England) [24]. Bacterial strains were cultured in Luria-Bertani (LB) broth or agar at 37 ± 100 μg/ml ampicillin. Induction of the *nir*15 anaerobic promoter was performed using a BBL GasPak 100 system, for 4–6 h at 37 °C (Becton Dickinson Diagnostic, Franklin Lakes, NJ). To prepare vaccine inoculum, 15 ml cultures were grown overnight at 37 °C at 200 rpm, diluted 1:2 with fresh LB broth + 100 μg/ml ampicillin, then grown standing for 4–6 h at 37 °C under anaerobic conditions. Cultures were centrifuged at 3000 × *g* for 20 min at 4 °C, and pellets resuspended in 10 ml fresh medium without antibiotic. Inoculum dose was determined to be 10^9–10^{10} cfu/ml by plating serial dilutions.

2.3. Construction of expression plasmid

Polymerase chain reaction (PCR) cycle parameters were: 94 °C for 3 min followed by 35 cycles at 94 °C for 15 s, 55 °C for 15 s, 72 °C for 40 s to 2 min and a final extension at 72 °C for 15 min. Amplified PCR products were separated according to standard protocol, and fragments were purified using the QIAEX II Gel Extraction Kit (Qiagen Co., Valencia, CA), plasmid DNA purified using the QIAprep Spin Miniprep kit (Qiagen Co., Valencia, CA), and PCR products purified using a QIAquick PCR purification kit (Qiagen Co., Valencia, CA).

The HA1 gene sequence was obtained from HPAI virus strain A/Whooper swan/Mongolia/3/2005 (H5N1) (GenBank accession number: AB233322). The open reading frame (ORF) of the DNA sequence was codon-optimized based on *S. typhimurium* LT2 codon usage and synthesized from synthetic oligonucleotides using the Gene Optimizer™ system (Geneart Inc., Toronto, Canada). The synthetic 1707 base pair HA sequence (GenBank accession number: GU143823) was PCR amplified using primer pair HaBamHI/HaSmaI (Table 1), cloned into plasmid pUC19 (Geneart Inc., Toronto, Canada) to generate pUCHA, and transformed into *E. coli* DH5α-TIR TOP10F' (Invitrogen, Carlsbad, CA). The anaerobically inducible promoter *nir*15, the ribosomal binding site Shine-Dalgardo (SD) sequence, and restriction sites ApaI and BamHI were incorporated into the HA sequence via two PCR steps; the HA ORF was amplified from pUCHA using primers pTETApaIBglISDHA1.1/pTETHABamHI, followed by amplification using primers pTETApaIBglISDHA1.3/pTETHABamHI (Table 1). The HA and pTET plasmid backbone were digested with *Apa*I/*Bam*HI, dephosphorylated with Antarctic phosphatase (New England BioLabs, Ipswich, MA), and ligated overnight at 14 °C using T4 DNA

Ligase (Invitrogen, Carlsbad, CA), creating plasmid pTET*nir*15HA. To confirm orientation, insert ends were sequenced using primer pairs optHA158/optHA157 and pTET3644/pTET281 (Table 1). Plasmids were transformed into bacterial cells using the Bio-Rad Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA) [25].

2.4. Evaluation of in vitro and in vivo protein expression

Bacterial strains were grown and pelleted as described for preparation of inoculum. Soluble and insoluble protein fractions were separated using the non-ionic detergent protein extraction reagent BugBuster® (Novagen, EMD Biosciences, San Diego, CA). Proteins were concentrated by addition of 10% trichloracetic acid (Fisher Scientific, Bridgewater, NJ), overnight incubation at 4 °C, and centrifugation at 15,000 × *g* for 30 min at 4 °C. Pellets were washed with ice-cold acetone, air-dried, and resuspended in 50 μl of sterile phosphate buffered saline (PBS) and protein content quantified using a bicinchoninic acid (BCA) protein assay (Thermo Fisher, Rockford, IL).

Western blot analyses were performed according to standard procedures. Proteins were separated on 12.5% Tris–HCl sodium dodecyl sulfate-polyacrylamide (SDS-PAGE), Criterion® (BioRad, Hercules, CA), and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) using a Criterion® blotter (BioRad, Hercules, CA). The Rainbow ladder® was used as the molecular weight marker (BioRad, Hercules, CA). Membranes were blocked overnight at 4 °C in PBS + 1% polyvinyl pyrrolidine and 1% Tween-20. Polyclonal ferret-anti-Turkey/Wisconsin/68 (H5N9) serum (Dr. Terrence Tumpey, CDC, Atlanta, GA), was the primary antibody, and the secondary antibody was mouse-anti-Ferret-horseradish peroxidase (HRP) conjugate (Bethyl Laboratories, Montgomery, TX). Peroxidase activity was detected with Sigma FAST OPD substrate (Sigma–Aldrich Biotechnology, St. Louis, MO).

2.5. Bacterial colonization and plasmid stability

To assess colonization of bacterial strains and plasmid stability *in vivo*, fecal samples were collected from five chickens 24 and 48 h after inoculation. One gram fecal samples were added 10 ml LB broth, and aliquots were spread onto LB or XLT4 agar plates ± ampicillin, plates incubated overnight at 37 °C, and colonies counted. Two weeks after inoculation, chickens were sacrificed liver and spleen combined into one sample, which was stomached for 5 min in10 ml LB broth, and aliquots were spread onto LB or XLT4 plates ± ampicillin. One milliliter sample was placed into 9 ml tetrathionate broth + iodine, incubated overnight at 37 °C, and aliquots were spread onto LB plates ± ampicillin, incubated overnight at 37 °C, and colonies counted.

2.6. Serologic assays

Hemagglutination (HA) assays were performed according to standard protocol. Samples were serially diluted 2-fold in sterile PBS pH 7.3 and 50 μl was added to the wells along with 50 μl of fresh 0.5% chicken red blood cells in Alsever's solution. Plates were incubated at room temperature for 30 min until complete agglutination occurred in positive control wells. The last dilution well giving full agglutination was designated as the $\log_2$ HA unit measurement for the sample. Hemagglutination-inhibition (HI) assays were performed according to standard protocol. LPAI virus A/Ck/PA/1370/83 (H5N2) was used in Experiment I, and HPAI A/chicken/Queretaro/14855-19/95 (H5N2) virus was used as the antigen in experiments II and III.

2.7. Statistical analysis

Kaplan–Meier survival curves were generated with Prism 5 (GraphPad Co., San Diego, CA). The Mantel-Cox log-rank test was used to compare survival curves between two experimental groups (Prism 5). Statistical differences in mean and standard error between mean HI titers were analyzed using Tukey one-way ANOVA (Prism 5). The Fisher Exact test was used for pair-wise comparison of group mean HI titer or frequency of virus isolation between groups (SigmaStat 2.0.3, SPSS Inc., Chicago, IL). Lower case letters indicate statistical significance between compared groups. All statistical tests were performed using $p < 0.05$.

2.7.1. Animal experiments

For all experiments, mixed-sex specific pathogen free (SPF), White Leghorn (WL) chickens were obtained from, and housed at the Southeast Poultry Research Laboratory in a BSL2 facility, and transferred to a BSL3E facility for challenge. Birds were maintained in Horsfall isolation units with feed and water *ad libitum*. Oral vaccine was administered via crop gavage at 1 week intervals, with the last dose 1 week prior to challenge or sampling. Crop gavage samples were collected as previously described [26]. Challenge groups received ocular and nasal administration of 100 μl BHI containing challenge virus delivered equally between inoculation sites as previously described [3]. Unchallenged groups received the same volume of PBS delivered via the same route. Serum samples were taken via wing vein prior to vaccination, on the day of challenge (0 dpc), and 10 dpc, and were stored at −20 °C. Oro-pharyngeal and cloacal swab samples were collected on 0, 2, 4, 7, and 10 dpc. Swab samples were placed in 2.0 ml BHI broth (1000 units/ml penicillin G, 200 μg/ml gentamicin sulfate, 4 μg/ml amphotericin B) and stored at −70 °C (Sigma Chemical Company, St. Louis, MO). After low speed centrifugation to remove debris, 10-fold dilutions of collection media were inoculated into eggs and viral titers determined. The accuracy of the viral challenge doses administered was confirmed by immediate back-titer in embryonated eggs. Following challenge, groups were monitored twice daily for 10 days for clinical signs, and those with severe clinical signs disease were humanely euthanized by approved protocol and counted as mortalities for that day.

2.7.2. Experiment I – production of HI specific antibodies, vacine safety, in vivo survival of vaccine strains

Twenty, 4-week-old SPF WL chickens were divided randomly into four groups of five birds each. Birds were inoculated via crop gavage with 1 ml volume of bacterial cultures (*E. coli* pTET*nir*15HA: EC, *S. typhimurium* BRD 509 pTET*nir*15HA: S509, *S. typhimurium* pTET*nir*15HA: S5010), or sterile culture media (NV). Birds were vaccinated twice and observed for signs of diarrhea or distress caused by inoculation. Fecal samples were collected 1, 2, 3, and 7 dpc, and 3×10^3 cfu/ml feces were cultured on XLT4 + 100 μg/ml ampicillin. Two weeks after last inoculation birds were bled, crop gavaged, and liver and spleen were collected after euthanasia for bacterial culture. Twenty-four hours after initial inoculation with the attenuated *Salmonella* strains (S509, S5010) containing the expression plasmid.

2.7.3. Experiment II – clinical protection of vaccinated chickens afterhomologous or heterologous challenge

Seventy, 2-week-old mixed-sex SPF WL chickens were divided randomly into 11 groups. Three unvaccinated groups of four birds designated NV, received culture medium via crop gavage. Eight experimental groups of six birds each were inoculated with live vaccines. Four groups designated S509 received *S. typhimurium* BRD509 pTET*nir*15HA, and four groups designated S5010 received *S. typhimurium* LB5010 pTET*nir*15HA. All groups were vaccinated or mock-vaccinated four times at 1 week intervals. Challenged

groups received ocular and nasal administration of challenge virus. Groups designated ML were challenged with low dose (10^3 EID_{50}) of WM05, and groups designated MH were challenged with high dose (10^5 EID_{50}) of WM05. Groups designated QL were challenged with low dose (10^4 EID_{50}) of CQ95, and groups designated QH were challenged with high dose (10^6 EID_{50}) of CQ95. Unchallenged groups designated NC received PBS via the same route. Serum samples were taken via wing vein prior to vaccination, 0, and 10 dpc. Oro-pharyngeal and cloacal swab samples were collected on; 0, 2, 4, 7, and 10 dpc.

2.7.4. Experiment III – survival of heterologous challenge

Fifty six, 2-week-old mixed-sex SPF WL chickens were divided randomly into 12 groups of six. Groups were vaccinated or mock-vaccinated four times at 1 week intervals. Unvaccinated groups were designated NV, and unchallenged groups were designated NC. Groups designated EC, S509, and S5010, were inoculated with bacterial cultures. Three groups designated MEC, MS 509, MS 5010, were inoculated with bacterial strains without expression plasmid. Groups designated QL were challenged with low dose (10^4 EID_{50}) of CQ95, and groups designated QH were challenge with high dose (10^6 EID_{50}) of CQ95. The groups vaccinated with the untransformed bacterial strains (MEC, MS 509, MS 5010), received only low dose challenge (QL).

3. Results

3.1. Expression of HA protein in bacterial strains

To ensure accurate expression of the HA protein in bacterial strains the gene encoding HA1 protein was codon-optimized based on *S. typhimurium* LT2 codon usage, resulting in expression of a full-length HA protein with 100% amino acid identity with the original viral protein (Table 2 Supplemental). The full-length gene encoding the 64 kDa HA1 was cloned under control of the anaerobicaly inducible promoter *nir*15 in the pTET*nir*15 plasmid backbone, creating the expression vector pTET*nir*15HA. The Plasmid was transformed into bacterial strains *E. coli* DH5α, *S. typhimurium* LB5010, and *S. typhimurium* BRD509, to create live-attenuated vaccine strains.

The codon-optimized HA ORF encodes 569 amino acids, and has a theoretical product size of 64 kDa. Bacterial expression of the protein from pTET*nir*15HA was confirmed in both the soluble and insoluble protein fractions using Western blot (Fig. 1). A protein of approximately 65–70 kDa was recognized by a polyclonal serum ferret-anti-Turkey/Wisconsin/68 (H5N9) in the insoluble protein fractions from the bacterial stains transformed with the expression plasmid (Fig. 1). No proteins were recognized by the AI antiserum in protein extracts from cultures of the parent bacterial strains not containing the expression plasmid (Fig. 1). The recombinant HA1 protein was found largely in the insoluble fraction of lysates from bacterial cultures induced under anaerobic conditions.

An HA assay to assess the ability of AI antiserum to recognize the recombinant protein in bacterial cultures. When compared to positive and negative control reactions, dilutions up to 6-fold (1:64) of insoluble protein fractions from bacterial extracts containing the expression plasmid resulted in positive agglutination reactions, while soluble protein fractions did not result in positive agglutination reactions. Samples of soluble and insoluble protein fractions from bacterial cultures without expression plasmid did not give positive agglutination reactions.

3.2. Experiment I – production of HI specific antibodies, vaccine safety, in vivo survival of vaccine strains

To assess the ability of the two attenuated *Salmonella* strains to persistently colonize inoculated chickens, and the stability of

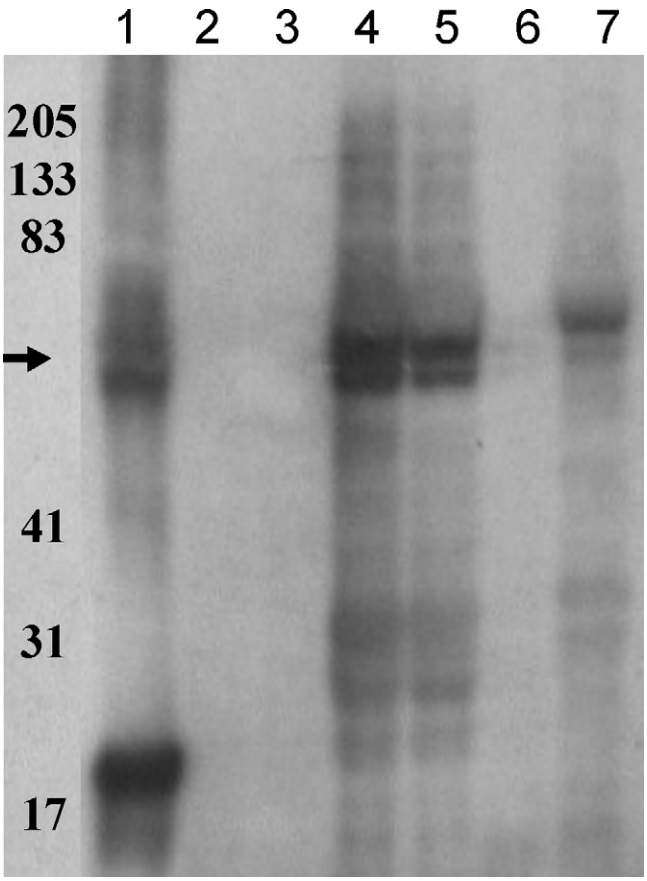

Fig. 1. Western blot showing expression of sequence-optimized avian influenza HA1 protein by *Salmonella typhimurium* strains LB5010 and BRD509, and *Escherichia coli*. Cell lysates were separated with SDS-PAGE, proteins transferred to PVDF membrane, and probed with polyclonal anti-A/turkey/Wisconsin/68 (H5N9) antiserum. Arrow indicates viral HA1 protein. Lane 1: A/whooper swan/Mongolia/3/2005 (H5N1); lane 2: *S. typhimurium* LB5010; lane 3: *S. typhimurium* BRD509; lane 4: *S. typhimurium* LB5010 pTET*nir*15HA; lane 5: *S. typhimurium* BRD509 pTET*nir*15HA; lane 6: *E. coli* DH5α; lane 7: *E. coli* DH5α pTETnir15HA.

the plasmid *in vivo*, fecal samples were collected from five chickens inoculated with each of the transformed bacterial strains on days 1, 2, 3, and 7 dpv, for culture and enumeration of the vaccine strain bacteria on selective media. No clinical signs of diarrhea or distress from inoculation with live bacterial vaccine were noted at any time during the experiment. Twenty-four hours after inoculation 10^6 cfu/ml ampicillin resistant organisms were cultured, and 48 h after inoculation 10^2 cfu/ml ampicillin resistant organisms were cultured. No ampicillin resistant organisms were isolated from fecal samples taken 3–7 days after inoculation. No *Salmonella* was isolated from liver, spleen, or cecal tissue samples collected from inoculated birds 14 days after second inoculation.

The presence of antibodies recognizing HA protein in serum and crop gavage samples collected 2 weeks post-vaccination was assessed by HI assay. The *E. coli* vaccinated group (EC) produced a mean serum HI titer of less than 1:16, 2 weeks post-second vaccination (Fig. 2). The mean serum HI titers for *Salmonella* vaccinated groups (S09, S5010) were 1:64 (Fig. 2). All three vaccinated groups produced HI titers that were significantly higher than the unvaccinated group, although only the *Salmonella* vaccinated groups produced HI titers considered protective. An HI titer of 1:32 (6 $\log_2$) is considered positive according to the Office International de Epizooites (OIE), and is indicated by the dashed line. No positive AI HI titer was measured in serum from birds in the control group (NV) (Fig. 2). The crop gavage samples from groups EC, S509, and S5010 collected 2 weeks post-vaccination had HI titers of 1:16, 1:32, and

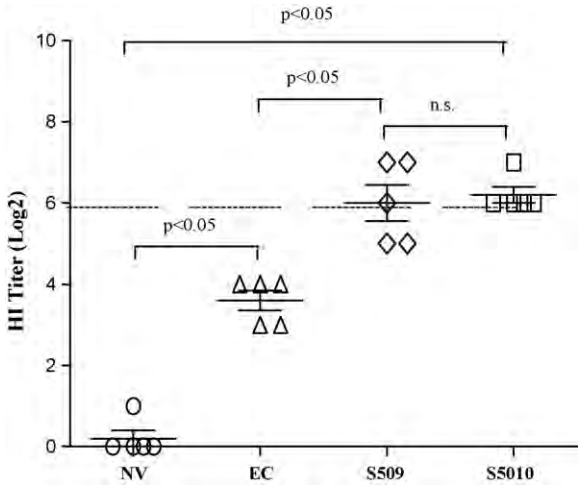

Fig. 2. Mean $\log_2$ HI titer and standard error for groups in Experiment I, 1 week post-second vaccination. The dashed line indicates the HI (2^5 $\log_2$) reported to be required for survival from challenge. Lower case letters indicate statistical significance between mean titers as determined with the Wilcoxon signed rank test ($p < 0.05$). NV NC: unvaccinated unchallenged; EC: *E. coli* pTET*nir*15HA; S509: *S. typhimurium* BRD509 pTET*nir*15HA; S5010: *S. typhimurium* LB5010 pTET*nir*15HA.

1:64, respectively, versus the control group NV, which produced no HI titer (data not shown).

3.3. *Experiment II – clinical protection of vaccinated chickens against homologous or heterologous challenge*

The low and high challenge doses of WM05 were determined to be 10^2 EID_{50} and 10^4 EID_{50} and the low and high doses of CQ95 were determined to be 10^4 EID_{50} and 10^6 EID_{50} by back-titration of challenge inoculum in eggs. No clinical signs were observed in birds prior to challenge. The percent survival of experimental groups versus dpc are represented as a Kaplan–Meier survival plots, with lower case letters indicating statistically significant ($p < 0.05$) differences in survival between groups at a time point (Figs. 3 and 4).

The survival of groups receiving homologous challenge with WM05 is shown in Fig. 3a and b. The unvaccinated unchallenged controls (NV NC) did not display clinical signs of disease and had no mortality during the course of the study. The unvaccinated group challenged with a low dose of WM05 (NV ML) experienced 50% mortality within 5 dpc (Fig. 3). Both of the *Salmonella* vaccinated groups receiving low dose homologous challenge (S509 ML, S5010 ML), had 100% survival, however there was significantly lower survival in both the *Salmonella* vaccinated groups (S09 MH,

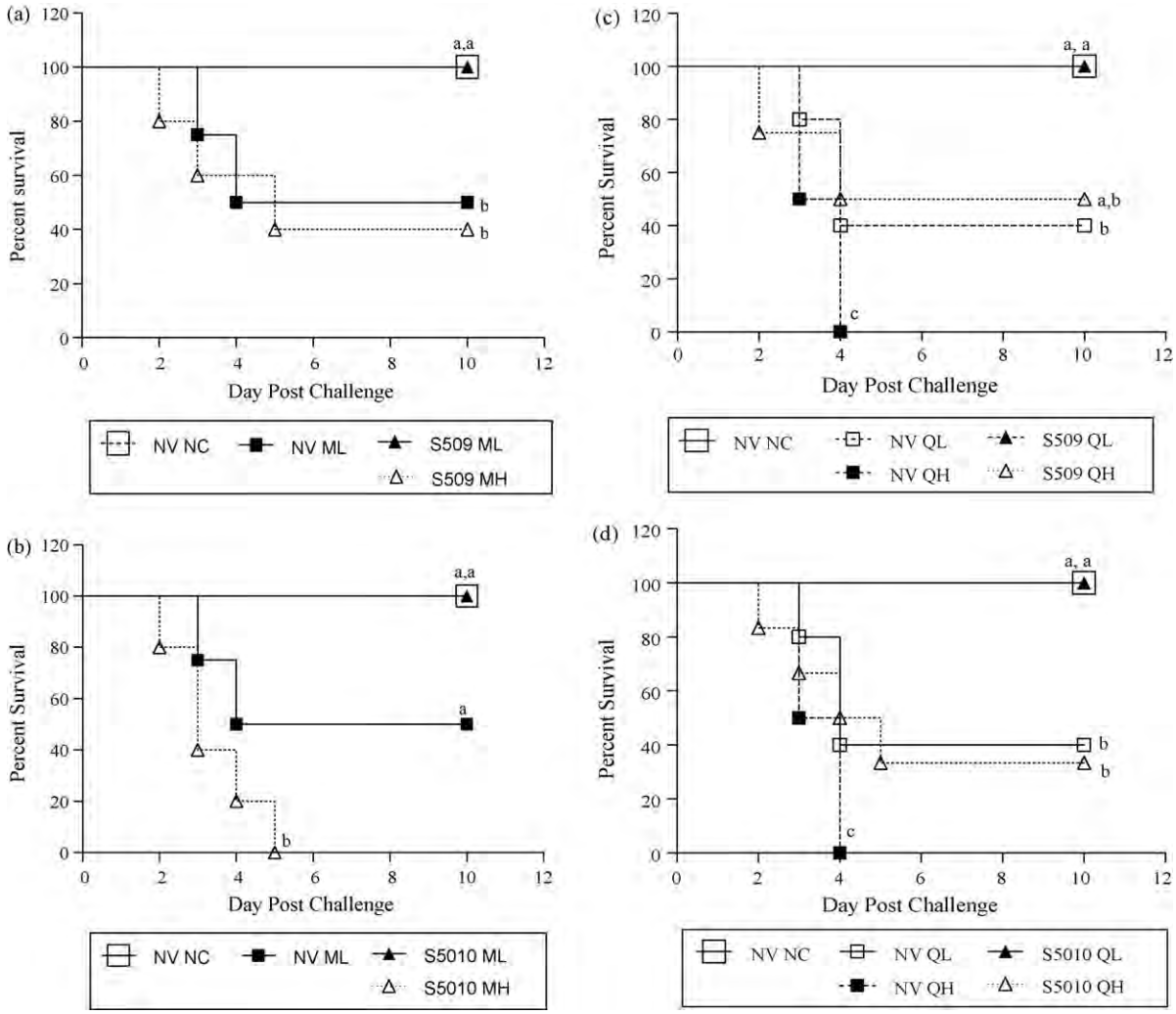

Fig. 3. Kaplan–Meier survival plots for Experiment II. The percent survival versus day post-challenge with low dose (10^2 EID_{50}) (designated ML), or high dose (10^4 EID_{50}) (designated MH), of HPAI A/Whooper swan/Mongolia/3/2005 (H5N1) for groups vaccinated with *S. typhimurium* BRD509 pTET*nir*1HA (designated S509), is shown in (a), for birds vaccinated with *S. typhimurium* LB5010 pTET*nir*15HA (designated S5010), is shown in (b). The percent survival versus day following lethal challenge with a low dose (10^4 EID_{50}) (designated QL), or high dose (10^6 EID_{50}) (designated QH), of HPAI A/chicken/Queretaro/14855-19/95 (H5N2) for birds vaccinated with S509 is shown in (c), and for birds vaccinated with S5010 is shown in (d). Unvaccinated groups are designated NV, and unchallenged NC. Lower case letters indicate statistical significance in survival curves between vaccine groups as determined by Tukey one-way ANOVA ($p < 0.05$).

S5010 MH) when faced with high dose challenge (Fig. 3). There was significantly higher survival of the S509 group (40%) versus the S5010 vaccinated group (0%) when challenged with the high dose of WM05 (Fig. 3).

The survival of groups after heterologous challenge with CQ95 is shown in Fig. 3c and d. Again, unvaccinated unchallenged control birds (NV NC) did not display clinical signs of disease during the study, and had no mortality. The unvaccinated control group challenged with low dose CQ95 (NV QL) reached 60% mortality, and that receiving high dose challenge (NV QH) reached 100% mortality within 4 dpc (Fig. 3c and d). There was a significant difference in survival between S509 vaccinated groups challenged with low (S509 QL) (100%) versus high (S509 QH) (50%) doses of CQ95 (Fig. 3c and d). There was a significant difference in survival of the unvaccinated control groups (NV QL, NV QH) receiving low and high challenge doses, with 40% of unvaccinated birds surviving low dose challenge (Fig. 3c and d). There was significantly less mortality in the S5010 vaccinated groups challenged with either low (S5010 QL) (0%) or high (S5010 QH) (60%) doses of CQ95, versus the unvaccinated group (NV QH) (100%) challenged with the high dose of CQ95 (Fig. 4a and b). There was a significantly higher survival of both *Salmonella* vaccine groups after low dose challenge versus high dose challenge (Fig. 4a and b).

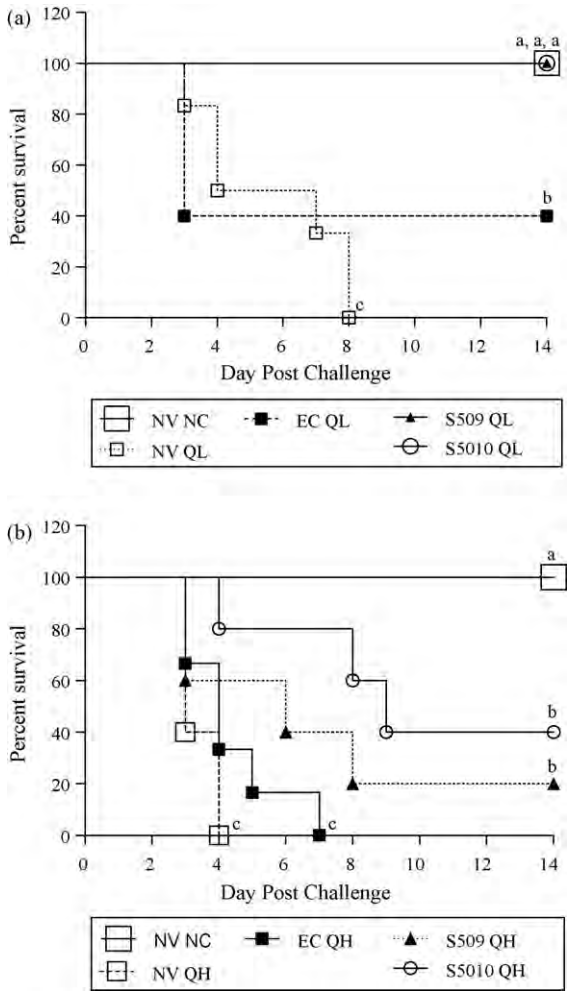

Fig. 4. Kaplan–Meier survival plots for groups in Experiment III. The percent survival versus day following lethal challenge with a low dose (10^4 EID_{50}) (designated QL), is shown in (a), and following a high dose (10^6 EID_{50}) (designated QH), of highly pathogenic H5N2 HPAI A/chicken/Queretaro/14855-19/95 (CQ95) is shown in (b). Vaccinated groups received *S. typhimurium* BRD509 (designated S509), *S. typhimurium* LB5010 (designated S5010), or *E. coli* DH5α (designated EC), containing expression plasmid pTET*nir*15HA. Unvaccinated groups are designated NV, unchallenged groups designated NC. Lower case letters indicate statistical significance in survival curves between groups as determined by Tukey one-way ANOVA ($p < 0.05$).

There was a significant increase in group mean HI serum titer between the all vaccinated groups (S509 Pre, S5010 Pre), and the unvaccinated control groups (NV NC, NV ML) prior to challenge with WM05 (Fig. 1 – Supplemental). Both *Salmonella* vaccinated groups had mean titers of 6 $\log_2$ prior to challenge, and the group mean titers of survivors increased post-challenge to 8 $\log_2$ after both low and high dose challenge with WM05 (Fig. 1 Supplemental). There was a significant increase in group mean HI serum titer between the unvaccinated control groups (NV) and the *Salmonella* vaccinated groups (6 $\log_2$) prior to challenge with CQ95 (Fig. 1 Supplemental). The mean serum titer of survivors increased to 8 $\log_2$ in the NV QL, S509 QL, and S5010 QL groups after low dose challenge with CQ95 (Fig. 1 Supplemental).

The frequency of virus isolation from oro-pharyngeal and cloacal swab samples from groups challenged with WM05 or CQ95 is shown in Table 1 (supplementary). The titers of AI virus shed from the oropharynx and cloaca during the course of challenge are shown in Table 1 (supplementary).

All birds were negative for AI virus prior to challenge and no virus was isolated from the unchallenged controls at any time point. The S509 vaccine group challenged with a low dose of WM05 (S509 ML) shed virus from the cloaca for the entire 10 dpc, with significantly increased frequency of isolation on days 2, 4, and 10 dpc compared to the S5010 vaccine group (S5010 ML) receiving the same challenge dose (Table 1 Supplemental). Both *Salmonella* vaccine groups receiving high dose challenge shed virus from the cloaca for 10 dpc, with no statistically significant difference in the frequency of virus isolation (Table 1 Supplemental). There were also no significant differences in titer of virus shed from either mouth or cloaca, between the *Salmonella* vaccine groups (S509, S5010) challenged with either low or high doses of WM05 (Table 1 Supplemental).

The unvaccinated control group (NV QL) challenged with a low dose of CQ95 shed virus from both the oropharynx and cloaca until 7 dpc (Table 2 Supplemental). There was no significant difference in the titer of virus shed from either the mouth or cloaca between experimental groups at any time point after challenge with either low or high dose CQ95 (Table 2 Supplemental).

3.4. Clinical protection of vaccinated chickens vaccinated after heterologous challenge with differential doses of the HPAI strain A/chicken/Queretaro/14588-19/95 (H5N2) – Experiment III

Percent survival of experimental group versus day post-challenge is represented as Kaplan–Meier survival plots (Fig. 4a and b). No clinical signs of avian influenza infection were observed in any bird prior to challenge with AI. Birds in the unchallenged control group (NVNC) did not display signs of clinical disease during the study. All birds in unvaccinated control groups (NVQL, NVQH) challenged with low or high doses of CQ95 displayed severe clinical signs and died or were euthanized 2–8 dpc (Fig. 4a and b). As a control, groups were vaccinated with bacterial strains without the expression plasmid (MEC QL, MS509 QL, MS5010 QL). After challenge with low dose CQ95, all birds in these vaccine control groups displayed severe clinical signs, and died or were euthanized within 5 dpc (data not shown). As a control, groups were vaccinated with *E. coli* containing the expression plasmid pTETnir15HA (EC). After challenge with CQ95, the low dose challenge group had 60% mortality, and the high dose challenge group had 100% mortality (Fig. 4a and b). *Salmonella* vaccine groups (S509, S5010) did not show any clinical signs of disease after low dose challenge and had 100% survival (Fig. 4a and b). There was significantly lower survival in the S509 and S5010 vaccinated groups after challenge with high

dose (QH) versus low dose (QL), with 20% and 40% survival, respectively (Fig. 4a and b). However, there was no significant difference in survival between the *Salmonella* vaccine groups after low dose challenge, and the unvaccinated unchallenged control group.

Serum collected from the unvaccinated control group (NV NC) was negative for antibodies against AI as measured by HI assay (Fig. 2 Supplemental). One week after last inoculation, prior to challenge, there was a significant increase in mean HI titer in all vaccinated groups (EC Pre, S509 Pre, S5010 Pre), versus the unvaccinated control group (NV Pre) (Fig. 2 Supplemental). Post-vaccination, the EC group had a mean titer of 3 $\log_2$, while the *Salmonella* vaccine groups (S509, S5010) had mean titers of 6 $\log_2$ (Fig. 2 Supplemental). The mean titers of all survivors increased after challenge.

4. Discussion

In this study, we constructed live-attenuated *Salmonella typhimurium* vaccines expressing an H5 hemagglutinin gene from a highly pathogenic avian influenza virus which was codon-optimized for expression in *Salmonella*. To ensure accurate expression of the hemagglutinin protein in bacterial strains, the HA gene was codon-optimized based on *S. typhimurium* LT2 codon usage which resulted in full-length protein expression and 100% amino acid identity with the hemagglutinin protein from the HPAI A/whooper swan/Mongolia/3/2005 (H5N1).

We demonstrated by serology that the recombinant HA protein was expressed in bacteria both *in vitro* and *in vivo*. The recombinant HA protein was found largely in the insoluble fractions of bacterial lysates after induction by anaerobic conditions, indicating the formation of inclusion bodies within cells. It has been hypothesized that despite the importance of glycosylation for antigen recognition, absence of these branching structures may unmask antigenic sites usually hidden, thereby increasing the variety of antibodies produced against the antigen [27]. Despite the lack of eukaryotic glycosylation and the formation of inclusion bodies, the recombinant HA protein was recognized by polyclonal chicken anti-turkey/Wisconsin/68 (H5N9) antiserum in both a hemagglutination assay, and in Western blots. That immunized birds produced partially protective immunity to challenge indicates that folding of the protein in the bacterial expression system was adequate to provide enough epitopes capable of inducing a protective antibody response, and justifies codon-optimization of the HA1 gene sequence.

The use of live-attenuated *Salmonella* to deliver recombinant antigens decreases potential pathogenicity while taking advantage of the ability to invade intestinal cells and deliver expressed antigen directly to the immune system [12,15]. In culturing feces after inoculation, we did not find evidence of persistent colonization of the liver or spleen of SPF chickens when the organs were cultured 14 days after oral inoculation with either of the attenuated *S. typhimurium* strains; BRD509, or LB5010, in contrast to prior results in mice [8,13].

Live bacterial vectors have been shown to induce systemic immune responses in chickens and mice, inducing humoral, cellular, and mucosal immunity against a variety of pathogens [7–9,28]. *In vivo* expression of antigens inside M-cells, combined with antigen processing and presentation by macrophages present in the Peyer's patches, should generate dual immunity by activating both the humoral and cell-mediated arms of the immune system [28]. Differences in survival post-challenge between the two *Salmonella* vaccine groups may be due in part to differences in attenuation of these strains, which resulted in differences in invasion and survival of the strains in macrophages. The importance of *in vivo* expression of antigen inside the M-cells was demonstrated by the reduced anti-HA titer, and lack of survival after viral challenge, for chickens in the control group vaccinated with *E. coli* containing the expression plasmid, as *E. coli* lacks the ability to invade and survive in the M-cells. We demonstrated that both serum antibody responses and mucosal immune responses were produced against HPAI by oral vaccination of chickens with live-attenuated *Salmonella* expressing an AI HA antigen.

The challenge viruses, A/whooper swan/Mongolia/3/2005 (H5N1) and A/chicken/Queretaro/14588-19/95 (H5N2) were determined to have 87.3% deduced amino acid sequence similarity in their HA1 genes. These two viral strains represent the North American, and Eurasian lineages of highly pathogenic H5 avian influenza viruses, respectively [29]. It has been shown previously that inactivated North American lineage H5N2 avian influenza viral vaccines can protect chickens from HP Asian H5N1 AI viruses [29]. Oral administration of *S. typhimurium* LB5010 or BRD509 containing expression plasmid pTETnir15HA1 afforded complete clinical protection against low dose challenge with A/chicken/Queretaro/14588-19/95 (CQ95), and partial protection against low dose challenge with A/whooper swan/Mongolia/3/2005 (WM05). Neither vaccine afforded protection to chickens when challenged with high doses of either of these HPAI viruses, although survival was better against challenge with CQ95, which has a longer mean death time (4 days), compared to the 2-day mean death time of WM05. The longer mean death time may have allowed vaccinated chickens more time to mount an anamnestic immune response. Although it has been reported that closely matching the vaccine hemagglutinin to the challenge virus HA can significantly reduce the amount of virus shed from the oropharynx, we did not see any correlation between the similarity in HA sequence and reduction in either oral or cloacal shedding of challenge virus at the peak of viral shedding, 2 days post-challenge [3]. Induction of cellular immunity by $CD8^+$ T-cells can promote viral clearance, and is important in controlling influenza infection due to the faster response of this arm of the immune system [28]. It is possible that we did not stimulate adequate cellular immunity, resulting in the incomplete clinical protection observed.

Live-attenuated *Salmonella* vaccines could have potential for application in the poultry industry, where inexpensive mass immunization can be achieved through drinking water or spray cabinet administration. Because serum antibodies will be produced only against the viral hemagglutinin when using such a recombinant vaccine, this strategy may prove useful when trying to distinguish between immunized and naturally infected birds in a disease outbreak situation (DIVA). Live vaccines may also be advantageous when used in a prime-boost vaccination program. Using a live vaccine to boost birds previously primed with a killed vaccine may provide for production of cellular and mucosal immune responses in addition of serum antibody production. Mass production of bacterially expressed HA protein by fermentation in *E. coli* could be used to provide ample recombinant protein for incorporation into traditional emulsified oil-in-water vaccines, or may provide an inexpensive and safer source of viral protein for use in the development of diagnostic assays, without having to address safety issues common to protocols for viral inactivation.

In conclusion, we have demonstrated that oral administration of live-attenuated *Salmonella* expressing a codon-optimized HA sequence by can produce both serum antibody and mucosal antibody responses, and provide partial protection against challenge with highly pathogenic H5 avian influenza viruses of both North American and Asian lineage in chickens.

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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.vaccine.2010.04.009.

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Influenza Vaccines for Avian Species

Darrell R. Kapczynski and David E. Swayne

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Abstract Beginning in Southeast Asia in 2003, a multinational epizootic outbreak of H5N1 highly pathogenic avian influenza (HPAI) was identified in commercial poultry and wild bird species. This lineage, originally identified in Southern China in 1996 and then Hong Kong in 1997, caused severe morbidity and mortality in many bird species, was responsible for considerable economic losses via trade restrictions, and crossed species barriers (including its recovery from human cases). To date, these H5N1 HPAI viruses have been isolated in European, Middle Eastern, and African countries, and are considered endemic in many areas where regulatory control and different production sectors face substantial hurdles in controlling the spread of this disease. While control of avian influenza (AI) virus infections in wild bird populations may not be feasible at this point, control and eradiation of AI from

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commercial, semicommercial, zoo, pet, and village/backyard birds will be critical to preventing events that could lead to the emergence of epizootic influenza virus. Efficacious vaccines can help reduce disease, viral shedding, and transmission to susceptible cohorts. However, only when vaccines are used in a comprehensive program including biosecurity, education, culling, diagnostics and surveillance can control and eradication be considered achievable goals. In humans, protection against influenza is provided by vaccines that are chosen based on molecular, epidemiologic, and antigenic data. In poultry and other birds, AI vaccines are produced against a specific hemagglutinin subtype of AI, and use is decided by government and state agricultural authorities based on risk and economic considerations, including the potential for trade restrictions. In the current H5N1 HPAI epizootic, vaccines have been used in a variety of avian species as a part of an overall control program to aid in disease management and control.

1 Introduction

Avian influenza (AI) is a viral disease of birds that remains an economic threat to poultry throughout the world. AI viruses are classified in the family Orthomyxoviridae, genus Influenza A (Type A), and contain a segmented, negative-sense RNA genome (Swayne and Halvorson 2003). Type A influenza virions are enveloped, spherical to pleomorphic in shape, and approximately 80–120 nm in size (Swayne and Halvorson 2003). Type A influenza virus genomes consist of eight segments, which code for at least ten viral proteins, including the hemagglutinin (HA), matrix (M1), membrane-bound ion channel-like protein (M2), neuraminidase (NA), nucleocapsid (NP), nonstructural protein 1 (NS1), nuclear export protein (NS2), and three proteins associated with polymerase activity (PA, PB1, and PB2). The lack of proofreading mechanisms of the viral RNA polymerase results in a viral genome that is highly variable, with mutations readily occurring in the HA and NA genes. Antigenically, 16 HA (H1–H16) and 9 NA (N1–N9) subtypes have been described (Ada and Jones 1986; Fouchier et al. 2005; Lambkin and Dimmock 1996). The segmented nature of the virus genome allows for reassortment of genes when a susceptible host is coinfected with different influenza viruses. Thus, influenza viruses with up to 144 possible combinations of HA and NA subtypes can be isolated from birds.

Two clinical forms of the AI virus can be seen, including the less severe low pathogenic (LP) form that causes mucosal infections with variable morbidity and mortality, and the high pathogenic (HP) form, which causes high morbidity and mortality in gallinaceous poultry. A major virulence factor associated with the different clinical forms of AI is the presence of basic amino acids in at the HA fusion cleavage site. LPAI isolates do not contain multiple basic amino acids at this site or long insertions of extraneous nucleic acids, thus limiting their replication to

mucosal tissues. The presence of multiple basic amino acids found in HPAI isolates allows the virus to replicate in deeper tissues, resulting in increased virulence.

Wild aquatic birds are a primary natural reservoir for the LPAI viruses. The LPAI virus replicates in the intestinal tracts of these birds and is spread primarily by fecal contamination of the water environment. Although wild aquatic birds do not normally get sick from AI, they have on occasion transmitted the virus to domesticated birds, including chickens, ducks, and turkeys (Halvorson et al. 1985). Within the last several years, outbreaks of H5N1 HPAI in Southeast Asian, European, African, and Middle Eastern countries have resulted in one of the most severe animal disease outbreaks in recent history, with hundreds of millions of wild birds and poultry dead or depopulated (Capua and Marangon 2007a).

In the poultry industry, vaccines have been used for decades to combat production losses due to various infectious organisms. Some of these pathogenic organisms are reportable to the World Organization for Animal Health (OIE), including H5 and H7 HP and LPAI viruses, and thus their isolation in poultry has been linked to significant economic trade implications. Although infection with HPAI viruses can result in devastating morbidity and mortality of infected flocks, the use of vaccines to control AI in commercial poultry operations has been uncommon in most developed countries. In fact, extraordinary circumstances are generally required before approval is given to use H5 or H7 AI vaccines in the field. In the USA, the legal authority to conduct and implement H5 and H7 AI vaccination programs requires both state and federal approval. AI eradication programs, which include vaccination, have been previously used to control outbreaks of H5 or H7 AI in the USA (H7N3 and H7N2 LPAI), Mexico (H5N2 LPAI and HPAI), Italy (H7 LPAI), Hong Kong (H5N1 HPAI), Indonesia (H5N1 HPAI), Pakistan (H7N3 HPAI), Russia (H5N1 HPAI) and Vietnam (H5N1 HPAI) (Capua and Marangon 2007b; Sims 2007; Swayne 2003; Swayne and Halvorson 2008; Swayne and Kapczynski 2008; Villareal 2006).

1.1 History of Avian Influenza Vaccines

Avian influenza vaccines have their origins back in the late 1920s, when chickens infected with fowl plague virus (now known as HPAI virus) recovered from the disease and were resistant to re-exposure (Beaudette et al. 1932; Todd 1928). Initially, experimental vaccines against fowl plague were based on the experiences of Pasteur in rabies, who used spinal cords from infected animals as vaccines to protect against virulent rabies virus. Although many AI vaccine failures were observed, either from lack of immune response or inefficient viral inactivation, efficacious AI vaccines were eventually produced that protected birds from disease. However, by that point, the policy of "stamping out" HPAI virus infections of poultry through culling and depopulation in order to control the spread of disease had gained support throughout Europe and vaccines were generally not used as part of any control strategy.

More recently, vaccines have been developed and approved for use against LPAI virus infections of poultry. Beginning after the mid-1960s, when the economic impact of LPAI virus infections in poultry was realized, control strategies were implemented based on economic need. Early field management strategies included controlled exposure of pullets to LPAI viruses in order to produce immunity prior to egg lay (D. Halvorson, personal communication). In the USA beginning in 1979, AI vaccines were primarily used to prevent production losses in turkeys and egg-laying chickens (breeders and table-egg production). In the past decade, H1N1 and H3N2 swine influenza virus infections of turkeys have resulted in significant decreases in egg production and quality (McCapes and Bankowski 1987; Price 1981) (E. Gonder, personal communications). However, because these are low pathogenic isolates, limited conditional-use inactivated vaccines have been used in such turkey flocks as a management strategy. The first LPAI vaccine, a H4 and H6, was used in 1979 in the USA (Swayne and Kapczynski 2008). A multivalent inactivated AI vaccine containing H5N2, H6N2, and H10N2 along with Newcastle disease virus (NDV) was reported to have been used in Italy in 1980 to control multiple subtypes of LPAI virus (Zanella et al. 1981). Finally, following an outbreak of H7N2 LPAI in an isolated commercial chicken facility containing laying hens in Connecticut, agreements between state, federal, and industry representatives allowed the use of an inactivated LPAI vaccine as part of a comprehensive strategy to control the virus as an alternative to immediate depopulation (Adriatico 2005). As a part of the control strategy, vaccinated flocks were intensively monitored for virus shedding through dead bird testing, and serological surveillance using non-vaccinated sentinels and a neuraminidase (NA)-based DIVA (differentiation of infected from vaccinated animal) approach to detect infections in vaccinated birds. Taken together, vaccination can now be considered a valuable component in comprehensive AI control strategies. While situational or local outbreaks of HPAI may always require stamping out in nonendemic areas, in the face of an epizootic event, vaccines can be formulated and used based on field isolates recovered.

2 Avian Influenza Vaccines

Many different types of experimental AI vaccines have been described, and some have been licensed for commercial use (Swayne and Halvorson 2003; Swayne and Kapczynski 2008). Categories of vaccines include the following: inactivated, live, subunit, recombinant vectors expressing AI genes, and DNA vaccines. While many of these vaccines have been shown to induce protective immunity in the laboratory under optimal conditions, the final proof of protection and efficacy is still derived from field application. For field use, the overwhelming majority of AI vaccines produced and sold have been oil emulsion inactivated whole AI virus vaccines delivered via the parenteral route (subcutaneous or intramuscular) and, less frequently, recombinant vectored vaccines.

2.1 Protective Antigenic Component of Avian Influenza Vaccines

Protective immunity in poultry against AI viruses is primarily the result of an antibody response directed against the hemagglutinin (HA), of which there are 16 different subtypes (Swayne and Halvorson 2008). Antibodies produced against the HA are neutralizing and thus prevent attachment of the virus to host cells. When bursectomized chickens (i.e., unable to produce antibody responses) were vaccinated and challenged, no protection was provided, indicating the role of antibodies in protection from AI (Chambers et al. 1988). A minor contribution to immunity is provided against the NA protein, of which there are nine different subtypes (Swayne et al. 2000). Our current knowledge of protective immunity against AI is derived from experimental HPAI challenge studies (Alexander and Parsons 1980; Allan et al. 1971; McNulty et al. 1986; Rott et al. 1974), in which the NA in a whole AI virus vaccine provided mostly partial protection from mortality. In addition, immunization with NA protein alone produced only partial protection following 2–3 vaccinations (Sylte et al. 2007). AI vaccines are generally custom-made against the specific HA subtype and/or NA subtypes of the current field virus. Because protection is provided through an immune response to the HA, the more efficacious vaccines target the specific phylogenetic lineages of the virus within a HA subtype (Fig. 1). Tumpey et al. (2004) showed that antigenically related H7 AI viruses provide protection in turkeys through decreased challenge virus shedding (Table 1). In addition, H5 vaccines have been demonstrated to provide broad protection against diverse H5 viruses when the vaccine and challenge virus vary by up to 13% in HA-deduced amino acid sequence (Swayne et al. 1999).

Vaccines composed of internal AI proteins, including the matrix or nucleoprotein, did not provide significant protection in poultry, although a measurable immune response occurred (Brown et al. 1992; Webster et al. 1991). Because many different serotypes of AI viruses exist through different combinations of the HA and NA (potentially 144), there is no one universal vaccine which will protect against all AI viruses. However, because the M2 transmembrane protein is highly conserved between both avian and human influenza isolates, current research on human influenza is targeting this protein in the hope of producing such a vaccine (Huleatt et al. 2008; Watanabe et al. 2008). In mice, vaccination with the ectodomain of M2 (M2e) and hepatitis B virus core protein (HBc) resulted in protection against homologous and heterologous human influenza A virus challenge (De Filette et al. 2005, 2006). In a limited study, partial protection was provided following two vaccinations of chickens with a recombinant salmonella vaccine expressing the M2e against LPAI virus (D. Kapczynski, unpublished data). The mode of protection appeared to be humoral; purportedly the antibodies interfere with the ion-transport function of the protein required for virus uncoating. However, this type of vaccine is likely years away from any potential field application, and so, before adoption, there must be a demonstration of consistent protection that is comparable to HA-based vaccines.

