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February 2013

Southeast Poultry Research Laboratory

Research Publications 2012

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Southeast Poultry Research Laboratory

Introduction:
The Southeast Poultry Research Laboratory (SEPRL) scientific expertise brings together an integrated research approach that includes Virology, Molecular Biology, Pathology, and Immunology that is key to developing prevention, management and control and eradication strategies that protects the $25 billion/year U.S poultry industry ($2.5 billion in U.S exports). It is a major international resource on research to control highly pathogenic avian influenza and virulent Newcastle diseases, which poses a major threat to the U.S poultry industry and also a severe risk to public health. Research has also been expanded in the last five years to address priority domestic viral diseases that are impacting the U.S poultry industries. SEPRL has two research units: 1) Exotic and Emerging Avian Viral Diseases Research Unit, and 2) Endemic Poultry Viral Diseases Research Unit. To effectively respond to the research needs of the U.S, SEPRL requires new laboratory and animal housing facilities if it is to remain competitive and continue providing rapid responses to new and emerging disease threats.

Established: 1960

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:
The objectives of the research program are to produce new research knowledge and technology to: 1) prevent, reduce or eliminate losses from impaired performance, and increased deaths and condemnations; 2) develop more sensitive, specific and faster diagnostic tests; 3) improve vaccines, and vaccine delivery methods; 4) improve our understanding of the ecology and epidemiology of viruses at the wild bird-domestic poultry interface; and 5) improve our understanding of the genetic and pathobiological basis of virulence. This research provides government regulatory agencies and the poultry industries with improved intervention strategies against poultry viral diseases.

Priority Diseases:
High pathogenicity Avian Influenza Virus – Select Agent
Virulent Newcastle disease Virus – Select Agent
Low pathogenicity Avian Influenza Virus – Non-select Agent
Low virulent Newcastle disease virus – Non-select agent
Marek's disease herpesvirus - Non-select agent
Enteric viruses of poultry - Non-select agent

Stakeholders:
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
Food and Drug Administration
Centers for Disease Control and Prevention
University diagnostic laboratories
Our nation's veterinary colleges

Research Facility:
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 & Decontamination Procedures
- BSL-2/3E, ABSL-2/3E & 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

- Development and validation of rapid, Real-time Reverse Transcriptase-Polymerase Chain Reaction (RRT-PCR) diagnostic tests used in live bird markets and Virginia during 2001-2002
- Collaborative research with corporate partners resulted in the license and commercialization of two avian influenza vaccines: fowlpox recombinant-avian influenza H5 gene insert and inactivated H5 avian influenza
- Collaborative research with corporate partners resulted in two licensed ELISA test kits for avian influenza diagnostics
- Molecular epidemiology in tracking sources and links in Mexican H5 outbreak (1994-95), H7N2 outbreak in LBM of Northeastern USA (1994-2002), Hong Kong H5N1 outbreaks (1997-2001), Chilean H7N3 outbreak (2002) and Central American H5N2 outbreaks
- Provided scientific data in USDA negotiations on trade in poultry and poultry products – validation of pasteurization to inactivate LPAI virus in egg product and determined the low risk of LPAI virus passing in broiler meat
- Demonstrated susceptibility of turkeys to 2010 pandemic H1N1 and modified RT-PCR diagnostic tests for biosurveillance in poultry
- Molecular epidemiology in tracking sources of the virulent Newcastle Disease (NDV) in the Mexican outbreak of 2000, and the California and Texas outbreaks of 2002-2003.