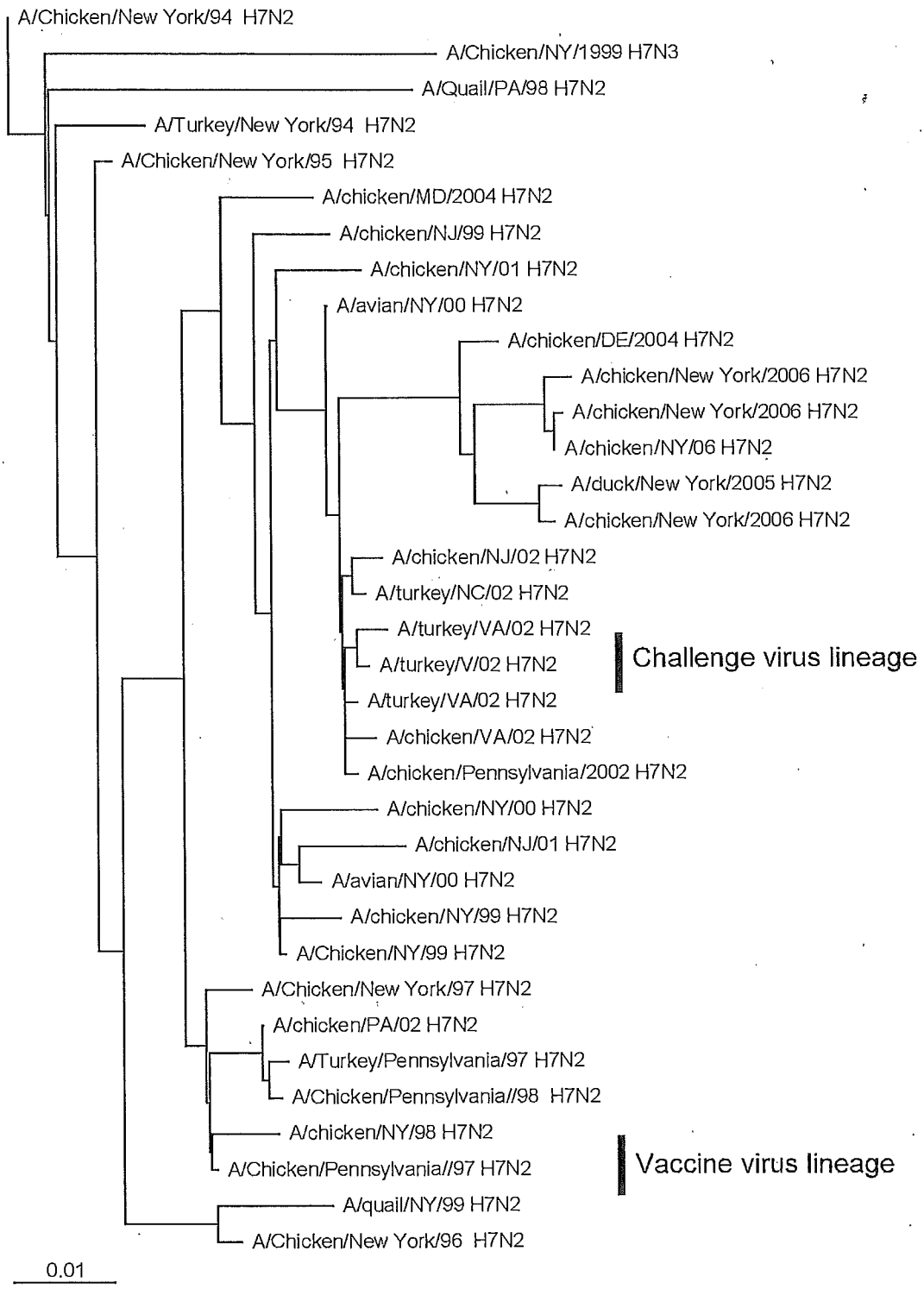

Fig. 1 Phylogenetic analysis of the HA gene of H7N2 subtype AIVs isolated in North America. The tree was generated by the neighbor-joining method. The names of the states where the viruses were isolated are represented by their standard two-letter postal codes

Limited data exist on the contribution of cellular immunity to protection against AI in birds. In mice, live influenza virus vaccines induce both humoral and cellular immunity, but the overall contribution of the cellular aspect to protection remains

Table 1 AI vaccine protection, as measured by the decrease in viral shedding following vaccination of eight-week-old turkeys with a commercial AI H7N2 oil emulsion vaccine

Inactivated vaccine dose	Viral titer from oropharyngeal swabs	
	2 days postchallenge[a]	4 days postchallenge
Sham control	6.4[A]	5.6[A]
H7N2 1X	4.0[B]	2.5[B]
H7N2 2X	2.8[B]	2.3[B]

[a] Virus end-point titers are expressed as mean $\log_{10}$ EID_{50} per ml. Numbers with different uppercase letters indicate significant differences between the H7N2 vaccinated groups and the sham-control group by analysis of variance

Turkeys received a single (at four weeks of age) or double (boost at six weeks of age) injection with a commercial AI vaccine H7N2 (A/chicken/PA/97), applied according to the manufacturers recommendations. Birds were challenged intranasally with 10^7 EID_{50} at eight weeks of age with a current H7N2 field isolate (A/turkey/VA/02). Taken from Tumpey et al. (2004)

unknown, since it takes 5–7 days for virus-specific CD8+ thymocyte-derived T cells to migrate and localize in lungs (Lawrence et al. 2005). However, the benefit of a secondary cellular response against influenza virus resulted in decreased duration and amount of viral shedding, which could reduce transmission potential to susceptible cohorts and which decreases severity of disease (Flynn et al. 1998). In chickens, Seo and Webster reported that adaptive transfer of AI-primed CD4+ and CD8+ T cells protected birds against lethal H5N1 challenge (A/chicken/Hong Kong/97), suggesting that cellular immunity alone could protect against HPAI virus challenge (Seo and Webster 2001). However, because recent H5N1 HPAI viruses caused rapid, sudden mortality (mean death times of 2–3 days), and mobilization of memory T cells to target organs may take 3–10 days, protection from disease may not be reasonable without a supporting innate and/or humoral immune response. Vaccine-induced cellular immunity requires either active viral replication in host cells for major-histocompatibility complex (MHC)-class I presentation or a recombinant-vectored or DNA vaccine expressing AI genes in host cells. However, the use of live AI virus vaccines in poultry is universally discouraged because of the potential for reassortment with genes from other AI viruses (Swayne and Halvorson 2008). While inducing both humoral and cellular immunity represents the best overall protective qualities in a vaccine, limitations on vaccine formulations capable of inducing cellular immunity and licensure for field application make this an achievable goal only when using non-AI virus vaccines, such as recombinant virus vectors.

2.2 *Inactivated Virus Vaccines*

Inactivated AI vaccines are prepared by first propagating the virus in embryonated chicken eggs, followed by chemical or physical inactivation (Swayne and Kapczynski 2008). The most widely used inactivation chemical is formalin, which crosslinks the viral proteins such that viral replication cannot occur. Other chemicals

such as B-propiolactone or binary ethyleneimine can be used as inactivants (King 1991). Inactivated human influenza vaccines are usually detergent treated, which allows for partial purification of the viral surface proteins, HA and NA, into a subunit vaccine. In contrast, AI vaccines for poultry use nonpurified allantoic fluid (i.e., crude preparation), which keeps the production costs lower; however, as well as inducing a protective immune response against HA and NA, it also induces non-neutralizing antibodies against internal viral proteins.

To increase the immunogenicity of inactivated AI vaccines, most are blended with oil to form an emulsion. The emulsions are prepared by mixing a water-based antigen phase with an oil phase, normally containing surfactant for stabilization (e.g., sorbitan monoleate and Tween 80) to produce a water-in-oil emulsion. Without other components, oil-based adjuvants stimulate mainly antibody responses, although under some circumstances water-in-oil emulsions may be able to activate CTLs (Aucouturier et al. 2001; Beck et al. 2003; Folitse et al. 1998; Stone 1986, 1989; Xie and Stone 1990, http://www.seppic.com). The type of oil used in vaccine production can affect the overall immune response to the vaccine such that nonmetabolizable oils (e.g., mineral oil) enhance antibody responses over biodegradable oils (e.g., vegetable) (Stone 1993, 1997). Besides having adjuvant properties, oil emulsion vaccines slowly release antigen over time, resulting in higher immune responses than would be produced by the antigen alone.

Besides oil, different types of adjuvants have been added to vaccine formulations to enhance immune reactions (Fatunmbi et al. 1992; Jansen et al. 2006; Katz et al. 1993; Stephenson et al. 2005). Vaccine adjuvants are chemicals, microbial components, or mammalian proteins that enhance immune responses to vaccine antigens (Vogel 2000). In general, the antigen itself is an inert element which must be taken up by host cells for presentation to the immune system. The adjuvant, which represents the stimulating signal, is responsible for the activation and mobilization of professional lymphocytes to the vaccine antigen. Many different adjuvants have been described in experimental and commercial vaccines, including aluminum salts, bacterial derivates, cytokines, emulsions, ISCOMS, liposomes, nonionic block copolymers, polysaccharides, and saponins (Aucouturier et al. 2001; He et al. 2002; Hu et al. 2001; Roth 1999; Sambhara et al. 2001; Schijns 2003). Despite their extreme relevance and the demand for novel vaccine adjuvants, the various processes explaining how the immune system "knows" when to become activated by the adjuvant remain unknown (Schijns and Tangeras 2005).

Both homologous and heterologous inactivated AI vaccines have been produced. Homologous vaccines contain the same HA and NA subtypes as the field virus, whereas heterologous vaccines contain a matched HA with a different NA subtype. Heterologous vaccines can be used in a DIVA strategy (differentiating infected from vaccinated animals) that enables serological screening of flocks to determine if vaccinated flocks become infected from field virus exposure (Capua et al. 2003).

Because of their safety and immunogenicity, inactivated AI vaccines have been used in multiple avian species, including commercial poultry (e.g., chickens, turkeys, ducks, and geese), exotic (e.g., zoo and pet birds) and endangered species (Oh et al. 2005; Philippa et al. 2007). A minimum of two vaccinations, which can

be adjusted based on weight, may be necessary to induce protective immunity, although subsequent boosting is needed in long-lived birds (Bertelsen et al. 2007).

2.3 *Live Virus Vaccines*

No live AI virus vaccines have been approved for use in poultry, although the use of live LPAI vaccines has been studied experimentally in poultry species (Beard and Easterday 1973; McNulty et al. 1986). One example of the latter is a laboratory-passaged, attenuated AI virus that contains a truncation of the NS1 gene in A/turkey/Oregon/1971 (H7N3). In chickens, this virus exhibited decreased replication and attenuation of infectivity (Cauthen et al. 2007). Although this virus has the potential for vaccine use, its safety would still need to be examined in the field because of the potential to reassort with NS1 genes from another AI virus, resulting in a field virus with increased infectivity. Because of this reassortant potential, live LPAI virus strains are not recommended for use as poultry vaccines, especially H5 and H7 subtypes, which have shown the ability to mutate into HP forms. LPAI vaccines may also be associated with increased losses due to respiratory disease caused by viral replication and may spread to surrounding farms (Beard 1981; Lee and Suarez 2005).

2.4 *AI Subunit Vaccines*

Most AI-expressed subunit vaccines are based on expression of the HA gene in animal or plant cells, bacteria, viruses, or yeast (Chambers et al. 1988; Crawford et al. 1999; De et al. 1988; Schultz-Cherry et al. 2000). The resulting protein is then purified and quantified prior to application. Following purification, the antigen can be emulsified in oil with or without adjuvant as described for inactivated vaccines. In studies with mice, yeast-expressed influenza HA and NA protected against lethal challenge (Martinet et al. 1997; Saelens et al. 1999). Other vectors, such as baculovirus, have been used to express HA in cell-culture supernatants, which was utilized for experimental vaccine preparation in poultry (Crawford et al. 1999). One advantage of this vaccine platform is that it can be utilized in a DIVA strategy. However, because of the time and cost involved with production, none of these have yet been commercialized.

2.5 *Recombinant Virus-Vectored Vaccines*

Using recombinant DNA technologies, these vaccines utilize noninfluenza virus vectors to express influenza genes in vivo so as to induce protective immunity in the host (Beard et al. 1992; Boyle and Coupar 1988; Boyle et al. 2000; Bublot et al. 2006, 2007;

Jia et al. 2003; Qiao et al. 2003, 2006; Swayne et al. 2000; Webster et al. 1991). Because these are live viruses, they can provide the benefits of live virus vaccines, including the stimulation of humoral, mucosal, and cellular immunity. Unlike live AI virus vaccines, recombinant vectored vaccines do not have the potential for AI virus gene reassortment, making them safer to apply in the field. However, pre-exposure of the host to the vector may render the vaccine ineffective, as the vaccine virus may not be capable of sufficient replication and thus adequate immunological stimulation. The use of Newcastle disease virus (NDV) as a vectored vaccine for AI has shown promising results (Swayne et al. 2003). However, as most commercial birds are vaccinated multiple times during the course of their lives with NDV vaccines, and progeny contain maternally derived antibodies in ovo, the timing, route of administration and antibody levels for the NDV vector must be considered before the vaccine can be used in the field (Ge et al. 2007; Park et al. 2006; Veits et al. 2006). In China, a commercially licensed NDV-vectored H5 AI vaccine has been used in the field since 2006. Recombinant poxvirus-based AI vaccines have also been licensed and used in the field, most notably in China, Mexico, Guatemala, and El Salvador, against H5 subtype viruses (Bublot et al. 2006; Ge et al. 2007; Swayne et al. 1997, 2000). As with other AI vaccines, matching the HA sequence in the vaccine construct to the field-circulating virus resulted in decreased morbidity and morality, decreased duration of virus shedding, and decreased titer levels shed of challenge virus. A major advantage of recombinant AI vectored vaccines is the ability to rapidly change the AI gene insert into the recombinant backbone to compensate for the drift of the field virus. The production capacity and size of the vaccine market are limiting factors that a manufacturer uses before deciding to make and sell an AI vaccine. Other vectored viruses have been constructed to express AI genes as experimental vaccines, and include infectious laryngotracheitis virus (*Herpesviridae*), vaccinia virus (*Poxviridae*), human adenovirus 5 (*Adenoviridae*), Venezuelan equine encephalitis virus (*Togaviridae*), and retrovirus (*Retroviridae*) (Brown et al. 1992; Chambers et al. 1988; De et al. 1988; Gao et al. 2006; Hunt et al. 1988; Luschow et al. 2001; Schultz-Cherry et al. 2000; Toro et al. 2007; Veits et al. 2003). Finally, the ability to mass administer vaccines to commercial poultry, including spray and drinking water, offer significant cost savings to poultry producers and may improve field usage.

2.6 DNA Vaccines

DNA vaccines containing *E. coli*-derived plasmid DNA expressing viral gene(s), under the control of a eukaryotic promoter, have demonstrated protective immunity in chickens against AI viruses (Fynan et al. 1993; Le Gall-Recule et al. 2007; Robinson et al. 1993; Suarez and Schultz-Cherry 2000). Following vaccine application and cellular uptake, the DNA vaccine is transcribed into RNA and exported to the cytoplasm for translation. The in situ expressed protein antigen can either be processed intracellularly with the resulting peptides bound to MHC class I for cytotoxic T lymphocyte stimulation, or concomitantly released from the cell and taken up by

antigen-presenting cells for MHC class II presentation to stimulate humoral immunity. The ability to elicit both humoral and cellular adaptive immune responses is a major advantage of DNA vaccines, and mimics an active viral infection. Although different viral genes have been tested, the HA appears most protective, since it contains epitopes for inducing virus-neutralizing antibodies. Recently, a small number of DNA vaccines have been licensed for veterinary use, including West Nile virus for use in horses (see http://www.aphis.usda.gov/lpa/news/2005/07/wnvdna_vs.html) and infectious hematopoietic necrosis virus in salmon (see http://www.ah.novartis.com).

2.7 *Efficacy of Avian Influenza Vaccines in Birds*

Under ideal conditions, AI vaccination of commercial poultry would stimulate a protective immune response that would prevent infection and disease following a single dose. Vaccine-induced protection from AI virus challenge is measurable in vivo using various avian models, including those bird species most likely to experience AI virus infection during an epizootic, such as chickens, turkeys, and other avian species. Because initial infection occurs at respiratory or alimentary mucosal surfaces, and parenteral vaccines primarily induce a humoral immune response, sterilizing immunity with complete inhibition of infection is not realistic in the field.

Generally, protection from mortality and morbidity are the easiest measurable (qualitative) outcomes following challenge. In addition, antibody titers (quantitative) following vaccination, but prior to challenge, can be used to predict protection from lethal challenge. It is generally believed that HI antibody titers of 1:32–40 or greater will protect birds against mortality following challenge. Other parameters of protection include decreasing virus shed titers from the respiratory and intestinal tracts following challenge, as well as decreases in the duration of shedding. These parameters can be indirect measures of the efficacy of the vaccine in limiting environmental contamination and thus decreasing transmission to susceptible cohorts (Capua et al. 2004; Swayne 2003; Swayne et al. 1997, 1999). A hundredfold reduction in challenge virus shedding in vaccinated vs. nonvaccinated animals has been recommended as the minimum standard of vaccine-induced immune potency (Suarez et al. 2006; Swayne et al. 1997). In some cases, protection from decreases in egg production and quality is associated with vaccine efficacy (D. Kapczynski, personnel observation) (Fig. 2).

Most vaccine studies involving the protection of birds against both LPAI and HPAI viruses have been performed in chickens and to a lesser extent turkeys and ducks (Cristalli and Capua 2007; Middleton et al. 2007; Philippa et al. 2005; Steensels et al. 2007; Swayne 2006; van der Goot et al. 2005, 2007). Generally, wild waterfowl are less susceptible to mortality following HPAI virus infection. However, the most recent H5N1 isolates of Southeast Asian origin have demonstrated high virulence for young domestic waterfowl, which have been identified as contributors to the spread of the disease (Hulse-Post et al. 2005). For this reason, it is important to establish

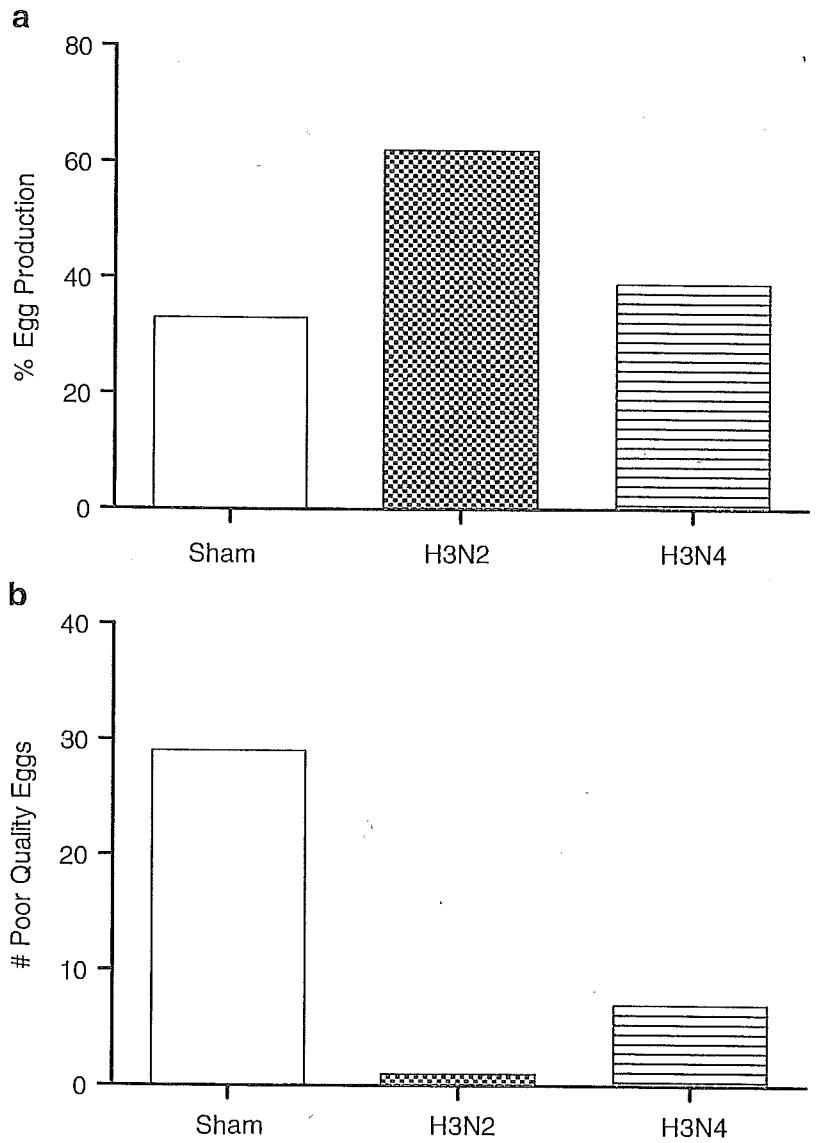

Fig. 2a–b AI vaccine protection against egg production losses in vaccinated turkey breeder hens following AI H3N2 challenge. Commercial egg-laying turkey hens received two doses of autogenous killed oil emulsion vaccine containing either H3N2 (A/turkey/North Carolina/05) or H3N4 (A/duck/Minnesota/79/79) at 26 and 30 weeks of age, followed by a challenge with a recent H3N2 field virus (A/turkey/NC/16108/03). Hens receiving phylogenetically matched H3N2 vaccine displayed increased production (**a**) and egg quality (**b**) over sham and H3N4-vaccinated hens

vaccine potency and efficacy for domestic ducks and other minor poultry species. Although the immune system of chickens is the best studied of that of any bird species, ducks are a distant second in terms of anatomical and immunological understanding and knowledge. While many features of their immune systems are similar, one dramatic anatomical difference is the presence of lymph nodes in ducks, which are absent in chickens. The overall impact of lymph nodes in vaccine-induced protection is unclear, although at least one study suggests that ducks may require twice the antigen content to produce a protective immune response when compared to chickens

(Middleton et al. 2007). All vaccinated ducks were protected from disease, and very few (2/15) shed virus at day 4 postchallenge. However these differences (and likely others) in other bird species underscore the need to evaluate a vaccine's potency and efficacy in each host species before licensing. Extrapolating the data generated in one species and applying it to another should be pursued with caution. Each species has distinct differences in immune response that impact on the protection induced by vaccines.

In the event of an avian influenza epizootic, zoo birds would constitute a potential source of infection and transmission among wild birds and poultry, and would likely need prophylactic vaccination. Only a few studies have examined the use of AI vaccination in zoo birds. Within the European Union (EU), following an outbreak of H7N7 HPAI in poultry, vaccination with an inactivated H7N1 virus was undertaken at a number of zoological parks (Philippa et al. 2005). Of the 211 birds vaccinated, 81.5% seroconverted (HI ≥ 1:40) after two doses of a commercial inactivated H7 AI poultry vaccine, with birds of the orders Anseriformes, Galliformes, and Phoenicopteriformes exhibiting better responses than birds in other orders. In Singapore during 2005, inactivated H5N2 vaccine was applied to zoo birds following a H5N1 HPAI outbreak, with 84% of birds exhibiting seroconversion after two doses of commercial inactivated H5N2 AI vaccine (Oh et al. 2005). A similar study utilizing commercial inactivated H5N2 virus vaccine in 334 zoo birds resulted in the seroconversion of 80.4% of birds (Philippa et al. 2007). Finally, a third research study with commercial inactivated H5N9 in zoos produced an 84% seroconversion rate among all bird orders tested, with black winged stilts, parrots, Congo peafowl, rheas, ibis, flamingos, and kookaburras displaying greater seroconversion rates than penguins, pelicans, ducks, geese, herons, Guinea fowl, and cranes (Bertelsen et al. 2007). In that study, the dose of commercial vaccine was applied based on body weight (1–99 g: 0.1 ml; 100–2,999 g: 0.3 ml; 3–20 kg: 0.6 ml; >20 kg: 1 ml), which may have influenced some of the differences observed in seroconversion; however, it underscores the need to adjust the antigenic load based on bird weight.

2.8 *Scenarios for Avian Influenza Vaccine Application*

Several situations lend themselves to the application of vaccines for AI control. Although not every outbreak requires the use of vaccine, in the event of an epizootic, vaccines must be safely and effectively applied to curb the spread of the virus in susceptible birds. When vaccination campaigns begin, an increased level of biosecurity must be practiced to reduce the risk of personnel mechanically spreading the virus from farm to farm on their shoes and clothing or by moving the virus as fomites on equipment and supplies. In addition, a planned eradication program must be developed, as well as surveillance and monitoring of all vaccinated flocks. An exit strategy for withdrawal of vaccine use should be developed to avoid overdependence on vaccination for control and to allow true eradication of the virus from poultry sectors. Vaccination can be used in three different ways as part of an

AI control strategy, including prophylactic or preventive vaccination, endemic (routine) vaccination, and emergency (outbreak) vaccination (Marangon et al. 2008).

Prophylactic vaccination is applied when an area has high risk for the introduction of H5 or H7 virus to susceptible bird species. Under this scenario, a country, geographical zone or production compartment that is free of AI may be bordered by an AI outbreak zone. The use of preventive vaccine increases the resistance of poultry to infection and reduces the risk of introduction within the country, zone, or compartment. Although vaccination can decrease the potential for virus introduction, this is highly dependent upon the population immunity within the area. Appropriate surveillance measures, including either sentinel bird placement, DIVA testing, and/or appropriate diagnostics, are needed to assess the effectiveness of vaccination and to detect any potential virus introductions, because vaccination could mask the clinical detection of infection and allow the potential spread of AI virus. In the event of virus introduction, prophylactic vaccination will decrease the duration and level of virus shedding, resulting in decreased risk of secondary outbreaks, but biosecurity is necessary in all vaccinated flocks to make vaccination an effective tool in AI control (Capua and Marangon 2007a; Swayne and Kapczynski 2008).

When HPAI is endemic in a country, vaccination can be applied as an overarching control measure to reduce environmental load and spread, in preparation for future stamping out of the disease. In this situation, all birds that can be accessed should be vaccinated, with multiple doses applied throughout the period of potential virus exposure. The goal is to increase flock immunity to the level of 60–80%, which should prevent the spread of the virus between well-vaccinated flocks and allow the eradication of the virus within the area. However, it may also be necessary to apply stamping-out procedures to infected flocks to reduce the spread of the virus and prevent the development of a reservoir. Surveillance and increased biosecurity of all bird species and premises, including poultry flocks, waterfowl, and at-risk zoo birds, are critical to stamping out the disease from endemic areas. Constant veterinary medical vigilance, supported by government, commercial, and local officials, is needed to achieve this goal.

Emergency vaccination may be needed when a HPAI epizootic is spreading rapidly, causing large numbers of outbreaks on farms with susceptible poultry within a defined area or country, and may be necessary to contain the disease and reduce the risk of further virus spread. An initial step would be to establish a movement control zone around the index case and all noninfected birds; within the ring, all birds would be vaccinated or euthanized. Outside the primary ring, a secondary ring would be established, and all birds within that ring would be vaccinated and monitored for presence of virus. Because immunity may require 10–14 days to develop postvaccination, a rapid decision among government officials, along with input from local and commercial entities, is required for the emergency release of vaccines. Preplanning among officials is critical for the successful implementation of emergency vaccination due to inherent delays in vaccine application. Such planning should include (1) assessments of the number of vaccine doses on hand, (2) vaccine production, (3) mobilization of vaccination crews, (4) establishing routes of vaccination crews, (5) surveillance measures and reagents, and (6) development of

the administrative control structure. While emergency vaccination may be considered a worst-case scenario for any country, the decision to implement the procedure must be developed based on cost–benefit ratios for each situation.

3 Conclusions

AI vaccines provide protection by stimulating host immunity, which is largely based on antibody production against the HA glycoprotein. These antibodies are capable of neutralizing the virus, thereby preventing infection, and reducing disease and virus transmission. A successful vaccine campaign will use a vaccine of the same HA subtype as that of the field virus. The establishment of neutralizing antibodies prior to HPAI virus infection is critical for disease control because these viruses induce rapid mortality within days of exposure and the immune system of the nonimmune bird does not have time to mobilize an efficient defense against the virus. Although many AI vaccine constructs have been described, only two are licensed for use in the field: inactivated AI virus and recombinant vectored vaccines. Until recently, inactivated vaccines were prepared from historically available LPAI virus isolates which grew to high titers in embryonating chickens eggs and required low biocontainment during manufacture because of the low escape risk. However, reverse genetic techniques are creating vaccine viruses with HA glycoproteins that are genetically a closer match and that have high growth properties in eggs but none of the safety concerns associated with of HPAI viruses. To standardize vaccine potency, quantification of HA per dose or seroconversion postvaccination can be used. Minimal protective titers described for birds should be >1:32–40. However, new research is needed to determine if these levels are adequate for all bird species, as only limited information is available on vaccine efficacy and protection outside of commercial chickens.

Avian vaccines applied during an AI event can be utilized as an integrated component of a comprehensive control strategy to limit the spread of the virus within animal and human populations and provide a safe, economical food source for the world's population. The strategic objectives should also include surveillance, biosecurity and quarantine, and education. While no single strategy exists to cover all outbreak situations for all countries and all susceptible bird species, appropriate programs should be developed by government officials, veterinary researchers, commercial industry and local entities to provide the leadership and science-based knowledge needed to limit the impact of AI influenza.

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Review

Newcastle disease: Evolution of genotypes and the related diagnostic challenges

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ABSTRACT

Since the discovery of Newcastle disease virus (NDV) in 1926, nine genotypes of class I viruses and ten of class II have been identified, representing a diverse and continually evolving group of viruses. The emergence of new virulent genotypes from global epizootics and the year-to-year changes observed in the genomic sequence of NDV of low and high virulence implies that distinct genotypes of NDV are simultaneously evolving at different geographic locations across the globe. This vast genomic diversity may be favored by the large variety of avian species susceptible to NDV infection and by the availability of highly mobile wild bird reservoirs. The genomic diversity of NDV increases the possibility of diagnostic failures, resulting in unidentified infections. Constant epidemiological surveillance and pro-active characterization of circulating strains are needed to ensure that the immunological and PCR reagents are effective in identifying NDV circulating worldwide. For example, in the United States, the widely used real-time reverse transcription polymerase chain reaction (RRT-PCR) matrix gene assay for the identification of NDV often fails to detect low virulence APMV-1 from waterfowl, while the RRT-PCR fusion gene assay, used to identify virulent isolates, often fails to detect certain virulent NDV genotypes. A new matrix-polymerase multiplex test that detects most of the viruses currently circulating worldwide and a modified fusion test for the identification of virulent pigeon viruses circulating in the U.S. and Europe have recently been developed. For newly isolated viruses with unknown sequences, recently developed random priming sequencing methods need to be incorporated into the diagnostic arsenal. In addition, the current system of classifying NDV into genotypes or lineages is inadequate. Here, we review the molecular epidemiology and recent diagnostic problems related to viral evolution of NDV and explain why a new system, based on objective criteria, is needed to categorize genotypes.

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1. Newcastle disease (ND)

Newcastle disease (ND) results from infections with virulent Newcastle disease viruses (NDV), having intracerebral pathogenicity indices (ICPI) of ≥0.7 in day-old chickens (*Gallus gallus*) and/or

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having multiple basic amino acids (at least three arginine (R) or lysine (K) residues) at the C-terminus of the fusion protein cleavage site, starting at position 113, along with a phenylalanine at position 117 (OIE, 2009). NDV potentially infects most species of birds, and for susceptible poultry it is highly contagious and usually fatal (Alexander, 1988). This disease is one of the most important infectious diseases of poultry because of its worldwide distribution and the potential for devastating loses. It occurs on at least six of the seven continents of the world and is enzootic in many countries. In the United States (U.S.) virulent NDV (vNDV) strains are classified as select agents. These strains are not endemic in poultry and disease caused by them is often referred to as exotic Newcastle disease (END) (USDA, 2006). Worldwide, outbreaks of vNDV are a constant threat to poultry with clinical infection confirmed in 2008 from the Dominican Republic, Belize, Peru, Finland, Germany and Japan and likely other outbreaks not reported from countries throughout Africa, and Asia with endemic vNDV.

Clinical manifestation of ND in chickens varies significantly among isolates. Infection with virulent viruses causes three well-defined clinical forms: (1) viscerotropic velogenic, which is characterized by acute lethal infections, usually with hemorrhagic lesions in the intestines of dead birds, (2) neurotropic velogenic, which is characterized by high mortality following respiratory and neurological disease, but where gut lesions are usually absent, and (3) mesogenic, which causes respiratory and neurological signs, but low mortality. The presence of virulent strains (velogenic and mesogenic strains) in poultry requires monitoring and control measures even in countries where they are endemic because the existence of the virus severely impacts commercial productivity and the international trade in poultry and poultry products. Low virulence NDV (loNDV) are often referred to as lentogenic and are endemic in the U.S. In addition to being classified as being of low virulence, causing mild infections of the respiratory tract, lentogens can also be classified as asymptomatic enteric in which replication appears to be primarily in the gut without respiratory signs.

2. Newcastle disease virus (NDV)

Newcastle disease virus, also known as avian paramyxovirus serotype-1 (APMV-1), a member of the genus Avulavirus within the Paramyxoviridae family (Fauquet and Fargette, 2005; Mayo, 2002), is a negative-sense, single stranded, non-segmented, enveloped RNA virus (Alexander and Senne, 2008). The NDV genome is composed of six genes and encodes their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L) (Chambers et al., 1986). RNA editing of the P protein produces additional non-structural proteins V and possibly W (Chambers and Samson, 1982; Collins et al., 1982; Locke et al., 2000; Mebatsion et al., 2001; Steward et al., 1993). While the virulence of NDV is dependent on multiple genes, the fusion protein cleavage site is the critical site responsible for major changes in virulence (de Leeuw et al., 2005; Peeters et al., 1999). The less virulent viruses (loNDV) have fewer basic amino acids at this site and a leucine instead of a phenylalanine at position 117 (Alexander and Senne, 2008). Using reverse genetic techniques it has been shown that the phenylalanine at position 117 and basic amino acids surrounding Q 114 are necessary for virulence (de Leeuw et al., 2003; Glickman et al., 1988).

3. Newcastle disease virus classification

Different genotypes of APMV-1 circulate in different parts of the world. Although all NDV are members of APMV-1, antigenic and genetic diversity is recognized (Aldous et al., 2003; Alexander et al., 1997; Kim et al., 2007a). Two different systems of classifying NDV are currently utilized worldwide with no consensus as to which is more appropriate. A system suggested by Aldous groups NDV into six lineages and 13 sublineages and later, three additional sublineages were added (Snoeck et al., 2009; Aldous et al., 2003). A second system classifies NDV into two major divisions represented by class I and class II, with class I being further divided into nine genotypes and class II into ten when comparing the sequences isolated over time (Fig. 1A and B) (Ballagi-Pordany et al., 1996; Czegledi et al., 2006; Kim et al., 2007b). Both systems of classification are based on similar genomic information therefore discrepancies between the two classification systems are nominal. Fig. 1B, showing class II viruses, illustrates the most significant differences between the two systems. For example, genotypes III, IV, V and VIII are grouped in the Aldous system to create the very large lineage 3. Problems with both nomenclature systems are illustrated in Table 1, which lists the estimates of the average evolutionary divergence among genotypes. The overall distances between genotypes range widely from to 0.041 (between III and IV) to 0.181 (between II and VII) (Table 1). In the case of genotype VI the distances between sub-genotypes VIc and VIf (0.061) or between VIf and VIb (0.054) are larger than the distances between genotype III and IV (0.041) suggesting that genotype VI may need further separation. Estimates of average evolutionary divergence over sequence pairs within genotypes demonstrate the existence of a large variation of distances among viruses within each genotype (Table 2). For example, a distance within genotype IX is 0.004 and it is 0.085 for viruses within genotype III. These wide differences in distances separating genotypes and the lack of any

Table 1
Estimates of evolutionary divergence over sequence pairs between genotypes.

	Genotype									
	II	IIwfl	III	IV	V	VI	VII	VIII	VIb	VIc
I	0.103	0.070	0.067	0.080	0.131	0.115	0.133	0.114		
II		0.111	0.124	0.153	0.176	0.164	0.181	0.166		
IIwfl			0.100	0.122	0.160	0.144	0.147	0.157		
III				0.041	0.100	0.099	0.108	0.090		
IV					0.089	0.082	0.092	0.087		
V						0.075	0.084	0.078		
VI							0.065	0.075		
VII								0.079		
VIc									0.047	
VIf									0.054	0.061

The number of base substitutions per site is shown for genotypes of class II viruses. All results are based on the pairwise analysis of 1003 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA4 (Tamura et al., 2004, 2007). Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 372 positions in the final dataset. wfl = waterfowl isolates also called genotype IIa.

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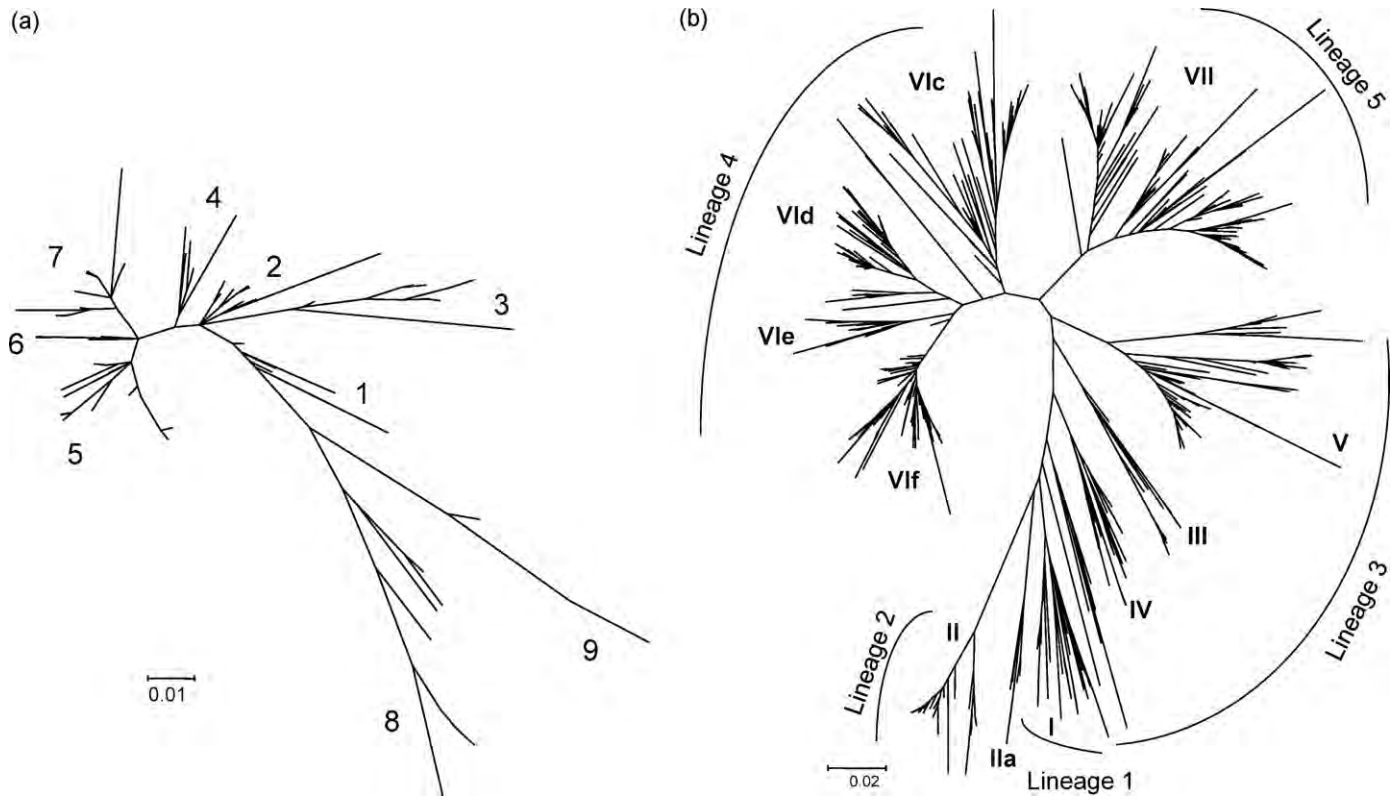

Fig. 1. Phylogenetic trees of Newcastle disease viruses. Tree construction was done using the Neighbor Joining method with the maximum composite likelihood substitution model for the 372 bp region encoding the amino terminal end of the fusion protein (Tamura et al., 2004, 2007). (a) Class I viruses ($n = 216$). (b) Class II viruses ($n = 956$) and genotypes are labeled with arabic numberals for (a) and roman numerals for (b). The sub-genotypes of genotype VI are included. Genotypes VIII, IX, and X for class II viruses are not included.

other additional criteria (e.g. antigenic distances) to separate genotypes suggest the need for a scientific conference to discuss and establish the objective standards that would be needed to create a widely recognized and consistent classification.

In this review, we will utilize the second classification system. APMV-1 viruses have at least three genome lengths; 15,186, 15,192 and 15,198 nucleotides (Czegledi et al., 2006). Class I viruses are avirulent in chickens (except for one known virulent virus) and historically have been recovered from waterfowl (Family Anatidae) and shorebirds (Alexander et al., 1992; Kim et al., 2007a). Class I viruses have the longest of the APMV-1 genomes at 15,198 nucleotides (Czegledi et al., 2006). These viruses comprise at least nine (1–9) genotypes that may be distributed worldwide in wild birds (Kim et al., 2007a) and are frequently isolated in live bird market (LBM) samples (Fig. 1A) (Kim et al., 2007a,b).

Table 2
Estimates of evolutionary divergence over sequence pairs within genotypes.

Genotype	Distance
I	0.079
II	0.027
IIwfl	0.029
III	0.085
IV	0.057
V	0.056
VI	0.076
VII	0.071
VIII	0.068
IX	0.004

The number of base substitutions per site is shown for genotypes of class II viruses. All results are based on the pairwise analysis of 1003 sequences. Analyses were conducted using the maximum composite likelihood method in MEGA4 (Tamura et al., 2004, 2007). Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 372 positions in the final dataset. wfl = waterfowl isolates also called genotype IIa.

Class II viruses have been studied in more detail and comprise ten (I–X) genotypes. The genotypes that are considered "early" (1930–1960) I, II, III, IV and IX contain 15,186 nucleotides (Czegledi et al., 2006). Viruses that emerged "late" (after 1960), V, VI, VII, VIII, and X contain 15,192 nucleotides. Except for recent vNDV responsible for the 1998–2000 Australian outbreak, all other known class II, genotype I viruses are of low virulence and some are often used as live vaccines (chicken/Australia/QV4/1966 and chicken/N. Ireland/Ulster/1967). Class II, genotype II includes viruses of low virulence that are used as vaccine viruses worldwide, such as LaSota, B1 and VG/GA. It also includes the neurotropic virulent chicken/U.S. (TX) GB/1948 (TXGB) isolate, which was isolated in 1948 and is used in the USA as a challenge to show efficacy of ND commercial vaccines before production. In the U.S., in the 1940s and 1950s the above-mentioned vaccines were highly effective against the circulating neurotropic vNDV, such as TX/GB and Kansas-Manhattan strains. In fact, virulent neurotropic viruses of genotype II no longer circulate in the U.S. Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and in Zimbabwe in 1990 (Yu et al., 2001). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi et al., 2006).

Genotypes V, VI, VII, and VIII are the predominant genotypes circulating worldwide and contain only virulent viruses. Genotype

V viruses emerged in South and Central America in 1970 and caused outbreaks in Europe that same year (Ballagi-Pordany et al., 1996). These viruses also caused outbreaks in North America in Florida (1971, 1993) and California (1971, 2002) (Wise et al., 2004a) and are still circulating in Mexico (Perozo et al., 2008). Genotype VI emerged in the 1960s and continued to circulate as the predominant genotype in Asia until 1985 when genotype VII became more common (Mase et al., 2002). Genotype VI is further divided into sub-genotypes VIa through VIg with VIb being commonly isolated from pigeons.

Genotype VII was initially divided in to two sub-genotypes: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous et al., 2003). The two sub-genotypes of VII are further divided into VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa (Bogoyavlenskiy et al., 2009; Wang et al., 2006), and VIIf, g, and h, which represent African isolates (Snoeck et al., 2009). Genotype VIII viruses have been circulating in South Africa since 1960s (Abolnik et al., 2004a) and continue to circulate in Southeast Asia. Genotype IX is a unique group that includes the first virulent outbreak virus from China from 1948 and members of this genotype continue to occasionally be isolated in China (Wang et al., 2006). Genotype X viruses have been isolated exclusively from Taiwan in 1969 and 1981 (Tsai et al., 2004). Fig. 2 illustrates the yearly distribution of genotypes separated by decade based on sequences reported to GenBank. It indicates that multiple genotypes are circulating simultaneously worldwide and reveal an increase in the number of (reported) genotypes circulating at the present time. With the exception of genotype IV, which has not been reported to GenBank since 1989, viruses from most genotypes still continue to circulate at the present time.

4. The evolution of NDV of low virulence

Very little is known about the evolution of NDV of low virulence (loNDV) as most NDV research has been performed on virulent isolates. Globally, loNDV from all genotypes of class I and from genotypes I and II of class II are commonly isolated from domestic poultry and wild bird species (Huovilainen et al., 2001; Jorgensen et al., 1999; King and Seal, 1997; Marin et al., 1996; Rosenberger et al., 1975; Seal et al., 2005; Takakuwa et al., 1998). In the U.S., where vNDV are not endemic in poultry, the primary reason for vaccination is to protect against economic losses from respiratory disease caused by endemic loNDV (Miller et al., 2009a). In addition, if a poultry flock is not uniformly vaccinated with a live NDV vaccine or if another vaccine interferes with the NDV vaccine, the birds sub-optimally vaccinated for ND may "break" with respiratory disease days later. These vaccine reactions are called "rolling reactions." As the virus passes from bird to bird, the respiratory disease seen may be more severe than that originally seen with the initial application of the vaccine (J.A. Smith, personal communication, 04/2009). Although viruses involved in rolling reactions are presumed to be of vaccinal origin, without the genomic characterization of these isolates, the possible involvement of loNDV from wild birds in rolling reactions cannot be discounted.

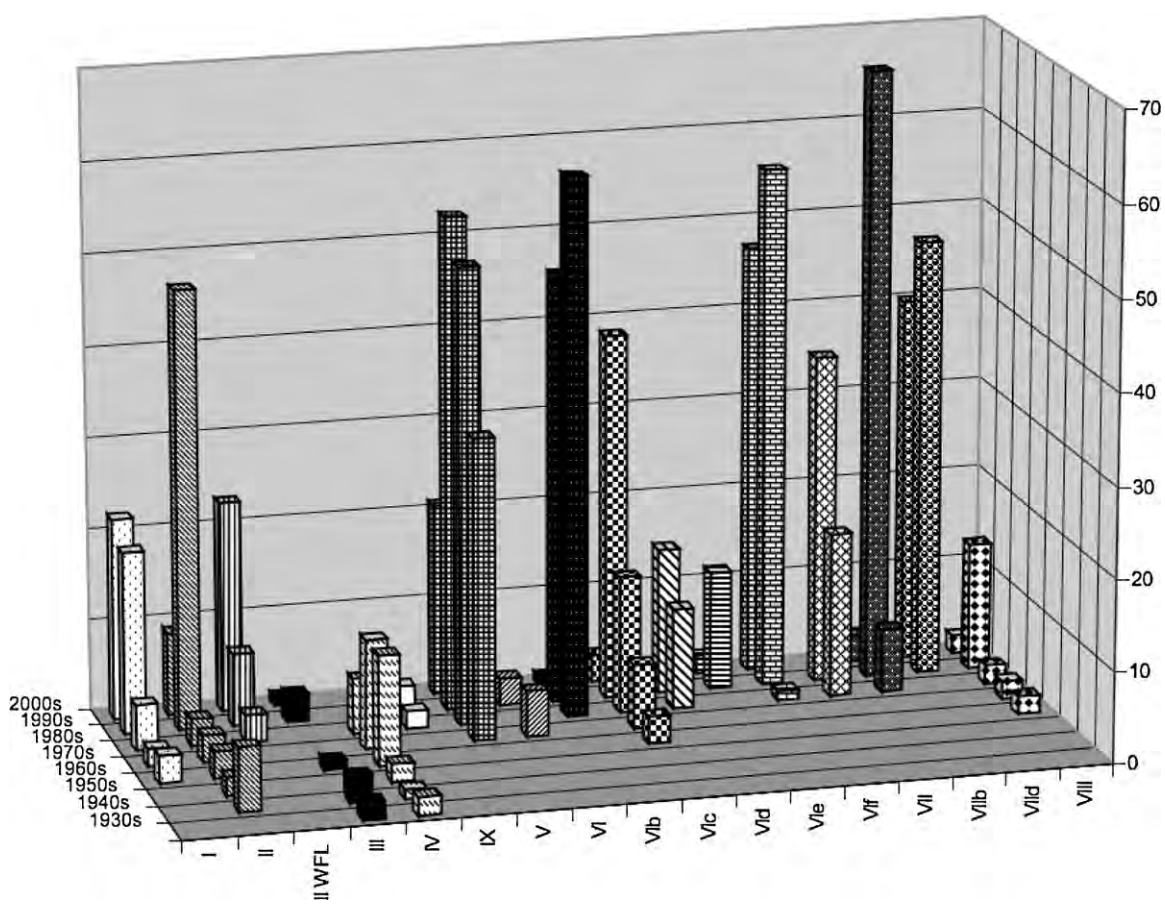

Fig. 2. Class II viruses used in Fig. 1B were sorted by date of isolation and the number of viruses reported to GenBank for each of the genotypes per decade was graphically represented. *X* axis represent genotypes, *Y* represent decades and *Z* represent the number of isolates. The number of virus isolates reported for each genotype per decade: G I (1962–2004) $n = 52$; G II (1945–2001) $n = 78$; G III (1930–2002) $n = 14$; G IV (1933–1989) $n = 37$; G V (1970–2003) $n = 158$; G VI (1968–2002) $n = 343$; G VII (1981–2002) $n = 250$; G VIII (1965–2000) $n = 24$; G IX (1985–1997) $n = 11$. Genotype X is not included.

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Wild waterfowl and shorebirds (WS) are infected with a large and diverse group of avirulent viruses that normally do not produce any clinical signs in poultry. A recent phylogenetic evaluation of avirulent NDV isolates from U.S. LBM and WS found that about 70% of these viruses belong to class I and 30% to class II (Kim et al., 2007a). Almost half of the class I viruses came from the Mallard duck (*Anas platyrhynchos*). In this single species, six of the nine class I genotypes were represented, suggesting that certain species are highly susceptible to NDV infection and likely to be reservoirs. Wild WS were carrier of class II viruses however no virulent virus was present. These class II viruses grouped into genotype I and two new sub-genotypes: IIa and Ia. The Ia viruses were related to viruses endemic to Australia, including the virulent virus isolated from the 1998 Australian outbreak. The IIa isolates grouped with viruses previously isolated from Argentina (AY727881, duck and AY727882, swan) and Spain (AY175642, chicken). None of the WS viruses clustered into genotype II with the common vaccine viruses, B1 and LaSota. This is surprising considering the amount of live virus that is applied annually to poultry in the U.S and worldwide.

As previously described with virulent class II viruses diversity was observed at the antigenic level by comparing monoclonal antibody (mAb) binding patterns among different ND isolates (Alexander et al., 1997). For example, when a panel of nine mAb was tested against 58 U.S. viruses isolated from Anatidae species from 1986 to 1999, seven different binding patterns were revealed (Kim et al., 2007a). Considering that in this study, only avian WS species from the orders Anseriformes and Charadriiformes were studied and that NDV has been documented to infect at least 241 different bird species (Kaleta and Baldeuf, 1988), the existence of NDV with different mAb binding patterns in other birds species is plausible. The broad phylogenetic diversity of NDV discovered in this limited wild bird population emphasized the potential for more genotypes yet to be discovered.

Class I viruses, in general, are viruses of wild birds. The discovery of a large number of class I ND viruses from LBMs in the U.S. and Asia, and the existence of genetically related viruses in wild birds suggests that epidemiological connections exist between these populations. A close phylogenetic relationship was found between some class I LBM isolates and WS isolates collected in the U.S. from 1986 through 2005 (Kim et al., 2007a). Class I viruses were also isolated from LBM domestic poultry in Hong Kong (HK) from 2003 to 2005, suggesting that these viruses were circulating in LBM birds and wild birds worldwide (Kim et al., 2007b). The identification of a class II genotype Ia virus from a 12-week-old domestic turkey in Ontario in 2003 (AY289194), which clustered closely with U.S. shorebird isolates from New Jersey in 2001 (EF564885, EF564816, EF564888–EF564889), may indicate that these viruses occasionally transmit between wild bird and domestic poultry populations. Class II genotype Ia and I viruses have also been isolated previously from chickens at LBM in the U.S. (AY130861, AY626268) (Kommers et al., 2003; Seal et al., 2005).

While NDV isolated from WS are predicted to exhibit low virulence phenotypes, concern exists regarding possible genetic changes from loNDV to vNDV upon replication in poultry. At least one virulent class I virus has been identified as the etiology of an outbreak in the past (Alexander et al., 1992). Virulent viruses of similar genotypes to those found in WS and at LBM have previously been recovered (Alexander et al., 1992; Gould et al., 2001). The 1990 Ireland outbreak was caused by a virulent form of a class I virus (IECK90187/1990; accession AY135757), and the Australian outbreak of 1998–2000 was caused by a virulent class II genotype Ia virus. It was theorized that low virulence viruses circulating in and among the waterfowl of Ireland were transmitted to poultry and mutated to form quasi-species from which a virulent class I virus arose (Collins et al., 1993). Genomic analysis of the class II genotype Ia viruses from the Australian outbreak provided additional evidence that low virulence viruses have the potential to become virulent over time (Gould et al., 2001), and demonstrated that a change in the cleavage site of the fusion protein of the native virus resulted in increased virulence (Gould et al., 2003). Virulent isolates from outbreaks in Australia were shown to be genetically similar to the viruses of low virulence that were known to be previously circulating in the country (Kattenbelt et al., 2006). These endemic low virulence viruses required only two point mutations to become virulent (Westbury, 2001). In addition to field conditions, some loNDV have shown to have the capacity to become virulent under experimental conditions (de Leeuw et al., 2003; Shengqing et al., 2002; Zanetti et al., 2008). Three factors that may increase the risk of an outbreak and predict the need to conduct studies on the evolutionary mechanism affecting NDV genomes are: (1) only a few nucleotide changes in the fusion gene are sufficient to change NDV from low virulence to high virulence, (2) there are large and highly mobile reservoirs of low virulence viruses in nature that may come into contact with poultry, and (3) billions of doses of low virulence live vaccine virus are inoculated into poultry annually, thereby likely releasing the vaccine virus into the environment. For the purposes of disease prevention it would be ideal to be able to predict the potential of each genotype of low virulence NDV to mutate into a virulent form.

Recently, a comprehensive dataset of NDV genome sequences was evaluated using bioinformatics to characterize the evolutionary forces affecting NDV genomes and it was found that there was no evidence of positive selective pressures at the fusion protein cleavage site (Miller et al., 2009a,b). This study demonstrated negative or neutral selective pressure at the fusion protein cleavage site for both vNDV and loNDV. Specifically, regardless of the genotype or the virulence of the virus, the cleavage site motif was highly conserved, implying that changes in virulence were unlikely to occur frequently. Interestingly, the complete coding sequence of the fusion gene of diverse isolates and the complete genomes of viruses from wild birds displayed higher yearly rates of change in virulent viruses than in viruses of low virulence, indicating that higher virulence may confer some other positive selective advantages to NDV (Miller et al., 2009b).

5. Virulent NDV and the role of ND vaccines on their evolution

Although the most likely reservoir of vNDV is the vaccinated poultry population there is evidence that wild birds may represent natural reservoirs of mesogenic viruses (Aldous et al., 2007; Czegledi et al., 2006). Phylogenetically related vNDV of genotype V have been isolated from double-crested cormorants (*Phalacrocorax auritus*) from 1975 through 2008 and they have been implicated in earlier ND outbreaks (Allison et al., 2005; Blaxland, 1951; Heckert et al., 1996). Virulent pigeon paramyxovirus-1 (PPMV-1) isolates, which are clinically neurotropic in chickens, were first isolated in 1981 in pigeons (*Columba livia*), and continue to circulate in feral birds of the Columbidae family worldwide (Kaleta et al., 1985; Kim et al., 2008a; Mase et al., 2009; OIE, 2003). Although an earlier isolation of viscerotropic vNDV was documented in pigeons (Pearson and McCann, 1975), typical pigeon isolates are variants from genotype VIb, that cause neurological signs, have specific monoclonal antibody binding patterns, and often do not hemagglutinate chicken red blood cells well (Ujvari et al., 2003). Other virulent viruses have been isolated sporadically from imported tropical bird species but the existence of a reservoir in these other species remains to be confirmed. In fact, it has been shown that some wild birds were not natural reservoirs of vNDV, but infected at quarantine stations prior to export (Kaleta and Baldeuf, 1988).

The vNDV isolated from cormorants and pigeons are considered mesogenic because their ICPI values in chickens vary from >0.7 to

<1.5 and they do not usually cause significant disease in poultry. However, recently the U.S. designated all NDV with ICPI values >0.7 or containing an amino acid sequence consistent with virulent strains of NDV as virulent and classified them as select agents, to follow the World Organization for Animal Health (OIE) and the European Union standards (EU) (APHIS, 2008; OIE, 2004). Although cormorant and pigeon viruses continue to evolve and display year-to-year genomic changes, no significant changes in virulence have been observed in samples obtained from wild birds (Weingartl et al., 2003).

The almost exclusive predominance of low virulence class I and mesogenic viruses of class II, genotypes V or VI in cormorants and pigeons, in contrast to the prevalence of viscerotropic vNDV (class II, genotypes V–X) in vaccinated poultry (Czegledi et al., 2006), suggests that the immune pressure from vaccination may be selecting variant forms of vNDV. This puzzling persistence of vNDV in poultry despite intensive vaccination efforts has been a recurrent phenomenon in endemic countries of Asia, Africa and Central America (Alexander, 2001; Czegledi et al., 2006). It is possible that the combined characteristics of current poultry production systems including: (1) host genetic homogeneity (with few host adaptive bottlenecks), (2) high density rearing (allowing close animal-to-animal contact, and favoring transmission of highly virulent virus over milder forms), and (3) intensive vaccination programs (which provide selective immune pressures and may be executed improperly in developing countries), may contribute to the evolution of virulent viruses (Higgins and Shortridge, 1988). However, to date, well-controlled studies have not been performed to demonstrate a role of vaccination on the evolution of virulent NDV.

Large phylogenetic and antigenic distances between vaccines and current circulating virulent strains may facilitate the evolution of virulent NDV (Miller et al., 2007). Many have demonstrated that current vaccines prevent disease but cannot stop viral shedding (Kapczynski and King, 2005; Miller, 2009; Utterback and Schwartz, 1973). In addition, evidence that using genotype-matched vaccines can significantly reduce viral shedding is emerging. More recently two antigenically matched vaccines have demonstrated increased capacity to prevent viral shedding of viruses of genotype VII and V, respectively (Hu et al., 2009; Miller, 2009).

Existence of field-isolated variants that escape vaccination has been presented (Cho et al., 2008a). NDV variants were isolated at multiple locations and time points in Korea with an increase in the frequency of isolation from 21% in 2002 to 85.7% in 2003–2006. Unfortunately, as the total number of isolates analyzed was small, the epidemiological significance is uncertain (56 total samples). These authors further identified one variant epitope in the HN gene and developed a recombinant vaccine utilizing the variant virus HN gene in a LaSota strain backbone (Cho et al., 2008a). A killed vaccine virus containing a matched HN gene was used and demonstrated to be equally protective when compared to commercial vaccines against morbidity and mortality. However, their recombinant vaccine, homologous to the challenge virus, was more effective in preventing decreases in egg production. Unfortunately, no evaluation of viral shedding was done in this study.

Genetic variants may also be occurring in Mexico. NDV was isolated for the first time in Mexico in 1946, and the last detailed report of a field outbreak caused by a highly virulent strain dates from the year 2000, when 13.6 million birds were slaughtered and 93 farms quarantined (OIE, 2003). After these outbreaks extensive vaccination programs were implemented in the affected areas. A phylogenetic comparison of the complete coding region of the fusion gene from recent Mexican viruses to previously published NDV from other known class II genotypes indicated that all the viruses isolated in Mexico between 1998 and 2006 grouped with the class II, genotype V viruses (Perozo et al., 2008). However, a clear phylogenetic separation of two groups of viruses analyzed in this study into either an early (1998–2001) or a recent (2004–2006) group, along with the ICPI and mean death time (MDT) results (traditionally, used to evaluate the pathogenicity of any new NDV isolate), suggested that some distinct selective pressure on the 1998–2001 viruses may have led to the appearance of the new group. The 1998–2001 isolates showed a 98% very high percent identity with other Mexican viruses reported between 1996 and 2001; but these were only 93–94% identical to recent isolates. Unfortunately, no studies were performed to characterize the antigenic differences between both groups of viruses.

Evidence that NDV vaccines may not be very effective under specific conditions is provided by field observations from Mexico (www.sagarpa.gob.mx). In addition, to the continued vNDV outbreak in backyard flocks, a drop in egg production levels in well-vaccinated birds displaying high levels of antibody to NDV has been observed since 2005 (Castilla and colleagues, 2009 ANECA abstract), similar to the egg production problems described in South Korea (Cho et al., 2008b). With the absence of clinical signs, the presence of vNDV has been confirmed by viral isolation and histopathology.

Evidence that current ND vaccines fail to protect against morbidity and mortality caused by new variants from genotype VII, is more controversial. One report, based on cross protection experiment suggests that at least two recent Chinese isolates from 2001 and 2003 are antigenic variants (Qin et al., 2008). In this report, 50, 40 and 10 percent of the birds vaccinated with a live LaSota vaccine displayed disease symptoms after being challenged with possible antigenic variants, in comparison to all birds being without clinical disease after being challenged with the viruses that were not identified as variants. However, evidence of the immune status (hemagglutination-inhibition assay or ELISA antibodies) of the vaccinated animals prior to and after challenge was not presented. In a similar experiment, in which no corroborating antibody data was presented, a 1996 isolate was reported as an antigenic variant (Yu et al., 2001). In contrast to these studies, two manuscripts demonstrate that current vaccines have good protective efficacy for morbidity and mortality against viruses currently circulating in Asia (Jeon et al., 2008; Liu et al., 2003). In one study, chickens vaccinated with either a live or a killed oil-emulsion LaSota (genotype II) vaccine, were fully protected against heterologous challenge strains of genotypes VIg, VIb, VIId and IX (Liu et al., 2003). Similarly, the other study demonstrated protective efficacy of two commercial vaccine strains in SPF chickens against two virulent challenge viruses of genotype VII (Jeon et al., 2008). Despite the controversy, enough evidence exists to suggest that NDV variants may be created in poultry as a result of suboptimal vaccination.

6. Diagnostic challenges

6.1. Detection of all NDV

Newcastle disease is generally diagnosed by isolation of NDV in SPF embryonating chicken eggs (ECE), by serology using the hemagglutination-inhibition (HI) test, or by real-time RT-PCR (RRT-PCR). All NDV isolates are known to replicate in ECE and the MDT to kill the embryo varies depending on the virulence of the virus. The HI test is used to identify a virus as NDV. Monoclonal antibody (mAb) testing can further be used to characterize NDV. While no single mAb can determine the virulence of NDV, a panel of mAb can be used to characterize differences, for example viruses of loNDV from PPMV-1. In addition, the use of mAb assays for rapid characterization of these viruses is not optimal for class I viruses as most of the mAb were developed and optimized to recognize class II viruses and fail to recognize viruses of class I (Collins et al., 1998;

Kim et al., 2007b). Because mAbs are directed against single epitopes, individually their ability to detect a broad spectrum of viruses is often limited. Other serologic assays such as ELISA are also used in diagnostic laboratories to assess antibody response following vaccination, but have limited value in surveillance and diagnosis because of the almost universal use of vaccines in domestic poultry.

Following identification, pathotyping of isolates is required to determine virulence characteristics. The methods used to pathotype newly isolated strains of the virus include the intracerebral pathogenicity index (ICPI) test, MDT and determining the amino acid motif at the cleavage site of the fusion protein. As pathotyping tests are time consuming and expensive and serological tests are complicated by the universal use of live-virus vaccines in poultry, rapid nucleic acid based assays have been developed. Multiple single-tube, sensitive, rapid real-time reverse transcription polymerase chain reaction (RRT-PCR) assays have been developed in the last decade around the world to detect the viruses circulating in those locations (Antal et al., 2007; Fuller et al., 2009; Pham et al., 2005; Tan et al., 2004; Wise et al., 2004b). In addition, other techniques and variations of RT-PCR increase the number of techniques available to detect NDV (Farkas et al., 2007; Wang et al., 2008).

In the United States, two different RRT-PCR assays have been used extensively and field validated to identify APMV-1 viruses (matrix gene assay) and to differentiate virulence (fusion gene assay), respectively (Wise et al., 2004b). They were developed to detect class II NDV in diagnostic clinical samples from birds using fluorogenic probes and were validated by the U.S. Department of Agriculture in response to the 2002 END outbreak in the state of California, which was contained in 2003 (OIE, 2004). Depending on assay conditions, these RRT-PCR tests can be equally or more sensitive than virus isolation, but are always faster than virus isolation (VI) and have been adopted as the standard method for surveillance in the U.S. Although these assays were used and shown to be effective during the California outbreak for screening flocks (Crossley et al., 2005), at the time of the assay's development and testing, not all clinical samples that were virus isolation (VI) positive were detected by the assay, which led the developers to reiterate that the assay should be used for screening flocks and not for individual samples.

6.2. RRT-PCR to detect all NDV

The primers and probe for the M-gene assay were designed to detect the highly conserved matrix (M) gene of NDV and, as such, will detect most NDV genotypes of class II, regardless of pathotype. However, due to the heterogeneous genetic nature of this virus, class I viruses tested often fail to be detected (Kim et al., 2007a). More recently, the M-gene assay was used to characterize hemagglutination-positive surveillance samples from waterfowl and shorebirds. It was found that approximately 70% of these isolates that corresponded to class I viruses were poorly or not detected (Kim et al., 2007a,b). Evaluation of the nucleotide sequence alignment of the M-gene assay probe site of class I and II viruses revealed a high number of mismatches between the two classes, and this is likely the reason that the class I viruses escape detection by this assay.

A new matrix-polymerase multiplex RRT-PCR was developed for the detection of a broad range of class I and II NDV isolates (Kim et al., 2008b). A conserved region from the polymerase (L) gene of class I NDV genomes was identified and used in the design and evaluation of a multiplex RRT-PCR assay (L-TET) that identifies a broad range of NDV. This L-TET assay, which demonstrated a ten-fold increase in sensitivity over the previously reported L-gene assay, identifies previously missed class I isolates, and works in conjunction with the existing M-gene assay using the same protocol (Kim et al., 2007b). Differential detection of mixed class I and II viruses down to 100 fg was possible because L-TET uses an alternate fluorophore from the M-gene assay. The multiplexed assay was capable of detecting a broad range of class I and II NDV ($n = 108$) genotypes 1–2, 4–9 and ($n = 32$) genotypes I to VII, respectively, all of which were correctly identified by both the single- and multiplex formats.

6.3. RRT-PCR to detect virulent isolates

The ability to detect virulent viruses quickly is key to containing an outbreak. The F gene probe created specifically to detect virulent NDV from field swabs during the outbreak of 2002 in the U.S. is widely used because it was field validated. The primers and probe match the genome of the California 2002 virus closely, however the test was and was not intended to detect vNDV worldwide (Wise et al., 2004b). During the initial testing, this assay was able to identify a wide range of isolates from different worldwide locations, except for one Dove/It virus collected from a collard dove (*Streptopelia decaocto*) in Verona, Italy in 2000 (AF520965) (Terregino et al., 2003). Dove/It is phylogenetically related to other pigeon adapted NDV and has a unique cleavage site motif with a lysine (K) replacing a glutamine (Q) at residue 114 that has been reported in other NDV isolated from pigeons in Finland and Germany (Huovilainen et al., 2001; Oberdorfer and Werner, 1998). The isolates with this cleavage site motif represent a distinct subset of PPMV-1 NDV (Aldous et al., 2004). Because all of the other PPMV-1 tested had been detected by the F-gene assay, further studies were performed to determine why Dove/It escaped detection. Sequence analysis of the Dove/It isolate identified four nucleotide differences with the F gene probe sequence corresponding to positions 1, 6, 13, and 14 of the probe (Kim et al., 2006). Four nucleotide differences between the Dove/It isolate genome and the fusion test probe appeared to be responsible for the test failure.

More recently, NDV isolates recovered from dead birds from the order of Columbiformes in Rhode Island and eastern Texas between 2000 and 2007 were positively identified as NDV by the RRT-PCR M-gene assay (Kim et al., 2008a). Monoclonal antibody patterns of nine of the 15 isolates showed the typical pattern seen with PPMV-1 and due to their ICPI values being greater than 0.7 or by their fusion cleavage sites having the ^{112}RRKKRF^{117} motif, all 15 isolates were defined as virulent. All of these isolates were negative by the F-gene assay. A new probe, designed to account for the three mismatches in the Dove/It isolate probe site previously identified, was able to identify these virulent PPMV-1 (Kim et al., 2008a).

Pigeon paramyxoviruses are distributed worldwide. Although many countries maintain compulsory vaccination of racing pigeons, there is no form of disease control in wild pigeons, which frequently have contact with backyard and free-range poultry (Alexander et al., 1984, 1985; Toro et al., 2005). As virulent PPMV-1 isolates continue to circulate and evolve in pigeons and doves worldwide (Abolnik et al., 2008; Kim et al., 2008a; Liu et al., 2007), outbreaks of PPMV-1 in chickens are feasible, as they have occurred in the past (Abolnik et al., 2004b; Alexander et al., 1984). It is imperative that diagnostic laboratories using the USDA validated or other fusion protein based PCR assays continue to monitor genomic changes and re-design alternate primers and probes to prevent the failure of detection of PPMV-1. Alternatively, the use of virus isolation in eggs in conjunction with the PCR assays will identify these isolates with embryo mortality.

6.4. Random priming methods

The use of random sequencing methods for diagnostic purposes is based on the following premises. The high capacity for mutation in RNA viruses (genetic drift) and the large diversity of NDV genotypes often makes it difficult to predict the genetic composition of new

isolates. Efforts to identify new isolates have focused on the development of DNA microarrays, real-time PCR, or rapid sequencing of RRT-PCR products (Hoffmann et al., 2007; Kiss et al., 2006; Romano et al., 1996; Spackman et al., 2002; Townsend et al., 2006). The common pitfall for each of these methods is that detection relies upon primers that are designed from existing sequences, which presumes that the unknown virus will resemble previously sequenced viruses. In addition, with the exception of complete genomic sequencing, most of these methods characterize small genomic regions, thus offering a partial view of the virus.

Another issue that affects the rapid and precise NDV characterization is the frequent occurrence of mixed NDV infections in birds with viruses of different genotypes. In these cases, any primer-specific method will amplify the viral product most homologous to the primer(s) of choice, but not necessarily the most abundant or representative of the viral population infecting the birds. Random genome sequencing represents an unbiased and thorough alternative that has been widely used to characterize the genomes of large DNA viruses (Afonso et al., 2006), but has only recently been used with NDV (Djikeng et al., 2008). A protocol that follows two basic principles: (i) random amplification of total RNA, and (ii) random selection of colonies followed by sequencing and assembly has been developed.

This protocol, a modification of the sequence-independent, single-primer amplification, SISPA method, first developed by Reyes and Kim (Reyes and Kim, 1991), and recently adapted for NDV entails the directional ligation of an asymmetric primer at either end of a blunt-ended DNA molecule (Djikeng et al., 2008). Following several cycles of denaturation, annealing and amplification, minute amounts of the initial cDNA are enriched and then cloned, sequenced and analyzed. Several modifications of the SISPA method have so far been implemented including random-PCR (rPCR) (Froussard, 1992). The rPCR method combines reverse transcription primed with an oligonucleotide made up of random hexamers tagged with a known sequence, which is subsequently used as a primer-binding extension sequence. This initial modification was first used to construct a whole cDNA library from low amounts of viral RNA.

A more recent modification, the DNAse-SISPA technique (Allander et al., 2001; Allander et al., 2005; Breitbart and Rohwer, 2005), includes steps to detect both RNA and DNA sequences. The combining of sample filtration through a 0.22 μm column and a DNAse I digestion step led to the identification of viruses from clinical samples. In addition, to its utility for viral discovery, the DNase-SISPA method has utility in obtaining full genome sequence from uncharacterized viral isolates or viral isolates from highly divergent families. The SISPA method was used as a rapid and cost effective method for generating full genome coverage of NDV. A total of 349 sequences were needed to completely sequence the LaSota NDV genome, with a redundancy of 13.72. A limitation to the method includes the need for samples that contain a large number of viral particles (10^6 particles in 0.2 ml samples). Moreover, because the capsid structure renders the viral genomes nuclease-resistant, this protocol requires encapsidated viral genomes to allow the removal of most extra-viral contaminants. For samples with high levels of host nucleic acid contamination, we have used 5 μg of RNAse A to treat 500 μl of filtered virus for 1 hr. We have found that RNAse A treatment eliminates the majority of host RNA derived sequence contaminant in these cases (Afonso et al., unpublished observations). In addition, DNAse I can be used to reduce host contaminants (Djikeng et al., 2008).

7. Conclusions

Because of the highly contagious nature of NDV and its clinical similarity to highly pathogenic avian influenza, accurate monitoring and rapid diagnosis of bird infections are crucial to any control and eradication program. Active surveillance of wild birds, LBM, and poultry production sites should increase our understanding of the predominance and evolution of NDV. Although surveillance should continue in some recently found reservoirs of NDV (including waterfowl and shorebirds), the search for additional natural reservoirs of virulent viruses should continue. Live bird markets are specifically significant because they promote the commingling of multiple bird species in close quarters and provide an environment in which reservoir species such as waterfowl are closely housed with gallinaceous hosts. Many challenges still remain on the detection of vNDV. Prompt detection and differentiation is challenging due to the broad genetic diversity of NDV and because vaccine and endemic viruses are often serologically indistinguishable. Rapid differentiation of loNDV strains from vaccines and identification of new forms of vNDV needs to be investigated further. In addition, phylogenetic analysis reveals that loNDV and vNDV are continually evolving. For improved surveillance and to increase our understanding of the ecology of NDV among wild bird and domestic poultry populations, specific diagnostic assays capable of identifying the full range of circulating NDV genotypes are needed. Similar to the situation with diagnostic assays for highly pathogenic avian influenza (HPAI), continual optimization must be carried out to ensure that all NDV are detected, whether they are from a wild bird species or from poultry. While continued genetic characterization of circulating strains by traditional methods is likely to provide evolutionary information and to help expand the arsenal of PCR based detection methods, random sequencing should be incorporated as a tool to identify new genotypes. Finally, consideration to include objective and uniform criteria to classify NDV into genotypes should be made.

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Phylogenetic and Biological Characterization of Newcastle Disease Virus Isolates from Pakistan▽†

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Eight Newcastle disease virus isolates from Pakistan were sequenced and characterized. A PCR matrix gene assay, designed to detect all avian paramyxovirus 1, did not detect four of the isolates. A new matrix gene test that detected all isolates was developed. Phylogenetic analysis and pathotyping confirmed that virulent viruses of different genotypes are circulating in Pakistan.

Newcastle disease (ND) is a devastating infection of chickens caused by Newcastle disease virus (NDV), an avian paramyxovirus (1). Since ND is highly contagious and is clinically similar to the highly pathogenic avian influenza (2), accurate and rapid diagnosis of an outbreak is important. Prompt detection and differentiation from lentogenic NDV are necessary.

Eight previously uncharacterized NDV isolates were obtained from commercial and backyard poultry flocks surrounding Karachi, Pakistan, showing clinical signs of ND and experiencing relatively high rates of mortality. The samples were propagated in 9- to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. RNA was extracted from infected allantoic fluid samples with TRIzol LS (Invitrogen, Carlsbad, CA) as described previously (3, 4). Clinical isolates were tested for NDV using the USDA-validated fusion (F) gene real-time reverse transcription-PCR (rRT-PCR) assay (F-gene assay) protocol (8). The F-gene assay detected all of these Pakistani isolates, except for the LaSota vaccine strain, as expected (data not shown). The F-gene test is designed to differentiate lentogenic (low-virulence) NDV from mesogenic (moderate-virulence) and velogenic (high-virulence) NDVs based on sequence changes at the fusion cleavage site (8), a well-characterized virulence determinant for NDV (6). The F-gene test is negative against avian influenza.

The 8 isolates and 49 additional ones were also tested for NDV using the USDA-validated matrix (M) gene rRT-PCR assay (M-gene assay) protocol (Table 1; see also Table S1 in the supplemental material) (8), designed to detect all NDV class II strains. In contrast to the results from the F-gene test, only five of eight Pakistani field isolates and the LaSota vaccine strain, but not the Mukteswar vaccine strain, were detected (Table 1). Because the validated M-gene assay is typically used to screen clinical samples, the failure of this test could result in an unacceptable false-negative diagnosis. While the test has failed to detect class I viruses before (5), this is the first case where the test has failed to detect class II NDVs and shows that constant monitoring of NDV genomic sequences is needed to detect future variability worldwide. This outcome is not surprising, given the difficulty of developing a single test for such a large, diverse group of viruses that continue to evolve (reviewed in reference 7). The test was modified by lowering the annealing temperature from 56°C to 52°C and 50°C, which allowed for the detection of NDV/27 and NDV/32, respectively. However, validated M-gene tests using annealing temperatures as low as 48°C were negative for the two remaining undetected viruses.

The sensitivity of the standard M probe (M+4169) was compared to that of a new M probe. The new probe, M+4169Pak (5′–[6-carboxyfluorescein] TTY TCT AGC AGY GGG ACA GCY TGC [black hole quencher_1]–3′) (Fig. 1), was designed using the consensus from an alignment of 50 samples with different origins and hosts (data not shown). For all class II viruses, the performance of the new M-gene assay was comparable (average threshold cycle [C_T] value for M+4169Pak = 16.7; average C_T value for M+4169 = 16.5). For the detection of class I viruses, a slightly improved performance for the new test was observed; however, the test does not provide dependable detection because of its very high C_T values (average C_T value = 36.5). Sequencing of the matrix genes of the Mukteswar vaccine strain (1974/PK/1) and 2007/PK/33 indicated that mismatches to the probe were likely responsible for the test failure (Fig. 1). These strains have four nucleotide differences relative to the probe (two of the mismatches are shared). The two other matrix genes sequenced were from strains that were detected by the validated M-gene assay, 2008/PK/43 and 2005/PK/26. Both of these strains had fewer mismatches with the matrix probe, three and two, respectively, than the two strains that failed the test. Moreover, the test did not likely fail because of differences in the primer sequences, since there was only one mismatch, and it was not near the 3′ end (data not shown).

Pathotyping showed that all of the Pakistani isolates from chickens collected between 1995 and 2008 are velogens (Table 1). The pathotypes of NDV isolates are determined by either

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† Supplemental material for this article may be found at http://jcm.asm.org/.

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TABLE 1. Comparison of new and validated (old) matrix rRT-PCR assays

Isolate name[k]	Fusion tree name[a]	Species/strain	Year	Matrix rRT-PCR		Pathotype results	
				Old	New	MDT (h)	ICPI
SPVC/Karachi/NDV/1[e]	G/1974/PK/290918495	Mukteswar	1974[d]	0	18.42	50	1.4
SPVC/Karachi/NDV/2	G/1995/PK/290918503	LaSota	1995[d]	12.26	12.28	110	0.4
SPVC/Karachi/NDV/16	G/1995/PK/290918487	Chicken[b]	1995	13.2	13.13	48.2	2
SPVC/Karachi/NDV/22	G/2004/PK/290918499	Chicken[b]	2004	22.5	18.07	48	1.7
SPVC/Karachi/NDV/23	G/2004/PK/290918491	Chicken[b]	2004	18.56	13.3	48.3	1.9
SPVC/Karachi/NDV/26[f]	G/2005/PK/290918497	Chicken[b]	2005	29.68	23	51.6	1.75
SPVC/Karachi/NDV/27	G/2006/PK/290918505	Chicken[b]	2006	19.42[i]	17.14	64.8	1.7
SPVC/Karachi/NDV/32	G/2007/PK/290918493	Chicken[b]	2007	14.88[j]	12.24	50	1.8
SPVC/Karachi/NDV/33[g]	G/2007/PK/290918501	Chicken[b]	2007	0	12.56	54.5	1.85
SPVC/Karachi/NDV/43[h]	G/2008/PK/290918489	Chicken[c]	2008	14.16	12.57	49	1.7

[a] Tree name shown for isolates included in phylogenetic analysis trees (see Fig. S2 in the supplemental material).
[b] Collected from commercial poultry.
[c] Collected from a backyard flock.
[d] Years when Mukteswar and LaSota vaccines were first used by Sindh Poultry Vaccine Center (SPVC), Karachi, Pakistan.
[e] The matrix tree name (see Fig. S2B in the supplemental material) for this isolate is G/1974/PK/290918481.
[f] The matrix tree name (see Fig. S2B) for this isolate is G/2005/PK/290918485.
[g] The matrix tree name (see Fig. S2B) for this isolate is G/2007/PK/290918483.
[h] The matrix tree name (see Fig. S2B) for this isolate is G/2008/PK/290918479.
[i] At 52°C, negative at 56°C.
[j] At 50°C, negative at 56°C.
[k] All isolates are class II NDV isolates from the vicinity of Karachi, Pakistan.

the intracerebral pathogenicity index (ICPI) found in 1-day-old chicks, mean death time (MDT) for embryonated eggs, or sequence of the fusion protein cleavage site. The ICPI ranged from 1.7 to 2.0, and the MDT ranged from 48 to 64 h; therefore, no clear change in pathogenicity over time was measured.

The complete M gene and the 374-bp region (cleavage site) of the F gene were sequenced. Gene sequences were obtained by RT-PCR using the Qiagen OneStep RT-PCR kit with gene-specific primers. The USDA-validated matrix probe (M+4169) sequence was compared to all available ($n = 167$) NDV matrix gene sequences in GenBank (Fig. 1). These sequences were aligned and analyzed for mismatches at the probe site. All of the sequences analyzed had less than four mismatches, with the exception of samples from class I waterfowl viruses with lentogenic cleavage sites, which had four or more mismatches. These viruses have already been shown to fail the test. Several isolates had three mismatches, and these should be followed more carefully, as they, too, will likely not be detected with the M-gene test.

Phylogenetic analysis of the eight NDV isolates in comparison to two vaccine strains and other reference isolates was done to determine the relationship between the Pakistani isolates and other class II sequences. The maximum likelihood PHYML version 2.4.4 software was used with a general time-reversible (GTR) model of nucleotide substitution, as shown previously (4), for 50 matrix and 71 partial fusion coding sequences.

Phylogenetic analysis using the 374-nucleotide partial fusion gene sequence shows that the recent Pakistani isolates 2007/PK/32, 2007/PK/33, 2006/PK/27, and 2008/PK/43 form a distinct cluster within genotype VII viruses and are most related to a 1989/Japan isolate (see Fig. S2A and Table S2 in the supplemental material). The Mukteswar (1974/PK/1) vaccine virus is distant from these and grouped with many recent Asian

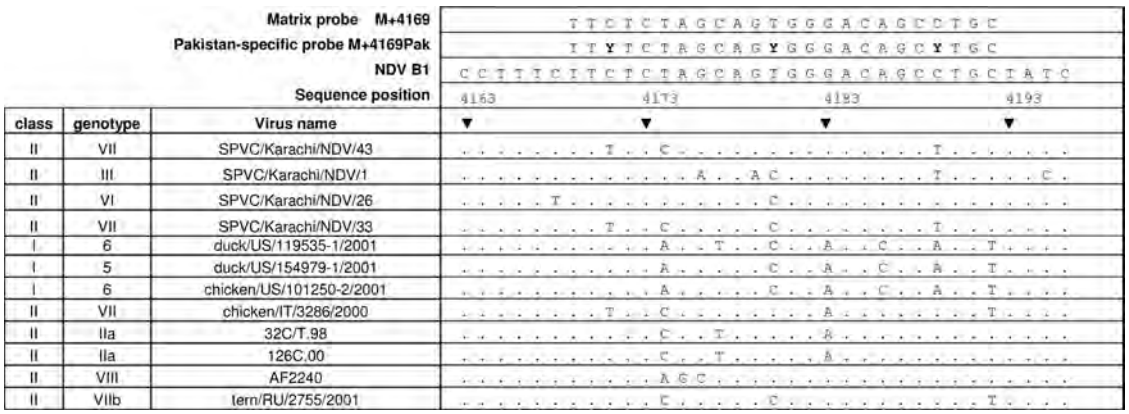

FIG. 1. Comparison of sequences at the validated M-gene assay probe site. Four Pakistan isolate sequences and eight previously characterized sequences with significant numbers of mismatches (≥3) with the validated M-gene assay probe are shown. Conserved nucleotides are shown as dots; nucleotide changes introduced into the Pakistan-specific M-gene assay probe are in boldface.

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genotype III to IV viruses. The remaining four isolates obtained from 1995 to 2005, 2004/PK/23, 1995/PK/16, 2005/PK/26, and 2004/PK/22, are closely grouped among genotype VI viruses.

Phylogenetic analysis of the full matrix sequence shows a grouping similar to that found with analysis of fusion gene sequences, with some differences due to the availability (or lack) of sequences in GenBank. Of the four Pakistan NDV viruses for which matrix sequences were available (see Fig. S2B and Table S2 in the supplemental material), 2007/PK/33 and 2008/PK/43 are tightly grouped in genotype VII viruses near a 2001/Russia tern isolate. Mukteswar (1974/PK/1) is distant from recent isolates and groups with other vaccine strains, as was seen with the 374-nucleotide partial fusion tree. 2005/PK/26 is grouped with genotype VI pigeon viruses and not with the other Pakistani viruses.

Overall, these results indicate that there are multiple velogenic genotypes circulating in Pakistan and causing outbreaks in poultry. Newcastle disease viruses have historically showed great mobility with viruses of Asian, American, or European origin, eventually achieving worldwide distribution (7). This report represents the first failure of the validated M-gene assay to detect virulent viruses. Therefore, providing sequences of isolates and the development of an rRT-PCR assay should aid diagnostic labs worldwide if these viruses move out of Pakistan. The need for continued modification of rRT-PCR test primer/probe sets should be expected.

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Mention of trade names or commercial products in this work 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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Virulent Newcastle disease virus elicits a strong innate immune response in chickens

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Newcastle disease virus (NDV) is an avian paramyxovirus that causes significant economic losses to the poultry industry worldwide. There is limited knowledge about the avian immune response to infection with virulent NDVs, and how this response may contribute to disease. In this study, pathogenesis and the transcriptional host response of chickens to a virulent NDV strain that rapidly causes 100 % mortality was characterized. Using microarrays, a strong transcriptional host response was observed in spleens at early times after infection with the induction of groups of genes involved in innate antiviral and pro-inflammatory responses. There were multiple genes induced at 48 h post-infection including: type I and II interferons (IFNs), several cytokines and chemokines, IFN effectors and inducible nitric oxide synthase (iNOS). The increased transcription of nitric oxide synthase was confirmed by immunohistochemistry for iNOS in spleens and measured levels of nitric oxide in serum. *In vitro* experiments showed strong induction of the key host response genes, alpha IFN, beta interferon, and interleukin 1β and interleukin 6, in splenic leukocytes at 6 h post-infection in comparison to a non-virulent NDV. The robust host response to virulent NDV, in conjunction with severe pathological damage observed, is somewhat surprising considering that all NDV encode a gene, V, which functions as a suppressor of class I IFNs. Taken together, these results suggest that the host response itself may contribute to the pathogenesis of this highly virulent strain in chickens.

INTRODUCTION

Newcastle disease virus (NDV) is an avian paramyxovirus that causes significant economic damage to the poultry industry. NDV is a negative-stranded RNA virus with a 15 kb genome that contains six genes: nucleoprotein (NP), phosphoprotein (P) (RNA editing of the P gene can also result in expression of V and W), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and polymerase (L). Over 200 avian species are naturally or experimentally susceptible to NDV. Isolates are of a single serotype, but have a wide range of naturally occurring pathogenicities from avirulent (lentogen), to mildly virulent (mesogen) and highly virulent (velogen) (Alexander & Senne, 2008).

While the highly virulent strains of NDV are not normally found in poultry in the USA, an outbreak of Newcastle disease (ND) in the South-western USA in 2002 required extensive culling and surveillance for control and resulted in a loss of millions of dollars (Yu *et al.*, 2001). This outbreak was caused by a highly virulent isolate that typically causes 100 % mortality in susceptible poultry 3–6 days post-infection (p.i.) (Wakamatsu *et al.*, 2006a). Virulent NDV is endemic in a large number of countries around the world, and there are reports of disease occurring in vaccinated birds (Yu *et al.*, 2001). In addition, some NDV strains are now becoming particularly virulent in avian species, e.g. geese, which had generally been resistant to clinical disease with virulent NDV infections (Liu *et al.*, 2003).

The host innate immune response to virus infection is an immediate reaction designed to retard virus growth and aid the host in developing specific protection from the adaptive immune responses. Research has shown that, in chicken cells, NDV can induce nitric oxide (NO) *in vitro* in heterophils (Sick *et al.*, 2000) and peripheral blood mononuclear cells (Ahmed *et al.*, 2007), alpha (IFN-α) and beta (IFN-β) interferon mRNA in macrophages (Sick *et al.*, 1998) and gamma interferon (IFN-γ) mRNA in peripheral blood mononuclear cells (Ahmed *et al.*, 2007). Yet this existing knowledge of the *in vitro* response to NDV

Supplementary material is available with the online version of this paper.

is insufficient to understand the nature of the host response to virulent NDV *in vivo* or to understand the underlying mechanisms of pathogenesis. Despite decades of research characterizing pathogenesis of different isolates, little is known about the underlying molecular mechanisms of disease caused by NDV. Here, we report a global analysis of the host response to infection *in vivo* with a virulent NDV strain from the 2002 outbreak in California (NDV-CA02), and characterize the early host response of splenocytes.

RESULTS

NDV-CA02 induces a robust host response in spleens *in vivo*

To measure the early host response to NDV *in vivo*, microarray experiments using complete chicken genome array slides were performed on spleens from specific-pathogen-free (SPF) chickens inoculated bilaterally intranasal and into the conjunctiva with an embryo infectious doses (EID_{50}) per 0.1 ml dose of $10^{5.3}$ NDV-CA02 or 0.1 ml of PBS control. The host gene expression profiles in the spleens of birds inoculated with PBS were compared with NDV-CA02 at both 1 and 2 days p.i. (five birds per treatment per time point). Spleens were chosen for analysis because these organs are known to be infected early and are lymphoid in nature. At 1 day p.i., the microarray analysis identified 125 genes that were induced by NDV-CA02 at least twofold, and 65 genes that were repressed at least twofold out of 6772 genes with significant data ($P<0.05$). By 2 days p.i., out of 6317 genes with significant data ($P<0.05$), 704 were induced at least twofold and 405 were repressed at least twofold (complete results presented in Supplementary Table S1, available in JGV Online). For certain genes, the microarray analysis generated no data. This occurs when appropriate probes representing genes are not present on the array or when the raw hybridization of the fluorescently labelled cDNA to the probe is too weak to produce a reliable signal. Of the significantly induced or repressed genes detected, genes specifically involved in the host inflammatory response, the innate antiviral response and well-characterized cytokines were chosen for further discussion in this study.

Many genes associated with an early innate host response were induced by NDV-CA02 at 1 day p.i., including the pro-inflammatory cytokine interleukin 6 (IL-6), chemokine macrophage inflammatory protein-3 alpha (MIP-3α), myxovirus resistance (Mx), lysozyme, interferon-induced protein with tetratricopeptide repeats 5 (IFIT-5), interferon-stimulated gene (ISG)12-2, and melanoma differentiation associated protein-5 (MDA-5) and IFN-γ precursor (Table 1). Changes in mRNA expression at 1 day p.i. of four early innate response genes were confirmed by quantitative RT-PCR on spleens from infected chickens (Supplementary Fig. S1, available in JGV Online). By 2 days p.i., each of these genes were further upregulated. In addition, several other markers of the innate immune response that were not induced at 1 day p.i. were upregulated at 2 days p.i., such as, iNOS, the pro-inflammatory cytokines IL-1β, IL-18, IL-8 and IFN-γ. The fact that expression of a subset of genes associated with an early host response increased from 1–2 days p.i. concomitant with an increase in virus titres in the spleen is evidence that these times are early in the infection. Further changes after 2 days p.i. were not measured in this study because they could be complicated by the host's transition from an innate to an adaptive immune response.

In addition to upregulating Mx, NDV-CA02 induced expression of the antiviral IFN effector genes protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS) (Table 1). Also induced were numerous cytokines: K203, ah221, CXCL13/BCA-1, CCL21, MIP-3α and MIP-1β. Several of these cytokines are chemokines, most notably MIP-3α and MIP-1β that function to set up later cell-mediated responses by recruiting effector leukocytes like neutrophils. Finally, many genes that were significantly induced by NDV-CA02 infection are part of the innate response signalling processes: regulator of G-protein signalling 1 (ADORA), suppressor of cytokine signalling (SOCS)-1 and -3, N-myc and STAT interactor (signal transducer and activator of transcription), STAT4 and IFN regulatory factors (IRF) 1, 7 and 10 (Table 1).

NDV-CA02 infection *in vivo* induces a strong iNOS response

With evidence from microarrays that NDV-CA02 was inducing iNOS expression in spleens, immunohistochemistry (IHC) for iNOS was done. Fig. 1(a), left panel shows that intense immunoreactivity for iNOS was seen in spleens of NDV-CA02-infected birds at 3 days p.i. The immunostaining is most intense in areas where lymphocytic depletion is most severe and, not surprisingly, the accumulated fixed macrophages are positive for iNOS antigen. Spleen tissue sections from PBS-inoculated birds demonstrated no positive signal (Fig. 1a, right panel). Also, an assay to measure the product of active iNOS, NO, was done (Fig. 1b). Serum from NDV-CA02-infected birds were assayed for nitrites (the assay converts NO and nitrates to stable nitrites) at 1, 2 and 3 days p.i. and compared with PBS-inoculated bird serum. NDV-CA02 induced NO production in serum as early as 2 days p.i. with greater than a twofold induction that increased to over fourfold by day 3.

Characterization of early pathogenesis of NDV-CA02 *in vivo*

Virulent NDV from the 2002 outbreak in California virus was administered to 15 naïve, SPF chickens bilaterally intranasal and into the conjunctiva with a $10^{5.3}$ EID_{50} per 0.1 ml dose. Eyelids, caecal tonsils and spleens harvested from five birds at 1, 2 and 3 days p.i. were used to characterize early infection and pathogenesis. These organs

Table 1. Selected genes significantly upregulated in the spleen during infection with NDV-CA02

GenBank best hit	24 h*		48 h		Gene description
	Fold increase	Probability	Fold increase	Probability	
BU138064	21.75	0.00132	37.9	2.76e−05	IFIT-5
X05343	11.35	0.00747	29.03	0.000223	Mature avidin
AB088533	18.6	0.00814	25.84	7.87e−06	Mx protein
BX275358	12.24	2.48E-05	17.22	0.000277	Putative ISG12-2 protein
CO765511	13.77	0.00832	14.41	0.000934	Lysozyme
AJ309540	5.584	0.014	12.45	0.000489	IL-6 (IFN-β2)
BX933162	9.837	0.00177	12.21	0.00104	Chemokine ah221
BX929857	8.891	0.000164	11.39	4.68e−05	Chemokine (C-C motif) ligand 19 (MIP-3β)
Y18692	3.364	0.0247	8.97	0.00243	Chemokine K203
TC218839	6.602	0.0201	8.886	1.67e−05	MDA-5
TC190629	3.377	0.000447	8.65	0.000635	Chemokine ah221
CR389044	2.291	0.037	7.412	0.00345	Chemokine (C-C motif) ligand 4
CR522995	0.854	0.019	6.992	0.000635	Small inducible cytokine A21 isoform 1
Y14971	1.609	0.00391	6.691	0.00344	IL-8
BX933215	4.462	0.00296	6.029	3.04e−05	SOCS-1
AF424806	2.578	0.0106	4.936	0.00118	SOCS-3
AB002586	2.472	0.0219	4.833	5.45e−05	OAS
BX950337	4.138	0.000152	4.656	1.97e−05	N-myc and STAT interactor
AB125660	2.794	0.0145	4.591	3.04e−05	PKR
AY775780	1.713	0.0635	4.269	0.00229	IL-18
NM_205372	2.433	0.00434	4.258	0.0011	IRF7
BX264625	0.969	0.71	4.217	0.0115	Chemokine CXCL13/BCA-1
NM_205415	1.572	0.000654	3.397	0.00011	IRF1
U46504	0.769	0.017	3.233	0.00174	iNOS
BX935231	0.789	0.699	3.088	0.00266	MHC class I antigen
AB101005	0.948	0.544	2.862	0.00185	Chemokine (C-C motif) ligand 20 (MIP-3α)
BX932486	1.811	0.0276	2.815	2.96e−05	Phorbol-12-myristate-13-acetate-induced protein 1
BX933123	†		2.658	0.00997	STAT4
AY705909	1.861	0.0315	2.611	0.00614	IFN-γ precursor
Y15006	1.515	0.04	2.238	0.00617	IL-1β
CF255763	1.556	0.111	2.166	0.0012	ADORA
AF380350	1.364	0.0709	2.143	0.000904	IRF10

*24 h data for all genes in the 48 h list are included regardless of fold-change or *P*-value.
†Raw signal of fluorescent cDNA hybridized to probe was insignificant.

have been shown previously to be sites of early replication for NDV, and all have abundant lymphoid cells (Alexander & Senne, 2008; Wakamatsu *et al.*, 2006b). Results of histopathological evaluation and IHC for virus are presented in Table 2. Overall, there were minimal pathological changes seen histologically at 1 day p.i. The spleen and eyelids both had very mild lymphocyte depletion, while the caecal tonsils did not show lesions. The IHC for NDV at 1 and 2 days p.i. were weakly positive in the spleen of each bird. A mean NDV-CA02 titre of $10^{4.2}$ EID_{50} per 1 mg tissue (with a range of $10^{2.9}$–$10^{4.3}$) was detected in the spleen by 1 day p.i. and titres increased by 2 days p.i. to $10^{6.8}$ EID_{50} per 1 mg tissue (with a range of $10^{5.9}$–$10^{7.1}$). By day 2 the disease progressed, and moderate to severe lymphocyte depletion was observed by haematoxylin and eosin (H&E) in the spleens of three birds, and lesions were found mostly in the periarteriolar lymphoid sheaths (Fig. 2a, b). The splenic lesions progressed to severe multifocal to coalescing splenic necrosis and severe lymphocytic depletion by 3 days p.i. (data not shown). The lymphocyte depletion was associated with accumulation of macrophages and scattered heterophils, and the necrosis was characterized by areas of homogeneous amorphous cellular debris especially in the periarteriolar lymphoid sheaths. IHC of the spleens was weakly positive for NDV-CA02-infected birds at 2 days p.i., and nucleoprotein was detected mainly in the macrophages near the periarteriolar lymphoid sheaths. By 3 days p.i., NDV antigen was no longer confined to the periarteriolar region, consistent with dissemination of the virus to the spleen. Virus titres in the spleen remained high with a mean EID_{50} mg^{-1} of tissue of $10^{7.2}$ (with a range of $10^{5.7}$–$10^{7.7}$).

In the caecal tonsils, the pathological changes were similar but occurred slightly later than those in the spleen.

Table 2. Histological analysis of spleens of NDV-CA02-infected chickens

ND, Not determined.

		Day 1		Day 2		Day 3	
	Bird #	H&E*	IHC†	H&E	IHC	H&E	IHC
Spleen	1	−	−	+	ND	+++	ND
	2	+	ND	+	+	+++	ND
	3	−	ND	++	ND	+++	ND
	4	+/−	ND	++	ND	++	+++
	5	−	−	++	+/−	++	+++
Caecal tonsil	1	−	−	+	ND	++	ND
	2	−	ND	+	++	++	ND
	3	−	ND	+	++	+++	ND
	4	ND	ND	++	ND	+++	ND
	5	ND	−	++	ND	+++	+++
Eyelid	1	−	ND	+	+	+++	+++
	2	+/−	+	+	+	+++	ND
	3	+/−	ND	+	ND	+++	ND
	4	−	ND	+	ND	+++	+++
	5	−	−	+	ND	++	+++

*−, Normal; +/−, minimal lymphocytic depletion; +, mild to moderate lymphocytic depletion and increase in histiocytes; ++, moderate to severe lymphocyte depletion, necrosis, histiocytosis ; +++, severe lymphocytic depletion, severe necrosis, haemorrhages, histiocytosis and heterophilic infiltration.
†−, Negative; +, scattered-positive cells in the tissue for NDV nucleoprotein (N); ++, moderate amount of viral N detected; +++, large amount of NDV nucleoprotein detected (N).

Pathological changes in these tissues were also characterized by severe lymphocytic depletion, marked macrophage accumulation and moderate to severe heterophil infiltration by 3 days p.i. (Fig. 2d). IHC revealed moderate staining in the macrophages by day 2, in greater amounts than what was detected at the same time in the spleen. By 3 days p.i., the caecal tonsils were strongly immunopositive for NDV. The areas with severe lymphocyte depletion were those that had

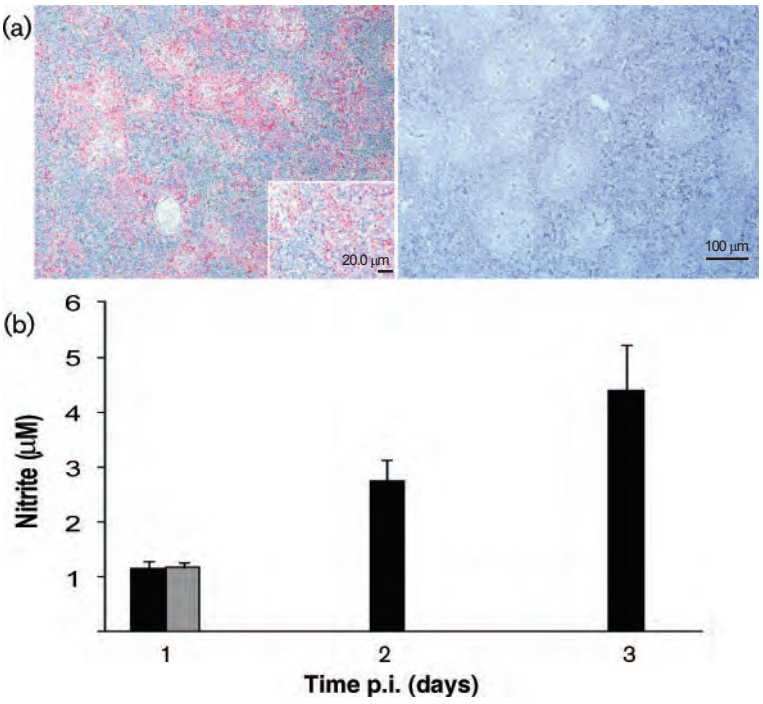

Fig. 1. iNOS response to NDV-CA02 infection in chicken spleen and serum. (a) IHC using anti-iNOS antibody on spleen tissue sections 3 days p.i. Image of spleen section from NDV-CA02-inoculated birds on the left (CA02) and PBS-inoculated birds on the right (PBS). Fast Red chromogen and haematoxylin counterstain. CA02 image includes ×5 magnified insert. (b) NO levels in the serum of NDV-CA02-infected birds. Serum from each of five NDV-CA02 (CA02)-inoculated birds at each time point was assayed for nitrites by a colorimetric assay and compared with values from serum of PBS-inoculated birds. Hatched bar represents PBS control and solid bar represents NDV-CA02.