- Demonstrated recent NDV isolates and those recovered during disease outbreaks since the 1970s as well as new clades of virus emerging in Asia are phylogenetically distinct from current vaccine viruses and standard challenge strains
- Genetic characterization of the first U.S. avian pneumovirus isolate – resulted in classification as type C, different from European types A and B.
- Demonstrated waterfowl outside of Minnesota can carry and potentially disseminate avian metapneumoviruses.
- Isolated the first astrovirus and demonstrated its involvement in poult enteritis mortality syndrome (PEMS)
- Developed and transferred to diagnostic laboratories a multiplex PCR test for simultaneous demonstration of astrovirus, reovirus and coronavirus
- Demonstrated astrovirus was resistant to inactivation by common disinfectants with Virkon S being the only effective disinfectant
- Demonstrated that inoculation of turkeys did not produce disease and the West Nile Virus (WNV) replicated poorly in turkeys such that minimal potential exists 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 DISEASES 56:601–604, 2012

Research Note—

Serum and Egg Yolk Antibody Detection in Chickens Infected with Low Pathogenicity Avian Influenza Virus

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Received 25 February 2012; Accepted 16 May 2012; Published ahead of print 17 May 2012

SUMMARY. Surveillance for low pathogenicity avian influenza virus (LPAIV) infections has primarily relied on labor-intensive collection and serological testing of serum, but for many poultry diseases, easier-to-collect yolk samples have replaced serum for surveillance testing. A time-course LPAIV infection study in layers was performed to evaluate the utility of antibody detection in serum *vs.* egg yolk samples. Layers inoculated with the LPAIV A/Bobwhite Quail/Pennsylvania/20304/98 (H7N2) were tested for antibody levels in the serum and egg yolk by using the agar gel immunodiffusion test (AGID), hemagglutination-inhibition test (HI), and a commercially available enzyme-linked immunosorbent assay (ELISA). Anti-influenza specific antibodies were detected in the serum as early as 7 days postinoculation (DPI), and the majority of the hens remained positive until 42 DPI. Antibodies in the egg yolk were first detected by AGID at 7 DPI, which was also the first day of detection in serum. However, the majority of the eggs were positive by all techniques at 11 DPI and remained positive until 42 DPI, at which time the number of AGID+ and HI+ samples declined slightly as compared to ELISA+ samples. These results suggest that egg yolk can be an alternative to serum for flock serological surveillance against LPAIV infections, and the three methods (AGID, HI, and ELISA) will give similar results for first 42 days after infection, although AGID may give earlier positive response.

RESUMEN. *Nota de Investigación*—Detección de anticuerpos en el suero y en la yema de huevo en los pollos infectados con el virus de la influenza aviar de baja patogenicidad.

La vigilancia de las infecciones por el virus de la influenza aviar de baja patogenicidad se ha basado principalmente en la recolección de muestras de suero y en su análisis serológico que representan un trabajo intensivo, pero para muchas enfermedades de las aves, la recolección más fácil de muestras de la yema ha reemplazado al suero para pruebas de vigilancia. Un estudio del curso temporal de la infección por cepas del virus de la influenza aviar de baja patogenicidad se realizó para evaluar la utilidad de la detección de anticuerpos en suero en comparación con las muestras de yema de huevo. Aves de postura inoculadas con el virus de influenza aviar de baja patogenicidad de codorniz cepa A/ Bobwhite Quail/Pennsylvania/20304/98 (H7N2) fueron analizadas en relación a sus niveles de anticuerpos en el suero y en la yema de huevo mediante la prueba de inmunodifusión en gel de agar (AGID), mediante la prueba de inhibición de la hemaglutinación (HI), y mediante un estuche comercial del ensayo de inmunoabsorción con enzimas ligadas (ELISA). Se detectaron anticuerpos específicos contra influenza en el suero tan pronto como a los siete días después de la inoculación, y la mayoría de las gallinas se mantuvieron positivas hasta los 42 días después de la inoculación. Los anticuerpos en la yema de huevo fueron detectados por primera vez mediante AGID a los siete días después de la inoculación, que también fue el primer día de la detección en el suero. Sin embargo, la mayoría de los huevos fueron positivos por todas las técnicas a los 11 días después de la inoculación y se mantuvieron positivos hasta los 42 días después de la inoculación, momento en el que el número de muestras positivas por AGID y por inhibición de la hemoaglutinación se redujo ligeramente en comparación con las muestras positivas por ELISA. Estos resultados sugieren que la yema de huevo puede ser una alternativa al suero para la vigilancia serológica en las parvadas contra las infecciones por virus de influenza aviar de baja patogenicidad, y los tres métodos (AGID, HI y ELISA) proporcionan resultados similares en los primeros 42 días después de la infección, aunque la AGID puede dar una respuesta positiva más temprana.