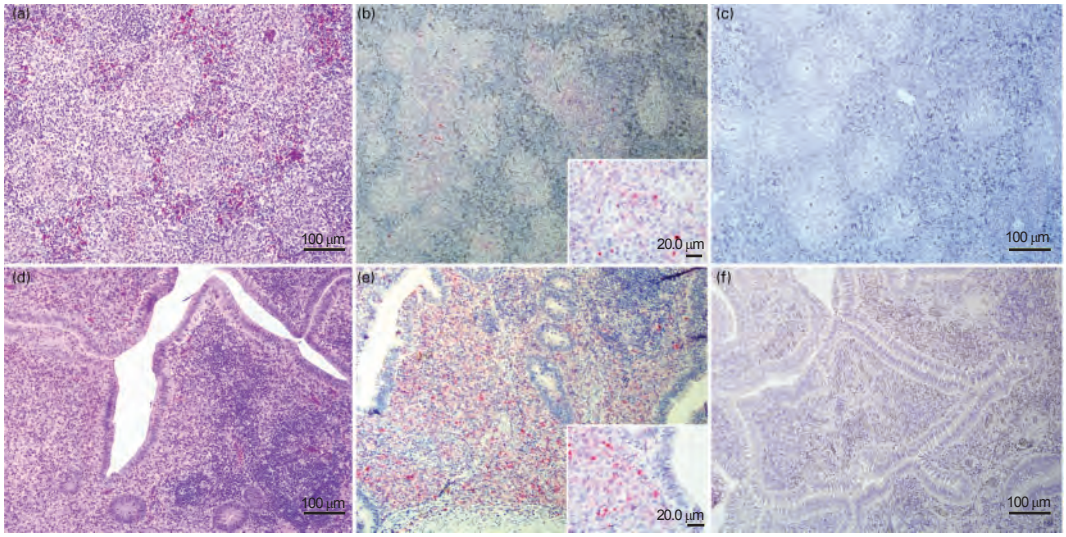

Fig. 2. Histological analysis of NDV-CA02-infected birds. Two days p.i. spleen (a–c) and 3 days p.i. caecal tonsil (d–f) sections were stained with H&E alone (a, d) or anti-NDV IHC with Fast Red substrate and haematoxylin counterstain (b, c, e and f). Control PBS-inoculated sections (c and f) are also shown. (b and e) Include ×5 magnified inserts. At 2 days p.i., there is extensive depletion of lymphocytes. At this same time period, using IHC, there is abundant viral antigen.

the greatest numbers of cells positive for NDV antigen (Fig. 2e) in comparison to the uninfected control (Fig. 2f).

Pathological changes in the eyelids followed a similar pattern of disease progression. At 3 days p.i., lymphocyte depletion, macrophage accumulation, necrosis and heterophil infiltration were predominant. IHC for NDV was mildly positive by 2 days p.i., while at 3 days p.i. the immunoreactivity was intense. The nucleoprotein appeared mainly confined to the macrophage population and some of the muscular cells of the eyelid.

Overall, the lesions observed in all the three organs are consistent with a necrotizing pattern of inflammation, and for all the tissue, the detection of the antigen by IHC was greatest at 3 days p.i. Both the anatomical lesions and the IHC results follow an increasing severity pattern from 1 to 3 days p.i.

NDV-CA02 infection of chicken splenocytes *in vitro* induces strong early cytokine response

To confirm that the most relevant early response genes were induced at early times post-infection, real-time RT-PCR was performed with RNA samples from chicken splenocytes following infection with NDV-CA02 *in vitro*. Splenocytes were infected with purified NDV-CA02 or LaSota (vaccine strain) at an m.o.i. of 1 and RNA was collected at 6 h p.i. Real-time RT-PCR was done with primers against IFN-α, IFN-γ, IL-1β and IL-6 (Fig. 3). Both type I and type II interferons, IFN-α and IFN-γ, were induced significantly by NDV-CA02, six- and sevenfold, respectively, while LaSota did not induce them over twofold relative to mock (Fig. 3). The pro-inflammatory cytokines, IL-1β and IL-6 were induced to a much greater degree by NDV-CA02, 18- and 35-fold, respectively. LaSota did not induce IL-1β over twofold and IL-6 was only modestly induced sixfold. These results corroborate the microarray and RT-PCR data from spleens *in vivo*, and show that NDV-CA02 induces a strong cytokine response in chickens.

DISCUSSION

The use of a whole chicken genome array (42 000 probes) to analyse the host response to infection *in vivo* allowed the identification of a large number of genes (704) that are part of the host early innate response to NDV. This included groups of functionally related genes, such as ISGs and pro-inflammatory chemokines, thus providing an additional level of confidence to the results. At 1 and 2 days p.i. *in vivo*, both type I and type II IFN responses were detected using microarrays. The induction of IRF1 and 7 are consistent with a type I IFN response since these proteins are upstream signals in the Toll-like receptor response to viruses that initiate IFN-α and IFN-β production (Au *et al.*, 1998; Parrington *et al.*, 1993). In addition, MDA-5, an RNA helicase that recognizes viral dsRNA and initiates a type I IFN response to many viruses [although reportedly not NDV (Loo *et al.*, 2008)], is induced by NDV-CA02. Downstream effectors of IFN action include several IFN-stimulated-response genes (ISRGs) including, Mx, OAS and PKR that were upregulated following NDV-CA02 infection.

In vitro experiments suggest that a pronounced and rapid innate immune response may be induced by virulent

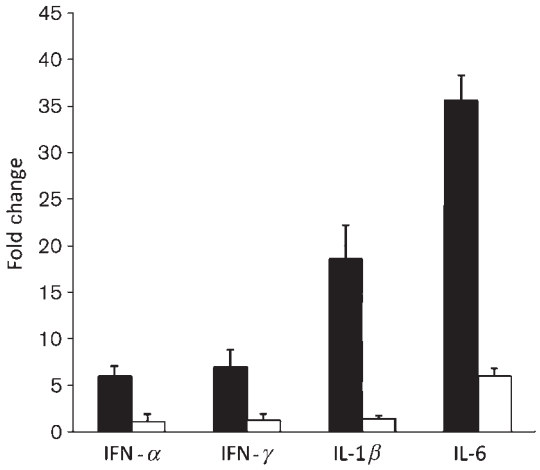

Fig. 3. Real-time RT-PCR of NDV-CA02 and LaSota vaccine strain-infected chicken splenocytes *in vitro*. Total RNA isolated from chicken splenocytes infected with NDV-CA02 and LaSota for 6 h was used for real-time RT-PCR using SYBR Green for IFN-α, IFN-γ, IL-1β and IL-6. Results were normalized against 28S rRNA and fold-changes relative to mock-infected samples are shown. Open bar represents LaSota and solid bar represents NDV-CA02 (CA02).

viruses in splenocytes. Using real-time RT-PCR of RNA isolated from NDV-infected splenocytes, we have demonstrated that the virulent CA-NDV, but not the lentogenic LaSota virus, is capable of rapidly and strongly inducing IFN-γ, IFN-α, IL-6 and IL-1β at 6 h p.i., genes integral to a pro-inflammatory response. Unfortunately, since LaSota does not reach the spleen but only replicates at the site of inoculation (Wakamatsu *et al.*, 2006b, c), parallel *in vivo* experiments to compare the relative host response to velogenic and lentogenic viruses were not possible. Our results confirm and extend *in vitro* analysis of gene expression patterns from chicken embryo cells in response to virulent NDV reported previously (Munir *et al.*, 2005). There, the effect of a low m.o.i. (0.01) was analysed throughout a 36 h period, and a significant but late induction of 22 genes was noted (probably as a result of the low m.o.i. used). Upregulated genes included some similar functions to the IFN and pro-inflammatory response that was observed here, including some ISGs, IL-8 and STAT1.

NDV encodes the V protein that blocks various components of IFN pathways. Specifically, the NDV V protein targets STAT1 for degradation and recombinant NDVs that do not express V have drastically lower virulence *in vivo* (Huang *et al.*, 2003; Wakamatsu *et al.*, 2006a, b, c). STAT1 is a particularly important signalling molecule for ISRG regulation because unlike STAT2, which is targeted by the V proteins of other paramyxoviruses (Parisien *et al.*, 2001), STAT1 is a transactivator for both IFN types I and II pathways (Darnell, 1997). Our data would indicate that at least at the mRNA level, a block of IFN pathways is not occurring with this particular NDV isolate. Here, we show that transcription of the types I and II IFNs and many ISGs are being induced by NDV-CA02, therefore further studies are necessary to determine the expression level and effectiveness of NDV-CA02 V protein during infection in both lymphoid and epithelial cells. In addition to detecting upregulation of IFN and pro-inflammatory genes, we also measured increased iNOS expression in tissues and increased levels of NO in serum. These results are in agreement with the observed upregulation of IFN-γ in microarray since IFN-γ induces iNOS expression (Kapczynski & Kogut, 2008; Koide *et al.*, 1993). Assaying NO levels was particularly valuable because it allowed for confirmation of the microarray data at a functional level, while using a validated commercially available assay. Constitutive low-level expression of NO in the vascular endothelium plays a beneficial role in maintaining blood vessel homeostasis (Palmer *et al.*, 1987), but high levels of NO produced by macrophages in response to pathogens can have toxic effects on the host. NO can indiscriminately modify many chemical groups and result in well-characterized decrease of protein function and cellular processes (Pacher *et al.*, 2007). The nitrite (μM) concentrations measured in NDV-CA02-infected chicken serum are well within its range required for detrimental effect (Shiva *et al.*, 2001). The significant upregulation of iNOS in spleens and NO in serum is a potentially destructive innate response of chickens to NDV infection, but in light of the robust replication and rapid mortality of this virus, it seems quite possible that NO is contributing to mortality not recovery.

Our data are consistent with a strong innate immune response that is unable to protect birds from disease and death, as we observed a clear innate IFN and cytokine response to NDV-CA02 *in vivo*. In spleens, there was a strong correlation between virus titres, pathological changes, and host innate immune response at 1 and 2 days p.i. The spleens and caecal tonsils of infected birds showed significant lymphoid depletion and infiltration of fixed macrophages by 2 days p.i. that progressed to necrotic lesions. This histopathology provides a good visual correlation to the microarray and PCR assays of cytokine expression levels. The histology and IHC of the spleens demonstrate that the cytokine increases are associated with tissue destruction and increasing quantities of virus.

The spleen was chosen for analysis of the early innate host response because it is a lymphoid organ that the virus reaches early in disease (Brown *et al.*, 1999b). However, analysis of the response to infection in the spleen may not be sufficient to completely understand NDV mechanisms of disease or death. The global analysis of the innate response to NDV *in vivo* and analysis of select innate genes *in vitro* provide a good overview of the host response; however, the roles of individual cell types in this response cannot be effectively measured in this way. Several major cell types present in spleens (macrophages, heterophils, B-cells and T-cells) appear to be permissive to NDV (Brown *et al.*,

1999a; Dalgaard *et al.*, 2010; Lam, 1996; Lam *et al.*, 1996; von Bulow & Klasen, 1983). Our data highlight the important role that splenocytes have in the host response to NDV. IL-6 and IL-1β were greatly induced in spleens by NDV-CA02 and are two well-characterized early innate response genes of macrophages to viruses through Toll-like receptor activation (Alexopoulou *et al.*, 2001). Additionally, the iNOS induction measured at the transcript and functional (NO) levels is mainly produced by macrophages. Given the proclivity of macrophages to be present at the sites of infection, often harbouring virus (Fig. 1 and Brown *et al.*, 1999b), future analysis of the role of individual leukocyte cell populations in the innate response to NDV are warranted.

The existence of new virulent NDV genotypes throughout the world and the susceptibility of wild avian species are cause for concern over the continued effectiveness of the currently used 50-year-old vaccine. The development of better vaccines and control strategies will require a greater understanding of the mechanisms of pathogenesis. Our report of the first global analysis of the natural host's response to an avian paramyxovirus *in vivo* provides basic information needed to extend our understanding of the nature of the virus–host interactions.

METHODS

Viruses. NDV from chicken isolate California/S0212676/2002 (NDV-CA02) was originally isolated from the field during the 2002–2003 California exotic ND outbreak (Pedersen *et al.*, 2004). This isolate was plaque-purified three times on chicken embryo fibroblasts and amplified by inoculation of SPF 9–11-day-old embryonating chicken eggs (ECE). The pathotype of the plaque-purified isolate was confirmed as a virulent by having a mean death time of 54 h in ECE (<60 being virulent) and intracerebral pathogenicity index of 1.85 (>1.5 being virulent) using standard procedures (Alexander, 1998). The low virulence NDV vaccine virus, LaSota, was received from Lohmann Animal Health International (Gainesville, Ga). For *in vitro* splenocyte infections, viruses were first purified by ultracentrifugation for 1 h (35 000 ***g***) at 4 °C through a 10 % sucrose cushion. Pellets were resuspended in PBS with 1 % BSA, and mock infections were done with PBS with 1 % BSA. Virus inoculated into birds was harvested from infected allantoic fluid, titrated in ECE and diluted in PBS.

Birds and eggs. The source of SPF chickens and ECE was the Southeast Poultry Research Laboratory's SPF White Leghorn chicken flock.

Cells. Splenocytes used for *in vitro* infections and real-time RT-PCR assays were isolated from 2-week-old SPF White Leghorn chickens. Spleens were collected aseptically and placed in sterile 4 °C PBS. Single-cell suspensions were prepared by gently pushing the splenic pulp through a sterile nylon mesh with a pore size of 70 μM (Fisher Scientific). Cells were washed and resuspended in 3 ml cold PBS and then layered over 3 ml Histopaque 1077 (Sigma). The preparations were enriched for leukocytes by centrifugation (450 ***g***) for 30 min at 18 °C. Cells were recovered from the interface, resuspended in cold PBS, and washed twice in 3 ml cold PBS.

Animal experiment and tissue collection. Each of 15 chickens was inoculated with a total dose of $10^{5.3}$ 50 % EID_{50} in 0.1 ml of virus divided into the right nares (50 %) and both eyes (25 % each). PBS was used as inocula for non-infected controls. Five infected birds were sampled each day. The five non-infected control birds were sampled only on 1 day p.i. Birds were anaesthetized and blood samples were drawn from the heart. Birds were then euthanized with an intravenous injection of pentobarbital (Sigma-Aldrich). Immediately post-mortem, eyelid, spleen and caecal tonsils were harvested aseptically for histology, IHC and RNA extraction. Spleens harvested for analysis were cut into three pieces, with one piece of each used for histology, RNA isolation and virus titration. Virus titres of spleens were determined after homogenization in 10 % (w/v) PBS. Confirmation that sampled birds were infected with virus was based on virus recovery from oral or cloacal swabs collected before or on the day tissue samples were collected. Oral and cloacal swabs from all birds were positive by haemagglutination assay for NDV-CA02 by 2 and 3 days p.i., respectively.

RNA isolation, reverse transcription, labelling and microarray hybridization. Spleen cell suspensions were generated by pushing freshly isolated tissue through a 70 μM pore-size mesh cup, immediately into Trizol (Invitrogen) and stored at −80 °C after collection of all samples for that day. Samples were later thawed at room temperature and chloroform was added according to the manufacturer's protocol. Samples were incubated for 10 min on ice. Following centrifugation at 2400 ***g*** for 15 min, the aqueous phase was removed, mixed with an equal volume of 70 % ethanol, and applied to Qiagen RNeasy midi prep columns. RNA quantity was assessed using a Nanodrop spectrophotometer on the RNA-40 setting and quality was assessed using a nanochip on a Bioanalyser (Agilent Technologies). All RNA samples had an RNA integrity number greater than 8.6 with a mean of 9.7. Total RNA (50–500 ng) was reverse transcribed to cDNA, amplified, and labelled with Cy3 or Cy5 using the Two-colour Linear Amplification kit (Agilent Technologies). Complete chicken genome microarray slides (42 000 probes) were purchased from Agilent and labelled cRNAs were hybridized for 17 h at 65 °C with Gene Expression Hybridization Solution (Agilent Technologies). Slides were disassembled from their hybridization chambers in Gene Expression Wash Buffer 1 then washed for 1 min in Gene Expression Wash Buffer 1, 1 min in Gene Expression Wash Buffer 2, 1 min in acetonitrile, and 30 s in Stabilization and Drying Solution (Agilent Technologies) following manufacturer's specifications. RNA from individual NDV-CA02-inoculated animals (n=4) were considered as separate experiments and compared with pooled RNA from the PBS-inoculated animals (n=4 and n=3 for 24 and 48 h experiments, respectively).

Microarray data collection and analysis. Differential expression measurements based on simultaneous two-colour hybridizations were performed with a GenePix 4200A scanner using a 5 μm resolution and the GenePix Pro 6.1 data acquisition and analysis software (Axon Instruments; Molecular Devices). GeneSpring GX computer software (Agilent Technologies) was used for all normalization and statistical analysis of the GenePix output. RNA from PBS-inoculated birds was pooled and compared with RNA from individual NDV-CA02-infected birds for a total of four arrays. The intensity ratio of expression for each gene was calculated by dividing the measured infected bird values (test channel) by the intensity of the mock-infected bird values (control channel). All outputs with control channel values of less than 300.0 were not considered. Intensity-dependent normalization was used to reduce the ratios to the residual of the Lowess fit of the intensity-versus-ratio curve. Per-chip normalization was done by creating an artificial-positive control for each sample with the 50th percentile of all measurements across the entire chip. Chips were scanned with high laser strength and low laser strength independently to generate non-saturated data for the maximum number of spots. Individual sample t tests were calculated as the mean of replicate normalized values for a single gene – 1/standard error. This analysis produces low P-values when the mean of replicate normalized values (fold-changes) is significantly different from one and the standard error is small.

Quantitative RT-PCR. *In vitro* determination of cytokine induction of chicken splenoytes following NDV-CA02 infection was performed as previously described (Kapczynski & Kogut, 2008). Briefly, isolation of total RNA from infected splenocytes was performed using the Qiagen RNeasy Mini kit following the manufacturer's recommendations. Standard cycling conditions were used with iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) with annealing temperatures optimized for each primer pair. Each RT-PCR experiment was run in triplicate with a no-template control, test samples and a $\log_{10}$ dilution series of standard RNA. Regression analysis of the mean values of three replicate RT-PCRs for the $\log_{10}$ diluted RNA was used to generate standard curves. Primer pairs used were (forward, reverse): IFN-α (5′-GACAGCCAACGCCAAAGC-3′, 5′-AATGCTTGAGCAGCAGCGAC-3′); IFN-γ (5′-GCCAGACTTACAACTTGTTTG-3′, 5′-AGAACTGGACAGAGAGAGAAATGAG-3′); IL-1β (5′-ACATGTCGTGTGTGATGA-3′, 5′-GCTTCATCTTCTACCGCCTG-3′); IL-6 (5′-GCGAGAACAGCATGGAGATG-3′, 5′-CTGTTCGCCTTTCAGACCTAC-3′); 28S (5′-GGCGAAGCCAGAGGAAACT-3′, 5′-GACGACCGATTTGCACGTC-3′).

NO assay. Blood was drawn into S-monovette Z Serum V 4.5 ml syringes containing the clotting stimulant kaolin (Sarstedt) and centrifuged at 1000 ***g*** for 15 min to separate the serum. Serum samples (250 µl each) were applied to pre-rinsed 10 kDa molecular mass cut-off microfuge filters (Millipore) and centrifuged at 5000 ***g*** for 40 min. The flow-through was used in the nitric oxide synthase assay kit (Calbiochem) according to the manufacturer's instructions. Serum from each of five birds for each condition were read and averaged. NO levels were calculated using a standard curve.

Histopathology and IHC. Sample tissues were immediately fixed in 10 % neutral buffered formalin for approximately 52 h. All sampled tissues were routinely processed into paraffin blocks, and 3 µm sections were cut onto positively charged slides (Probe On Plus; Fisher Scientific) both for H&E staining and IHC. All tissues were examined histologically, with recording of severity of lesions. For IHC, two infected birds from each sampling day were assayed for the presence of NDV nucleoprotein. For this procedure, after deparaffinization, tissue sections were subjected to antigen retrieval by microwaving in citrate buffer solution (Antigen Unmasking Solution; Vector Laboratories) for 10 min at full power. Blocking was performed with a universal blocking reagent (Biogenex) as recommended by the manufacturer. The primary antibodies, polyclonal rabbit anti-NDV nucleoprotein (peptide) (Kapczynski & Kogut, 2008; Kommers *et al.*, 2001) and polyclonal rabbit anti-iNOS (Thermo Scientific Anatomical Pathology) were diluted in 10 % normal sheep serum, and incubated at 37 °C for 1 h. The dilutions used were 1 : 8000 for rabbit anti-nucleoprotein, and 1 : 75 for rabbit anti-iNOS. After washes, the slides were incubated with an alkaline phosphatase-based polymer system specific for the Fc portion of rabbit IgG (Ultra Vision One; Thermo Scientific Anatomical Pathology). The reaction was revealed with a naphthol-based chromogen, Fast Red (Biogenex). Sections were counterstained lightly with haematoxylin and cover-slipped with Permount for a permanent record.

Quantitative RT-PCR confirmation of mRNA expression changes seen on microarray. RNA was isolated from spleens 24 h p.i. as described above. Changes in mRNA gene expression of IL-6, IFN-γ, iNOS and IFN-β were monitored as changes in SYBR Green fluorescence after amplification with specific primer sets. Primers for amplifying IFN-γ, IL-6, iNOS and IFN-β were derived from previously published sequences (Degen *et al.*, 2005; Kaiser *et al.*, 2003). β-Actin primers were manually selected according to the available nucleotide sequences available in GenBank (accession no. X00182) and properties were verified using the Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html). The primers were designed to span introns in the genomic DNA to prevent DNA contamination.

All primers were synthesized by Integrated DNA Technologies. Sequences are as follows: β-actin (5′-AGAGGCTCCCCTGAACCCCAAAGC-3′, 5′-CTGGATGGCTACATACATGGCTGG-3′); IFN-γ (as above); IL-6, (as above); IFN-β (5′-GGTAGGTCTGAAAGGCGAACAG-3′, 5′-TTGATGCTGAGGTGAGCGTTG-3′); and iNOS (5′-TTGGAAACCAAAGTGTGTAATATCTTG-3′, 5′-CCCTGGCCATGCGTACAT-3′).

One-step quantitative RT-PCR amplification and detection were performed using a 7500 FAST real-time PCR System (Applied Biosystems) with SYBR Green fluorescence detection of PCR product on 20 µl reaction mixtures containing: 10 µl Power SYBR Green RNA-to-C *1*-Step kit (Applied Biosystems), 500 nmol l^{-1} each of forward and reverse specific primer and 60 ng RNA. Thermocycler conditions were as follows: one cycle at 48 °C for 30 min, one cycle at 95 °C for 15 min, 40 cycles at 95 °C for 15 s and at 58 °C at 32 s. One cycle for dissociation curve for all reactions was added and the melting curve analysed.

Relative changes in gene expression were calculated according to the Pflaffl method (Pflaffl, 2001) using LinRegPCR version 12 (Ramakers *et al.*, 2003) to calculate individual specific primer efficiency by linear regression. All cycle threshold fluorescence (C_t) values were corrected to the mean C_t of PBS, mock-infected control for each gene of interest and β-actin was used as the endogenous control.

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VIROLOGY JOURNAL

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Metagenomic analysis of the turkey gut RNA virus community

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Abstract

Viral enteric disease is an ongoing economic burden to poultry producers worldwide, and despite considerable research, no single virus has emerged as a likely causative agent and target for prevention and control efforts. Historically, electron microscopy has been used to identify suspect viruses, with many small, round viruses eluding classification based solely on morphology. National and regional surveys using molecular diagnostics have revealed that suspect viruses continuously circulate in United States poultry, with many viruses appearing concomitantly and in healthy birds. High-throughput nucleic acid pyrosequencing is a powerful diagnostic technology capable of determining the full genomic repertoire present in a complex environmental sample. We utilized the Roche/454 Life Sciences GS-FLX platform to compile an RNA virus metagenome from turkey flocks experiencing enteric disease. This approach yielded numerous sequences homologous to viruses in the BLAST nr protein database, many of which have not been described in turkeys. Our analysis of this turkey gut RNA metagenome focuses in particular on the turkey-origin members of the *Picornavirales*, the *Caliciviridae*, and the turkey Picobirnaviruses.

Introduction

Enteric disease syndromes such as Poult Enteritis Complex (PEC) in young turkeys and Runting-Stunting Syndrome (RSS) in chickens are a continual economic burden for poultry producers. The only reliable method to reproduce the clinical signs of these syndromes in experimental birds is oral inoculation with crude preparations of intestinal contents from naturally infected birds. Further, the full spectrum of the field signs observed associated with these syndromes is difficult to reproduce experimentally with isolated viruses [1,2]. Numerous viruses are known to be circulating in turkey flocks in the United States, with recent research efforts targeting RNA viruses such as the turkey astroviruses, novel turkey-origin reovirus, and avian rotavirus, and DNA viruses such as the recently described turkey parvovirus [3-6]. However, there remains a possibility that an unidentified virus or combination of viruses may play a role in poultry enteric disease. Despite the isolation and characterization of many of these suspect viruses, the etiology of the poultry enteric disease syndromes remains elusive, and many enteric viruses can be detected in otherwise healthy turkey and chicken flocks [3,4]. Regional and national enteric virus surveys have revealed the ongoing presence of avian reoviruses, rotaviruses and astroviruses in turkey and chicken flocks, with combinations of viruses often present in the poultry gut [3,4]. A non-biased, comprehensive approach to virus discovery that would not require viral cultivation would reveal a great deal about the complex viral community in the turkey gut. Further, a community-based understanding of the viruses in the poultry gut will be an invaluable asset in ongoing studies of the enteric disease syndromes and would be welcome knowledge to poultry producers who rely upon efficient conversion of feed in the gut to produce an economically important commodity. A recent study utilizing a sequence-independent molecular screen of virus particle associated nucleic acid (PAN) in chicken enteric samples identified a novel chicken parvovirus (ChPV). This parvovirus is a member of the *Parvovirinae* sub-family within the *Parvoviridae*, and a PCR-based diagnostic test has been developed that targets the ChPV non-structural (NS) gene [6,7].The success of this PAN procedure suggests that similar approaches can be used for virus discovery in the poultry gut [7]. Ultra high-throughput nucleic acid pyrosequencing has emerged as a powerful

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diagnostic technology that can be applied to determine the full genomic repertoire present in a complex environmental sample [8]. Viral metagenomics can be specifically utilized to analyze viral sequences in just about any sample type, and is a powerful tool for virus discovery [9-13]. Further, viral metagenomics can be specifically applied to the problem of determining etiology in diseases and disease syndromes with no known cause [14-16]. In order to characterize the un-described viruses present in the turkey gut, we utilized the Roche/454 Life Sciences GS-FLX pyrosequencing platform to compile an RNA virus metagenome from turkeys experiencing enteric disease. The present analysis focused on RNA viruses in the turkey gut due to the large number of RNA viruses that have been identified to date as possibly contributing to enteric disease and poultry production problems. This approach yielded numerous sequences homologous to viruses in the National Center for Biotechnology Information (NCBI) BLAST non-redundant (nr) protein database, many of which have not been described in turkeys. These results validate this metagenomic approach to identifying known and novel RNA viruses in the poultry gut. The sequence data generated via this approach will prove useful in the molecular characterization of the viral constituency of the poultry gut, and will inform the selection of molecular diagnostic tests for enteric viruses. This will facilitate the development of updated molecular diagnostic tests, and a more thorough knowledge of the viral constituency in the poultry gut will lead to a better understanding of the role viruses play in enteric disease and in the performance of poultry flocks in general.

Results and Discussion

The initial pyrosequencing runs produced in excess of 139,000,000 bases of high quality nucleotide sequence with an average read length of 362. The sequence data was used to assemble 6526 contigs ranging in size from 97 to 2578 bp, with the majority of contigs falling in the range of approximately 250 to 450 bp. 4563 contigs produced no hits in the nr protein database using the blastx search parameters and the MEGAN default settings. 724 contigs had similarity to sequences from cellular organisms, including bacteria, fungi and avian species. 788 contigs had similarity to RNA viral sequences, including sequences from the dsRNA viruses (*Reoviridae* and Picobirnaviruses), and the ssRNA viruses (*Caliciviridae*, *Leviviridae*, *Picornavirales*, and *Astroviridae*) (Figure 1). The number of cellular sequences in the present dataset are likely due to the use of intestinal homogenates, which included intestinal tissue in order to ensure the discovery of cell-associated viruses in the submitted samples. The tblastx search output produced a MEGAN taxon tree similar to the one presented in Figure 1 and revealed that many of the unassigned contigs were similar to avian sequences.

The majority of the assigned viral contigs (620) showed similarity to database sequences from the *Picornavirales* order and other picorna-like viruses, viruses that, as a group, contain a positive sense single-stranded RNA genome and a virion approximately 30 nm in diameter [17]. Recently, a retrospective study of electron micrographs of enteric viruses from California turkeys experiencing enteric disease revealed a large number of "small round viruses" ranging in size from 15 to 30 nm, like most members of the *Picornavirales* [18]. These small round viruses are present in turkeys across a range of ages, but they have only been identified morphologically, making specific identification difficult. Avian enterovirus-like viruses have been described for years in domestic poultry; again this designation has historically been made based primarily upon morphological characterization, and little is known about their pathogenicity or their transmission characteristics[19]. It is unclear what role these picornaviruses and picorna-like viruses may play in turkey enteric disease or in turkey performance in general, but the presence of picornaviruses in other agricultural species has been closely associated with enteric disease, namely in pigs and cattle [20-23]. Members of the *Picornavirales* also infect avian species, with the etiologic agents of duck hepatitis (DH) in ducklings and avian encephalomyelitis (AE) in several poultry species both being picornaviruses [24,25]. The present metagenomic analysis has identified RNA sequences with homology to seven of the nine recognized picornavirus genera [26], with the largest proportion of the sequences bearing homology to the *Kobuvirus* genus.

The picobirnaviruses (PBVs) are a relatively recently described group of viruses that contain dsRNA, bi-segmented genomes and have non-enveloped capsids generally around 35 nm in diameter [27]. Since their initial description, the PBVs have been detected in enteric samples from several mammalian hosts, including humans [28-30]. A PBV has been described in chickens based upon morphological characterization and electropherotyping, along with a similar virus with an apparent tri-segmented genome [31]. The chicken PBV was not specifically associated with enteric disease. PBVs have been associated with gastroenteritis in humans [32,33], but a recent metagenomic analysis of the viral community in feces from healthy human volunteers revealed a relatively large number of PBV sequences [11]. Interestingly, PBVs have been detected in humans together with rotaviruses and astroviruses [32,34]. Phylogenetic analysis of a portion of the RNA-dependent RNA polymerase gene (RdRp) reveals that the putative turkey-origin PBV

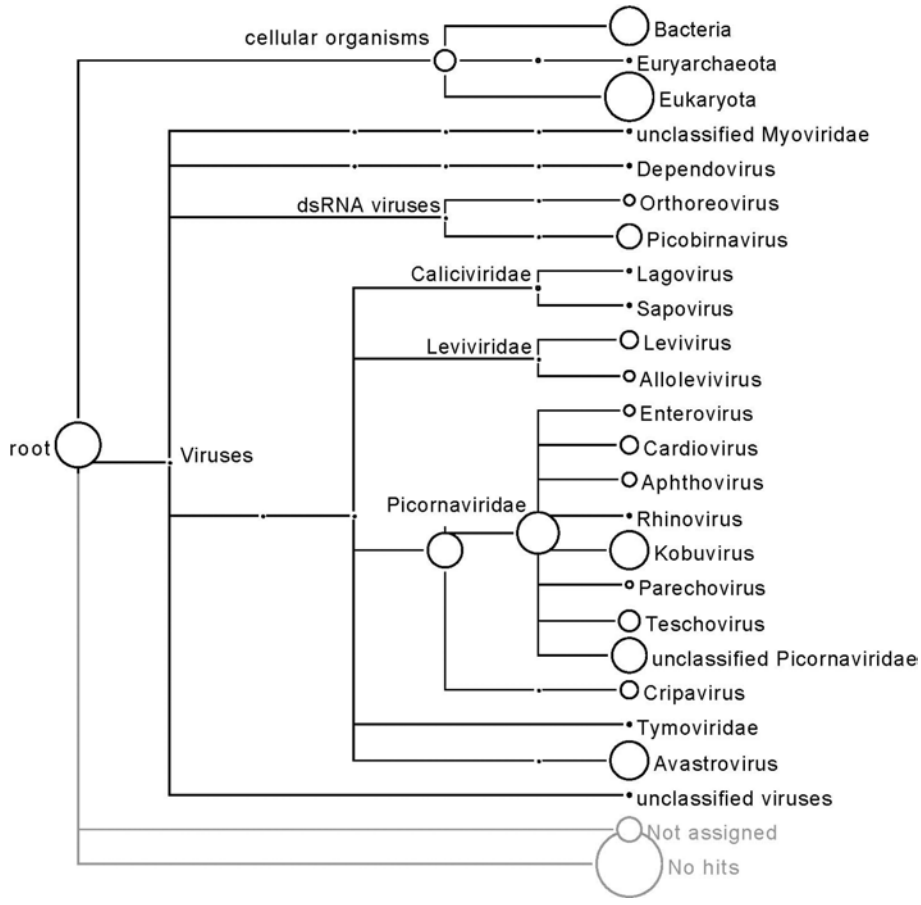

Figure 1 MEGAN tree with taxonomic assignments. The blastx output of the total contigs was assembled using the gsAssembler software. Circles located next to taxa are proportional to the total number of contigs identified in the pyrosequencing run and subsequent assembly (see Materials and Methods).

identified in the present metagenomic analysis is unique among the available PBV sequences, which include PBVs from humans, pigs, dogs, rats, snakes and municipal raw sewage (Figure 2). At the nucleotide level, the portion of the turkey-origin PBV used for the alignment and subsequent phylogenetic analysis shared from 49.5 to 70% sequence identity with the PBV sequences selected from the database; the highest identity was with the PBV detected in raw sewage in Washington state.

The family *Caliciviridae* includes four genera: *Norovirus*, *Sapovirus*, *Lagovirus*, and *Vesivirus* [26]. The present metagenomic analysis revealed nucleic acid with homology to database sequences from the *Sapovirus* and *Lagovirus* genera. The *Sapovirus* genus includes viruses that cause enteritis in swine and humans, and the Lagoviruses infect rabbits and hares (lagomorphs) [26]. In a phylogenetic analysis using a ~300 amino acid portion of ORF1 polyprotein homologous to the conserved P-loop NTPase superfamily, the putative turkey-origin Calicivirus grouped with the porcine enteric Sapoviruses (Figure 3). In general, the Sapoviruses are a very genetically heterogeneous genus [35], with numerous genogroups recognized, and many porcine Sapoviruses are related to human strains [36]; it will be interesting to determine the place the novel turkey caliciviruses hold in this genus, and it is notable that the portion of the ORF polyprotein analyzed in this study shared only 23.2 to 35% amino acid sequence identity with the Calicivirus isolates included in the alignment and subsequent phylogenetic analysis.

The appearance of avian reovirus and avian astrovirus sequences in the present analysis is not surprising, since these viruses are widespread in turkeys and chickens [3-5], and a good deal of recent research has helped to characterize the roles these viruses play in the poultry enteric syndromes [1,2,37-39]. The turkey astrovirus revealed using this metagenomic approach was most similar to the previously described type 2 turkey astroviruses (TAstV-2), which are very common in the turkey intestine [3]. The avian reovirus genes revealed using

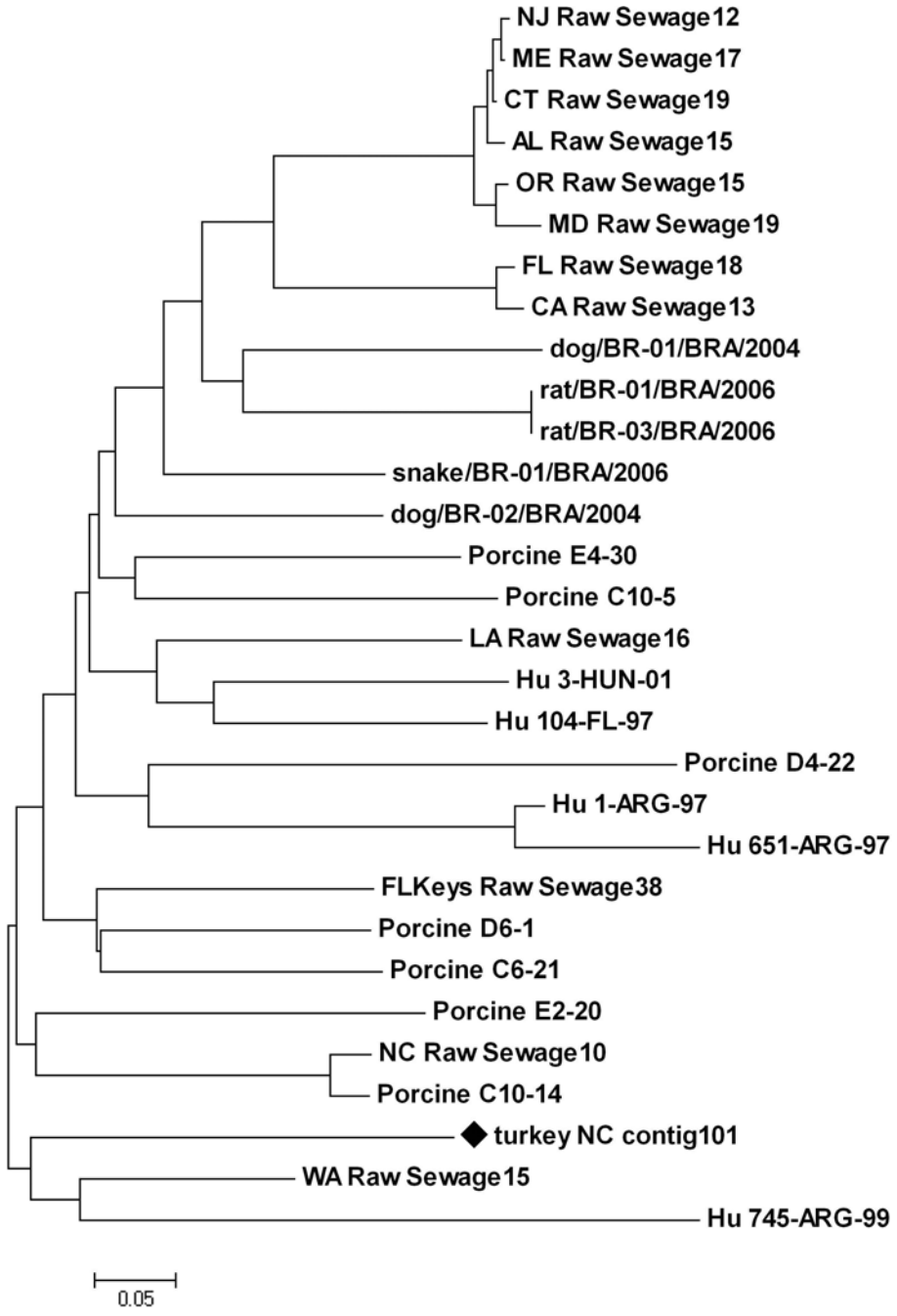

Figure 2 Picobirnavirus neighbor-joining tree. The evolutionary history for a 201 bp portion of the PBV RdRp gene was inferred using the Neighbor-Joining method, the optimal tree with the sum of branch length = 4.85654838 is shown. The nucleotide tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The turkey-specific sequence used in the analysis is indicated with a black diamond; its accession number is HM803965. Hu = Human.

this approach–namely the lambdaA core protein gene, the non-structural protein muC, and the structural protein muB–have not been described in turkeys previously, but they have homology to previously described avian reovirus genome segments [40,41].

This first look at the turkey gut viral community using metagenomics has revealed a great deal of novel RNA virus sequence, and this analysis is a step toward identifying some of these undescribed, small enteric viruses. The dataset includes samples from areas that historically

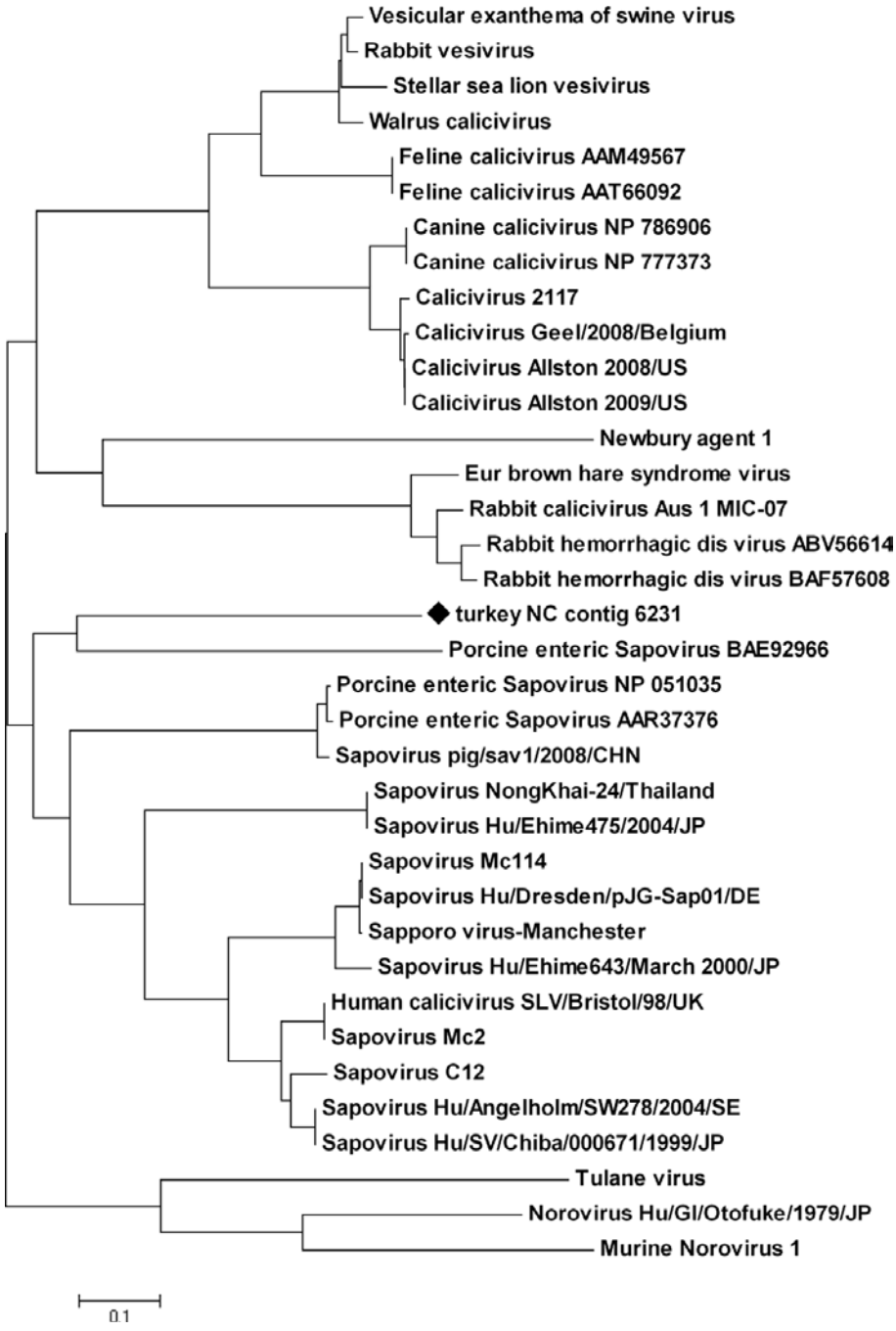

Figure 3 Calicivirus neighbor-joining tree. The evolutionary history of a ~300aa portion of the calicivirus ORF1 polyprotein was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 5.99935819 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The turkey-specific sequence used in the analysis is indicated with a black diamond; its accession number is HM803966.

have had problems with enteric disease and includes a range of flock ages. The samples were collected regardless of the present enteric disease status of the flocks to ensure that the viral flora had not been perturbed by advanced enteric disease signs and due to the numerous observations that enteric viruses are often present in healthy birds [3,4]. The sequence data generated via this approach will prove useful in the molecular characterization of the viral constituency of the poultry gut, and will inform the selection of molecular diagnostic tests

for enteric viruses. Further, this study sets the stage for subsequent comparative metagenomic analyses to determine the viruses commonly found in flocks with enteric syndromes versus the viral constituency of healthy flocks, for regional comparisons of circulating enteric viruses, and for comparing specific management and nutritional techniques and their effect on the gut microbiome.

Materials and methods

Gut samples receipt and preparation

With the cooperation of industry stakeholders, complete intestinal tracts (from duodenum/pancreas to cloaca, including cecal tonsils) from five turkey farms in North Carolina, U.S.A. with histories of enteric disease problems were received at the Southeast Poultry Research Laboratory in October 2008. Five complete intestinal tracts from each farm were collected. The intestines were processed promptly via blending into a ~20% homogenate in sterile phosphate-buffered saline (PBS) and were pooled into a single sample. This pooled intestinal homogenate represented turkeys ranging in age from 7 days to 34 days. After 5000 rpm (2400 × G) and 7500 rpm (5500 × G) centrifugation steps (15 min, 4°C) to clarify the homogenate (SLA 1500 SuperLite rotor, Sorvall), a stepwise filtration process involving 0.8 μm, 0.45 μm, and 0.2 μm cutoff bottle filters (Nalgene) was used to remove large particles and bacteria. Virus-sized particles were pelleted by ultracentrifugation (5 hr., 113,000 × G, 4°C).

Viral RNA isolation and cDNA synthesis

The virus particle pellet was resuspended in Tris-HCl buffer (pH 7.5) and treated with RNAse A (Invitrogen) to remove unencapsidated (non-viral) RNA. Total RNA was extracted from the pellet using TRIZOL-LS reagent (Invitrogen) and RNA was further purified from the TRIZOL-LS aqueous phase using the MagMax Viral RNA isolation kit (Ambion)[42]. This RNA sample was treated with Turbo DNAse (Ambion) to minimize any remaining DNA. cDNA was generated with random hexamers using the Invitrogen SuperScript Choice System and was ligated to the included EcoRI/NotI double-stranded oligonucleotide adapters and sephacryl column purified per the manufacturer's instructions. PCR using an EcoRI/NotI adapter primer (5'-CGG CCG CGT CGA C-3') was used to amplify the cDNA. An initial PCR reaction of 50 μl was split into 5 equal reactions prior to thermal cycling (94°C for 30s, 55°C for 30s, 72°C for 2 min, times 35 cycles, followed by an extension at 72°C for 7 min). The reactions were pooled and purified (Qiagen MinElute PCR cleanup kit).

High throughput sequencing and analysis

The amplified cDNA was utilized in high-throughput nucleic acid sequencing using Genome Sequencer FLX

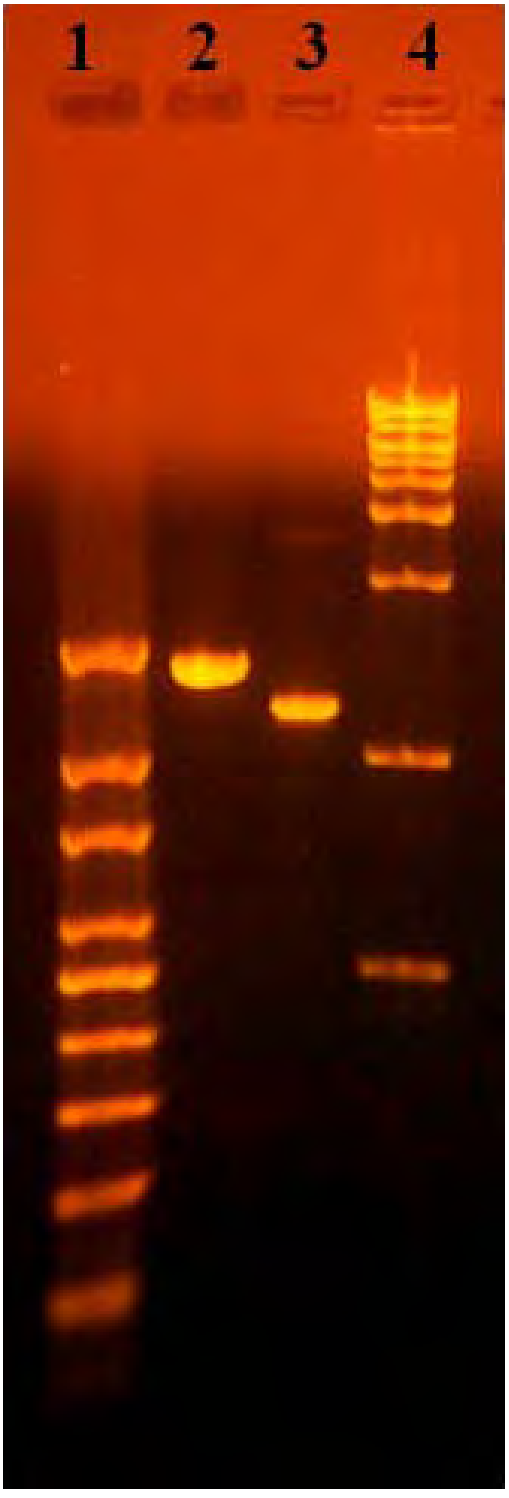

Figure 4 Turkey picobirnavirus and calicivirus diagnostic agarose gel. 1% agarose gel with RT-PCR amplicons from the turkey calicivirus ORF1 polyprotein region (1369 bp, lane 2) and the turkey picobirnavirus RdRp gene (1135 bp, lane 3). RNA was isolated from the intestinal contents collected from commercial North Carolina turkeys in 2009 (calicivirus positive) and 2010 (picornavirus positive).