Key words: avian influenza, diagnostic, egg yolk

Abbreviations: ABSL = animal biosafety level; AGID = agar gel immunodiffusion test; AI = avian influenza; AIV = avian influenza virus; DPI = days postinoculation; ELISA = enzyme-linked immunosorbent assay; ELD_{50} = mean embryo lethal doses; HI = hemagglutination-inhibition test; HP = high pathogenicity; LP = low pathogenicity; OD = optical density

Avian influenza (AI) virus is an important pathogen for avian species and humans (11). In poultry, AI is a highly fatal disease when birds are infected with high pathogenicity avian influenza (HPAI) virus (HPAIV) and low mortality when infected with a low pathogenicity avian influenza (LPAI) virus (LPAIV). Cases of HPAI and H5 and H7 LPAI are usually associated with severe economic losses and, for the latter, a barrier to commercialization of serological-positive birds (3,6,8).

Serological surveillance programs are constantly used to screen for antibodies in commercial chickens, especially associated with outbreak control programs (8). Even though serum is a traditional material used for surveillance for avian influenza, due to the increasing concern in animal welfare and labor-intensive and costly collection, the validation of other less invasive source of samples is important. In laying hens, immunoglobulins from the serum, mainly IgY, are transferred to the egg follicles (5), and can potentially be used for specific AIV antibody detection.

The egg yolk detection has been used in LPAI outbreaks to identify infected flocks, as in the H7N2 LPAI outbreak in Pennsylvania from 1996 to 1998 (8), and egg yolk has been

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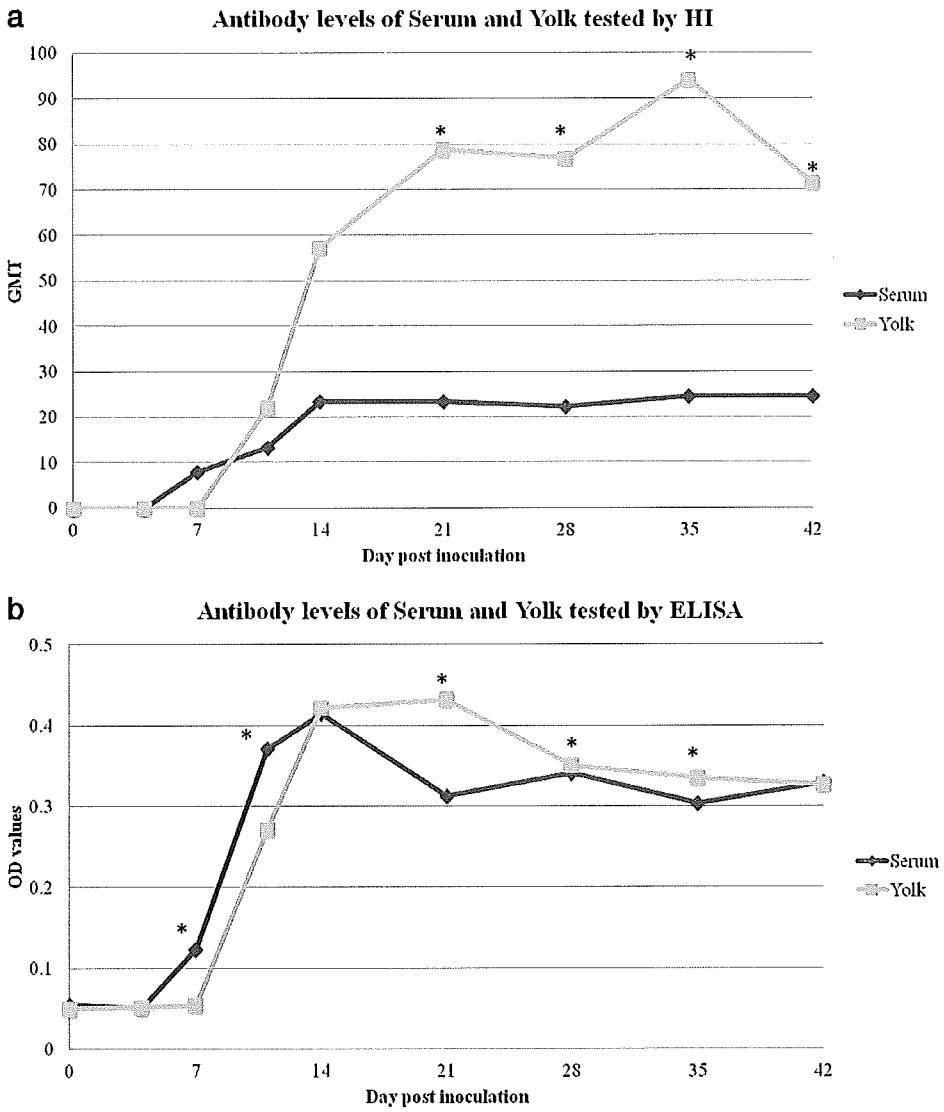