Titanium pyrosequencing technology and reagents (Roche). Contigs were assembled using the gsAssembler software (454 Life Sciences) using stringent parameters (50 bp overlap with 95% identity). Using the assembled contigs as query sequences, the blast non-redundant (nr) protein database (GenBank) was searched using the blastx and tblastx programs. The blastx and tblastx output was compiled and contigs were assigned to taxa with MEGAN using the default LCA algorithm parameters [43]. Nucleotide and amino acid sequences were aligned using ClustalW and phylogenetic trees were prepared using MEGA4; the relationship of the sequences was inferred using the Neighbor-Joining method, which was determined to be a computationally efficient method to deal with datasets of this size and produced single trees to illustrate an initial placement of these novel viruses among available sequences [44]. In order to confirm directly the presence of picobirnavirus and calicivirus sequences in gut samples, the metagenomic contigs were utilized to design RT-PCR primers to amplify portions of the picobirnavirus RNA-dependent RNA polymerase (RdRp) and calicivirus ORF1 polyprotein. These primers were subsequently used to amplify viral sequences in the original RNA prep used to create the metagenome and in archived turkey intestinal samples from North Carolina, U.S.A (Figure 4).

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Authors' contributions
JMD conceived and coordinated the study, performed the virus isolation and cDNA production, and wrote the paper. LLB performed the bioinformatic analyses. MVD prepared the samples and performed the 454 pyrosequencing. BES coordinated the bioinformatic analyses and pyrosequencing. LZ provided technical input and intellectually contributed to the study design. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Case Report—

Parvovirus-Associated Cerebellar Hypoplasia and Hydrocephalus in Day Old Broiler Chickens

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SUMMARY. Cerebellar hypoplasia and hydrocephalus were identified in day old broiler chickens showing nervous signs, impaired mobility, and diarrhea. At postmortem examination, brains of chickens were misshapen and cerebellums were smaller than normal. Microscopically, cerebellar folia were reduced in size and irregularly shaped, and the ventricles were widely distended. Affected cerebellums had focal areas along the base of folia where the internal granular cell layer had been lost, and Purkinje cells were disorganized and located within the molecular layer. Parvovirus DNA was detected by polymerase chain reaction in three of nine brains with oligonucleotide primers designed for amplification of chicken and turkey parvoviruses. On the basis of phylogenetic analyses, the detected virus was most closely related to chicken parvoviruses. These findings suggest that a chicken parvovirus might cause a neurologic disease of young chickens characterized by cerebellar hypoplasia and hydrocephalus; however, its role as the cause of the disease remains to be confirmed.

RESUMEN. *Reporte de Caso*—Hipoplasia cereberal e hidrocefalia asociadas con un parvovirus en pollos de engorde de un día de edad.

Se identificó hipoplasia cerebelar e hidrocefalia en pollos de engorde de un día de edad que mostraban signos nerviosos, dificultad de movimiento y diarrea. Al examen postmortem, los cerebros de las aves estaban deformes y los cerebelos estaban más pequeños de lo normal. Microscópicamente, la folia cereberal estaba reducida en tamaño y de forma irregular, mientras que los ventrículos estaban ampliamente distendidos. Los cerebelos afectados presentaron focos a lo largo de la base de la folia donde la capa granular interna se había perdido y las células de Purkinge estaban desorganizadas y se localizaban dentro de la capa molecular. Mediante la prueba de reacción en cadena de la polimerasa, se detectó ADN de un parvovirus en tres de nueve cerebros utilizando iniciadores diseñados para amplificar parvovirus de pollo y de pavo. Con base en el análisis filogenético, los virus detectados estaban más cercanamente relacionados a los parvovirus de pollos. Estos hallazgos sugieren que los parvovirus de pollo pueden causar enfermedad neurológica en pollos jóvenes, caracterizada por hipoplasia cereberal e hidrocefalia, sin embargo, su papel como la causa de la enfermedad debe confirmarse.

Key words: parvovirus, broiler chickens, cerebellar hypoplasia, hydrocephalus

Abbreviations: AV = Aino virus; CA = cerebellar abiotrophy; CH = cerebellar hypoplasia; CNS = central nervous system; FFPE = formalin-fixed, paraffin-embedded; FGV = fowl glioma–inducing virus; NS = nonstructural

Neurologic disease is a common cause of early culling and death in both chickens and turkeys. Established etiologies include avian encephalomyelitis (picornavirus), bacterial encephalitis (e.g., *Salmonella* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* sp.), nutritional encephalomalacia (vitamin E deficiency), encephalocele/exencephaly, and pox vaccination reaction (1,7,20,21). These diseases can usually be differentiated by gross and microscopic lesions in the brain. Hypoglycemia and poult flip-over syndrome result in neurologic signs with less well understood etiologies and absence of lesions in the central nervous system (CNS).

Two congenital neurologic diseases characterized by grossly small cerebellums are cerebellar hypoplasia (CH) and cerebellar abiotrophy (CA). Although these are common in mammalian neonates (27), reports in birds are rare. Cerebellar hypoplasia has been experimentally induced by yolk sac inoculation of chicks with both fowl glioma–inducing virus (FGV), a member of avian leukosis virus subgroup A (31), and Aino virus (AV), a virus of the family *Bunyaviridae* known to cause fetal deformities, CH, and hydranencephaly in cattle and sheep (19). Naturally occurring cerebellar hypoplasia in chickens or turkeys has not previously been reported.

Parvovirus is known to cause cerebellar hypoplasia in both kittens and puppies, although in the dog, the enteric form of the virus is most prevalent (24,26,27). Parvovirus is also a well-characterized enteric pathogen of young geese and Muscovy ducks (6,14) and has been described in both turkeys and chickens (12,13,15,16,17,18). Parvovirus has been associated with poult enteritis complex in turkeys and runting-stunting syndrome in chickens (32,33). The partial genome sequences of both the turkey and chicken enteric parvoviruses have recently been described and are closely related to each other, representing novel members of the *Parvoviridae* subfamily (33). Zsak *et al.* (32) have also characterized the ubiquitous nature of enteric parvovirus in commercial flocks in the United States. The virus is transmitted both vertically and horizontally and can become systemic (15); however, detection of the virus in neurologic tissues has not been previously reported.

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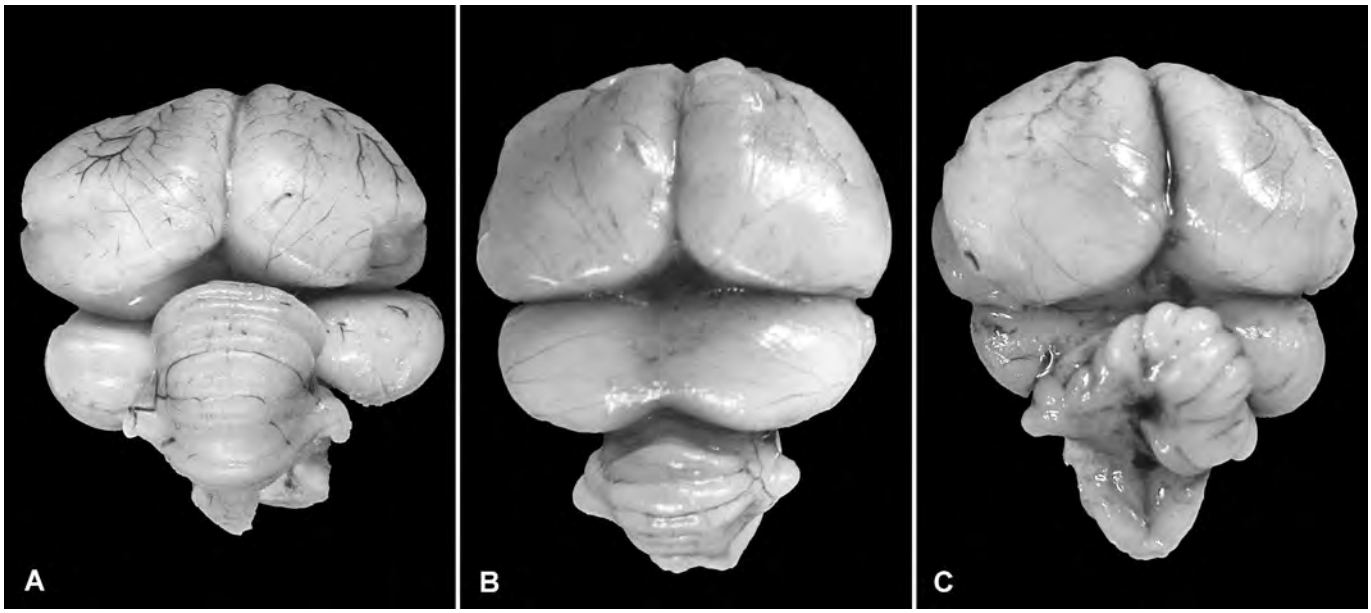

Fig. 1. Brains from day old chicks. (A) Normal; (B) small cerebellum and dorsally fused optic lobes; (C) malformed cerebellum.

This paper describes the first case of CH and hydrocephalus in commercial broiler chickens showing both enteric disease and neurologic signs. Evidence for a possible etiologic association of chicken parvovirus in this disease was provided by detection of the virus in affected brains by the PCR procedure.

CASE REPORT

Case history. Fourteen 1-day-old broiler chickens were presented for diagnostic evaluation to the Rollins Animal Disease Diagnostic Laboratory in Raleigh, North Carolina. Eleven chicks were alive and three were dead. Chickens were weak and abnormally small, had pasty vents, and mobility was impaired (''bad legs''). Eight of the 11 live birds were lying on their sides, with paddling of the legs. The birds were also experiencing increased mortality and diarrhea (enteric disease). Chickens were from one of four houses of a North Carolina farm, each house holding approximately 22,500 birds; no problems were seen in the other three houses. The percent morbidity in the house was not assessed; however, the grower noted morbidity was most prevalent during the first 3 days and declined after this time. The farm average weekly mortality was 1.23% (week 1), 0.4% (week 2), 0.39% (week 3), 0.25% (week 4), and 0.14% (week 5), consistent with the grower's observations.

Gross pathology. Five chickens had abnormal brains; both optic lobes were swollen and fused on the dorsal surface of the brain. In general, cerebellums were smaller than normal, and in one case, the cerebellum was grossly disfigured (Fig. 1). In seven chickens, the koilin layer of the gizzard was bile stained and had areas of hemorrhage. No other lesions were seen.

Histopathology. Histologic sections were prepared from the brains of nine live chicks. Of these, eight birds were on their sides and paddling. Consistent with the grossly small cerebellums, the folia were shortened and misshapen (Fig. 2A,B). Purkinje cells were usually organized in a single cell layer at the interface of the molecular and internal granular layers. However, in the most affected brains, there was focal loss of the internal granular cell layer along the base of some folia (Fig. 2B,C). In these areas, Purkinje cells were disorganized and located within the molecular layer. No active inflammation was associated with the lesions. In four of nine affected brains, the ventricles were dilated, indicating hydrocephalus (Fig. 2D). Lesions were also found in the ventriculus and included marked cavitation and focal hemorrhage in the koilin layer, but these were considered nonspecific because they are commonly seen in birds that are off feed.

PCR procedure. Formalin-fixed, paraffin-embedded (FFPE) sections of whole brain tissue from nine 1-day-old broiler chickens were used for DNA extraction. The procedure for whole brain extraction was performed with the QuickExtract™ FFPE DNA Extraction Kit (EpiCentre Biotechnologies, Madison, WI) according to the manufacturer's instructions.

A previously described PCR procedure that amplifies a 560-bp product within the nonstructural (NS) gene region of chicken and turkey parvoviruses was used (32). Positive control DNA consisted of an intestinal homogenate obtained from parvovirus-inoculated chickens (32). Negative controls included DNA extracted from brain sections of three normal chickens and a no-template control (nuclease-free water). The PCR procedure resulted in 560-bp products for three of nine brains. Direct nucleotide sequencing of the 560-bp products revealed that the nucleotide sequence corresponded to the NS gene region of chicken and turkey parvoviruses (GenBank GQ260159). Interestingly, the brain that appeared most normal histologically was most strongly positive for parvovirus by PCR, which might reflect the mild and temporal nature of the infection and difficulty identifying parvovirus postinfection by PCR.

Phylogenetic analysis. PCR amplicons were used for phylogenetic analysis, which was done according to described methods (32). Phylogenetic analysis demonstrated that the parvovirus amplicon in CH brains (ChbrNC) was closely related to chicken parvoviruses (Fig. 3).

DISCUSSION

In this study, parvovirus was detected by PCR analysis in day old commercial broiler chickens with CH and hydrocephalus. Fourteen commercial broiler chickens were submitted for postmortem

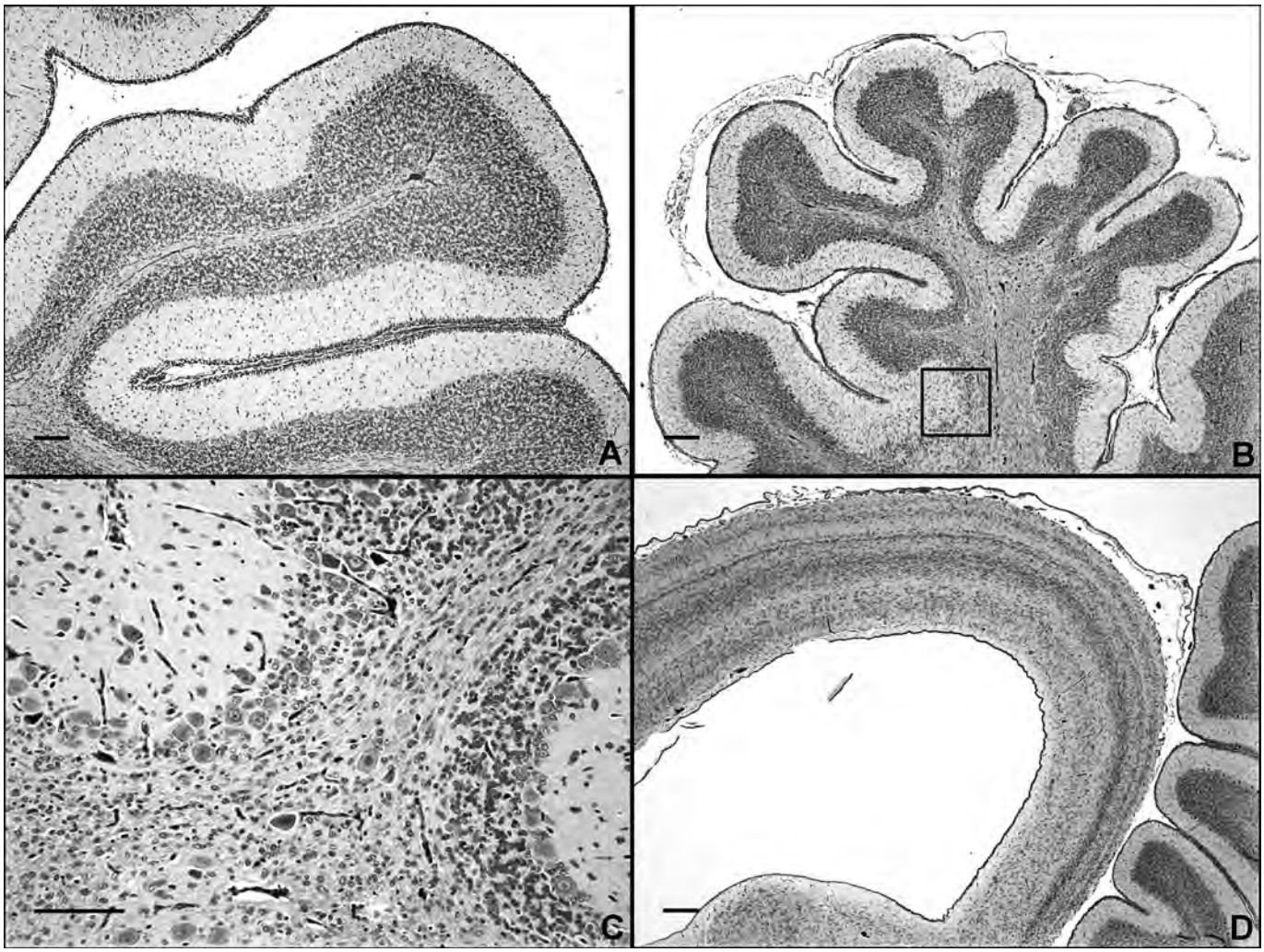

Fig. 2. Cerebellums from young chicks. (A) Normal folium of a 3-day-old chick. (B) Affected cerebellum, 1-day old chick. Folia are shortened and misshapen (compare with panel A taken at same magnification). Focal loss of the internal granular layer (box). (C) Higher magnification of area in box of panel B. Base of affected folium showing the lack of internal granular layer cells and displacement of Purkinje cells into the molecular layer. (D) Affected cerebellum, 1-day old chick. Marked dilatation of the third ventricle indicative of hydrocephalus. Bars in A, B, and D = 250 μm; bar in C = 150 μm.

examination to investigate runting-stunting, pasty vents, and impaired mobility. At postmortem examination, eight of 11 live birds showed neurologic signs (lying on their sides and paddling), prompting examination of the brains. Brains of nine birds (eight lying on their sides and paddling and one remaining upright) were examined. Gross brain lesions in five of the chicks were immediately obvious and included small or deformed cerebellums and swollen, dorsally fused optic lobes.

Two different early pathologic processes can result in small cerebellums: cerebellar hypoplasia and cerebellar abiotrophy. These can be initially distinguished by following the temporal pattern of clinical signs. Clinical signs are progressive with CA, and although CH can also be progressive (2,25), congenital CH is generally static, with signs remaining constant or diminishing as the animal adjusts. The grower's description of "bad legs" might have been the first neurologic sign seen in these chicks, which advanced to ataxia and paddling only under the stressful conditions of transportation. Because the grower reported that these clinical signs were most prevalent only during the first 3 days and had completely ceased after 1–2 wk, this further suggests congenital CH to be the most likely pathologic process.

Although a genetic anomaly could be responsible for the lesions seen, congenital CH is most often caused by viruses. This condition is well described in mammals and shows histopathologic lesions primarily in the cortex of the cerebellar folia, which vary with viral etiology. For example, bovine viral diarrhea virus, a pestivirus that causes CH in calves, is characterized by a nonsuppurative inflammation of the cerebellar cortex and adjacent leptomeninges, widespread necrosis of neuronal elements and neuropil, and hemorrhages and severe edema of the cerebellar folia and medullary white matter (23). Hog cholera, also a pestivirus, causes hypomyelinogenesis along with CH in pigs (3). In both dogs and cats, parvovirus targets rapidly dividing granular cells, causing necrosis of the external granular layer (24,26,27). The resulting hypoplastic molecular and granular cell layers cause CH. In cats, but not dogs, Purkinje cells also become infected by the virus via a different cytopathology and become haphazardly arranged adjacent to the white matter of the folia. Purkinje cell arrangement is normal in dogs.

The small cerebellums in these day old chickens are most consistent with virus-induced CH. Two viruses, Aino virus and fowl glioma–inducing virus have been shown to cause CH in

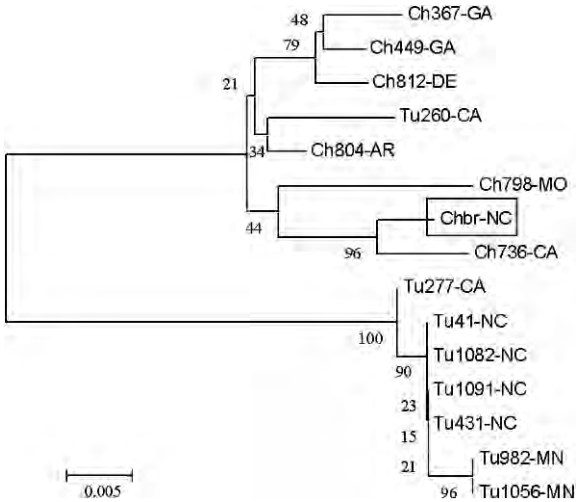

Fig. 3. Phylogenetic tree showing the relationship of the parvovirus DNA identified in affected day old chick brains (ChbrNC) to other chicken and turkey parvoviruses identified from intestinal samples on U.S. farms. The evolutionary history was inferred by the neighbor-joining method (22). The optimal tree with the sum of branch length = 0.12314265 is shown. Percentages of replicate trees, in which associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches (5). 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. The evolutionary distances were computed by the maximum composite likelihood method (29) and are in the units of the number of base substitutions per site. There were 561 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (28).

experimentally infected chickens. Gross and histopathologic brain lesions in chick embryos inoculated *in ovo* with AV are severe and result in early embryonic skeletal and brain abnormalities (19). In the cerebellum, differentiating cortical neurons become disorganized due to severe parenchymal liquefactive necrosis, thinning of the cerebellar parenchyma because of dilatation of the fourth ventricle and temporal gliosis; these lesions were not observed in the chickens in this study. In contrast, FGV infection results in cerebellar lesions that are similar to those seen with mammalian parvovirus, including a diffuse absence of internal granular cells and thinning of this layer. Disorganization of the Purkinje cell layer is also seen, but it is the result of decreased numbers and irregular arrangement of Bergmann's fibers rather than direct virus-induced cytopathology (31). Fowl gliomas were observed in the cerebra of all experimentally infected birds and in the cerebellum of one bird; these were not detected in chickens evaluated in this study. Neither AV nor FGV has been identified as a cause of naturally occurring CH in commercial flocks.

Chickens in this study had cerebellums that were, in general, smaller than normal, with folia that were shortened and irregular. The most distinctive cortical cerebellar feature was disruption of the architecture at the base of some folia, characterized by focal areas of missing internal granular layers and disorganization of Purkinje cells. Inflammation was not a feature of the lesions in the brain. Lesions in the cerebellums of these chicks were similar to both mammalian parvovirus–induced lesions and those that developed in chicks following *in ovo* infection with FGV.

Marked dilation of the third ventricle (hydrocephalus) also occurred in the day old chicks. Hydrocephalus can be internal, in which obstruction of CSF flow within the ventricular system causes ventricular enlargement and brain atrophy, or communicating, in which decreased resorption of CSF at the arachnoid villi causes expansion of the ventricular system and subarachnoid space. Communicating hydrocephalus caused by vitamin A deficiency has been reported in an adult Goffin's cockatoo (10), and internal hydrocephalus has been reported in Polish fowl because of abnormal brain growth changes (8). Viral infections during pregnancy are known to alter normal CNS development in offspring (9), and fetal parvovirus infection has been associated with hydrocephalus in both human beings (11) and cats (26,30). Aqueductal stenosis, a common malformation associated with the fusion of the two rostral colliculi (optic lobes in birds) that can follow prenatal inflammatory processes (27), is a likely cause for the dilated third ventricle seen in these chicks.

Both avian parvovirus (32,33) and leukosis virus serotype A (4) are common in the field and can be transmitted both vertically and horizontally. The very young age of the affected birds, the brain lesions, and the fact that runting-stunting syndrome, characterized by slow growth, diarrhea, and mortality was a major concern in the flock under consideration, prompted us to analyze the affected brains for parvoviral DNA. Parvovirus was detected in three of the nine brain samples. The inability to demonstrate parvovirus DNA in the other brain sections might have resulted from small amounts of virus in the tissue or possible DNA degradation from formalin fixation. Because the amount of tissue available for DNA extraction from day old cerebellum is very small, whole brain slices were used in the extraction process. This precluded localization of the virus to just the cerebellum and obtaining information about the etiology of the hydrocephalus.

Phylogenetic analysis indicated that the detected parvoviruses were similar to chicken parvoviruses previously identified in intestinal contents collected in the United States. This identification, along with neurologic signs and enteric disease at the time of diagnostic investigation, makes parvovirus a likely agent as the direct cause of the CH and hydrocephalus. However, FGV cannot be ruled out at this time. Immunohistochemical analysis for parvovirus localization and disease reproduction following Koch's postulates are needed to definitively establish parvovirus as the etiologic agent. Although parvoviruses appear to be common in chickens and turkeys, on the basis of previous findings, it seems strange that CH has not previously been described. However, infrequent evaluation or failure to closely evaluate the gross and microscopic appearance of brains of young chickens could explain the failure to recognize this condition in the past. Also, parvoviral infection with subsequent vertical transmission during lay might be rare because of the frequent infection of young chickens with the virus. Of particular interest would be to evaluate the genetics of the parvoviruses identified in the present case more fully to determine whether these viruses might represent unique genotypes associated with neurologic infection.

The congenital nature of this disease suggests that the etiologic agent was vertically transmitted; transovarial transmission of chicken parvovirus has been reported (17). Parvoviruses originating in the gut can become viremic and settle in multiple organs (6,14), however, and horizontal transmission from hatchery contamination during *in ovo* vaccination should be considered.

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ORIGINAL ARTICLE

Molecular characterization of avian astroviruses

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Abstract Astroviruses are frequently associated with enteric diseases in poultry, being isolated from cases of runting-stunting syndrome (RSS) of broiler chickens, poult enteritis complex (PEC), and poult enteritis mortality syndrome (PEMS) of turkeys. Currently, five types of avian astrovirus have been identified: turkey astroviruses 1 and 2 (TAstV-1, TAstV-2), avian nephritis virus (ANV), chicken astrovirus (CAstV) and duck astrovirus (DAstV). The objective of this study was to molecularly characterize the different types of avian astroviruses circulating in commercial poultry. Sequence analysis of a region of ORF2, which encodes the capsid precursor protein associated with serotype and viral pathogenesis, revealed extensive variation in amino acid sequence within each subtype: TAstV-2 (81.5%-100%), ANV (69.9%-100%), and CAstV (85.3%-97.9%). However, this region was more conserved in TAstV-1's (96.2%-100%). Furthermore, a novel astrovirus was detected in chicken samples and found to be <64% similar to ANV and <30.6% similar to CAstV. The results of this study underline the great genetic variability of avian astroviruses and indicate that there are most likely multiple serotypes of each avian astrovirus circulating in commercial poultry.

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Introduction

Astroviruses have been associated with enteric disease in humans and young animals such as calves, lambs, pigs, deer, mice, dogs, cats, and minks [28]. In avian hosts, astroviruses have been linked to enteritis and increased mortality in young turkeys, chickens and guinea fowl [4, 5, 15, 18–20, 25, 34, 37, 40, 45, 46, 54, 60], to nephritis in chickens [17, 23], and to fatal hepatitis in ducklings [11, 13].

The family *Astroviridae* is divided into two genera: *Mamastrovirus* (mammal astroviruses) and *Avastrovirus* (avian astroviruses). Within each genus, numerous groups have been described based on serotype and genetic differences [1]. Astroviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses of 28-30 nm diameter and have a star-like morphology [24]. Their genome is 6.4-7.7 kb in length and has three open reading frames (ORFs) and a poly-A tail [11, 28]. ORF1a and ORF1b encode the non-structural proteins, and ORF2 encodes a capsid precursor protein [28]. The greatest sequence variability in the astrovirus genome is found in ORF2; however, a high degree of conservation of the N-terminal 415 amino acids of ORF2 has been shown for HAstVs and other mammalian astroviruses. The avian astroviruses vary considerably from the mamastroviruses, although the basic character of the ORF2 amino terminus is

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also conserved [28]. Downstream of the conserved 415 residues, considerable sequence variability is seen among astroviruses isolated from different species and even among different serotypes from the same animal species. Based on structural predictions, the conserved amino-terminal half of the astrovirus capsid protein could play a role in assembly of the capsid core, whereas the hyper-variable carboxy-terminal region could form the spikes of the virion and participate in the early interactions of the virus with the cellular receptors on the host cell [28].

Enteric diseases cause substantial economic losses of commercial turkeys and broilers due to reduced weight gain, increased morbidity and mortality, poor condition of the flock, and increased production costs from poor feed conversions and increased use of therapeutic anti-microbial treatments. Three enteric disease syndromes have been described: runting-stunting syndrome of broilers (RSS), poult enteritis complex (PEC), and poult enteritis mortality syndrome (PEMS). These diseases are considered to be polymicrobial, and numerous viruses have been associated with them; however, no definitive cause has been identified. Astroviruses are among the most common viruses found in cases of PEC and PEMS [2, 3, 19, 21, 35, 36, 38, 39, 60]. Astroviruses have also been isolated from recent cases of RSS in broilers and have been associated previously with poor weight gain, enteric disease and kidney disease in chickens [4, 12, 21, 41–43, 53]. To date, five different astroviruses have been identified in avian species based on the species of origin and viral genome characteristics: two turkey-origin astroviruses (TAstV-1 and TAstV-2); two chicken-origin astroviruses, avian nephritis virus (ANV) and chicken astrovirus (CAstV); and a duck-origin astrovirus (DAstV). ANV has also been detected in commercial turkey poults [30], and TAstV-2 in guinea fowl [5]. Molecular characterization of these viruses has been done mostly by sequencing ORFs 1a and 1b. However, with the exception of TAstV-2 viruses [31, 47], little or no characterization has been done of the ORF2 or capsid gene.

The study of the capsid protein genes is important for understanding the relatedness of these viruses. Antigenic characterization of avian astroviruses has been limited by the difficulty in isolating and growing these viruses. Currently, two serotypes of ANV and two of TAstV-2 have been described [17, 50], but sequence data suggest that there may be many more [47]. There also appears to be more than one serotype of CAstV [31, 55]. No serological characterization of TAstV-1 isolates has been reported.

In this study, we sequenced new astrovirus isolates from chickens and turkeys and analyzed a region of the ORF2 (capsid proteins) of all available astroviruses detected in poultry.

Material and methods

Viruses

The viruses sequenced were obtained from the Southeast Poultry Research Laboratory (SEPRL) repository of intestinal contents collected from commercial turkeys and chickens throughout the United States between 2003 and 2007 [32, 33]. Specimens were from both healthy flocks and poorly performing flocks (Table 1). All samples were stored at -80°C until utilized for RNA extraction.

Viral RNA extraction and RT-PCR

Two-hundred microliters of intestinal contents was diluted in 1.2 ml of PBS, homogenized with sterile glass beads in a FastPrep homogenizer (Thermo-Savant, Inc., Waltham, MA) and centrifuged for 10 min at 800 × g. Total RNA was extracted directly from 250 µl of the supernatant using TRIzol LS reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions. For the initial detection and characterization of the viruses, a segment of the polymerase gene (ORF1b) was amplified by standard RT-PCR using a QIAGEN OneStep RT-PCR Kit (QIAGEN Inc., Valencia, CA). The primers, which amplify all four avian astroviruses, have been reported previously [51]. Sequence data for the capsid gene (ORF2) were obtained by designing primers based on published sequence data from reference isolates of TAstV-1 (Y15936.2), TAstV-2 (AF206663.2), ANV-1(AB046864) and ANV-2(AB033 998.1). All primers were designed using the PrimerSelect program (DNASTAR, Madison, WI). Sequence data from the capsid gene was also obtained by RT-PCR using an oligo dT primer and specific primers directed toward the polymerase gene. The variation found in the capsid sequence among the astroviruses studied was so high that more than 96 sequencing primers had to be developed in order to obtain sequence data. Rarely could one set of primers be used on more than one astrovirus, even if it belonged to the same type. Amplifications were performed in a MJ Research DNA Thermal Cycler (Waltham, MA). The RT-PCR products were separated on an agarose gel by electrophoresis, and amplicons of the appropriate size were excised from the gel and extracted using a QIAquick Gel Extraction Kit (QIAGEN Inc.). Purified RT-PCR products were sequenced directly using a BigDye Terminator Kit (Applied Biosystems, Foster City, CA) run on an ABI 3730 sequencer (Applied Biosystems). Sequences were assembled and edited with LASERGENE 7.2 (DNASTAR) and then aligned to previously published astrovirus ORF2 sequences.

Table 1 Chicken and turkey astroviruses examined in this study

Isolate name	Flock condition	Age in days	Genotype	GenBank accession number
NC-TK-SEP T1-489-05*	Healthy	28	TAstV-1	HQ185551
NC-TK-SEP T1-552-05	Healthy	36	TAstV-1	HQ185552
NC-TK-SEP T1-703-05	Healthy	84	TAstV-1	HQ185553
MO-TK-SEP T1-822-05	Healthy	15	TAstV-1	HQ185554
NC-TK-SEP T1-928-06	Healthy	14	TAstV-1	HQ185555
VA-TK-SEP T2-33-03*	PEC	22	TAstV-2	HQ185556
NC-TK-SEP T2-222-03*	Unknown	57	TAstV-2	HQ185557
TX-TK-SEP T2-311-04*	Unknown	Unknown	TAstV-2	HQ185558
CA-TK-SEP T2-269-05*	Enteric disease	8	TAstV-2	HQ185559
GA-CK-SEP-CA-457-05*	RSS	6	CAstV	HQ185560
DE-CK-SEP CA-651-05*	Poor	7	CAstV	HQ185561
DE-CK-SEP CA-811-05	RSS	10	CAstV	HQ185562
GA-CK-SEP-CA-364-05*	RSS	7	CAstV	HQ185563
MO-CK-SEP-CA-883-06	RSS	15	CAstV	HQ185564
DE-CK-SEP ANV-651-05*	Poor	7	ANV-2	HQ188692
DE-CK-SEP ANV-811-05	RSS	10	ANV-2	HQ188693
GA-CK-SEP ANV-368-05*	RSS	10	ANV-2	HQ188694
NC-TK-SEP ANV-537-05*	Healthy	56	ANV-2	HQ188695
GA-CK-SEP ANV-451-05*	RSS	11	ANV-2	HQ188696
NC-TK-SEP-ANV-670-05*	Healthy	84	ANV-2	HQ188697
GA-CK-SEP- ANV-364-05*	RSS	7	ANV-2	HQ188698
GA-CK-SEP- ANV-458-05	Healthy	11	ANV-2	HQ188699
GA-CK-SEP-ANV-792-05	RSS	Unknown	ANV-like	HQ185565

*Partial ORF1b reported previously [30, 31]

Sequence analysis

An 861-894-nucleotide region of ORF2 was used to evaluate phylogenetic relationships among avian astroviruses. This region corresponded to the following regions of the reference viruses: TAstV-1, nucleotides (nt) 5329-6204; TAstV-2, nt 5474-6340; ANV-1, nt 5054-5945; DAstV, nt 5844-6710. There is no full-genome sequence published for CAstV. The following capsid sequences from avian astrovirus published in GenBank were included in the phylogenetic analysis: TAstV-1 (Y15936.2); TAstV-2 (AF206663.2), AK/98, CA/00, CO/01, MN/01, MO/01, PA/01, MI/01, TX/00, VA/99 (EU143843.1 to EU143851.1), TAstV-1987 (AY769616.1), TAstV-2001 (AY769615.1); ANV-1 (AB046864); ANV-2 (AB033998.1); DAstV (FJ434664.1); HAstV-1 (L123513); HAstV-2 (Q82446.1); HAstV-3 (AAD17224.1); HAstV-4 (BAA93446.1); HAstV-5 (AAA56750.1); HAstV-6 (CAA86616); HAstV-7 (CAA 69922); HAstV-8 (AAF85964); Dog AstV (CAR82569.1); porcine AstV (CAB95000); sea lion TAstV (ACR54274.1); Bat AstV (EU847155); feline AstV (AAC13556.1); ovine AstV (Y15937); bottlenose dolphin AstV (ACR54280.1); mink AstV (AY179509). Also included was the sequence of CAstV-3 (US Patent 209009263) and GA-UGA_Ast1-Oct2006 (CAstV-UGA-2006, Patent WO/2010/059899), a CAstV isolated at the University of Georgia, Athens, GA, from a flock of chickens with RSS (GenBank accession number HQ185566).

The sequence information was compiled using the Seqman progam (Lasergene V. 6.01, DNASTAR, Madison, WI), and multiple alignments of nucleotide and amino acid sequences were constructed with the Megalign application of the same software package using the Clustal W alignment algorithm (Slow/Accurate, Gonnet). Pairwise sequence alignments were also performed with the Megalign program to determine nucleotide and amino acid sequence identity.

In addition, a 2.5-kb region of the ANV-2's corresponding to nt 3710 to 6569 of ANV-1, spanning part of ORF1b and ORF2, was analyzed using SimPlot software version 3.5.1 [22]. The polymerase-capsid region was aligned using Clustal W of the MEGA 4 software [49], and ANV-1 and GA-CK-SEP- ANV-364-05 were used as the query sequences for similarity analysis. Similarity was calculated in each window of 200 bp using the Kimura two-parameter method.

Phylogenetic trees

Sequences were aligned with Clustal W (Lasergene, V. 8.0.2 DNAStar, Madison WI). Nucleotide trees were

constructed with merged duplicate runs of BEAST v. 1.4.8 [9] using HKY substitution, empirical base frequency, gamma heterogeneity, codon 2 partitions, relaxed lognormal clock, Yule process tree prior with default operators with unweighted pair group mean with arithmetic average starting tree, and a Markov chain Monte Carlo length of 10^6. Protein trees were constructed with the same program and parameters, except the Blosum 62 substitution matrix and the gamma + invariant site heterogeneity model were used.

Sequence accession numbers

The assigned GenBank accession numbers for the sequences generated from the capsid region of TAStV-1, TAstV-2, CAstV, and ANV viruses are HQ185551-65, and the ANV sequences encompassing ORF1b and capsid are HQ1888692-99 (Table 1).

Results and discussion

The capsid precursor protein gene is the most variable astrovirus gene, containing sequence differences that certainly reflect variation in antigenicity and possibly also in pathogenicity [45]. In this study, we compared the nucleotide and predicted amino acid sequences of a section of the hypervariable region of the capsid (ORF2) of 19 avian astroviruses from chickens and turkeys, along with capsid gene sequences of 22 other avian astroviruses retrieved from the database. Pairwise comparisons of the nucleotide and amino acid sequences of the ORF2 region of the viruses analyzed in this study showed great variability among all groups of avian astroviruses, with the exception of TAstV-1, which had between 96.2% and 100% amino acid identity (Table 2). TAstV-2 had between 81.5% and 100% amino acid identity, and CAstV had between 85.3% and 97.9% amino acid identity. ANV strains were most closely related to the reference ANV-2 strain, with 85.8%-100% amino acid identity among them, and 69.9% to 74.0% amino acid identity with ANV-1. One astrovirus detected in chickens (GA-CK-SEP ANV-792-05) did not group with any of the known avian astroviruses but was most closely related to ANV-1, with which it had 64% amino acid identity. DAstV was closest to TAstV-2 in amino acid sequence similarity, with 72.8%-75.7% identity. This is interesting, considering that these two viruses differ in their genome organization [11].

The amount of variation observed in most of the avian astroviruses groups studied suggests that there is antigenic variation among them, since for HAstV, Walter *et al.* [56] found that when two strains had less than 95% nucleotide homology, they could be distinguished serologically. TAstV2001 and TAstV1987 have been reported to represent distinct serotypes, sharing only 82.8% amino acid sequence identity for the complete capsid region [52]. This level of sequence conservation is close to that of HAStV capsid genes from different serotypes (<80% nt similarity). These sequence distances suggest that many of the astroviruses studied may represent different serotypes in each group. However, in order to corroborate this, serological cross-reactivity analysis of the viruses still needs to be conducted. Similar remarkably high genetic diversity has also been observed within bat astroviruses [6, 61].

The degree of similarity at the amino acid level to mamastroviruses in the region examined was 20.7%-29.3% for TAstV-2, 21.1%-26.7% for TAstV-1, 20.3-28.9 for CAstV, and 22.4%-27.9% for ANV. The mamastroviruses analyzed were 31.4% to 61.2% similar among themselves,

Table 2 Range of percent amino acid and nucleotide (*italics*) identity among avian astrovirus isolates analyzed in this study. One ANV-1, nine ANV-2, seven CAstV, six TAstV-1, fifteen TAstV-2, one DAstV, and isolate ANV792 viruses were compared

	ANV-1	ANV-2	CAstV	TAstV-1	TAstV-2	DAstV	ANV792
ANV-1	-	69.9-74.0	31.6-33.7	30.4-38.1	28.6-31.7	28.1	64.0
	-	*66.0-67.5*	*42.9-44.8*	*45.6-46.0*	*42.6-45.2*	*42.0*	*65.1*
ANV-2		85.8-100	29.4-33.7	28.6-39.2	29.4-33.7	26.1-29.9	61.5-64.4
		85.4-98.2	*42.7-47.2*	*45.8-48.0*	*42.7-47.2*	*42.8-44.9*	*61.9-63.6*
CAstV			85.3-97.9	38.7-42.9	39.0-42.0	41.3-42.4	29.5-30.6
			74.0-91.8	*49.2-51.0*	*48.2-53.2*	*48.5-50.0*	*40.7-43.5*
TAstV-1				96.2-100	45.0-48.2	45.3-45.9	29.3-37.2
				95.5-100	*52.1-54.6*	*51.2-51.6*	*45.5-46.4*
TAstV-2					81.5-100	72.8-75.7	28.2-45.2
					81.4-100	*65.2-70.1*	*43.7-45.2*
DAstV						-	29.2
						-	*42.5*

with the exception of human and feline astroviruses, which were > 72.6% similar (data not shown).

Phylogenetic analysis based on the partial ORF2 amino acid sequences shows that the avian astrovirus form four groups (Fig. 1): TAstV-1-like, TAstV-2-like, CAstV-like and ANV-1-like viruses, similar to previous reports analyzing ORF1b sequences [30]. The phylogenetic relationships of the nt sequences were similar to those of the aa sequences. The ANV group contained both chicken-origin and turkey-origin isolates. The TAstV-1, TAstV-2 and CAstV groups contained only turkey and chicken-origin viruses, respectively. The TAstV-2 group was distant from TAstV-1 and was closely related in this region to DAstV. A phylogenetic tree based on amino acid sequences of the ORF2 region of the avian and selected mammalian astroviruses shows that the avian astroviruses form distinct groups consistent with the nucleotide tree and are separate from the mammalian isolates (Fig. 2). The mammalian isolates vary widely in their similarity to one another. The HAstV isolates clustered together with the feline astroviruses on a branch that was distant from other astrovirus groups. Several other isolates formed species-specific branches, including the porcine, canine, bottlenose dolphin and sea lion astroviruses. Conversely, some disparate isolates including mink, ovine and bat astroviruses clustered together.

A 2.5-kb nucleotide sequence spanning a region of the polymerase and capsid genes of eight ANV was also analyzed by comparison to the ANV-1 reference strain. The SimPlot computer program [22] was used to analyze the alignment of the eight ANV using a window size of 200 nucleotides that was moved along in 20-nucleotide steps. The percent identity was calculated for each window and plotted in a line chart. Figure 3 shows that the ANV-1 reference strain shared a lower level of sequence identity in the capsid region with the ANV analyzed, whereas, based on the phylogenetic analysis, these viruses were more closely related to ANV-2, a different ANV serotype. Because there are no sequence data available for the ORF1b of the ANV-1 virus, it was not included in the analysis. Alignment of the ORF1b-ORF2 region sequences of eight ANV2 strains showed that they shared high levels of sequence identity in the conserved domain (N-terminal 415 amino acids), whereas the carboxy-terminus of ORF2 showed higher levels of variation (Figure 4). This is consistent with results reported previously for HAstV [14, 27, 57]. Recent work by Strain *et al.* involving the analysis of nine complete turkey astrovirus genomes provided evidence for recombination between astrovirus genomes and

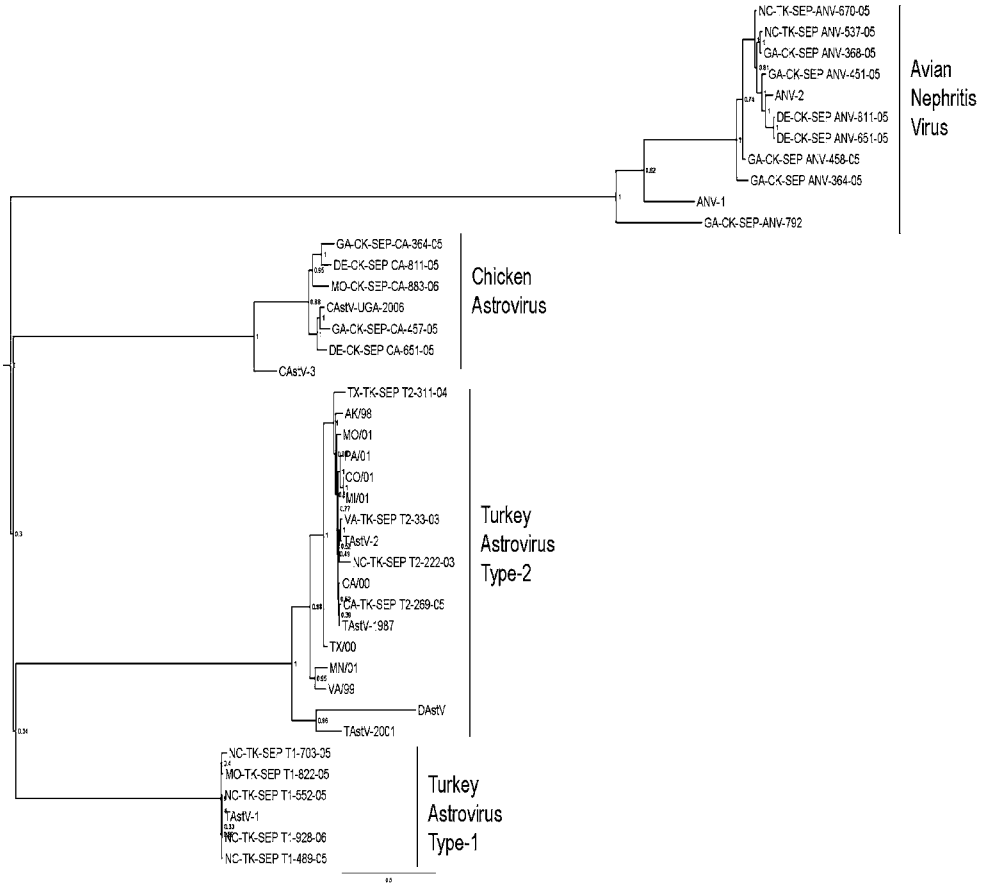

Fig.1 Phylogenetic tree of the nucleotide sequences of avian astrovirus capsid genes. Node posterior values are given

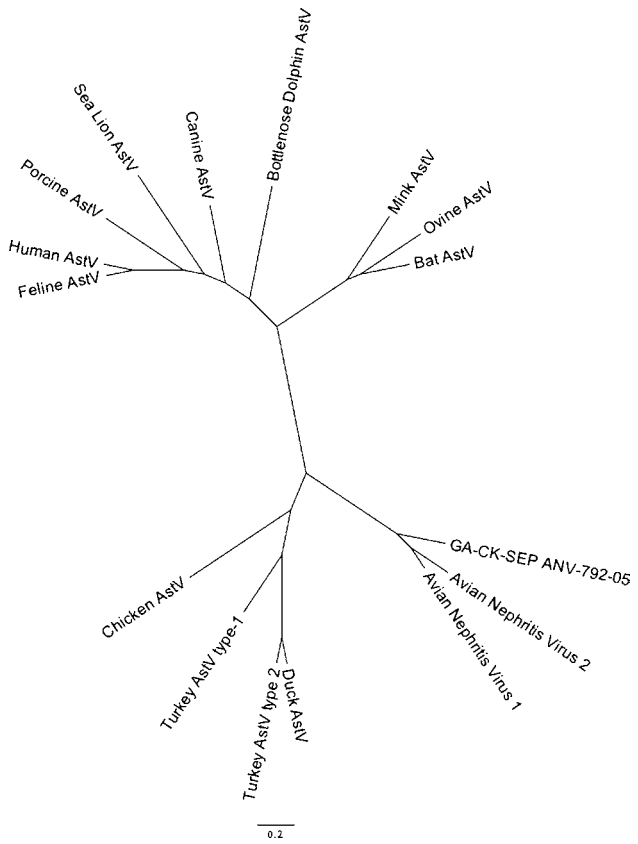

Fig. 2 Unrooted phylogenetic tree of the amino acid sequences of phylogenetically representative isolates from different species groups

supported the view that sequences from different regions of the astrovirus genome should be considered for phylogenetic analysis on which taxonomic subdivisions might be based [47]. With recombination, it is possible that different ANV-1 ORF1b sequences could be contiguous with ANV-1 capsid protein sequences, or conversely, they could have different ORF2 sequences, as was observed with the ANV-2 isolates analyzed. These data suggest that potential recombination could occur within ORF2, resulting in the emergence of new viruses.

Astroviruses appear to be widely disseminated and endemic in poultry in the US, and concomitant infection of flocks with two or more enteric viruses is common [19, 30, 32, 33]. The high incidence of astroviruses in turkeys affected with enteric disease has been documented previously [37]. Astroviruses have also been found in turkeys with poult enteritis mortality syndrome (PEMS), a disease that has caused severe economic losses to the turkey industry [2, 3, 35, 59, 60]. Although TAstV-2 is frequently detected in turkey poults, it is not clear how many serotypes exist. Phylogenetic analysis of TAstV-2 isolates revealed a high level of genetic variation, particularly in the capsid gene, suggesting the possibility of several serotypes [31, 47, 52]. Tang and Saif antigenically characterized two TAstV-2 isolates, TAstV1987 and TAstV2001, showing that they belonged to different serotypes [50], and in our analysis, these viruses are clearly of different genotypes. Until more serology is done to determine how many TAstV-2 serotypes exist, genetic characterization of the virus serves as a useful alternative for type assignment of avian astroviruses and permits the characterization of the astroviruses circulating in poultry. TAstV-1 strains, on the other hand, appear to be more closely related to each other and are less commonly detected on turkey farms. The reason for this is not clear, but it could be due to sample bias or real biological features of the virus.

Avian nephritis virus (ANV), previously classified as a member of the genus *Enterovirus*, family *Picornaviridae*, has been isolated from chickens and was re-classified as an astrovirus following the complete sequencing of the viral genome [17]. This virus was first isolated in 1976 and is associated with growth depression and kidney lesions in young chickens [17]. Avian nephritis virus has been shown to be widely distributed in chicken flocks in Japan [16], some European countries [7, 8, 29], and even in specific-pathogen-free flocks [26]. Under field conditions, clinical signs associated with this virus infection in broiler chickens have varied from subclinical to outbreaks of RSS and baby chick nephropathy [10, 16, 23, 42, 44, 48, 58]. At least two serotypes of ANV and different pathotypes have been reported [43, 54]. Based on sequence comparisons of part of the non-structural protease gene (ORF 1a), ANV could be grouped into three genotypes [23]. ANV has also been reported in turkeys [30], and antibodies to ANV have been detected in turkeys in Northern Ireland and England, but nothing has been reported about clinical signs of infection [7, 29]. The ANVs analyzed in this study, although closely related to ANV-1 in ORF1b, were mostly ANV-2-like based on ORF2, indicating the possibility of recombination. It is not clear if the GA-CK-SEP ANV-792-05 is in fact an ANV. Further genetic analysis of these viruses is required.

A second type of chicken astrovirus was shown to be antigenically and genetically distinct from ANV [4]. Although the sequence of this virus is still not available, we will refer to it as the reference CAstV (it has been patented as CAstV-2). CAstVs sharing high levels of nucleotide sequence identity with this first characterized CAstV have been detected in the United States in broiler chickens affected with RSS [30]. Recently, molecular and antigenic characterization of entero-like viruses (ELVs) demonstrated that they were CAstV, some of them closely related to the reference CAstV, and some of them more distantly related [54]. CAstV was detected by serology in broiler flocks in the United Kingdom, the Netherlands, Spain, Australia, and the United States [4]. Serological evidence also indicated that CAstV infections were common in

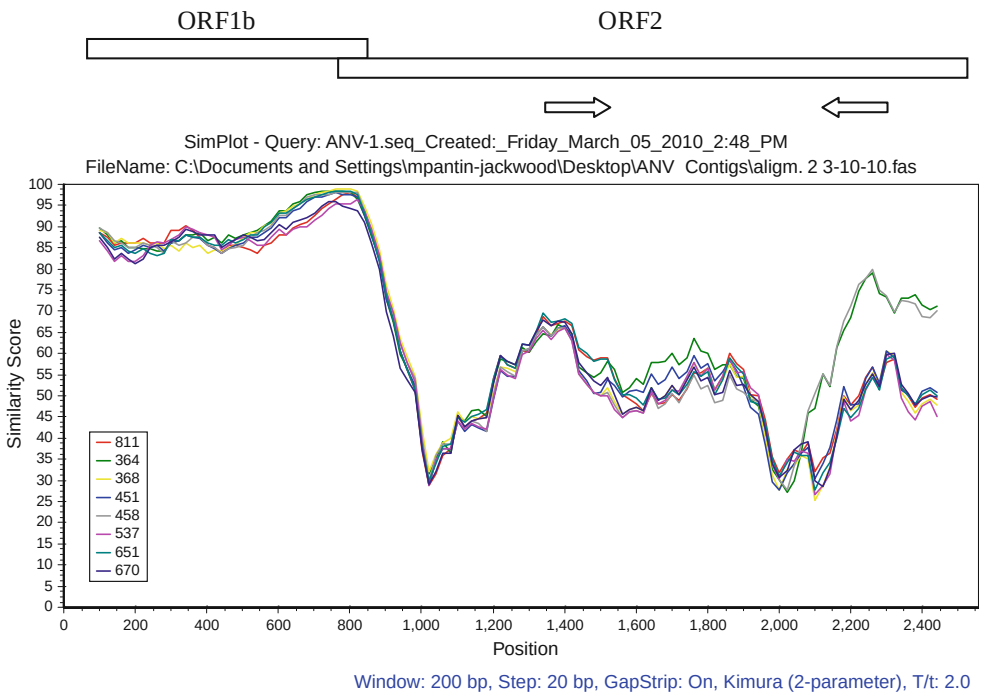

Fig. 3 Similarity analysis of ANV based on partial sequences of ORF1b and ORF2. Nucleotide identity plot of the 2.5 kb region comprising the amplified polymerase and capsid regions of the following ANVs compared with ANV-1: DE-CK-SEP ANV-651-05 (651), DE-CK-SEP ANV-811-05 (811), GA-CK-SEP ANV-368-05 (365), NC-TK-SEP ANV-537-05 (537), GA-CK-SEP ANV-451-05 (451), NC-TK-SEP-ANV-670-05 (670), and GA-CK-SEP-ANV-364-05 (364). The bar above the plot represents the regions corresponding to ORF-1b (polymerase) and ORF-2 (capsid). The arrows indicate the region amplified from the capsid gene and used in the phylogenetic analysis

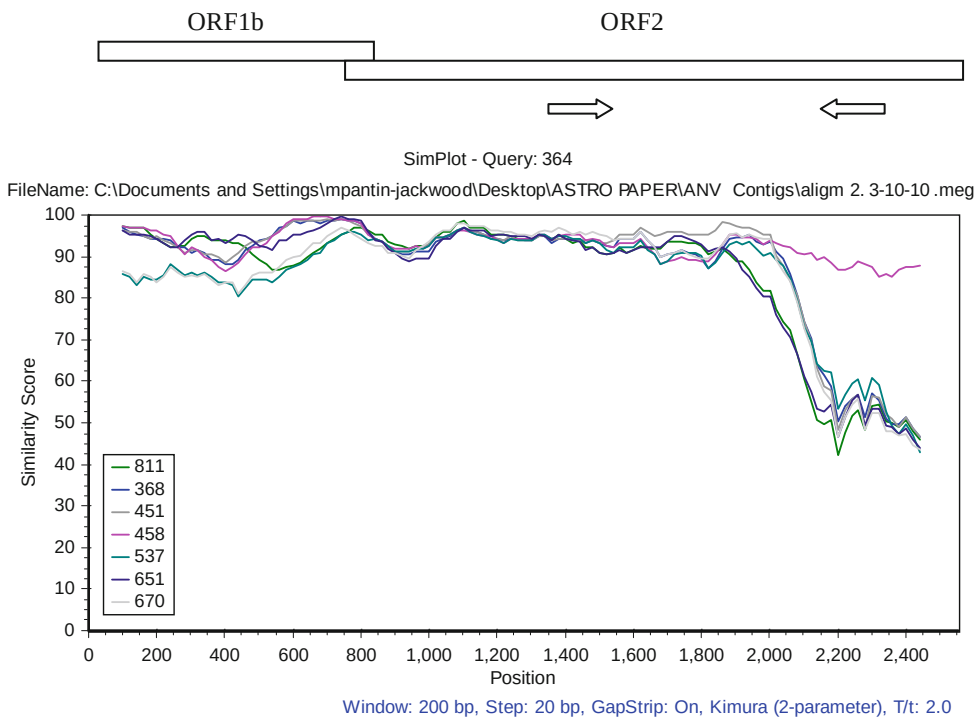

Fig. 4 Similarity analysis of ANV based on partial sequences of ORF1b and ORF2. Nucleotide identity plot of the 2.5-kb region comprising the amplified polymerase and capsid regions of the following ANVs: DE-CK-SEP ANV-651-05 (651), DE-CK-SEP ANV-811-05 (811), GA-CK-SEP ANV-368-05 (365), NC-TK-SEP ANV-537-05 (537), GA-CK-SEP ANV-451-05 (451), NC-TK-SEP-ANV-670-05 (670), and GA-CK-SEP-ANV-364-05 (364). The query isolate used was isolate 364. The bar above the plot represents the region corresponding to ORF-1b (polymerase) and ORF-2 (capsid). The arrows indicate the region amplified from the capsid gene and used in the phylogenetic analysis

broiler parent flocks within the United Kingdom and also appeared to be widespread in European breeder flocks [55]. CAstV was commonly detected by RT-PCR in broiler flocks with growth problems or ones that were affected by RSS [30, 45, 55]. Phylogenetic analysis based on a region of the ORF1b demonstrated the existence of two distinct clades [30, 45]. In this study, the CAstV isolates grouped separately from CAstV-3 and also formed two different groups (Fig.1). The complete genome of CAstV has not been published, so many question related to the genome structure and how they relate to other avian astroviruses remain to be answered.

A virus producing hepatitis in ducks (DHV-2) was identified to have astrovirus morphology and was renamed

duck astrovirus 1 (DAstV-1) [1, 13]. Sequence analysis of the ORF 1b region of this virus showed that it was closely related to CAstV. A second virus, DHV-3, was also identified as an astrovirus, but based on the amino acid identities in the ORF 1b region of this virus with that of DHV-2 (69%) it appears that they might be of different genotypes, and DHV-3 is more closely related to TAstV-2 [54]. The complete sequence of a duck astrovirus (DAstV) associated with an outbreak of fatal hepatitis in ducklings in China was published recently, and phylogenetic analysis revealed that DAstV was most closely related to TAstV-2 [11]. Our results also corroborate this.

A new taxonomy proposal for avian astroviruses has been submitted to the International Committee on Taxonomy of Viruses (ICTV) for approval (http://talk.ictvonline.org/files/proposals/taxonomy_proposals_vertebrate1/m/vert01/2358.aspx). This proposal is the result of the work done in agreement by all members of the *Astroviridae* Study Group during the preparation of the 9th ICTV Report. Based on new data, the *Astroviridae* Study Group states that a classification based on genetic criteria is more appropriate than classifying the viruses into species within the genus *Avastrovirus* based only on the host of origin. A phylogenetic analysis of avastroviruses based on the amino acid sequence of the full-length ORF2 results in three groups (genotype species). Mean amino acid genetic distances (p-dist) range between 0.576-0.742 and 0.204-0.284 between and within groups, respectively. This new classification establishes three new species within the genus: *Avastrovirus* GI.A, including turkey astrovirus 1; *Avastrovirus* GI.B, including avian nephritis virus 1 and 2; and *Avastrovirus* GII.A, including turkey astrovirus 2 and duck astrovirus. Some chicken astroviruses for which only partial sequences are available (chicken astrovirus 2, DQ324850), or no sequences at all, are deposited in GenBank (chicken astrovirus 3) and would be listed as "related viruses that might be members of the genus *Avastrovirus* but have not been assigned to a species".

As more sequence data become available, a clearer picture will emerge with regard to avian astroviruses. This will have to be complemented by serological and biological characterization of these viruses.

In conclusion, comparative analysis of avian astrovirus capsid genes revealed extensive genetic variation and the presence of distinct genotypes of TAstV-2, ANV and CAstV circulating in poultry. Changes in the capsid protein induced by mutations or recombination most likely have an effect on the pathogenicity and antigenicity of the viruses, and consequently, practical implications for virus detection methods, epidemiological studies and development of potential vaccines against astrovirus infections.

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Astrovirus, Reovirus, and Rotavirus Concomitant Infection Causes Decreased Weight Gain in Broad-Breasted White Poults

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SUMMARY. Turkey astrovirus type-2 (TAstV-2), turkey rotavirus (TRotV), and turkey reovirus (TReoV) have been implicated as possible causes of enteric diseases and poor production in turkeys; however, numerous studies with each individual virus have failed to reproduce the disease as observed in the field. Therefore, in this study we evaluated the pathogenesis of all possible combinations of one, two, or three viruses in comparison to sham inoculates in 3-day-old turkey poults. Body weights were recorded at 2, 4, 7, 10, and 14 days postinoculation (PI) and were decreased in virus-infected turkeys throughout the experiment as compared to sham inoculates. Although not significantly different from the other virus-exposed groups, the poults exposed to all three viruses had the lowest body weights throughout the experiment. Clinical signs, including huddling, diarrhea, and agitation, were only observed in groups exposed to TAstV-2 and/or TRotV. At 4 days PI, birds from each treatment group were necropsied, and pale intestines with watery contents and undigested feed were observed in the groups that were exposed to TRotV + TReoV or TRotV + TAstV-2 and the group exposed to all three viruses. Minimal microscopic lesions were observed in the intestines of turkeys infected with TAstV-2, TReoV, or a combination of both. In the turkeys infected with TRotV, either alone or in combination with other viruses, mild microscopic lesions were found in all sections of the small intestine and viral antigen was identified by immunohistochemical staining in mature enterocytes. No or very mild lesions were observed in other organs with the exception of the bursa of Fabricius, where mild to severe atrophy was observed in all virus-infected poults examined. Cloacal shedding of TAstV-2 and TRotV was evaluated by reverse-transcription PCR testing of cloacal swabs and minimal differences were observed among the treatment groups.

RESUMEN. Descenso en la ganancia de peso de pavipollos blancos de pechuga amplia por la infección simultánea de astrovirus, reovirus y rotavirus.

El astrovirus de los pavos tipo 2 (TAstV-2), el rotavirus del pavo (TRotV), y el reovirus de los pavos (TReoV) han estado implicados como posibles causas de enfermedad y baja producción en pavos. Sin embargo, numerosos estudios utilizando cada virus individualmente, han fracasado en la reproducción de la enfermedad tal como se presenta en el campo. Por lo tanto, en este estudio, se evaluó la patogénesis de todas las combinaciones posibles entre uno, dos ó tres virus en pavipollos de tres días de edad y se compararon con aves controles negativos. Se registraron los pesos corporales a los 2, 4, 7, 10, y 14 después de la inoculación y se observó disminución en el peso en las aves inoculadas durante el periodo experimental en comparación con las aves controles negativos. A pesar de que no existieron diferencias significativas con otros grupos con exposición viral, los pavipollos expuestos a los tres virus de manera simultánea, presentaron los pesos corporales más bajos a lo largo del experimento. Los signos clínicos incluyeron, amontonamiento entre las aves, diarrea y agitación, que fueron observados con los grupos expuestos a los virus TAstV-2 y/o TRotV. A los cuatro días postinoculación, se practicó la necropsia en las aves de cada grupo experimental, se observó palidez en intestinos con contenidos acuosos y alimento sin digerir en los grupos expuestos a los virus TRotV junto con TReoV o en las aves expuestas a TRotV junto con TAstV-2 y en el grupo expuesto a los tres virus. Se observaron lesiones microscópicas mínimas en los intestinos de pavos infectados con TAstV-2, TReoV, o con la combinación de los dos. En los pavos infectados con TRotV, solo o en combinación con los otros virus, se encontraron lesiones leves en todas las secciones del intestino delgado y el antígeno viral se identificó mediante inmunohistoquímica en enterocitos maduros. En otros órganos, no se observaron lesiones o éstas fueron mínimas exceptuando la bolsa de Fabricio, donde se observó atrofia leve a severa en todos los pavipollos infectados que fueron examinados. Se evaluó la eliminación cloacal de TAstV-2 y TRotV mediante la transcripción reversa y reacción en cadena de la polimerasa con hisopos cloacales y se observaron diferencias mínimas entre los grupos tratados.

Key words: astrovirus, enteric disease, reovirus, rotavirus, turkeys

Abbreviations: HRP = horseradish peroxidase; IHC = immunohistochemical staining; PEC = poult enteritis complex; PEMS = poult enteritis mortality syndrome; PI = postinoculation; RT-PCR = reverse transcription–PCR; TAstV = turkey astrovirus; $TCID_{50}$ = 50% tissue culture infectious doses; TReoV = turkey reovirus; TRotV = turkey rotavirus

Enteric disease in turkeys has an important economic impact because of production losses due to poor feed conversions and poor weight gain. Sporadic outbreaks of enteric disease are seen worldwide in commercial poultry and can vary widely in severity (reviewed by Reynolds [13]). The causes of enteric disease have never been definitively established because they are complex and polymicrobial and similar disease signs can likely be caused by different pathogens. Viruses from numerous virus families have been involved in causing enteric disease including avian astroviruses, particularly turkey astrovirus type 2 (TAstV-2) (10,14,26), avian reoviruses (6,7,18), and avian rotaviruses (23,24). However, because experimental infections with individual viruses are rarely reported to reproduce disease with the same severity observed in the field and surveys of chickens and turkeys have shown that infection with these viruses in "healthy" flocks is not uncommon (8), it has been difficult to definitively identify what is necessary and sufficient to cause poult enteritis complex (PEC) and poult enteritis mortality syndrome (PEMS).

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Table 1. Mean body weight in grams ± standard deviation by treatment group and days postinoculation. Values followed by different letters have statistically significant ($P < 0.05$) differences in body weights.

Treatment group[A]	Days postinoculation					
	0	2	4	7	10	14
Sham	53.4 ± 5.2a	73.5 ± 7.0a	99.1 ± 11.4a	149.4 ± 16.1a	252.0 ± 34.9a	350.0 ± 54.9a
TRotV	49.9 ± 3.7a	62.5 ± 5.9bc	81.5 ± 11.3c	128.3 ± 22.0bc	200.1 ± 34.5b	282.0 ± 45.9b
TAstV-2	50.2 ± 4.1a	66.4 ± 4.2bc	89.8 ± 8.5bc	126.8 ± 17.6bc	192.1 ± 30.9b	267.5 ± 45.3b
TReoV	50.1 ± 3.6a	68.0 ± 5.5b	95.5 ± 7.8ab	139.9 ± 17.7a	216.0 ± 32.2b	298.6 ± 45.7b
TRotV + TReoV	51.1 ± 3.8a	64.8 ± 5.7bc	80.0 ± 16.8c	136.7 ± 9.9ab	221.9 ± 25.8ab	303.5 ± 41.1ab
TRotV + TAstV-2	49.5 ± 2.8a	63.6 ± 3.4bc	81.0 ± 7.3c	124.7 ± 13.6bc	204.5 ± 19.5b	282.0 ± 29.3b
TAstV-2 + TReoV	49.3 ± 4.7a	61.6 ± 8.5bc	82.2 ± 11.6c	119.0 ± 19.9bc	195.7 ± 34.0b	273.1 ± 47.9b
TRotV + TAstV-2 + TReoV	50.0 ± 4.3a	60.2 ± 6.0c	72.1 ± 9.6c	111.6 ± 20.2c	189.2 ± 31.0b	263 ± 44.0b

[A]TAstV-2 = turkey astrovirus type-2; TRotV = turkey rotavirus; TReoV = turkey reovirus.

Since numerous surveys of virus incidence have shown that turkey flocks are frequently infected with both an astrovirus and a rotavirus (8,15,16), we evaluated the pathogenesis of concomitant infection of young turkey poults with both TAstV-2 and a turkey-origin rotavirus (TRotV) to determine if there is a synergistic interaction between the viruses. In addition, a turkey reovirus (TReoV) isolated from turkeys with enteric disease that has been shown to be immunosuppressive (4) was included as a third virus. Here we report the pathogenesis of isolates of TAstV-2, TRotV, and TReoV in all possible combinations in 3-day-old broad-breasted white poults. To our knowledge pathogenesis studies with all three viruses in all combinations have not been previously reported.

MATERIALS AND METHODS

Viruses. All three viruses were originally isolated from turkey flocks with unevenness and/or diarrhea. The TAstV-2 isolate was Turkey/CA/SEP-A270/04 (10) and was propagated for no more than three passages in embryonated turkey eggs. The TRotV isolate was Turkey/MN/SEP-996/07, which was isolated in MA-104 cells using previously reported methods (22) and was passaged three times in MA-104 cells. The TReoV isolate was Turkey/NC/SEP-R44/03 (20), which was passaged in VERO cells four times. TRotV and TReoV were titrated in cell culture using the same cell lines as those used for virus propagation as described previously (20) by inoculating the cells with 10-fold dilutions of virus prepared in serum-free media. Cells were observed for cytopathic effects to evaluate infection status. TAstV-2 was titrated in embryonated turkey eggs as previously described (10). Titers were calculated with the Reed–Muench method (12).

The TAstV, TReoV, and TRotV isolates used here were each tested for the following adventitious agents (excluding homologous viruses): adenovirus, astroviruses (including avian nephritis virus and turkey-astrovirus type-1 and type-2), coronavirus, reovirus, and rotavirus by PCR or reverse transcription (RT)–PCR (3,19,21) and were found to be negative.

Experimental infection of turkeys. Broad-breasted white turkeys were obtained from a commercial hobby-bird hatchery at 2 days post-hatch. The absence of TRotV, TReoV, and TAstV-2 infection were confirmed with RT-PCR methods as described previously (3,19). The poults were divided into eight treatment groups of 12 (Table 1) and housed in Horsfall isolators with *ad libitum* access to feed and water. Animals were humanely cared for in accordance with established institutional animal care and use procedures.

At 3 days posthatch the poults were inoculated with 0.2 ml of the appropriate material by oral gavage. The virus doses were TAstV-2 Turkey/CA/SEP-270/04 10^7 50% egg infectious doses, TRotV Turkey/MN/SEP-996/07 10^3 50% tissue culture infectious doses ($TCID_{50}$), and TReoV Turkey/NC/SEP-44/03 10^4 $TCID_{50}$. The highest dose possible was administered for TRotV and TReoV since these viruses do not grow to high titers in cell culture.

All birds were weighed and cloacal swabs were collected at 0, 2, 4, 7, 10, and 14 days postinoculation (PI) to evaluate virus shed by RT-PCR. The cloacal swabs were collected in phosphate buffered saline and frozen at −70 C and later tested by RT-PCR for each virus to which the birds where exposed. On day 4 PI, two birds from each group were necropsied and bursa, liver, spleen, thymus, proventriculus, ceca, jejunum, ilieum, and duodenum/pancreas were collected in 10% neutral buffered formalin for microscopic evaluation. At 21 days PI, serum was collected from the remaining birds. Since RT-PCR was not used with TReoV, infection was confirmed by homologous virus neutralization assay conducted as previously described (20) using serum collected from surviving birds at the termination of the experiment. Serum collected from five poults prior to inoculation was also tested for TReoV antibody and found to be negative.

Real-time RT-PCR and conventional RT-PCR. RT-PCR methods were used to evaluate cloacal shed of TAstV-2 and TRotV (TReoV was not included in the RT-PCR testing because it does not replicate well in the intestine and is shed poorly by the cloacal route) (9,19). Samples were tested for TAstV-2 by a real-time RT-PCR assay that targets the ORF 1B (POL gene) (19). TRotV was tested for with a conventional RT-PCR test that targets the NSP4 gene (3).

Statistical analysis of body weights. Body weights were compared among all groups by one-way repeated measures ANOVA (Student–Newman–Keuls method) (SigmaStat 3.1, Systat Software, Richmond, CA). A P value of <0.05 was considered to be significant.

Histopathology. Tissues were paraffin-embedded, sectioned, mounted, stained with hematoxylin and eosin, and examined by light microscopy. All samples were evaluated in a manner that blinded the examiner to the treatment groups.

Immunohistochemical staining (IHC) for the detection of TRotV in tissues. We focused on TRotV for the IHC assay because we have previously reported IHC results with the TAstV-2 and TReoV isolates utilized here (9,10). A peroxidase immunohistochemical technique using convalescent or hyperimmune sera from rotavirus-inoculated turkeys or chickens was used to identify rotavirus in all the tissues collected (bursa, liver, spleen, thymus, proventriculus, ceca, jejumun, ilieum, and duodenum/pancreas) from infected poults. All procedures were done at room temperature except when noted. Tissue sections were cut (4 μm thick) from paraffin-embedded tissue samples and mounted on charged glass slides (Superfrost/Plus; Fisher Scientific, Suwanee, GA). Deparaffinization, antigen retrieval, and blocking procedures have been previously described (9).

To ensure consistency, antibodies from two sources were utilized separately as primary antibodies: 1) turkey convalescent sera collected at 21 days PI as described above, and 2) sera from 14-wk-old chickens hyperimmunized with beta-propiolactone inactivated TRotV Turkey/MN/SEP-996/07. The primary antibodies were each diluted 1:100 in streptavidin peroxidase antibody diluent (Biogenex, San Ramon, CA) before use. Slides were incubated with the primary antibody overnight at 4 C. The secondary antibody was goat anti-turkey IgG, horseradish peroxidase (HRP) labeled or goat anti-chicken IgG, HRP labeled (Southern Biotechnology Associates, Inc., Birmingham, AL), diluted

Table 2. Distribution and severity of histological lesions in poults inoculated with turkey astrovirus type-2 (TAstV-2), turkey rotavirus (TRotV), or turkey reovirus (TReoV) and all combinations at 4 days postinoculation.

	TRotV	TRotV + TAstV-2	TAstV-2	TRotV + Reo	Reo	TAstV-2 + Reo	TRotV + Reo + TAstV-2
Duodenum	+[A]	+	−	+	−	+/−	+
Jejunum	+	+	+/−	+	+/−	+/−	+
Ileum	+	+/−	−	+/−	−	−	+/−
Bursa	+/−	+	++	++	++	+++	+++

[A]Microscopic lesions: − = no or sporadic lesions; +/− = minimal; + = mild; ++ = moderate; +++ = severe.

1:100 in antibody diluent (Biogenex), and incubated for 1 hr. The reaction was visualized with the DAB Substrate kit for peroxidase (Zymed Laboratories Inc., San Francisco, CA). After staining, the sections were counterstained with hematoxylin, air dried, cover-slipped, and examined by light microscopy. Tissues collected from sham inoculated poults served as negative controls. Additional primary antibody controls included nonimmune goat sera, and turkey convalescent antiserum specific for TReoV used in place of the TRotV-specific antibody (9).

RESULTS

Body weights. At 2 days PI, the mean body weights of all treatment groups were significantly different from the sham inoculated group. The TAstV-2 alone, TRotV alone, and TRotV + TAstV-2 groups and the group exposed to all three viruses remained significantly different from the sham inoculates for the duration of the experiment (Table 1). The TReoV only group body weights were similar to those of the sham inoculates at 4 and 7 days PI and the TRotV + TReoV group body weights were similar to the sham inoculates at 7, 10, and 14 days PI. Among the treatment groups exposed to viruses there was no group that was significantly different from the other virus infected groups at all sample times, although the group exposed to all 3 viruses did have the lowest body weights at all time points postinfection.

Clinical sign and gross lesions. Clinical signs which included huddling, diarrhea and agitation/increased vocalization, were observed 4-11 days PI, and were generally mild and similar among the groups exposed to either TAstV-2 or TRotV. Clinical signs were not observed in the sham inoculates or the group exposed to TReoV only. The severity of clinical signs was not clearly greater in any of the groups exposed to 2 viruses or all 3 viruses as compared to the groups exposed to a single virus. Mortality was minimal through-out the experiment; at 4 days PI one TRotV only exposed bird died, at 5 days PI one sham inoculate died and at 6 days PI one TRotV + TReoV–exposed bird died.

At necropsy at 4 days PI, gross lesions consisting of pale or translucent intestines with watery contents and undigested feed were seen in all birds from the groups inoculated with TRotV + TReoV, TRotV + TAstV-2, and TRotV + TReoV + TAstV-2. Gross lesions were not observed in any of the other groups.

All of the remaining TReoV-exposed birds were tested for antibody to confirm infection at the termination of the experiment by virus neutralization assay and all were positive.

Microscopic lesions. The distribution and severity of histological lesions by tissue and virus are summarized in Table 2. No lesions were observed in tissues collected from sham inoculated poults at any time. The cecum, liver, pancreas, kidney, thymus, and proventriculus, which presented no lesions or very mild lesions in all treatment groups, were excluded from the table. Intestinal sections were scored for degeneration, vacuolation, and sloughing of intestinal epithelial cells; villous atrophy, blunting, and fusion; crypt epithelial hyperplasia; and infiltration of the lamina propria with inflammatory cells. Bursa sections were scored for lymphoid depletion and fibroplasia. Lesions were scored in increasing levels of severity as follows: absent or sporadic, minimal, mild, moderate or severe.

Mostly minimal lesions were present in the intestines of turkeys infected with TAstV-2, TReoV, or a combination of both. Shrunken degenerate cells present in the villi and crypt epithelium and mild crypt hyperplasia resulting in increased crypt depth were the most common histopathological findings. Mild villus shortening and increased number of lymphocytes in the lamina propria were also present. These lesions occurred principally in the jejunum.

In the turkeys infected with TRotV, alone or in combination with other viruses, mild microscopic lesions were found in all sections of the small intestine. Villous atrophy with widening of the lamina propria and separation of enterocytes and desquamation at the distal third of the villi was observed (Fig. 1). Increased crypt depth with marked hypercellularity and numerous mitotic figures was present principally in the jejunum and duodenum. Vacuolated and degenerated enterocytes were commonly found in the crypt.

Bursal atrophy characterized by lymphoid depletion and fibroplasia were the major histological lesions associated with TReoV infection in poults. TAstV-2 also induced mild bursal atrophy, and the combination of all three viruses induced the most severe bursal damage with severe fibroplasia between bursal follicles (Fig. 2). Mild to moderate lymphoid depletion and histiocytic and ellipsoidal hyperplasia were present in the spleens of all infected turkeys. Mild lymphocytic infiltration was present in the liver and pancreas of some infected birds.

Detection of rotavirus viral antigen by IHC. Intestinal tissue from TRotV-infected poults, but not those from the control poults, contained detectable viral antigen. Positive staining by IHC was characterized by the presence of intracytoplasmic dark brownish granules. No specific peroxidase-positive staining was seen in the tissues of the control poults. Of the two primary antibodies used, the chicken hyperimmune sera gave the best results, with less background and stronger staining. In spite of the difference in quality of staining, both antibodies gave the same staining pattern. Positive staining was observed in the cytoplasm of the mature enterocytes at the tip of the affected villi (Fig. 1). Staining was present in all sections of the small intestine, but was observed more frequently in the jejunum and duodenum. No specific staining was observed in the bursa, liver, spleen, thymus, or proventriculus.

RT-PCR on cloacal swabs. Real-time RT-PCR and conventional RT-PCR on cloacal swabs was used to evaluate cloacal shed of TAstV-2 (Table 3) and TRotV (Table 4). The poults used in this study were negative for TAstV-2 and TRotV when they arrived from the hatchery. Infection with more than one virus did not clearly affect virus shed for either TAstV-2 or TRotV; however, TAstV-2 was generally shed for a longer period of time than TRotV. Briefly, all birds in treatment groups that received TAstV-2 were positive for TAstV-2 from 2 through 11 days PI, except TAstV-2 + TReoV at 4 days PI, for which only 80% of the poults were positive. At 14 days PI, the percentages of TAstV-2–positive poults in each group were 40% in TAstV-2 only, 60% in TRotV + TAstV-2, 70% in the group

Fig. 1. Photomicrographs of sections of the jejunum from a poult infected with rotavirus, 4 days PI. (a) Widening of the lamina propria of the villi (arrow) and increased crypt depth. Hematoxylin and eosin. Magnification, 200×. (b) Marked hypercellularity and numerous mitotic figures (arrows) and vacuolated enterocytes (arrowhead). Hematoxylin and eosin. Magnification 400×. (c) and (d) Separation of enterocytes from the lamina propria and desquamation at the distal third of the villi. Viral antigen staining present in the cytoplasm of the enterocytes at the distal section of the affected villi (arrows, dark staining). Immunoperoxidase labeling, hematoxylin counter stain. Magnification, 200× and 400×, respectively.

exposed to all three viruses, and 87.5% in the TAstV-2 + TreoV group.

Cloacal swabs from all poults that received TRotV were positive TRotV at 2 days PI (Table 4). At 4 days PI, TRotV was only detected in swabs from 45% of the birds exposed to all three viruses, 50% of the TRotV + TAstV-2 birds, 60% of the TRotV + TReoV birds, and 70% of the poults in the TRotV only group. By 7 and 11 days PI, TRotV was only detected in 10%–25% of the poults, and at 14 days

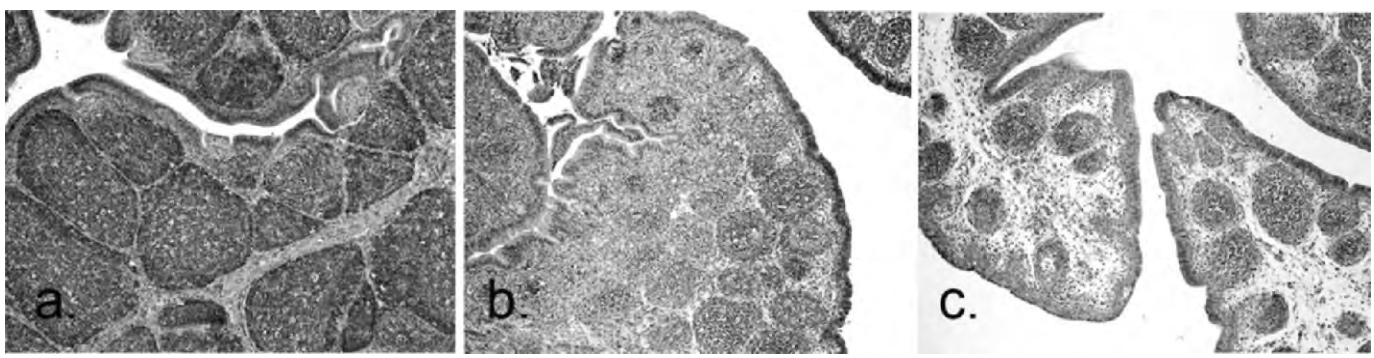

Fig. 2. (a) Normal bursa from a sham inoculated poult, 4 days PI. Hematoxylin and eosin. Magnification, 100×. (b) Bursa with cortical and medullary follicle lymphoid depletion from poult inoculated with reovirus, 4 days PI. Hematoxylin and eosin. Magnification, 100×. (c) Bursa with cortical and medullary follicle lymphoid depletion and severe stromal fibroplasia. Poult inoculated with rotavirus astrovirus and reovirus, 4 days PI. Hematoxylin and eosin. Magnification, 100×.

Table 3. Results of TAstV-2 RT-PCR testing of poults inoculated with TAstV-2.

Treatment Group	Days postinoculation				
	2	4	7	10	14
TAstV-2	100 (10/10)[A]	100 (10/10)	100 (10/10)	100 (10/10)	40 (4/10)
TAstV-2 + TRotV	90 (9/10)	100 (10/10)	100 (10/10)	100 (10/10)	60 (6/10)
TAstV-2 + TReoV	100 (10/10)	80 (8/10)	100 (10/10)	100 (8/8)	87.5 (7/8)
TAstV-2 + TRotV + TReoV	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)	70 (7/10)

[A]Percent positive (number positive/total tested).

PI, the only poults positive for TRotV were 20% (2 of 10) of the poults in the group exposed to all three viruses. The group that was exposed to all three viruses was the only group in which there were at least some individual TRotV-positive poults at all time points.

DISCUSSION

The roles of different viruses in enteric disease have not been well defined, and in commercial flocks it is not uncommon to find clinically normal birds infected with enteric viruses. Frequently, one flock will be infected with numerous viruses (8,11,16,17). In order to sort out the effects of concomitant infection of young turkeys with two of the most common enteric viruses (TRotV and TAstV-2) and a relatively common virus that is probably immunosuppressive (TReoV), 3-day-old broad-breasted white poults were exposed to TRotV, TAsTV-2, and TReoV in all possible combinations.

Body weight was used as a primary measure of disease severity because of its importance in production and because it can be quantified. Differences were observed between the sham inoculates and the virus-exposed groups. Among the groups that were exposed to viruses, groups with TRotV and TAstV-2 in any combination were more severely affected than TReoV alone. Furthermore, although differences among groups exposed to multiple viruses were not significantly different from each other, the group that was exposed to all three viruses consistently had the lowest body weights. This suggests that poults exposed to all viruses in commercial conditions could perform more poorly than poults exposed to fewer viruses, regardless of the severity of other clinical signs, which were similar among all TRotV- and TAstV-2–exposed groups.

The lesions and clinical signs observed here (huddling, diarrhea, agitation) are consistent with numerous previous reports of enteric virus pathogenesis (1,2). However, in contrast to a synergistic interaction observed between rotavirus and enterovirus or astrovirus shown by earlier reports (5,15), the severity of clinical signs, microscopic lesions, and body-weight differences were not clearly enhanced when the birds were exposed to more than one virus. Also the effect of immunosuppression caused by TReoV appeared to be minimal when the birds were exposed to the viruses at the same time. Perhaps if the birds were exposed to TReoV prior to exposure to the enteric viruses there would be more of an impact because the effects of the bursal damage would be greater.

Cloacal shedding of TRotV and TAstV-2 appeared to follow different patterns. TAstV-2 was shed by a high proportion of poults throughout the duration of the experiment, which is consistent with previous experiments with TAstV-2–infected SPF poults (10) and studies of commercial flocks in which TAstV-2 seems to persist (10). In contrast TRotV was shed by 100% of the inoculated birds at 2 days PI, after which the proportion of infected poults decreased through day 14 PI when only two of the birds exposed to all three viruses were positive. Corresponding with this, microscopic lesions observed in the intestines of TRotV-infected poults at 4 days PI were mostly reparative in nature, with numerous immature enterocytes being produced in face of the loss of mature enterocytes, indicating that the damage occurred earlier. TRotV antigen staining was present in the enterocytes at the distal section of the affected villi and sloughed epithelial cells. In previous studies, we showed that TAstV-2 and TReoV induced only mild microscopic lesions in the intestines, with mild damage to the intestinal epithelium and a mild inflammatory response (9,10). Viral antigen staining in tissues from poults infected with TAstV-2 was most commonly found in the enterocytes at middle section of the affected villi (10). Viral antigen staining in tissues from poults infected with TReoV was present in the bursa in the surface epithelial cells and macrophages, and to lesser degree, in splenic red pulp macrophages and intestinal epithelial cells (9). This indicates a difference in the basic pathogenesis of these viruses which needs to be further evaluated.

These results suggest that interactions among these viruses may have some effect on body weights, although the interaction was not sufficient to reproduce the full clinical disease syndrome seen in the field. Additionally, the relatively high prevalence of TRotV and TAstV-2 infection in turkey flocks regardless of flock disease status (8,16) indicates that other factors in the field, whether environmental, management, or microbial are necessary for the full clinical disease of PEC or PEMS to develop. For example, age at exposure may be a critical factor as Hayhow and Saif (5) reported more severe disease and synergism between an avian rotavirus and enterovirus when the poults were exposed at 2 weeks *vs.* 3 days of age. Similarly, Yason *et al.* (25) also reported more severe lesions in older turkeys exposed to an avian rotavirus than in young poults. Here we exposed the birds at 3 days posthatch in an attempt to simulate the early age at which poults would be exposed to environmental virus when placed at the farm.

Alternatively, there may be natural pathotypic variants of the enteric viruses that produce more severe disease than the isolates used

Table 4. Results of TRotV RT-PCR testing of poults inoculated with TRotV.

Treatment group	Days postinoculation				
	2	4	7	10	14
TRotV	100 (10/10)[A]	70 (7/10)	0 (0/10)	20 (2/10)	0 (0/10)
TRotV + TAstV-2	100 (10/10)	50 (5/10)	10 (1/10)	40 (4/10)	0 (0/10)
TRotV + TReoV	100 (10/10)	60 (6/10)	25 (2/8)	50 (3/6)	0 (0/6)
TAstV-2 + TRotV + TReoV	100 (10/10)	45 (4/9)	20 (2/10)	80 (8/10)	20 (2/10)

[A]Percent positive (number positive/total tested).

here. The particular astrovirus and rotavirus types that were utilized in this study were selected because they were relatively common (e.g., TAstV-2 is more common than TAstV-1) (11); only the TReoV TK/NC/SEP-44/03 isolate is known to be relatively virulent (4,20). Other considerations why disease in these experimental infections tends to be mild may be due to attenuation by cell-culture and egg propagation or to low challenge dose because accurate titration of some enteric viruses is difficult. Furthermore, in a laboratory setting, animal care facilities eliminate numerous environmental stressors, such as excessive cold, heat, dampness, or other pathogens that the birds may experience in the field that can nonspecifically contribute to disease severity through physiological stress. Because of these and other unknown factors, generalizations about the relative virulence of all TRotV and TAstV-2 strain can not be made. Further work needs to be done to elucidate the mechanism of TRotV and TAstV-2 pathogenesis and to understand their full impact on turkey production.

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Determination and analysis of the full-length chicken parvovirus genome

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ABSTRACT

Viral enteric disease in poultry is an ongoing problem in many parts of the world. Many enteric viruses have been identified in turkeys and chickens, including avian astroviruses, rotaviruses, reoviruses, and coronaviruses. Through the application of a molecular screening method targeting particle-associated nucleic acid (PAN), we recently described the detection and partial characterization of a novel enteric parvovirus in chickens. Subsequent surveys of intestinal homogenates from turkeys and chickens in the United States revealed widespread occurrence of parvovirus in poultry. Here we report the first full genome sequence of a novel chicken parvovirus, ChPV ABU-P1. ChPV ABU-P1 genome organization, predicted amino acid sequence, and phylogenetic relationships with other described parvoviruses are discussed.

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Introduction

Enteric disease syndromes are a continual economic burden for the poultry industry throughout the world. The major recognized enteric syndromes in poultry are poult enteritis complex (PEC) and poult enteritis mortality syndrome (PEMS) in turkeys and runting–stunting syndrome (RSS) in broiler chickens (Barnes and Guy, 2003; Barnes et al., 2000; Goodwin et al., 1993). Poultry enteric disease is marked by diarrhea, stunting, and increased time to market; the more severe forms of enteric disease are characterized by immune dysfunction and increased mortality.

Numerous viruses have been detected in or isolated from the intestinal tract of poultry (both diseased and healthy), and have subsequently been implicated in poultry enteric disease. These viruses include the avian reoviruses, rotaviruses, astroviruses and coronaviruses; despite these diagnostic efforts and viral descriptions, the role these viruses play in the enteric syndromes are still poorly understood (Guy, 1998; Pantin-Jackwood et al., 2008a; Pantin-Jackwood et al., 2007a; Pantin-Jackwood et al., 2008b; Pantin-Jackwood et al., 2007b; Reynolds et al., 1987; Spackman et al., 2005).

We recently reported the use of a molecular screening protocol to amplify and identify novel enteric viruses in the intestinal tract of poultry showing characteristic enteric disease signs (Zsak et al., 2008). This random amplification of viral particle-associated nucleic acid identified numerous parvovirus DNA sequences directly from the intestinal homogenates of chickens with signs of enteric disease. Initial analysis of this chicken-origin parvovirus (ChPV) revealed that it is a novel member of the *Parvovirinae* subfamily within the *Parvoviridae*, and led to the development of a specific molecular diagnostic test targeting the ChPV non-structural (NS) gene (Zsak et al., 2009).

Parvoviruses have been described that infect a wide range of invertebrates and vertebrates, and a thorough knowledge of the parvovirus genome and encoded proteins is essential to an understanding of the evolution of parvoviruses in various hosts, including tissue tropism during viral pathogenesis (Hueffer and Parrish, 2003). Parvoviruses are well known for causing enteric disease in mammals, with the canine parvoviruses recently emerging as important pathogens in the 1970s (Decaro et al., 2009; Truyen, 1999). Parvoviruses have been known for years as the causative agent of Derzsy's disease in young geese, and are also found in Muscovy ducks, where they cause multiple clinical signs ranging from enteritis to hepatitis and infectious myocarditis (Gough, 2008). Parvoviruses have been previously described in chickens based upon their morphology via electron microscopy and upon their genome size (Kisary et al., 1985; Kisary et al., 1984), and enteric disease signs have been attributed to parvovirus-like particles detected in turkey intestinal tracts (Trampel et al., 1983). A recent diagnostic survey of enteric samples collected from United States turkey and chicken flocks revealed widespread presence of parvovirus in U.S. poultry (Zsak et al., 2009). This is the first in-depth molecular characterization and analysis of the full-length genome sequence of ChPV. The analysis includes comparisons to other members of the *Parvovirinae* that infect mammals and birds, including two novel turkey-origin parvoviruses (TuPV) recently sequenced using a similar molecular approach.

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Results

The ChPV ABU-P1 genome

The fully assembled ChPV ABU-P1 genome is 5257 nucleotides (nt) in length and has a basic organization similar to previously described members of the *Parvoviridae* (Fig. 1). The genome is flanked on the 5′ and 3′ ends by 206 nt direct repeat sequences, each of which contains 39 nt inverted repeats presumed to form a hairpin structure similar to the structures common in the parvoviruses (Fig. 2) (Farkas et al., 2004; Muzyczka and Berns, 2001; Sukhumsirichart et al., 2006). The 206 nt direct repeats are identical and are found in the same orientation 5′ to 3′ in the genome. The genome contains 32.36% A, 21.95% G, 24.08% T, and 21.61% C, with an A + T content of 56.44% and a C + G content of 43.56%. The overall genomic organization of ChPV ABU-P1 is similar to other parvoviruses, with two major predicted open reading frames (ORFs). The 5′ ORF is 2085 nt long, and a blastx search using the putative amino acid sequence revealed a protein similar to the *Parvoviridae* non-structural (NS) proteins NS1, with the greatest similarity to human bocavirus NS1. The major 3′ ORF is 2028 nt long, and encodes a protein similar to the *Parvoviridae* capsid protein VP1, with the greatest similarity to avian adeno-associated virus, a member of the *Dependovirus* genus. The ChPV genome also contains a small (306 nt) ORF located between the major 5′ and 3′ ORFs. This ORF shows no homology to known protein coding regions in the databases.

NS1

The ChPV NS1 ORF is preceded upstream by the 39 bp inverted repeat sequence. The NS1 start codon is in a strong Kozak context (AAG*ATG*G) and is preceded directly upstream by a TATA box, an extended CAAT box, and a putative Inr-box (Hernandez, 1993; Rupp et al., 1990; Smale and Kadonaga, 2003). NS1 also contains a well-conserved phosphate-binding loop (P-loop) motif (GXXXXGKT…EE), distinguishing it as a member of the ATP and GTP-binding superfamily of proteins (Saraste et al., 1990). At the amino acid level, the ChPV NS1 protein exhibited from 45.2 to 53.9% amino acid similarity (17.1 to 19.0% identity) with NS1 from other parvovirus isolates. The NS1 similarity increased to 89.3 and 100% (99.3 to 83.7% identity) when ChPV was compared with two turkey-origin parvoviruses (TuPV), TuPV 260 and TuPV 1078 respectively. The lower identity noted when comparing the ChPV and TuPV 1078 NS1 coding sequences can be partially explained by the presence of a stop codon beginning at position 1882 in the TuPV 1078 NS1 coding region.

VP1

The predicted VP1 ORF begins at position 2998 in the ChPV genome. The predicted VP1 start codon (ATG) overlaps with the stop codon (AAT) of the predicted 306 nt ORF located between the ChPV NS1 and VP1 ORFs. VP1 is preceded upstream by a TATA box and a polyadenylation signal (AATAAA) follows the ORF; this poly-A signal immediately precedes the 39 nt inverted repeat at the 5′ end of the genome. One of two downstream ATG codons that begin 417 and 420 nt downstream to the start of the putative VP1 ORF may serve as the start codon for a VP2 capsid protein that shares a stop codon (TAA) with VP1. The second of these ATG codons has the stronger Kozak consensus sequence (ATG*ATG*G) compared to the translation initiation context of the first codon (GAA*ATG*A) and remains in frame (Kozak, 1987). A third start codon with a favorable translation initiation context (GAC*ATG*G) is present at position 3067 in the ChPV genome, and may represent the beginning of a putative VP3 ORF as has been described in the goose and Muscovy duck parvoviruses

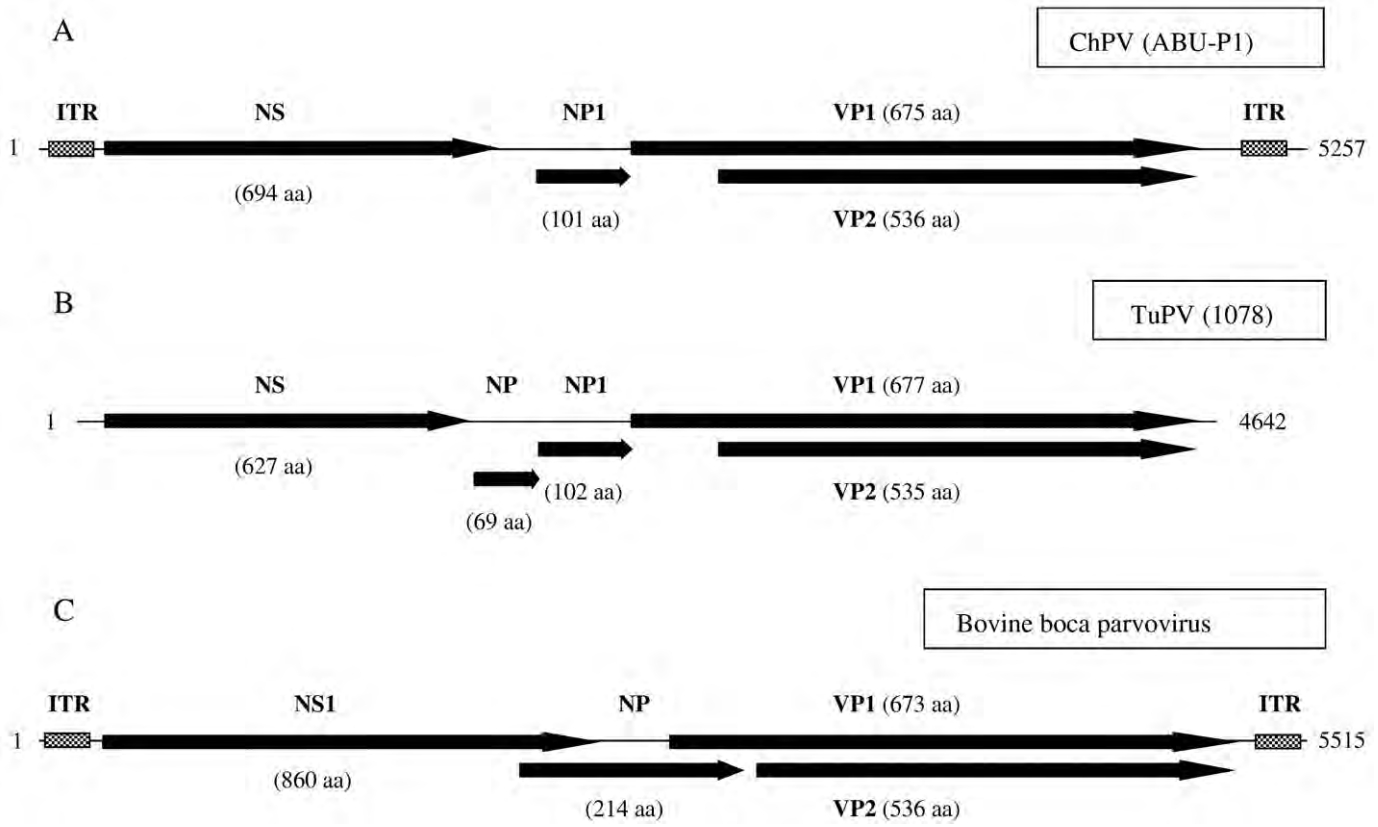

Fig. 1. A graphical representation of the genome organization of (A) ChPV ABU-P1, (B) the TuPV 1078, and (C) bovine bocavirus, a parvovirus with an overall genome organization similar to ChPV ABU-P1. The full genome length in nucleotides and the number of amino acids in each encoded protein are indicated. ITR = inverted terminal repeat.

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ChPV(ABU-P1)

   1    GAGTCTTGCTTTCTCTAGTCAACCTACTTCCATCCTCCATGGAAGTATTATTAAGTGTAGTTTTTATACCTTATCGCTAG
                                                                                          ---------->
  81    GGGAATCCCTGGCGCTTGCGAACAGCGGGCTTCGCCCGCGCCCGAAGGGCGTCTTAGAAGGCGTGACTATGCATATGAGG

 161    GATAACATGGAAGTTGGTGGTTGGTTGTAGAGAGAAAGCAAGACTCATAACGGGTGGTATGACCTATCAATGATTGACTA
                                                                                          <----------
                                                                  TATA Box                CAAT Box
 241    GCTGAGTCATGCTGACTTCGGATTCGCTGGGTATATAAGGCGGTGTCTGGAGCGGCCAATCACCCATCCACCTTAAGTGG
                 Inr Box
 321    TTCCGGGTCACTCTCAGAGCGCTCATTTGCCCTTTGACAGGCGAACAATCCGCACGTTGATTCGGCTGCCAGTGCTGCTG
                      M  A  F  P  T  R  G  G  F  S  A  I  L  N  L  P  S  D  L  I  V  G  Q
 401    AGCTGGTAAGATGGCATTCCCAACACGCGGTGGGTTTTCCGCTATTCTAAATCTTCCCAGCGATTTAATCGTGGGACAGA
        K  N  M  P  V  F  E  C  V  T  A  G  Y  T  S  P  D  S  G  E  F  V  A  S  R  F  P
 481    AAAATATGCCTGTATTCGAGTGTGTAACTGCCGGTTACACTTCTCCGGATTCAGGCGAATTCGTGGCTTCTAGATTTCCA
         P  N  H  E  L  R  Q  E  I  R  N  F  I  G  N  P  E  W  D  D  P  D  L  T  W  I  D
 561    CCCAACCACGAATTGAGACAAGAAATTAGAAATTTTATCGGGAACCCAGAATGGGACGATCCAGATTTGACATGGATAGA
          F  H  I  Q  L  A  K  H  A  I  D  V  E  T  K  L  N  P  N  L  Q  I  F  W  Q  M
 641    TTTCCACATTCAATTGGCGAAACACGCCATAGACGTGGAAACTAAACTCAATCCAAATTTACAAATTTTCTGGCAAATGG
        E  K  S  S  Q  S  G  R  Y  H  L  H  L  L  F  V  E  G  C  T  S  R  Q  I  T  W  I
 721    AAAAATCTAGTCAGTCGGGAAGATATCATCTCCATTTACTGTTCGTAGAAGGATGTACTTCCAGGCAAATTACATGGATA
         V  K  R  M  R  R  E  L  C  N  K  A  A  E  M  L  H  M  F  C  P  D  F  P  K  S  Q
 801    GTCAAAAGAATGAGAAGGGAACTTTGCAACAAAGCTGCAGAAATGTTGCACATGTTTTGTCCTGATTTCCCCAAAAGTCA
          I  W  G  S  L  V  N  I  Q  N  A  M  L  S  L  N  R  G  Y  S  P  R  I  G  K  P
 881    AATTTGGGGATCGCTGGTGAATATCCAAAACGCTATGCTTTCACTGAATAGAGGATACTCACCGAGAATTGGGAAACCAA
        I  P  Q  P  V  N  P  Y  S  F  L  K  Y  Y  M  F  N  S  A  K  M  S  F  L  R  G  A
 961    TCCCCCAACCAGTGAATCCATACTCATTTCTAAAATACTACATGTTCAATTCTGCTAAAATGAGCTTTCTGAGAGGGGCT
         T  P  K  L  A  E  K  L  P  I  G  T  R  Y  A  R  F  E  G  S  P  D  L  D  E  G  P
1041    ACTCCAAAATTAGCTGAAAAATTACCAATTGGTACAAGATATGCTAGATTTGAAGGGTCTCCTGACCTGGACGAGGGTCC
          E  I  P  S  Q  E  L  T  P  G  D  T  L  P  V  C  Y  V  S  T  E  T  G  W  G  N
1121    CGAAATTCCATCGCAGGAATTAACTCCAGGGGACACTCTACCTGTATGCTATGTAAGTACTGAAACTGGTTGGGGGAACG
        G  G  G  E  T  V  I  K  T  S  I  I  E  K  L  C  M  E  A  L  R  L  C  R  E  N  H
1201    GTGGGGGGGAGACCGTGATCAAAACCAGTATCATTGAAAAATTATGCATGGAAGCATTAAGATTATGCAGAGAAAATCAC
         I  F  T  M  K  A  F  K  L  K  F  P  D  K  F  M  Q  F  S  S  R  N  Q  G  L  V  K
1281    ATTTTTACCATGAAAGCATTTAAGCTAAAATTCCCAGACAAATTCATGCAGTTCAGTTCGCGAAATCAGGGGTTAGTGAA
          L  E  E  T  I  N  L  Y  C  E  T  I  I  N  E  H  D  A  W  E  I  A  K  S  I  H
1361    ATTAGAAGAAACAATAAACCTGTATTGTGAGACCATTATCAACGAGCACGATGCGTGGGAAATAGCTAAATCAATACATG
        G  D  V  D  T  A  D  L  D  D  N  L  A  I  R  L  C  S  Y  Q  G  Y  S  P  K  Y  V
1441    GAGATGTTGACACAGCTGATCTAGATGATAATTTAGCCATCCGGTTATGCAGCTACCAAGGATATAGTCCAAAGTACGTT
                                                                                  P-loop
         A  R  L  I  L  C  W  L  S  G  Q  A  G  K  K  N  A  L  Y  F  H  G  P  A  N  T  G
1521    GCTAGACTGATATTGTGCTGGCTATCCGGACAAGCAGGGAAAAAAAACGCATTATATTTCCACGGACCAGCCAATACTGG
          K  T  M  M  A  E  S  I  C  K  M  V  Q  I  Y  G  N  V  N  H  N  N  K  N  F  P
1601    TAAAACAATGATGGCAGAAAGCATATGCAAAATGGTACAAATATACGGTAACGTAAATCATAACAACAAAAACTTCCCAC
        L  N  D  C  H  N  K  A  V  L  W  W  E  E  C  S  M  T  E  E  H  V  E  S  A  K  C
1681    TCAACGACTGTCACAACAAAGCAGTACTTTGGTGGGAAGAATGTAGCATGACGGAAGAACACGTAGAATCTGCAAAATGT
         I  M  G  G  S  S  V  R  I  D  K  K  N  Q  D  S  V  L  L  C  K  T  P  I  V  I  T
1761    ATCATGGGGGGATCCTCAGTGAGAATAGATAAAAAAAATCAAGATTCAGTTCTACTATGTAAAACTCCAATCGTTATTAC
          S  N  N  D  I  T  Q  V  S  S  R  N  A  I  S  T  V  H  A  A  A  I  R  A  R  C
1841    TTCTAATAACGATATCACTCAAGTTTCAAGCAGGAATGCCATCTCAACAGTTCATGCAGCTGCAATAAGAGCAAGATGCT
        L  K  F  T  F  N  N  W  L  T  S  N  W  G  L  I  T  V  D  Q  M  Y  Q  F  L  C  W
1921    TAAAATTTACATTTAACAATTGGCTAACTTCAAATTGGGGACTGATAACCGTTGATCAAATGTATCAATTTTTATGCTGG
         G  E  L  E  G  I  P  T  V  D  S  L  L  K  D  H  P  E  F  N  G  T  L  P  Y  N  Q
2001    GGAGAATTAGAGGGCATTCCGACCGTGGATAGTCTCCTGAAAGATCATCCCGAATTTAACGGGACTTTGCCTTATAACCA
          P  K  G  K  F  C  A  D  C  V  N  Q  F  S  T  A  T  N  L  T  V  C  P  A  C  S
2081    ACCCAAAGGTAAATTTTGTGCCGATTGTGTTAACCAGTTCAGCACTGCAACTAACCTGACCGTGTGCCCAGCCTGCTCCG
        G  W  T  R  K  P  F  S  E  E  E  E  P  P  Y  T  G  S  E  S  G  L  F  E  K  A  N
2161    GATGGACAAGAAAGCCATTCTCCGAAGAAGAGGAACCCCCCTATACTGGTTCTGAATCCGGGCTCTTTGAGAAAGCCAAC
         E  G  K  N  E  V  P  G  I  Y  P  V  N  S  G  S  R  L  I  P  E  A  P  I  L  Q  K
2241    GAAGGTAAGAATGAGGTCCCAGGGATTTATCCCGTAAATAGTGGATCCAGGTTAATCCCTGAGGCCCCAATCTTACAGAA
          P  S  T  Y  W  E  T  E  I  P  S  E  S  S  G  F  P  D  E  P  D  F  T  A  S  A
2321    ACCATCGACCTATTGGGAGACGGAGATCCCCAGCGAATCGAGTGGATTTCCAGACGAGCCAGACTTCACCGCAAGCGCAA
        K  L  L  L  K  V  D  D  L  H  L  E  S  D  L  K  V  E  Q  D  P  L  E  C  K  K  C
2401    AACTTCTCCTGAAAGTGGACGATCTCCACCTAGAAAGCGATCTGAAAGTGGAGCAGGATCCTCTGGAGTGCAAAAAGTGC
         C  L  V  Q  *
2481    TGTCTGGTTCAATAGGCCAACTCGAAGGTAAAAGCATAACATTTACTGATGATCTACCTTCCCTTACTGAGTTATTTTGC
                                                  Inr Box
2561    GACGAAACGCTGCCCCCATCCCCGACATCCCCAATCAATCTCCCTGAACCCTCTCGAGTGGTACAATGGGGGGACTTCAA
                                                                          M  C  W  Y  N  A  N  I
```

Fig. 2. Full-length nucleotide sequence of the ChPV genome. The terminal repeat sequences are shaded gray, and the palindromic (hairpin-forming) regions are overlined with an arrow. TATA boxes, Inr's, and CAAT boxes are underlined and indicated with a label. Start and stop codons for each of the major and minor ORFs are boxed; a putative alternative start codon for the VP2 ORF is boxed and shaded. The P-loop motif and downstream residues involved in NTP binding are shaded and in bold type. Putative polyadenylation signals are underlined.

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2641  AGCTCAATGGGTGGAAGGATACATTGGAAAGTGGAGGGTTGCATTTTCTAATTTCATGTGTTGGTATAATGCTAATATTA
      T  Q  I  Q  T  V  E  G  D  P  C  T  N  P  D  V  F  H  E  L  Y  F  P  I  K  Q  H
2721  CACAGATTCAGACAGTAGAAGGTGATCCTTGTACTAATCCTGATGTTTTTCACGAGTTATATTTCCCTATCAAGCAACAC
       A  M  D  A  R  M  L  K  M  K  I  E  D  K  I  Q  K  G  D  F  T  Q  D  I  H  L  D
2801  GCCATGGACGCACGCATGCTGAAAATGAAAATCGAAGACAAAATACAAAAGGGCGACTTCACACAAGACATACACCTAGA
        M  T  L  T  Q  C  I  Q  T  A  L  S  D  D  P  A  G  I  W  P  C  V  D  R  L  A
2881  CATGACATTAACACAATGCATTCAAACAGCACTTAGCGACGACCCAGCAGGGATATGGCCATGTGTAGACAGGTTAGCTA
      N  Y  C  N  V  I  L  A  S  I  G  T  Q  *
                TATA Box                 M  K  Y  K  A  P  K  G  Y  V  P  S  L  P
2961  ATTACTGCAATGTTATATTGGCTTCTATAGGCACACAATGAAATACAAAGCTCCGAAAGGCTACGTCCCTTCACTGCCCA
      T  T  D  E  E  A  Y  H  H  T  R  W  W  L  D  H  V  E  N  Y  G  K  N  K  A  I  G
3041  CCACTGATGAAGAAGCATATCACCACACTAGATGGTGGCTGGATCATGTTGAAAATTATGGAAAAAATAAAGCTATTGGT
       R  G  K  G  K  A  R  K  E  L  T  P  Q  Q  K  A  E  R  K  R  F  F  I  T  Q  A  Q
3121  AGAGGCAAAGGAAAAGCTAGGAAAGAGCTCACACCACAACAAAAAGCTGAACGCAAACGCTTTTTTATCACGCAGGCACA
        K  N  K  K  P  N  Y  G  I  P  S  S  S  Q  H  K  E  F  F  K  N  H  Q  G  A  Q
3201  AAAAAATAAAAAACCTAATTACGGCATCCCATCTTCATCGCAGCACAAAGAGTTTTTCAAAAATCATCAGGGGGCGCAAG
      G  G  K  A  K  Q  K  T  K  H  I  E  N  Q  A  P  Q  R  Q  E  D  P  E  E  G  P  A
3281  GGGGAAAAGCAAAGCAAAAAACCAAACATATAGAAAATCAAGCACCACAAAGGCAAGAGGACCCAGAGGAAGGGCCCGCA
       P  K  Q  P  R  V  E  E  N  P  Y  N  Q  E  E  L  E  E  M  M  A  D  E  N  E  P  D
3361  CCTAAACAACCTAGAGTAGAAGAAAACCCATATAATCAAGAAGAATTAGAAGAAATGATGGCAGATGAAAATGAACCTGA
        Q  S  G  I  P  M  E  L  S  D  G  P  G  T  V  G  G  G  G  G  G  G  I  G  H  S
3441  TCAATCTGGGATCCCTATGGAATTAAGCGACGGGCCTGGGACTGTGGGAGGAGGTGGTGGTGGGGGAATCGGGCATTCTA
      T  G  N  W  N  C  D  T  I  W  A  E  N  T  C  K  T  Y  A  S  R  H  C  V  C  L  M
3521  CCGGTAATTGGAATTGTGATACTATATGGGCTGAAAATACATGTAAAACCTATGCAAGCCGCCATTGTGTCTGTCTTATG
       R  D  M  D  Q  Y  K  A  I  G  N  A  N  G  Q  H  G  L  D  T  E  N  Q  T  Q  Y  F
3601  CGTGACATGGATCAATATAAAGCAATTGGCAACGCTAACGGGCAACACGGGTTAGACACTGAAAACCAAACACAATACTT
        G  F  S  T  P  W  N  Y  I  D  F  N  Q  Y  S  V  H  F  S  P  R  D  W  Q  Q  L
3681  TGGTTTCTCTACACCATGGAATTATATTGACTTTAATCAATACAGCGTGCACTTTAGTCCACGAGATTGGCAACAACTAG
      V  N  N  Y  S  R  W  R  P  R  A  V  H  V  K  I  F  N  L  Q  V  I  Q  K  T  V  T
3761  TTAATAATTATTCGAGATGGCGCCCACGCGCCGTACACGTGAAAATCTTTAACCTGCAAGTCATCCAGAAAACCGTAACA
       D  S  G  T  Q  Y  S  N  D  L  T  G  T  I  Q  I  F  A  D  Q  E  G  R  Y  P  R  L
3841  GATAGCGGTACCCAGTACTCTAACGACTTGACAGGAACAATCCAAATATTTGCAGATCAGGAAGGCAGATATCCTAGATT
        L  Y  P  N  Q  T  T  L  M  G  P  F  P  S  N  I  Y  Y  L  P  Q  Y  A  Y  M  T
3921  GTTATACCCTAACCAAACAACTCTAATGGGTCCATTTCCATCCAATATATACTATCTGCCTCAATACGCATACATGACAA
      S  M  F  N  S  G  G  P  T  V  S  S  W  W  G  F  P  T  R  E  S  A  F  Y  C  L  D
4001  GCATGTTTAATTCGGGTGGACCAACAGTATCATCGTGGTGGGGATTTCCAACACGCGAAAGCGCATTTTACTGTCTAGAC
       E  S  P  S  Q  M  L  R  T  G  N  E  W  E  T  T  F  I  F  P  S  S  T  Q  W  C  S
4081  GAAAGCCCCTCGCAAATGTTAAGGACCGGTAATGAGTGGGAAACCACATTTATCTTTCCATCGAGTACACAATGGTGTAG
        N  K  I  S  T  V  S  I  T  Q  R  Q  N  P  L  Y  D  T  W  N  V  N  G  R  G  D
4161  CAATAAAATATCAACCGTGTCCATTACACAAAGACAAAACCCATTATACGATACATGGAACGTCAATGGCAGAGGCGATG
      D  A  K  R  G  N  F  C  T  W  R  S  P  W  Y  P  G  P  Y  I  Y  L  T  D  T  S  A
4241  ATGCTAAAAGAGGCAATTTCTGTACTTGGAGAAGCCCATGGTATCCAGGGCCTTACATTTACCTAACCGATACATCCGCA
       A  S  Q  R  L  E  N  L  A  N  V  A  V  G  P  D  G  M  P  L  A  P  G  M  P  Q  Y
4321  GCATCACAGCGTTTAGAAAATCTTGCTAACGTTGCCGTAGGCCCAGACGGCATGCCTCTAGCACCAGGAATGCCACAATA
        R  S  E  S  D  K  D  E  Y  L  H  T  F  W  V  P  K  K  V  K  M  N  E  G  D  I
4401  CAGATCAGAATCCGATAAAGACGAATATCTACATACATTTTGGGTACCTAAAAAAGTTAAGATGAACGAAGGAGATATAC
      R  N  A  Q  I  D  V  S  T  A  T  K  V  Q  V  D  T  S  T  L  Y  N  T  C  P  R  S
4481  GTAACGCGCAAATAGATGTATCCACTGCAACCAAAGTACAAGTAGATACATCGACACTATACAATACATGTCCTAGATCA
       G  Y  M  S  T  V  S  Q  G  G  A  P  A  P  I  V  W  S  G  C  V  P  G  M  I  W  D
4561  GGATACATGTCCACTGTTTCACAGGGAGGGGCACCCGCACCTATTGTCTGGTCAGGGTGTGTACCTGGCATGATATGGGA
        N  R  P  A  T  Y  F  D  P  I  W  Q  Q  F  P  E  T  D  E  Q  F  K  I  I  S  Q
4641  CAATAGGCCGGCCACCTACTTTGATCCGATATGGCAACAATTCCCAGAAACAGACGAACAATTCAAAATTATCTCTCAAT
      L  G  G  I  P  V  K  E  S  P  G  H  I  F  V  K  V  T  P  K  P  T  G  A  A  N  G
4721  TGGGGGGTATACCTGTTAAAGAATCACCTGGTCACATTTTTGTCAAAGTCACGCCTAAGCCAACCGGGGCTGCAAATGGT
       L  I  D  E  Y  C  T  F  T  C  T  V  E  I  E  W  E  L  E  P  L  N  T  H  R  W  N
4801  TTGATCGATGAATATTGTACTTTTACGTGTACTGTCGAAATAGAATGGGAATTAGAACCACTCAACACTCACAGATGGAA
        M  R  S  M  I  S  Y  E  T  T  D  A  K  A  G  R  Q  I  V  D  E  N  G  Q  Y  Q
4881  CATGAGAAGTATGATTTCGTACGAGACCACCGATGCCAAAGCTGGAAGACAAATTGTCGATGAAAATGGTCAATACCAGG
      V  N  V  N  S  A  D  I  T  R  L  Y  M  T  K  R  A  P  R  T  N  *
4961  TCAATGTTAACTCGGCTGACATTACTCGTCTGTATATGACCAAGCGCGCGCCGCGGACCAACTAATGTAACTTTCAAAAA

5041  ATTTCAATAAAGAGTCTTGCTTTCTCTAGTCAACCTACTTCCATCCTCCATGGAAGTATTATTAAGTGTAGTTTTTATAC
                                                  ------------------------------------>
5121  CTTATCGCTAGGGGAATCCCTGGCGCTTGCGAACAGCGGGCTTCGCCCGCGCCCGAAGGGCGTCTTAGAAGGCGTGACTA
                                                <------------------------------------
5201  TGCATATGAGGGATAACATGGAAGTTGGTGGTTGGTTGTAGAGAGAAAGCAAGACTC
```

Fig. 2 (*continued*).

(Zadori et al., 1995). The ChPV VP1 protein shares from 46.6 to 59.0% amino acid similarity (10.1 to 24.5% identity) with VP1 from other parvoviruses. The VP1 similarity increases to 100 and 95.1% (99.7 and 79.2% identity) when compared to VP1 from TuPV 260 and TuPV 1078, respectively.

Phylogenetic analysis

ClustalW was used to align the complete ChPV genome coding region with complete parvovirus sequences from mammals and birds available in the databases, and with two recently sequenced TuPV

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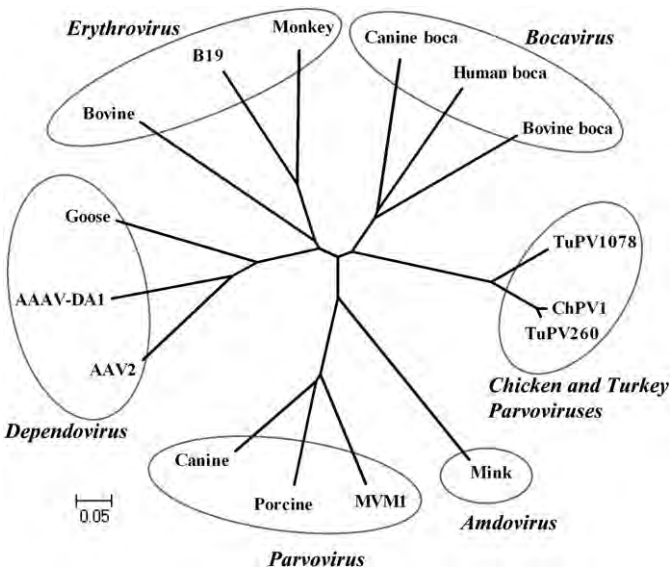

Fig. 3. A phylogenetic tree prepared using the full coding sequence of the indicated parvoviruses. Representative genera within the *Parvovirinae* are indicated. The evolutionary relationships were inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted using MEGA4. GenBank Accession numbers for ChPV, GU214704; TuPV 1078, GU214705; and TuPV 260, GU214706.

genome coding regions. The Neighbor-Joining tree was produced using MEGA4. The turkey and chicken sequences clustered together, and were clearly separate from the other members of the *Parvovirinae* (Fig. 3). A previous phylogenetic analysis using a conserved region of the ChPV and TuPV NS1 gene produced a similar tree (Zsak et al., 2008), and a phylogenetic analysis of the ChPV and TuPV VP1 gene produced an identical tree when compared to the full-length parvovirus coding region (data not shown).

Discussion

This is the first analysis of the complete nucleotide sequence from the ChPV ABU-P1 strain. Small viral particles (15 to about 25 nm) resembling parvovirus have been identified for years in the intestinal tracts of poultry (Kisary et al., 1984; Trampel et al., 1983; Woolcock and Shivaprasad, 2008). The use of a particle-associated nucleic acid (PAN) sequencing technique has recently allowed the partial molecular characterization of this novel ChPV (Zsak et al., 2008), and led to the identification and sequencing of two additional avian parvoviruses from turkeys: TuPV 260, originally detected in the intestinal tract of a turkey from California, and TuPV 1078, originally detected in the pooled intestinal tracts from a North Carolina turkey farm. All three of these poultry isolates are very similar to each other, yet each differs significantly from other members of the *Parvovirinae*. The high amino acid identities noted among the turkey and chicken isolates suggests that they diverged at sometime in the recent past from a common ancestor. It is known from studies of another member of the *Parvovirinae*, canine parvovirus (CPV), that the parvoviruses, despite their single-stranded DNA genome, have a mutation rate that approaches that of RNA viruses, which allows rapid evolution and host adaptation (Hoelzer et al., 2008; Shackelton et al., 2005). Interestingly, ChPV and TuPV do not group phylogenetically with the parvoviruses that infect geese (GoPV) and Muscovy ducks (MdPV), parvoviruses that are closely related to adeno-associated virus 2 (AAV-2), a member of the *Dependovirus* genus (Fig. 3) (Zadori et al., 1995; Zsak et al., 2008). Further, the ChPV NS protein shares only 19.1% amino acid identity with the MdPV NS protein.

The ITR sequences located at each end of the ChPV genome (Fig. 2) are each 206 nt long, somewhat shorter than those found in the prototypical human parvovirus B19 (383 nt) but longer than those found in some other autonomously replicating parvoviruses, namely the murine parvoviruses that have terminal palindromes of 115 and about 200 nt long in the same genome (Astell et al., 1979; Deiss et al., 1990). The terminal palindromes found at the 5′ and 3′ ends of most autonomously replicating parvoviruses bear no nucleotide identity with one another. Interestingly, this is not the case with ChPV, which has identical palindrome sequences at its 5′ and 3′ end (Fig. 2), a trait it shares with parvovirus B19 (Deiss et al., 1990; Muzyczka and Berns, 2001). It would be interesting to determine if the 5′ end of the ChPV genome exists in an alternative orientation, as is the case with B19, MdPV and GoPV, in which the 5′ sequence can be found in one of two “flip” or “flop” orientations, with one orientation being the inverted complement of the other (Deiss et al., 1990; Zadori et al., 1995).

The ChPV NS1 deduced amino acid sequence contains highly conserved motifs important for the initiation of parvovirus replication (Zsak et al., 2008), including a well-conserved phosphate-binding loop (“P-loop”) motif specifically involved in the binding of nucleoside triphosphates by this protein (Saraste et al., 1990). This motif is required for pathogenesis in the human parvovirus B19 and is found in many parvovirus isolates (Momoeda et al., 1994). The 5′ major ORF of ChPV appears to encode the viral capsid proteins VP1, VP2, and VP3. These three proteins together comprise the parvovirus virion (Muzyczka and Berns, 2001) and are responsible for the production of neutralizing antibodies during an infection (Saikawa et al., 1993).

The present analysis does not include members of the *Densovirinae* subfamily, which infect insects and other invertebrates (Muzyczka and Berns, 2001). ChPV and the closely related TuPVs were compared across their entire coding region with representative members of the *Parvovirinae* subfamily, which includes the *Dependovirus*, *Bocavirus*, *Erythrovirus*, *Amdovirus* and *Parvovirus* genera (Fig. 3). It is clear from the multiple sequence alignments and phylogenetic analysis that ChPV, along with the closely related TuPVs, represents a distinct member of the *Parvovirinae* subfamily and should be recognized as the prototypical member of a novel genus within the *Parvovirinae*.

Materials and methods

Assembly of the prototypical chicken parvovirus (ChPV ABU-P1) genome

The chicken parvovirus strain chosen for this analysis was originally isolated in Hungary from the intestines of chickens showing sings of a stunting syndrome (Kisary et al., 1984), and this original cesium chloride gradient-purified parvovirus (provided by J. Kisary) was propagated in specific pathogen free (SPF) chickens as described previously (Kisary, 1985; Zsak et al., 2009). A sequence-independent polymerase chain reaction (PCR) protocol was employed to amplify particle-associated nucleic acid (PAN) present in ChPV ABU-P1-positive chicken intestinal homogenates, and has been described elsewhere in detail (Zsak et al., 2008). The randomly amplified PAN was ligated into the TOPO-TA cloning vector (Invitrogen) and the ligation mix was used to transform competent TOP-10 *E. coli* cells (Invitrogen). Using this approach, a total of 768 clones were identified and sequenced using the M13 forward and reverse primers on an AB-3730 automated DNA sequencer. The sequenced clones were used as query sequences to search the GenBank non-redundant nucleotide (nr/nt) database using the blastn algorithm and the non-redundant (nr) protein database using the blastx algorithm. Four contigs with an average of eight-fold coverage and lengths of 1417 nt, 933 nt, 1069 nt, and 739 nt were assembled from 27, 24, 19, and 21 clones, respectively. These contigs had no significant nucleotide similarity to database sequences, but the deduced amino acid sequence from each contig

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revealed significant similarity to the members of the family *Parvoviridae* in the database. PCR primers were designed using the four assembled contigs and were subsequently used to close gaps of 58, 443, and 205 nt between the contigs and assemble the complete ChPV ABU-P1 genome. The right terminal region of contig 4 contained a portion of the right inverted terminal repeat (ITR). Primers designed using this partial ITR used in combination with forward and reverse primers within contig 1 (left terminal region of the genome) and contig 4 resulted in the cloning of the ITR regions at both the right and left genomic termini.

Sequence analysis

The ChPV and TuPV genomes and ORFs were aligned with each other and with selected parvovirus sequences available in the databases using ClustalW (Thompson et al., 1994). Searches for conserved domains and transcription factor binding sites within the ChPV genome were performed with GeneQuest (DNAStar/Lasergene8) and the Conserved Domain Database (CDD) Search Service v2.17 at the National Center for Biotechnology Information (NCBI) website (Marchler-Bauer et al., 2009). Phylogenetic analysis and tree construction were performed with MEGA4 using the Neighbor-Joining method (Tamura et al., 2007).

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Accumulation of attenuating mutations in varying proportions within a high passage very virulent plus strain of *Gallid herpesvirus* type 2

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ABSTRACT

Marek's disease (MD) is controlled through mass vaccination. Although these vaccines reduce or delay tumor formation they fail to induce sterilizing immunity and prevent virus shedding. Relatively little is known about the genetic changes that lead to attenuation. It has been established that serial passage of virulent strains in avian cell lines results in the generation of attenuated progeny at some undefined passage level. A detailed cataloging of the mutations needed for attenuation will be important for advancing our understanding of MD biology and should facilitate the development of better vaccines. Using deep sequencing, the complete nucleotide sequence of the very virulent plus (vv+) strain 648A representing the 101st passage was determined. Pathotyping studies have indicated that 648Ap101 is indeed attenuated. Comparative sequence analysis with the 648A strain at passage 11 has identified numerous gross genetic changes and subtle single nucleotide polymorphisms scattered throughout the genome. Relative to the strain (648Ap11), deletions were identified in MD-specific genes located in the repeat long (R_L), unique long (U_L) and repeat short (R_S) regions. A deletion in the R_L region, present in 33% of the p101 sequences, mapped to the genes encoding viral interleukin 8 (vIL8), RLORF4 and RLORF5. An R_S deletion was mapped to the Meq oncoprotein binding site within the ICP4 promoter and was present in 97.8% of the p101 sequences. A short deletion in the U_L region mapped to the 3′ terminus of the gene encoding vLipase and was present in 54% of the p101 sequences. The cis-acting sites involved in DNA replication and packaging also contained deletions in varying proportions (64% and 100%, respectively). Three mutations, present in 100% of p101 sequences, were identified in the overlapping genes encoding the Arg-rich protein (MDV002/079) and the virus-encoding telomerase (vTR). Varying proportions of 12 single nucleotide polymorphisms (SNPs) were identified within 11 open reading frames (ORFs) and 3 noncoding regions. This comparative sequencing study has provided a wealth of information regarding genetic changes which have occurred during the attenuation process and has indicated that serial passage results in the generation of mixed populations.

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1. Introduction

Marek's disease (MD) is a highly contagious neoplastic and neuropathic disease of chickens caused by Marek's disease virus-1, now called *Gallid herpesvirus* type 2 (GaHV-2) (Calnek et al., 1977). The disease has been controlled through mass vaccination of day-old chicks or chick embryos with either live attenuated strains of GaHV-2, a nononcogenic *Gallid herpesvirus* type 3 (GaHV-3), *Meleagrid herpesvirus* type 1 (MeHV-1) or a combination of the latter two (Witter, 2001). Since the discovery in the late 1960s that herpesvirus of turkey (MeHV-1) can protect chickens from clinical signs of Marek's disease and later the introduction of a protective serotype 1 vaccine (i.e. Rispens), many second generation vaccines have been developed through extensive serial passage of virulent field isolates in avian embryo fibroblasts (Dudnikova et al., 2009; Karpathy et al., 2002; Rispens et al., 1972; Witter, 2001; Witter and Kreager, 2004; Witter et al., 1995). However, few have been licensed and most fall short in comparison to the protection elicited by the gold standard Rispens (or CVI988) vaccine strain (Witter et al., 1995). To gain a better understanding of the genetic changes needed for attenuation the genome sequences of attenuated (CVI988-BAC, CVI988-699 and 548Ap80) and mildly virulent (CU-2) GaHV-2 strains have been determined (Spatz et al., 2007a, 2008; Spatz and Rue, 2008). Comparative genomics analysis with nucleotide sequences of virulent strains (Md5, Md11, RB-1B and GA) has provided some information on the genes and single nucleotide polymorphisms involved in virulence, but more data is needed (Lee et al., 2000; Niikura et al., 2005; Spatz et al., 2007b; Tulman et al., 2000). To this end we have embarked on a study to determine the mutations that accumulate during serial passage (101 times) of a very virulent plus (vv+) strain of GaHV-2, known

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as 648A (Witter, 2002). Biological characterization of passaged 648A clones has indicated that the ability to induce gross lesion in susceptible, maternal antibody-negative (ab−) chickens was lost after passage 70. Earlier passages (40–60) were still moderately oncogenic (Gimeno et al., 2001; Witter, 2002). This study details the accumulation of mutations in varying proportions within the genomes of passage 101 and identifies genes that contain mutations in common with other serially passaged attenuated vv+ strains.

2. Materials and methods

2.1. Virus and cells

Very virulent plus strain 648A of GaHV-2 isolated by Witter (2002) was used in these experiments. Virus stocks were generated at passage levels 10 and 100 in line O chicken embryo fibroblast (CEF) culture as described by Gimeno et al. (2001). Secondary CEF and duck embryo fibroblasts (DEFs) were used for nucleocapsid DNA isolation from passages 11 and 101 and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum. DEFs were used to propagate the near-field strain 648Ap11 since yields in CEF were extremely low. To propagate this passage, DEFs were infected with DMEM supplemented with 4% newborn calf serum (NCS) and maintained until CPE was greater than 80% in medium containing 2% NCS. Strain 648A at passage 101 was propagated on CEF in DMEM containing 2% fetal bovine serum. The concentration of the serum was reduced to 0.2% after an initial absorption period and the cells were harvested 4–6 days later when cytopathic effect was greater than 80%.

2.2. Isolation of high molecular weight GaHV-2 DNA

Nucleocapsid DNA for passages 11 and 101 was isolated using the hypotonic lysis/PEG precipitation methodology described by Volkening and Spatz (2009). The purity of the viral DNA after PEG precipitation was examined by gel electrophoresis of restriction endonuclease digested DNA preparations.

2.3. DNA sequencing

Five micrograms of DNA from passages 11 and 101 were processed according to the methodology as described by Margulies et al. (2005) with slight modifications. Briefly, DNA was sheared by nebulization to an average size of 500 bp. The ends were repaired and phosphorylated using T4 DNA polymerase and T4 polynucleotide kinase. Adaptor oligonucleotides were added using T4 DNA ligase. Purified DNA containing adaptors were hybridized to DNA capture beads and clonally amplified using emulsion PCR. DNA capture beads within emulsion bubbles were sieved onto a 40 mm × 75 mm PicoTiterPlate. Pyrosequencing was done on a Genome Sequencer 20 (GS20) System (454 Life Science Corporation) with an eight-lane gasket. DNA contigs were assembled using the GS De Novo Assembler (454 Life Sciences) and the Sequencher Program (Gene Codes, Ann Arbor, MI) from 42,589 and 67,307 reads for 648Ap11 and 648Ap101, respectively. With an average read length of 183 and 194 bp, this translated into an average of 43.9- and 73.6-fold coverage at each nucleotide for 648Ap11 and 648Ap101. Problematic regions containing mononucleotide reiterations were sequenced from PCR products generated in reactions containing Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) with 5% DMSO added. Sanger-based DNA sequencing was performed at the South Atlantic Area sequencing facility (Athens, GA) using the BigDye terminator cycle sequencing protocol and analyzed on a model ABI-3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA). DNA sequences were maintained and analyzed using Lasergene (DNASTAR, Madison, WI), NCBI Entrez and other web-based tools. Nucleotide sequence data reported in this paper have been submitted to GenBank nucleotide sequence database and have been assigned accession numbers.

3. Results

3.1. Genome organization of Gallid herpesvirus type 2 strain 648A

The complete nucleotide consensus sequences of the 648Ap11 and 648Ap101 genomes are 176,080 and 174,048 bp, respectively. The unique long (U_L) regions are 113,520 and 112,631 bp in length and extend from positions 13,448 to 126,967 and 13,333 to 125,963 for 648Ap11 and 648Ap101, respectively. The unique short (U_S) regions for 648Ap11 and 648Ap101 extend from positions 152,076 to 163,726 and 150,596 to 162,246 and both are 11,651 bp in length, a size identical to that found within the genome of the attenuated strain CU-2. For 648Ap11 the terminal repeat long (TR_L) and internal repeat long (IR_L) extend from positions 715 to 13,447 and 126,968 to 139,700, respectively, and each is 12,733 bp in length. The TR_L and IR_L for 648Ap101 extend from positions 462 to 13,332 and 125,964 to 138,834, respectively, and each is identical in length (12,871 bp). The internal repeat short (IR_S) for 648Ap11 is 11,669 bp in length and extends from positions 140,407 to 152,075 and the terminal repeat short (TR_S) is 11,668 bp in length and extends from positions 163,727 to 1,175,394. For 648Ap101 the IR_S is 10,966 bp in length and extends from positions 139,630 to 150,595 and the TR_S is 10,907 bp in length and extends from positions 162,247 to 173,153. The first 714 and last 686 nucleotides at the termini of the 648Ap11 genome contain the a-like sequences. This corresponds to the first 461 and last 895 nucleotides at the termini of the 648Ap101 genome. The size differences in the U_L and R_S regions can be attributed to 948 and 632 bp deletions, respectively, in the genome of 648Ap101. Differences in the R_L regions between 648Ap11 and 648Ap101 can be attributed to the duplications of the 132 bp repeat elements. As with most serially passaged attenuation studies (Maotani et al., 1986; Ross et al., 1993; Silva and Witter, 1985; van Iddekinge et al., 1999), the number of 132 bp repeats expand to high copy numbers upon passage. There are two varieties of the 132 bp with heterogeneity within the sequence CTCGC(T/C)TTAT. 648Ap11 contained only one CTCGC<u>T</u>TTAT- and one CTCGC<u>C</u>TTAT-containing 132 bp repeat. The sequences in passage 101 contained at least 10 copies based on PCR results (data not shown). In agreement with data reported by Silva and Gimeno (2007) and Silva et al. (2004), it is doubtful that these repeats play a significant role in attenuation.

3.2. Analysis of open reading frames

In order to investigate the open reading frames (ORFs) within the sequences of passage 101 containing mutations, consensus nucleotide sequences were generated and aligned using the MAFFT program (Katoh et al., 2005). This allowed for the identification of mutations that occurred in the majority of the sequences within passages 11 and 101. Mutations in the minority of the sequences were identified by manual examination of the aligned contigs. An inspection of the contigs and alignments revealed that mutations arose during serial passage of 648A in varying proportions. Few were present in 100% of the sequences at passage 101. This indicated that a mixed population of genomes was present in passage 101.

3.3. Open reading frames containing deletions

Alignment of the 648Ap11 and 648Ap101 consensus sequences quickly allowed for the identification of large deletions. These dele-

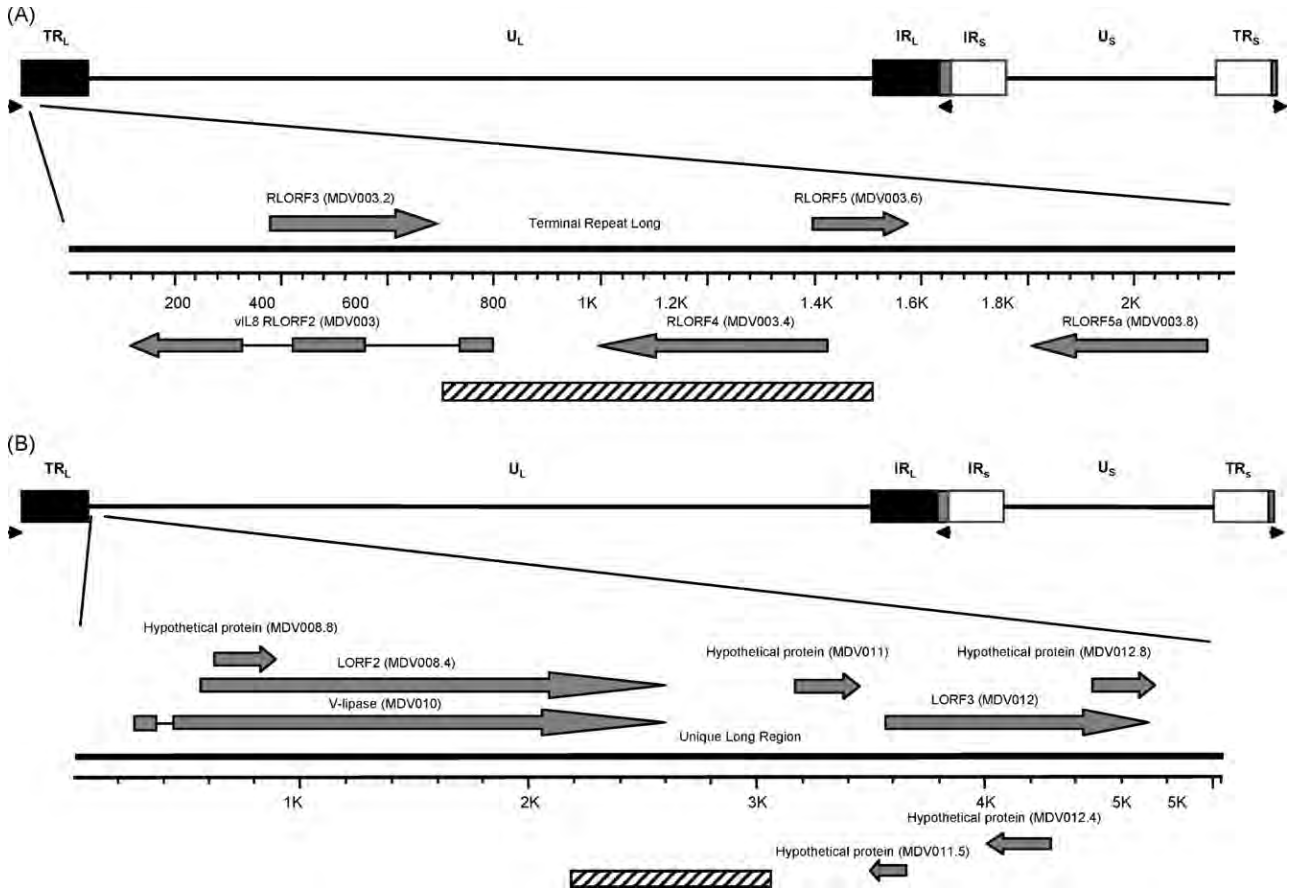

Fig. 1. Diagram of the 174 kb 648Ap101 genome with enlarged subgenomic regions where deletions were identified. Open reading frames (arrowheads) are indicated by the nomenclatures of Lee et al. (2000) and Tulman et al. (2000). The RLORF5a (Jarosinski et al., 2005) ORF encodes the protein known as L1 (Schat et al., 1998). The hatched boxes denote the position of the deletions: (A) the 820 nucleotide deletion in the terminal repeat long (TR_L) and (B) the 948 nucleotide deletion in the unique long (U_L).

tions were mapped to all subgenomic regions (repeat long [R_L], U_L and repeat short [R_S] except the U_S) (Fig. 1). A large deletion of 820 bp was identified within the R_L regions of 648Ap101 (Md5 coordinates 3455–4274 and 137,346–138,165). Roughly one-third of the sequences within this passage contained the deletion. Three genes (MDV003/MDV078, MDV003.4/078.3, and MDV003.6/078.2) encoding vIL8, RLORF4 and RLORF5, respectively were affected by the deletion. Both vIL8 and RLORF4 have been reported to be virulence factors (Jarosinski et al., 2005; Parcells et al., 2001). As show in Fig. 1, the deletion encompasses the entire RLORF4 reading frame, the first exon of vIL8 and the 5′ terminus of RLORF5.

A larger and more predominant deletion (in 54% of the p101 sequences) was identified within the U_L region and mapped to the gene MDV010 encoding the virulence factor vLipase (Kamil et al., 2005). The deletion removed 475 nucleotides from the 3′ end of the vLipase ORF and is predicted to generate a 609 aa polypeptide due to fusion with a downstream ORF.

The most predominant deletion, a 632 base pair deletion, was identified within the MDV084/100 (ICP4) promoter (Md5 coordinates 151,212–151,845 and 166,599–167,232). This deletion overlapped the 3′ end of the 10 kb latency associated transcript (LAT) and mapped 444 bp upstream of the ICP4 start codon within the Meq oncoprotein binding site (Levy et al., 2003). Five novel open reading frames (MDV084.5/099.5, 085/099, 085.3/098.6, 085.6/098.6 and 085.9/093.3) also mapped within the deleted region (Fig. 2). The deletion encompassed the 5′ termini of MDV085/099 and MDV084.5/099.5, the 3′ termini of MDV085.6/098.6 and MDV085.9/093.3, and the entire MDV085.3/098.6 gene. Whether these ORFs are expressed is not known. Nearly 98% of the sequences within the p101 population contained this deletion. Only 2.2% contained the intact ICP4 promoter.

Two small deletions were mapped within the R_L subgenomic region of 648Ap101. These deletions affected the novel diploid gene MDV05.5/75.91 and the overlapping genes MDV001/080 and MDV002/079 encoding the RNA component of the virus-encoding telomerase (vTR) and the Arg-rich protein RLORF1, respectively. A thymidine residue (after nucleotide 8062 in the Md5 genome) was missing in the MDV05.5/075.91 gene within the p101 sequences. This would result in a truncated protein of 81 aa. A 99 aa protein is predicted in the sequences of passage 11. It is not known whether this ORF expresses a protein. Seven polypeptides of varying length, from 36 to 99 aa, are predicted in GaHV-2 sequences found in GenBank.

Mutations affecting both MDV002/0079 and the vTR were identified in 100% of the p101 sequences. A series of 4 nucleotide deletions (at positions 382, 386, 394 and 398 on the Md5 vTR) were identified within these loci. Because of this a shift in the reading frame is predicted which would result in the generation of an elongated 230 aa MDV002/079 protein. This 4 nucleotide deletion mapped within the vTR gene (MDV001/080) to the stem structure within the CR7 domain and is predicted to significantly affect its structure (Fig. 3).

A single nucleotide deletion (thymidine) in the U_L gene encoding the DNA helicase–primase associated protein U_L5 (MDV017) was identified within 57% of the p101 sequences. This mutation mapped to a stretch of thymidine reiterations (after position 24,772 in the Md5 genome). A stretch of 7 thymidine residues within the U_L5

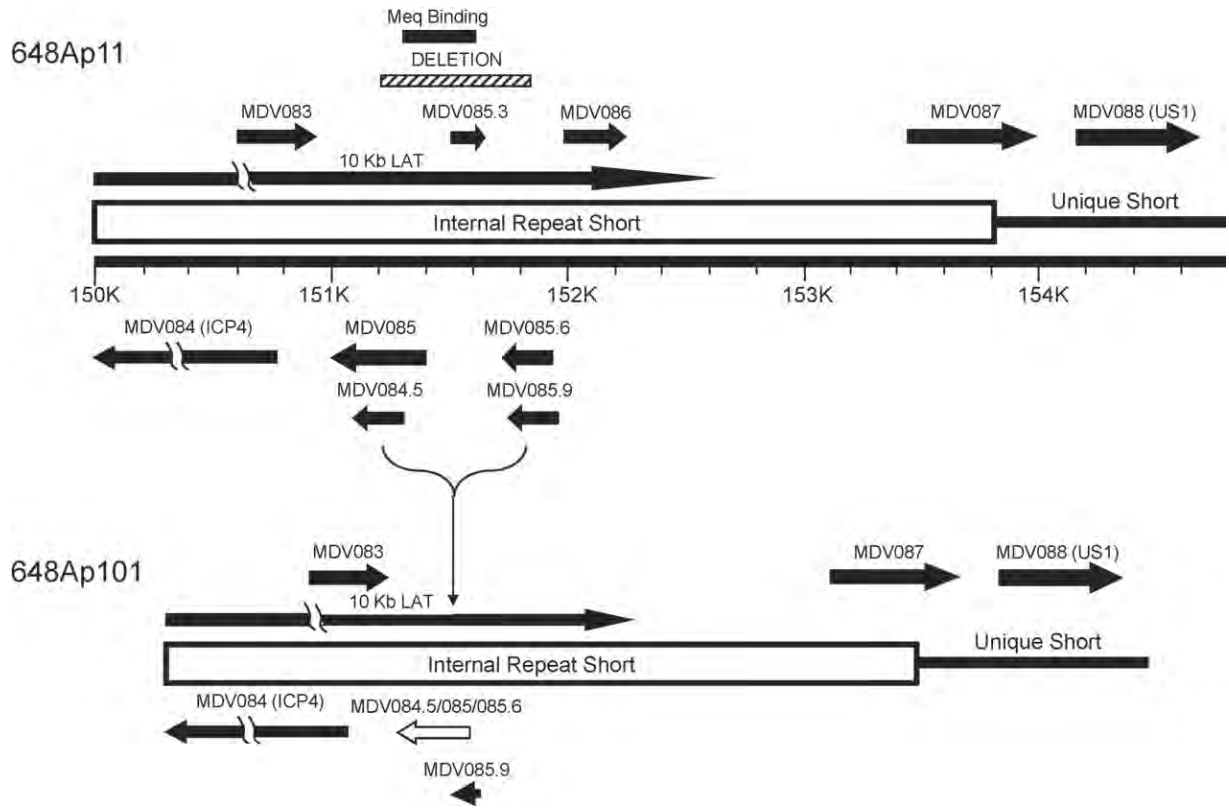

Fig. 2. Diagram of the internal repeat short (IR_S) of 648A showing the 632 bp deletion within the ICP4 promoter. The boundaries of the deletion are indicated with the hatched box. The position of the Meq binding site is indicated by the solid black box. A fusion polypeptide between MDV084.5, 085 and 085.6 is shown in the 648Ap101 genome as an open arrow.

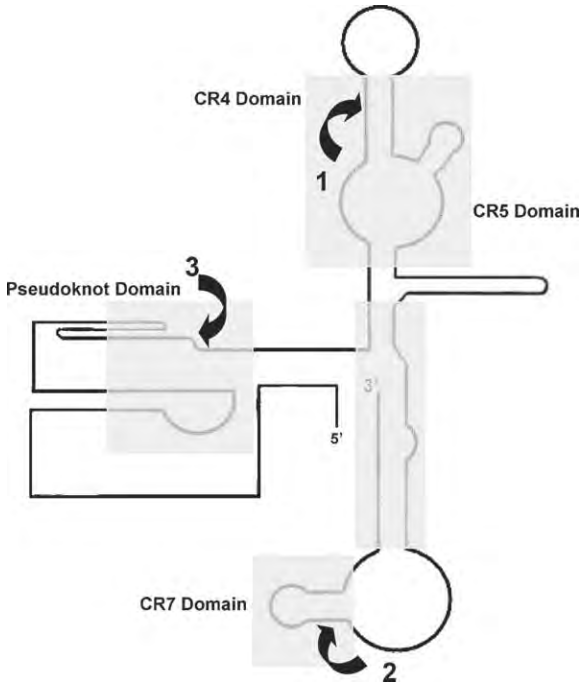

Fig. 3. The proposed structure of the GaHV-2 viral telomerase RNA component based on the vertebrate TR structure published by Chen et al. (2000, 2002) and Chen and Greider (2004). Within the genome of passage 101, a guanosine to cytosine transversion is indicated by the arrow at position 1 in the P6 stem structure of the CR4 domain; a four nucleotide deletion is indicated with the number 2 arrow at the P8A stem structure; and a two adenosine addition at the P3 structure within the pseudoknot domain is indicated with the number 3 arrow.

ORF was present in the minority of p101 sequences and in all the GaHV-2 U_L5 ORFs annotated in GenBank. The majority of the p101 sequences contained only 6 thymidines. Translation of the minority of U_L5 ORFs would result in a 858 aa protein, while translation of the majority of p101 U_L5 sequences would result in a 29 aa peptide. However, if a downstream start codon would be used then a 718 aa protein with proper COOH termini would be generated.

3.4. Open reading frames containing insertional mutations

In addition to the deletion mutations in the overlapping and MDV002/079 genes, two additional adenosine residues were identified at these loci within 100% of the p101 sequences. These residues mapped to the 5′ termini of MDV001/0080 (vTR) and 3′ noncoding region of MDV002/079 (after position 204 in the sequence of the Md5 vTR). On the predicted structure of the vTR (Fig. 3) the adenosine additions localize to the pseudoknot domain. It is unlikely that these insertions will have an effect on the function of vTR since multiple alignments of vTR sequences from 28 GaHV-2 strains suggest great sequence heterogeneity at this position.

Two nucleotide insertions (thymidine and cytosine) within the MDV002.6/078.5 diploid gene (after position 2562 in the Md5 genome) were identified within 100% of the p101 sequences. These sequences also contained a thymidine deletion after position 2541 (Md5). Because of these mutations a 60 aa MDV002.6/078.5 protein is predicted. Translation of this gene from the sequences of passage 11 as well as those of other GaHV-2 genomes annotated in GenBank would generated a 99 aa protein.

Similarly, a two nucleotide addition was identified in the MDV03.6/078.2 gene encoding RLORF5 within p101. These extra guanosine and thymidine residues mapped after nucleotide 4301 (Md5 coordinates). It is predicted that the additions would gener-

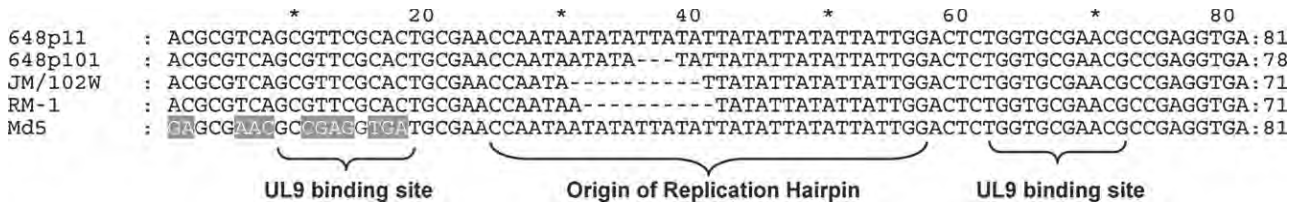

Fig. 4. Multiple alignment of the nucleotide sequence at the origin of replication within 648Ap11, 648Ap101, JM/102W, RM-1 and Md5 (Niikura et al., 2005; Spatz and Silva, 2007) at Md5 coordinates 128,253–128,331 (Tulman et al., 2000). The viruses 648AP101, JM/102W and RM-1 exhibit attenuated phenotypes.

ate a slightly larger protein (60 aa) than those RLORF5 homologues found within the p11 sequences (57 aa). This ORF is thought to be unimportant in virulence since the gene can be deleted without loss of virulence (Jarosinski et al., 2005). Furthermore, homologues in other sequenced GaHV-2 genomes contain proteins of varying lengths (57, 58, 59, 62 and 115 aa).

3.5. Identification of mutations in cis-acting elements

Besides deletions in protein coding regions, the cis-acting sites (origin of replication and the a-like sequence) also contained deletions within the sequences of passage 101. A TTA deletion within the hairpin structure at the origin of replication was identified within 64.2% of the p101 sequences. As shown in Fig. 4, other attenuated strains of GaHV-2 also contained deletions in the AT-rich hairpin structure. It is predicted that these deletions would negatively affect the thermal stability of the hairpin. Whether deletions in the hairpin structure affect the binding of the ssDNA binding protein UL9, thus affecting replication, remains to be determined.

Deletion mutations were also identified in sequences involved in packaging genome-length units into the capsid. These cis-acting sites are also known as a-like sequences and are characterized by DR1 sequences next to unique sequences (either *pac*1 or *pac*2) separated by telomeric repeat sequences (TRS). As shown in Fig. 5, an 89 nucleotide stretch between *pac*1 and the TRS was deleted in 64% of the p101 sequences. These sequences also contained a 34 nucleotide deletion which mapped downstream of the second DR1 site. Interestingly, a unique sequence (GTCATGTAGAGGGTCATGCGCG) was identified in 14 tandem repetitions within the p101 sequences. The origin of these repeats is unknown. They are exclusively found in p101 sequences and not present in any GaHV-2 sequences present in GenBank.

3.6. Cataloging of single nucleotide polymorphisms

With an average read length of 183 and 194 nucleotides resulting in an average of 43.9- and 73.6-fold coverage at each nucleotide for the 648Ap11 and 648Ap101 consensus genomes, a collection of single nucleotide polymorphisms that exist in differing proportions within the sequences of the two passages were cataloged (Table 1). SNPs were not only identified in comparing p101 to p11 sequences, but surprisingly occurred in sequences of passage 11 (Table 1: genes MDV001/079 and the overlapping vTR, and the overlapping genes within the MDV009 loci). Only one G to A transition within the promoter region of MDV006.1/075.1 encoding the hypothetical protein B68 (Peng et al., 1992, 1995) transitioned completely by passage 101. Some SNPs that occurred in MDV059, 3′ MDV082/102, MDV084/100(Val^{1871}Ala) and 5′ MDV086.1/097.9 transitioned to greater than 95%. Within the p101 sequences the majority of SNPs were of mixed populations. Based on the sequencing technology used (without cloning) it was impossible to map two (or more) SNPs on the same genome.

4. Discussion

This comparative genomics study involving the very virulent plus GaHV-2 strain 648A at passages that differ by 90 allowed for the identification of gross mutations and single nucleotide polymorphisms that accumulated over the year-long propagation in cell culture. Large deletions were identified within the genes encod-

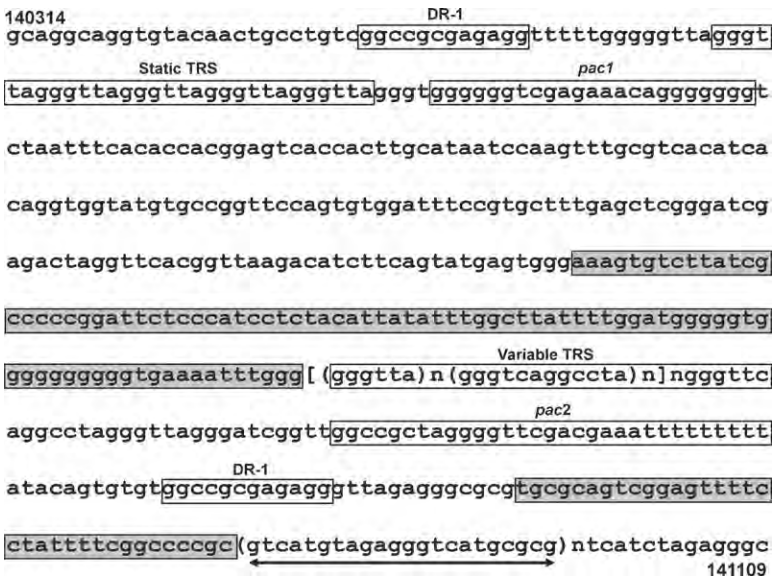

Fig. 5. The a-like sequence of the 648Ap11 genome. The shaded areas indicate the nucleotides that are deleted in 648Ap101. The 22 nucleotide stretch repeated 14 times in 648Ap101 is denoted by the double arrow.

Table 1
The identification of 14 single nucleotide polymorphisms within the sequences of passages 11 and 101 of 648A. The vertical bars represent the relative percentage of each SNP form. The common names and functions of the genes containing SNPs are also presented.

Location	Common name/function	SNP	648 passage number	
			11	101
MDV002/079 vTelomerase	RLORF1 Arg-rich protein contains a C_2H_2 domain unknown function RNA subunit of telomerase promotes malignant T cell lymphomagenesis	Arg^{180}Pro G to C		
MDV003/078	Spliced gene encoding vIL8 involved in chemotaxis of PBMC	Leu^{17}Ser		
MDV003/078	Spliced gene encoding vIL8 involved in chemotaxis of PBMC	Gln^{96}Arg		
5′ MDV006.6/075.1 3′ MDV007/074	B68, 14 kDa protein unknown function RLORF12 binds growth related translational controlled tumor protein	G to A		
MDV009	RLORF14 Early 24 kDa phosphoprotein, pp24	Gly^{9}Arg		
MDV008/073 MDV009.5/073.4	LORF1 unknown function RLORF13 binds C1q-binding protein	Pro^{161}Leu Arg^{45}Lys		
MDV054	UL41virionhostshutoff, RNase	Val^{359}Ala		
MDV059	UL46, tegument protein, VP11/12	Glu^{59}Lys		
3′ MDV082/102	RSORF1 unknown function	A to G		
MDV084/100	Immediate early ICP4 major transcriptional activator	Val^{1871}Ala		
MDV084/100	Immediate early ICP4 major transcriptional activator	Ser^{1641}Pro		
MDV084/100	Immediate early ICP4 major transcriptional activator	G^{189}A Silent		
5′ MDV086.1/097.9 3′ MDV086.4/097.3	Novel gene unknown function Novel gene unknown function	T to C		
MDV088	IE phosphoproein, ICP22, binds retinoblastoma-binding protein 4	Asp^{133}Gly		
MDV096	US8, glycoprotein E involved in cell-to-cell spread	Ser^{205}Pro		

ing RLORF5, viral interleukin 8 (vIL8), RLORF4 and vLipase. The latter three genes have previously been demonstrated to encode virulence factors in chicken experiments with knock-out viruses (Jarosinski et al., 2005; Kamil et al., 2005; Parcells et al., 2001). There is little evidence that RLORF5 encodes a functional polypeptide. Seven sequence length variations (57, 60, 62, 59 112, 113 and 115 aa) are predicted from sequences within GenBank and this gene can be deleted without notably affecting phenotype (Jarosinski et

al., 2005). Deletions were also identified in the noncoding regions of the ICP4 promoter, the origin of replication and the a-like sequence involved in cleavage and packaging. Interestingly, these deletions were present in varying proportions within the p101 sequences. Only 33% of the sequences contained the 820 bp deletion affecting RLORF4, RLORF5 and vIL8, 54% contained the vLipase deletion and 64.2% contained the deletion at the origin of replication. The deletion within the promoter region of ICP4 was present in nearly all (97.8%) of the p101 sequences. Overall this indicates that passage 101 contains a mixed population. The identification of the 14 single nucleotide polymorphisms presented in Table 1 add further support that GaHV-2 exists as a collection of mixed population. This discovery has been made only possible due to recent advances in sequencing without cloning and the ability to isolate relatively pure nucleocapsid DNA from this avidly cell-associated virus. The notion that GaHV-2 exists as mixed populations has been suspected for years since attenuated strains will often revert upon back passage in chickens (Karpathy et al., 2002; Witter and Lee, 1984), and because of the sheer number of GaHV-2 BACs that contain genetic aberrations, many of which when reconstituted in cell culture are avirulent in chickens (Hans Cheng, personal communication). In the construction of a RB-1B BAC that was subsequently phenotyped as incapable of horizontal transmission it was discovered that the parental DNA from transmission component viruses contained a mixed population genomes with wild-type and mutant UL13 genes (Spatz et al., 2007b).

Since 648Ap101 is attenuated and lacks the ability to form tumors (Gimeno et al., 2001) it was a goal of this study to identify the genes that may play a role in this phenotype. Only three genes [Meq, the viral encoded telomerase (vTR), and the ubiquitin-specific protease (USP) domain of U_L36] have been implicated in tumor formation in infected chickens (Jarosinski et al., 2007; Lupiani et al., 2004; Trapp et al., 2006). Surprisingly no mutations were identified in the genes encoding the oncoprotein Meq or the USP domain of the large tegument protein U_L36. Mutations were, however, identified in the diploid gene encoding the viral encoded telomerase RNA subunit (Fragnet et al., 2003, 2005; Trapp et al., 2006). Two of these mutations would affect stem structures within the vTR and it is possible that they may contribute to the lack of tumor formation. However data reported by Trapp et al. suggest that the vTR only contributes minimally in tumor formation. Lymphomas were still produced in animals receiving the vTR double knock-out virus although the tumors were significantly smaller in size and less disseminated. We postulate that the lack of tumors in animals inoculated with 648Ap101 is due to mutations within genes critical to efficient replication. Almost all of the sequences in passage 101 contained deletions within the promoter of ICP4, the major transcriptional activator of GaHV-2. This is likely to effect the activation of the early genes involved in DNA replication (Endoh, 1996; O'Hare and Hayward, 1985, 1987). Deletions were also detected within the origin of replication (71% of the p101 sequences), and 64% of the p101 sequences contained deletions in the a-like sequence needed for cleavage and packaging. Compounding this, SNPs were identified in two genes (U_L46 and U_S8, encoding the tegument protein VP11/12 and glycoprotein E) that most likely affect their structure/function. Nearly all of the translated U_L46 sequences of p101 contained the positively charged $lysine^{59}$ substitution within VP11/12 (translated p11 sequences contained the negatively charged $glutamate^{59}$ residue). Seventy-five percentage of the translate p101 sequences also contained the Pro^{205} SNP within glycoprotein E instead of the more common Ser^{205}. Glycoprotein E has been demonstrated to be essential for cell-to-cell spread (Schumacher et al., 2001) and probably plays a role in the lack of efficient replication of 648Ap101.

Previously we have sequenced a bacterial artificial chromosome (BAC) containing the 80th passage of a different very virulent strain, 584A, and have identified 17 ORFs containing genetic aberrations (Spatz et al., 2008). The nucleotide sequences of both 584A passage 80 and 648A passage 101 were examined for mutations shared in common since they both exhibited an attenuated phenotype at the passages that was sequenced. Three genes (encoding viral interleukin 8, RLORF4 and vLipase) contained mutations in both studies, suggesting that most mutations accumulate randomly during serial passage and are selected for based only on their cell culture adaptability. Simiarly Jarosinski et al. (2003) reported deletions within LORF 4 of additional attenuated strains (JM-16p48, JM-16p71and R2/23) and later proved that RLORF4 does indeed encode a virulence factor (Jarosinski et al., 2005).

The most revealing aspect of the research present in this paper is the discovery that Marek's disease virus exists as a collection of mixed populations. It would be interesting to determine the specific passage number at which the mutations first appeared and whether they were stable upon continued passage in cell culture. This study only examined the nucleotide sequences within passages 11 and 101, but aliquots of the intermediate passages are available. Deep sequencing of a limited number of these passages (p20, 30, 40, 50, etc.) might provide genotypic insight to other phenotypes (i.e. acute transient paralysis, persistent neurological disorder, red leg syndrome, ocular lymphomatosis) that are lost upon serial passage.

The fact that Marek's disease virus exists as a collection of mixed population not only at high *in vitro* passage but to a limited degree at low passage, opens the possibility that various genotypes probably exist in infected animals, and based on the complex cell tropisms of the virus within infected chickens it would be interesting to investigate whether different genotypes infect different organs and/or cell types. This will require generation of numerous GaHV-2 BACs with minimal *in vitro* propagation during virus reconstitution prior to *in vivo* experiments.

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Generation and biological assessment of recombinant avian metapneumovirus subgroup C (aMPV-C) viruses containing different length of the G gene

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ABSTRACT

Genetic variation in length of the G gene among different avian metapneumovirus subgroup C (aMPV-C) isolates has been reported. However, its biological significance in virus replication, pathogenicity and immunity is unknown. In this study, we developed a reverse genetics system for aMPV-C and generated two Colorado (CO) strain-based recombinant viruses containing either the full-length G gene derived from a Canadian goose isolate or a C-terminally truncated G gene of the CO strain. The truncated short G (sG) gene encoded 252 amino acids (aa), which is 333 aa shorter than the full-length G (585 aa). The biological properties of these two recombinant G variants were assessed in Vero cells and in specific-pathogen-free (SPF) turkeys. In Vero cells, the short G variant displayed a similar level of growth dynamics and virus titers as the parental aMPV-CO strain, whereas the full-length G variant replicated less efficiently than the sG variant during the first 72 h post-infection. Both of the G variants induced typical cytopathic effects (CPE) that were indistinguishable from those seen with the parental aMPV-CO infection. In SPF turkeys, both of the G variants were attenuated and caused little or no disease signs, but the full-length G variant appeared to grow more readily in tracheal tissue than the sG variant during the first 5 days post-infection. Both G variants were immunogenic and induced a slightly different level of antibody response. These results demonstrated that the large portion (333 aa) of the extracellular domain of the viral attachment protein is not essential for virus viability *in vitro* and *in vivo*, but may play a role in enhancing virus attachment specificity and immunity in a natural host.

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1. Introduction

Avian metapneumovirus (aMPV) is an economically important pathogen of turkeys with a worldwide distribution. It causes upper respiratory tract disease in turkeys and is also associated with "swollen head syndrome" (SHS) of chickens (Alexander, 2003; Cook, 2000a). aMPV is a non-segmented, single-stranded negative sense RNA virus, and belongs to the genus *Metapneumovirus* within the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Pringle, 1998). aMPV genome consists of eight genes flanked by a 3′ Leader and 5′ Trailer in the order 3′-N (nucleocapsid)-P (phosphoprotein)-M (matrix)-F (fusion)-M2 (second matrix)-SH (small hydrophobic)-G (glycoprotein)-L (large polymerase)-5′ (Ling et al., 1992; Yu et al., 1992). Based on the level of genetic variations and antigenic differences, aMPV isolates have been classified into four subgroups: aMPV-A, aMPV-B, aMPV-C and aMPV-D (Banet-Noach et al., 2005; Bayon-Auboyer et al., 2000; Juhasz and Easton, 1994; Seal, 2000).

The glycoprotein (G) of aMPV has been much of scientific curiosity owning its role in viral attachment, and possible role in viral virulence and protective immunity. The G protein of aMPV isolates exhibits extensive genetic and antigenic variations that are the primary criteria for aMPV subgroup classification and for consideration of vaccine development (Cook, 2000b; Juhasz and Easton, 1994; Patnayak et al., 2002). Among the aMPV subgroups, aMPV-C isolates possess the most extensive sequence divergence from other subgroups (Toquin et al., 2006). Furthermore, the G gene length reported by different researchers for aMPV-C strains isolated in the United States was strikingly different, ranging from 783 nucleotides (nts) coding for 252 aa to 1798 nts coding for 585 aa (Alvarez et al., 2003; Bennett et al., 2005; Govindarajan et al., 2004; Lwamba et al., 2005). Recent studies have shown that the G gene length variation for those U.S. isolates resulted from a truncation of the G gene during serial passages of the virus in Vero cells or circulation in a host in the field (Kong et al., 2008; Velayudhan et al., 2008). However, the biological significance of the G gene length variation in virus replication, pathogenicity and immunogenicity remains to be defined.

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During last two decades, a reverse genetics technology has been developed to study the molecular biology, pathogenicity and immunogenicity of many negative strand RNA viruses, including avian metapneumoviruses (Govindarajan et al., 2006; Naylor et al., 2004). In the present study, we developed a reverse genetic system for aMPV-C and generated two Colorado strain-based recombinant viruses containing either the full-length G gene derived from a Canadian goose isolate or a C-terminally truncated G gene of the CO strain. The biological properties of these two G recombinant variants were assessed in Vero cells and specific-pathogen-free (SPF) turkeys to elucidate the effect of different length of the G gene on virus replication, pathogenicity and immunogenicity.

2. Materials and methods

2.1. Viruses, cell cultures and viral RNA preparation

The aMPV-C Colorado strain (aMPV/CO/96/C) was kindly provided by Dr. Bruce Seal (USDA, ARS, RRC, Athens, GA, USA). The virus was propagated in Vero cells (ATCC, Manassas, VA, CCL-81) and maintained at passage level five as a stock. Viral rescue was performed on HEp-2 cell (ATCC, CCL-23) monolayers in 6-well plates. All cell monolayers were grown in high glucose Dulbecco's Modified Eagle Medium (D-MEM) supplemented with antibiotics and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and kept at 37 °C under a 5% CO_2 atmosphere unless otherwise indicated. A virulent challenge virus stock was prepared by three serial passages of the aMPV-CO stock in 1-week-old SPF turkeys as described previously (Velayudhan et al., 2007). At the third passage, the tracheas of infected turkeys were harvested and homogenized in brain heart infusion (BHI) medium (Becton Dickinson, Sparks, MD) containing antibiotics. The supernatant of the tracheal tissues was titrated in 1-week-old turkeys for 50% infective dose (ID_{50}) and used as a virulent challenge virus, designated as aMPV-C Tr. The modified vaccinia virus Ankara/T7 recombinant (MVA/T7, a gift of Dr. Bernard Moss, National Institute of Health) was used during viral rescue to provide the T7 bacteriophage polymerase (Wyatt et al., 1995). Total cellular RNA from infected Vero cells or tracheal tissues was extracted using either Trizol-LS reagent (Invitrogen, Carlsbad, CA) or a MagMAX™ AI/ND Viral RNA Isolation kit (ABI, Austin, TX) according to the manufacturer's instructions. Viral RNA of the Canadian goose isolate of aMPV-C was kindly provided by Dr. K. Nagaraja, University of Minnesota, USA (Bennett et al., 2005).

2.2. Construction of full-length cDNA clones

The total RNA extracted from Vero cells that were infected with the cell culture-adapted aMPV-CO strain containing a truncated G gene (Lwamba et al., 2005) was used for cDNA synthesis. Five overlapped cDNA fragments spanning the entire genome of the virus were generated by RT-PCR amplification of genomic RNA with five pairs of specific primers (Table 1) using a Superscript™ One-Step RT-PCR kit with Platinum *Taq* Hi-Fi Polymerase (Invitrogen, Carlsbad, CA) (Fig. 1). These cDNA fragments were cloned into a TOPO cloning vector (Invitrogen). One clone containing the M2, SH genes and the truncated G gene (783 nts), and four clones covering the rest of the genome were selected by sequencing analysis. Then these five cloned cDNA fragments were sequentially cloned into a modified pBluescript plasmid (Estevez et al., 2007) by taking advantage of the naturally occurred or engineered unique restriction-endonuclease sites in the genome. The assembled full-length cDNA clone (FLC) coding for the anti-genome of the Vero cell-adapted aMPV-C CO strain was designated as paMPV-C sG that contained the truncated short G gene (coding for 252 aa). The open reading frame (ORF) of the short G gene in paMPV-C sG was substituted by that of a full-length G gene (coding for 585 aa) from a Canadian goose isolate (Bennett et al., 2005) using an In-Fusion™ PCR Cloning kit (Clontech, Mountain View, CA) with specific primers (Table 1). The resulting FLC containing the full-length G gene was named as paMPV-C gG. A molecular tag was introduced to both FLCs by using a QuickChange site-directed mutagenesis kit (Stratagene) to create

Table 1
Sequences of primers used in the study.

Primer	Primer sequence	Primer name
1	5′gaagcggccgctaatacgactcactatagggACGAGAAAAAAACGCATATAAGACAACTTCCA	aMPV-C 1F
2	5′gatggcgccGATTTTCCACATCTCCAACCTCAAG	aMPV-C 1R
3	5′gaagcggccgcAGGGTACGAGATATGTCCTGAAGTC	aMPV-C 2F
4	5′gatggcgccAGGCCATCTGATCTATCTGTATTTC	aMPV-C 2R
5	5′gagcggccgcGAAGTACGGGGCAAGTGCAATAGAGG	aMPV-C 3F
6	5′gccggcgccACCCTTTAAATAAGAATCAGGTAGG	aMPV-C3R
7	5′ggggtaccTTAATTAAAAAGAAGGACCAAGTTAAAAATGGATCCACTAAATGAAGGGGT	aMPV-C 4F
8	5′TTAGGCCGGAAAGCAGAGAT	aMPV-C 4R
9	5′agcggccgctaggtaccatcccgagcttaattaACAACAACCTACCATGTCACGGGGAGGTAC	aMPV-C 5F
10	5′cagccggcgccagcgaggaggctgggaccatgccggccACGGCAAAAAAACCGTATTCATCCAAT	aMPV-C 5R
11	5′atggtaccACCATGTCTCTTCAGGGGATTCAGCTTAG	aMPV-N F
12	5′tcggatccTTACTCATAATCATTCTGGCCTTCCTC	aMPV-N R
13	5′atggtaccACCATGTCCTTTCCTGAGGGGAAAG	aMPV-P F
14	5′tcggatccTACATAGTAAGGGAGTATAGG	aMPV-P I R
15	5′tggaattccACCATGTCTCGCAAGGCTCCCTGC	aMPV-M2-1 F
16	5′agctcgagCTTAATCAGAGTTTGCACTATCTTGC	aMPV-M2-1 R
17	5′atggtaccACCATGGATCCACTAAATGAAGGGGTTGTG	aMPV-L F
18	5′gcaacgtctcggaTCCATCATTTCTTACTCATCAGCATGTAACC	aMPV-LR
19	5′GTTGACTTGTCCCGTTTTTCCTTCTCTGATCG	FLC G up
20	5′TTAATTAAAAAGAAGGACCAAGTTAAAAATGGA	FLC G down
21	5′AAAACGGGACAAGTCAACATGGAGGTCAAGGTAGAGAATG	gMPV G fo
22	5′GTCCTTCTTTTTAATTAACTAACTCCAGTTGTACGAGTTG	gMPV G rev
23	5′ATGTCTCTTCAGGGGATTC	aMPV-C 001
24	5′GCATCATTCAGCACACG	aMPV-C 067

Primers 1–10 were used to RT-PCR-amplify the complete genome of the parental aMPV-C CO strain virus into five fragments. Primers 11–18 were used to amplify the coding sequences of aMPV-C CO strain virus nucleoprotein (N), phosphoprotein (P), second matrix ORF 1 (M2-1), and polymerase (L) genes. Primers 19–22 were used to amplify the FLC vector and the G gene from the Canadian goose isolate for cloning the full-length G gene into the FLC. Primers 23 and 24 were used to amplify a part of the N gene for detection of viral RNA presence in tracheal tissues and infected cells. Nucleotides shown in lower case represent sequences from plasmid backbone or engineered restriction-endonuclease sites.

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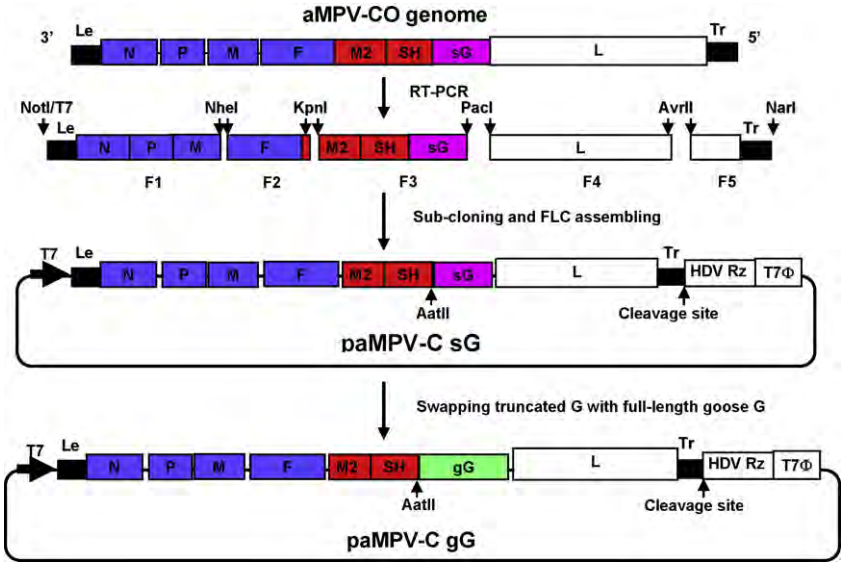

Fig. 1. Schematic presentation of aMPV-C full-length cDNA clone construction. Five cDNA fragments, marked with F1, F2, F3, F4, and F5, respectively, were generated by RT-PCR from genomic RNA of the Vero cell-adapted aMPV-CO strain containing a truncated G (sG) gene. These cDNA fragments were sequentially cloned and assembled into a modified pBluescript vector by using naturally occurred or engineered restriction-endonuclease sites that are marked by black arrows. The resulting full-length cDNA clone (FLC) containing the truncated short G gene, highlighted with the pink filling, is designated as paMPV-C sG. The open reading frame of the short G gene in paMPV-C sG was substituted with that of the full-length goose G gene, highlighted with the green filling, by using an In-Fusion™ PCR Cloning kit. The resulting FLC is named as paMPV-C gG. T7 promoter direction is indicated by a bold black arrow. Hepatitis delta virus ribozyme (HDV Rz) cleavage site is marked by a black arrow. T7Φ represents the T7 terminator sequence.

an *AatII* site in the intergenic region between the SH and G genes. The FLC plasmids were amplified in STBL2 cells (Invitrogen) at 30 °C, and purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

2.3. Construction of plasmids expressing the N, P, M2-1, and L proteins

cDNAs coding for the N, P, M2-1, or L protein of the aMPV-CO strain were generated by RT-PCR from viral RNA with specific primers (Table 1), and then cloned into a cloning site of pcDNA 3.1/Zeo(+) expression vector (Invitrogen, Carlsbad, CA) (Fig. 2). The viral gene expression was under the control of T7 RNA polymerase promoter. The plasmids were amplified in DH5α or STBL2 cells (Invitrogen), and purified using a QIAprep Spin Miniprep kit (Qiagen). These viral gene plasmids were designated as pN, pP, pM2-1, or pL, respectively.

2.4. Nucleotide sequencing

All RT-PCR products and purified plasmids were sequenced directly with M13 universal primers or gene-specific primers using the Applied Biosystems-PRISM fluorescent big dye sequencing kit and the ABI 3730 DNA sequencer (ABI, Foster City, CA). Nucleotide sequence editing, assembling and comparison were accomplished using the DNASTAR program (Madison, WI).

2.5. Virus rescue and propagation

A mixture of 1 μg of paMPV-C sG or paMPV-C gG, 1 μg of pN, 0.5 μg of pP, 0.1 μg of pL and pM2-1 plasmids was transfected into HEp-2 cells that were infected with MVA/T7 recombinant virus (Wyatt et al., 1995) using Lipofectamine™ 2000 reagent according to the manufacturer's protocol (Invitrogen). A negative control monolayer was transfected as described above, but omitting the pL

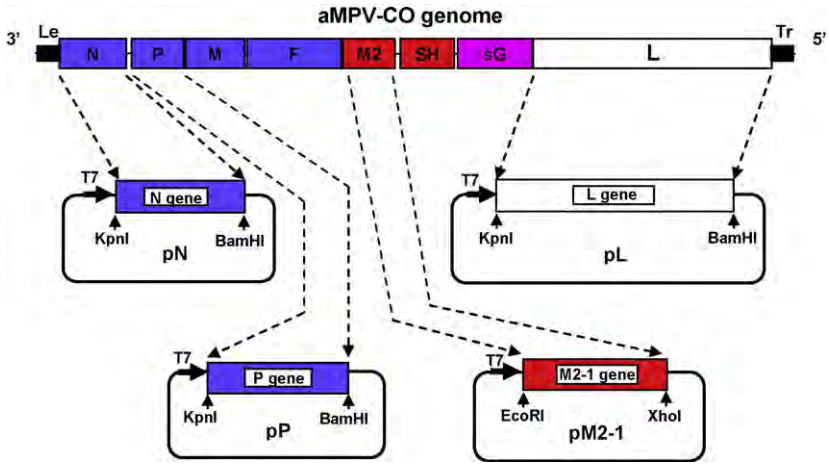

Fig. 2. Schematic presentation of viral gene expression plasmids. cDNAs coding for the N, P, M2-1, or L protein were generated by RT-PCR from viral RNA of the aMPV-CO strain, and cloned into cloning sites, marked by a black arrows, in pcDNA3.1/Zeo(+) expression vector. The viral gene expression was under the control of T7 RNA polymerase promoter (T7). T7 promoter direction is indicated by a bold black arrow. The resulting viral gene expression plasmids are designated as pN, pP, pM2-1, or pL, respectively.

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plasmid, thus not supplying the essential viral polymerase gene. At 6 h post-transfection, the cells were washed 1× with PBS and maintained in D-MEM supplemented with 2% FBS and antibiotics (Invitrogen). At 72 h post-transfection, the transfected HEp-2 cells were scraped and co-cultured on an existing 50% monolayer of Vero cells. The co-cultured cells were incubated at 37 °C in 5% CO_2 for 5 days. The rescued virus in the co-cultured cells was harvested by freeze and thawing three times and propagated in Vero cells for 3–5 passages until aMPV-induced cytopathic effects (CPE) were observed.

2.6. Virus titrations and growth curves

The titers of various aMPV-C virus stocks were determined by infecting Vero cell monolayers with 100 μL of serial 10-fold dilutions of the viruses as previously described (Kapczynski and Sellers, 2003). Titers were calculated by the Reed and Muench method (Reed and Muench, 1938) and are expressed as 50% tissue culture infective dose ($TCID_{50}$). Growth kinetics of the recombinant G variants and the aMPV-CO strain were examined in Vero cells. Briefly, Vero cell monolayers were infected with aMPV-CO, raMPV-C sG, or raMPV-C gG at 0.1 multiplicity of infection (MOI). At every 12 h post-infection, the monolayers were harvested. The mean virus titers of duplicates at each time point were determined in Vero cells and expressed as $\log_{10}$ $TCID_{50}$/mL.

2.7. Animal experimental design

Forty 1-week-old SPF turkey poults were randomly divided into four groups of 10 birds. Birds in group 1 received 100 μL of PBS via intranasal (IN) and intraocular (IO) routes per bird as a control. Birds in groups 2 and 3 were inoculated with 100 μL of raMPV-C sG (3.16×10^6 $TCID_{50}$/mL) or raMPV-C gG (3.16×10^6 $TCID_{50}$/mL) per bird, respectively, via IN and IO routes. Each bird in group 4 was infected with 100 μL of aMPV-C Tr (4.2×10^4 ID_{50}/mL), the challenge virus stock, via IN and IO routes as a positive control. After inoculation, birds were observed daily for recording clinical signs of aMPV disease. Intra-tracheal swabs were collected at 3, 5, and 7 days post-inoculation (DPI) from each bird for detection of virus replication or viral RNA shedding in the respiratory tract. Each of the swabs was stored in 1 mL of BHI medium containing antibiotics at −70 °C. At 14 DPI, serum was taken from each of the birds for detection of antibody responses by ELISA. Immediately after serum collection, birds in all groups were challenged with 100 μL (per bird) of aMPV-C Tr (4.2×10^4 ID_{50}/mL) via the IN/IO routes. The challenged birds were monitored daily for clinical signs. Tracheal swabs were collected from each of the challenged birds at 3, 5, and 7 days post-challenge (DPC) for detection of virus shedding.

2.8. Detection of viral RNA

Virus replication or viral RNA shedding in the turkey tracheal tissues was detected by RT-PCR with a pair of aMPV-C N-gene-specific primers (Table 1). Briefly, the tracheal swabs in the BHI medium were treated by one-round of freeze–thawing and vortexing. Fifty microliters of the tracheal swab medium were used for RNA isolation as described above. One fifth of the isolated RNA was used to detect the presence of aMPV-C viral RNA by RT-PCR using a Superscript™ One-Step RT-PCR kit (Invitrogen) and the N-gene-specific primers. The RT-PCR amplified products were analyzed and visualized by agarose gel electrophoresis.

2.9. Detection of antibody response by ELISA

Anti-aMPV-C antibody response of turkeys after infection was determined by an ELISA test as described by Kapczynski (2004) with some modifications. Sucrose gradient purified aMPV-CO virus was used as antigen. Turkey sera were diluted (1:100) and individually tested in triplicates. Coating, washing, blocking and diluting reagents were purchased from Immunochemistry Technologies (Bloomington, MN). Goat anti-turkey IgG horseradish peroxidase-conjugate and LumiGLO, a luminol-based chemiluminescent substrate, were supplied by KPL Inc. (Gaithersburg, MD). The chemiluminescence relative light unit (RLU) was measured on a Biotek Synergy HT Microtiter Plate Luminometer (Biotek Instruments, Winooski, VT). A standard negative serum pool (collected from turkeys inoculated with PBS) was included in each plate of the ELISA test as a negative control. The positive anti-aMPV-C serum pool (collected from turkeys following infection with the virulent challenge virus) was 2-fold diluted from 1:100 to 1:3200 and used to establish a standard curve for determining antibody titers of the test sera. Sera were deemed positive when the mean RLU value was greater than the mean RLU plus 2× standard deviation of the three dilutions (1:100 to 1:400) of the negative turkey serum pool.

3. Results

3.1. Generation of recombinant aMPV-C sG and gG gene variants

Two full-length cDNA clones containing a different length of the G gene (Fig. 1) and four supporting plasmids expressing the N, P, M2-1, and L proteins of the aMPV-CO strain (Fig. 2) were constructed. After co-transfection of the full-length clones and the supporting plasmids in HEp-2 cells and amplification in Vero cells, two aMPV-C infectious viruses were rescued. One of the rescued G gene variants, raMPV-C gG, contained the full-length G gene which was cloned from a Canadian goose isolate (Bennett et al., 2005). The goose G (gG) consists of 1798 nts, coding for 585 aa, which is the same length as that for the aMPV-C Colorado and Minnesota strains reported by Govindarajan and Samal (Govindarajan and Samal, 2004). Whereas the other recombinant G variant, raMPV-C sG, contained a truncated G gene (783 nts) coding for 252 aa, such that the C-terminal 333 aa of the G protein had been deleted. This short G (sG) gene possesses the same length and sequences as that of the Colorado strain reported by Lwamba et al. (2005). Comparison of the first 738 nts of the goose G gene with that of the Colorado strain showed 93% nucleotide sequence identity and corresponding 88.5% amino acids sequence identity (Bennett et al., 2005). To confirm the fidelity of the rescued viruses, the viral genomes were sequenced and the aa changes were identified (Table 2). Two aa changes occurred in the genome of the short G variant, raMPV-C sG, and six aa changes and one nucleotide insertion in a non-coding region were found in the genome of the rescued goose G gene variant, raMPV-C gG.

Table 2
Sequence changes in raMPV-C G variant genomes.

Position[a]	raAMPV-C sG	raAMPV-C gG	AA change	Gene/region
477		G to A	Asn to Asp	N
2528	A to T[b]		Met to Leu	M
5423		A insertion	Non-coding	M2/SH
6701		A to G	Ile to Met	G
6852		A to G	Gln to Arg	G
6861		A to C	Ile to Thr	G
7109		A to G	Arg to Gly	G
12504	G to A		Gly to Ser	L
13401		G to A	Gly to Lys	L

[a] Nucleotide positions are counted in relevant to the original positions in the genome.
[b] All nt sequences are presented in positive sense of the genome.

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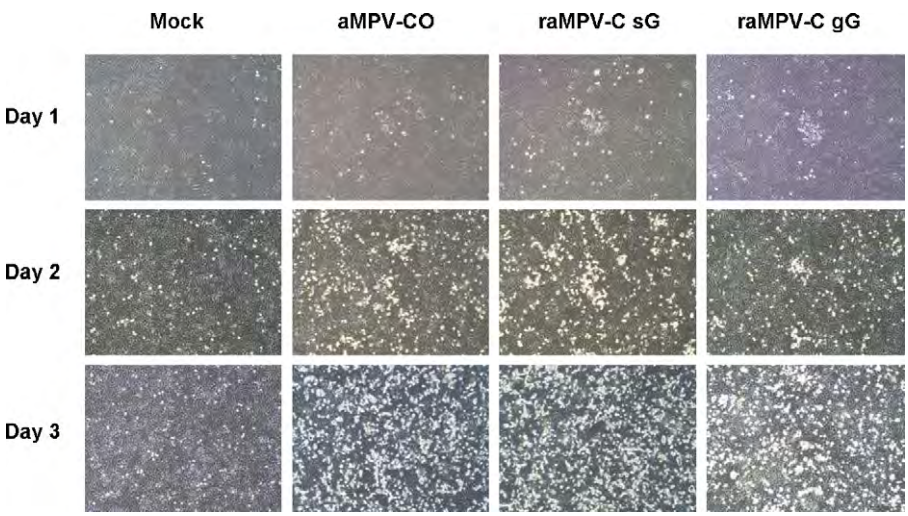

Fig. 3. Cytopathic effects induced by aMPV-C infection. Vero cell monolayers were infected with raMPV-C sG, raMPV-C gG, aMPV-CO Vero cell stock at 0.1 MOI $TCID_{50}$/cell, or mock infected with D-MEM. At days 1, 2, or 3 post-infection, the monolayers were digitally photographed using an inverted microscope at 100× magnifications (Olympus America, Melville, NY).

Table 3
Observation of clinical signs of turkeys at different days post-inoculation.

Inoculums	Number of birds showing clinical signs/number in group									
	1	2	3	4	5	6	7	8	9	10
PBS	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
aMPV-CO Tr	0/10	0/10	**7/10**	**7/10**	**9/10**	**10/10**	**8/10**	**4/10**	**2/10**	0/10
raMPV-C sG	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
raMPV-C gG	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

The bold values are used to emphasize the positive numbers of birds showing clinical signs.

3.2. Biological characterization of the recombinant G variants in cell cultures

To determine if the G gene length variation affects virus replication and growth characteristics *in vitro*, the recombinant G variants and the parental CO strain were examined in Vero cells for growth dynamics and cytopathic effects (CPE). As shown in Fig. 3, both G variants induced typical CPE that were indistinguishable from those seen with the parental aMPV-CO infection. Virus titers of samples collected at different time points post-infection showed that the sG variant displayed similar growth kinetics as the parental aMPV-CO strain. However, the gG appeared to replicate less efficiently (about one $\log_{10}$ titer lower) than the sG variant or the parental aMPV-CO strain during the first 72 h post-infection (Fig. 4).

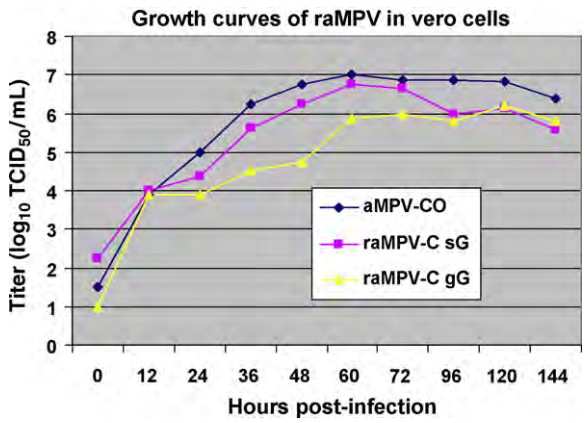

Fig. 4. Growth curves of the recombinant G variants and the aMPV-CO strain in cells. Vero cell monolayers were infected with raMPV-C sG, raMPV-C gG or aMPV-CO at 0.1 MOI $TCID_{50}$/cell. At every 12 h post-infection, the monolayers were harvested. Virus titers at each time point, in duplicate, were determined by $TCID_{50}$ titration in Vero cells. The mean titer of the two tests at each time point is expressed in $\log_{10}$ $TCID_{50}$/mL.

3.3. Pathogenicity of the recombinant G variants in turkeys

Experimental infection of 1-week-old SPF turkeys was carried out to evaluate the effects of the G gene length variation in viral pathogenicity *in vivo*. From 3 DPI, the turkey poults infected with the virulent aMPV-C Tr exhibited typical clinical signs of the disease, showing nasal exudates when squeezed, nasal discharge, or frothy eyes. These clinical signs gradually disappeared after 8 DPI. However, neither the short G nor goose G variant-infected birds showed any clinical signs (Table 3). RT-PCR data to detect viral RNA in tracheal tissues (Table 4) showed that 100% of challenge virus-infected birds became positive at 3 and 5 DPI and 60% of those birds were still positive at 7 DPI. However, only 55–75% of the gG variant-inoculated turkeys had detectable virus at 3 and 5 DPI, respectively, and only one of eight (13%) inoculated birds had virus in trachea at 7 DPI. The sG variant-infected group demonstrated a low level of virus replication at early times post-infection (22% at

Table 4
Virus shedding in turkey trachea at different days post-inoculation (DPI).

Viruses	No and % of birds in which viral RNA was detected in group		
	3 DPI	5 DPI	7 DPI
aMPV-CO Tr	10/10 (100%)	10/10 (100%)	6/10 (60%)
raMPV-C sG	2/9 (22%)	2/9 (22%)	5/8 (63%)
raMPV-C gG	5/9 (55%)	6/8 (75%)	1/8 (13%)

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Table 5
Serum antibody response in turkeys at day 14 post-inoculation.

Inoculums	No. of Ab positive sera and % in group	Number of sera with ELISA titer (log_2)					Mean ± S.D. (log_2)
		≤5.6	6.6	7.6	8.6	9.6	
PBS	0/10 (0%)	10					
aMPV-CO Tr	9/9 (100%)		1	4	4		7.9 ± 0.7
raMPV-C sG	6/8 (75%)	2	6				6.4 ± 0.4
raMPV-C gG	8/8 (100%)		8				6.6 ± 0

Table 6
Observation of clinical signs of turkeys at different days post-challenge (DPC).

Vaccines	Number of birds showing clinical signs/number in group									
	1	2	3	4	5	6	7	8	9	10
PBS	0/10	0/10	**9/10**	**10/10**	**9/10**	**6/10**	**2/10**	0/10	0/10	0/10
aMPV-CO Tr	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
raMPV-C sG	0/8	0/8	**1/8**	**1/8**	**1/8**	**1/8**	0/8	0/8	0/8	0/8
raMPV-C gG	0/9	0/9	0/9	**1/9**	**1/9**	**1/9**	**1/9**	0/9	0/9	0/9

Table 7
Virus shedding in turkey trachea at different days post-challenge (DPC).

Vaccines	No. and % of birds in which viral RNA was detected/number in group		
	3 DPC	5 DPC	7 DPC
PBS	10/10 (100%)	10/10 (100%)	0/10 (0%)
aMPV-CO Tr	0/9 (0%)	0/9 (0%)	0/9 (0%)
raMPV-C sG	5/8 (63%)	0/8 (0%)	0/8 (0%)
raMPV-C gG	8/9 (89%)	2/9 (22%)	0/9 (0%)

3 and 5 DPI) but the number of positive birds increased to 63% by 7 DPI.

3.4. Antibody response and protection of the recombinant G variant-vaccinated turkeys against challenge

As shown in Table 5, at 14 DPI, all birds inoculated with the gG variant or the challenge virus had positive serum conversion with a mean titer of 6.6 and 7.9 log_2, respectively. Six of eight (75%) turkeys inoculated with the sG variant had positive antibody response with a mean titer of 6.4 log_2. All sera collected from unvaccinated birds (inoculated with PBS) were negative.

To examine if the immune response of turkeys induced by the G variants was protective against aMPV-C disease, the vaccinated birds were challenged with aMPV-C Tr. As shown in Table 6, seven of eight (87%) birds vaccinated with the sG variant, and eight of nine (89%) birds vaccinated with the gG variant were protected against the challenge. Whereas all unvaccinated birds (PBS control) showed typical clinical signs of the disease. The birds inoculated with aMPV-C Tr were fully protected against virus challenge. Detection of challenge virus replication in tracheal tissue (Table 7) showed that 63% of the sG vaccinated birds and 89% of the gG vaccinated birds had a detectable level of virus RNA shedding at 3 DPC. At 5 DPC the challenge virus RNA shedding was decreased to 22% for the gG vaccinated birds or below a detectable level for the sG vaccinated birds. In contrast, all unvaccinated birds had challenge virus shedding at 3 and 5 DPC, whereas the aMPV-C Tr virus inoculated birds did not have any detectable virus shedding following challenge.

4. Discussion

In the present study, we developed a reverse genetics system for aMPV-C, and generated two recombinant G gene variants, representing the full-length G (585 aa) and a C-terminally truncated short G (252 aa) viruses. The *in vitro* biological characterization showed that both G variants were viable in Vero cells and induced typical cytopathic effects as seen with the parental virus although the gG grew slightly slower in the first 72 h of infection. This demonstrated that the C-terminal 333 aa, about 60% of the full size G protein, is not essential for virus viability in Vero cells. This is not unexpected since a complete G gene deletion from a rescued aMPV subgroup A recombinant virus did not decrease virus viability in Vero cells (Naylor et al., 2004). Interestingly, to date all pneumovirus G proteins studied by using the reverse genetic technology seem to be dispensable for virus viability in cell cultures (Biacchesi et al., 2004; Naylor et al., 2004; Techaarpornkul et al., 2001). This suggests that an alternative surface protein, most likely the F protein, of members of the pneumovirus subfamily may play a role in mediating virus attachment to the cultured cells for infection.

The clinical signs observed in the animal experiment have shown that both G variants were attenuated in turkeys and caused no clinical disease. The exact reasons for this attenuation are currently not known. It is possible that the unintentional amino acid changes which were introduced into the viral genome during construction of rescued viruses may have played a role in the attenuation. The sG variant has two aa changes found in the M and L proteins. Although they are not located in any known functional domains (Poch et al., 1990), we can not rule out the possibility of their contribution to the attenuation. Similarly, the gG variant contained aa changes that might have affected the virulence of the rescued virus. It has also been reported that cloned virus population for negative strand RNA viruses seems to be less virulent than its unpurified quasi-species population (Duarte et al., 1994a,b). Since our G variant stocks were prepared by propagation in Vero cells at a low level of passages (5–8 times), the virus population may still be relatively homogenous.

The detection of viral RNA shedding in the tracheal tissue revealed that both G variants were infectious in turkeys, but attenuated as evidenced by the lower percentage (22–75%) of birds with detectable viral RNA than those (100%) infected with the virulent challenge virus. The short G variant appeared to grow slower at early days of infection than the full-length G variant. These results demonstrated that the 333 aa extracellular domain of the G protein is not essential for virus viability in turkeys, but it may be required for efficient, rapid virus replication at early times post-infection. This is consistent with a previous report that a C-terminal truncation of the G gene in a rescued respiratory syncytial virus (RSV) mutant was viable but attenuated in mice (Elliott et al., 2004).

Our data clearly showed that both G variants were immunogenic and induced anti-aMPV-C antibody response. However, two of eight birds inoculated with the sG variant did not have a positive

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serum conversion. This can be explained with a lower level and delayed sG virus replication in these birds as it was detected by RT-PCR from the tracheal tissue. Together, the lower percentages of virus shedding and positive serum conversion for the sG variant compared to those observed with the gG variant indicated that the deleted region from the G gene may play a role in enhancing virus replication and antibody response, perhaps through increasing the attachment specificity. Importantly, both the sG and gG variants provided a significant protection against the virulent aMPV-C Colorado strain. About 90% of the challenged birds did not show any clinical signs, and challenge virus shedding was markedly reduced at 3 and 5 DPC. Despite the 100% positive serum conversion induced by the gG variant, it did not provide full protection against the virulent Colorado strain challenge. About 10% of the gG vaccinated birds showed clinical signs and 90% of the birds shed detectable challenge virus RNA at 3 DPC. It is possible that a small antigenic difference in the G protein between the goose isolate and the Colorado strain may account for the incomplete protection (Alvarez et al., 2004; Bennett et al., 2005).

In conclusion, we successfully developed a reverse genetics system for examining the effects of the G gene length variation among aMPV-C isolates on virus viability, virulence and immunity. Our results show that the C-terminal 333 aa (more than 60% of the extracellular domain) of the viral attachment G protein is not essential for virus viability *in vitro* and *in vivo*, but may play a role in enhancing virus attachment specificity and immunity in a natural host. Both the short and the full-length G containing variants were attenuated in turkeys, yet immunogenic and induced immune response that provided a significant protection against virulent aMPV-CO challenge.

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