Fig. 1. (a) HI titers for serum and yolk samples. (b) ELISA OD values for serum and egg yolk samples. Time points marked with * represent statistically significant difference in the HI titer or ELISA OD.

described as an effective source for detecting antibodies against several viral and bacterial diseases in poultry (1,2,4,7,13,14).

The aim of this study was to conduct a time course study of LPAIV infection in layers to determine the utility of egg yolk *vs.* serum samples for antibody detection using three different serological tests for detecting LPAIV specific antibodies. Hens were inoculated via the respiratory tract with an H7N2 LPAIV A/Bobwhite Quail/Pennsylvania/20304/98, and evaluated for antibody production in serum and egg yolk samples using the agar gel immunodiffusion test (AGID), hemagglutination-inhibition test (HI), and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Virus. LPAIV A/Bobwhite Quail/Pennsylvania/20304/98 (H7N2) was obtained from National Veterinary Services Laboratory (courtesy of Dennis Senne, Ames, IA) and propagated in 10-day-old embryonating chicken eggs by inoculation via the allantoic sac. The allantoic fluid was titrated and diluted in brain heart infusion media to a dose of $10^{7.0}$ mean lethal doses for eggs (ELD_{50}) per 0.1ml. Sterile allantoic fluid was prepared to be used in sham controls.

Chickens. Twenty-four specific-pathogen-free white leghorn hens (Southeast Poultry Research Laboratory, Athens, GA) were housed in biosafety level 2 enhanced animal (ABSL-2E) facilities and then moved to biosafety level 3 enhanced (ABSL-3E) animal facilities for H7N2 LPAIV inoculation. The sham controls were maintained in ABSL-2E. All birds were housed in individual laying cages, and eggs were individually collected and identified. Feed and water were provided *ad libitum.* General care was provided in accordance with 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.*

Serology. Serum and egg yolk were tested for antibodies against AI virus by AGID and HI as previously described (1). For AGID, the serum and egg yolk were tested at 1:1 phosphate buffered saline dilution. For the HI test, the egg yolk was diluted 1:1 with 5% sodium citrate and used four hemagglutination units of antigen prepared from chicken embryos inoculated with A/turkey/Oregon/71 (H7N3) virus. The ELISA tests were conducted using a commercial AI ELISA kit (IDEXX-ELISA, Westbrook, ME) at 1:500 dilution, according to the manufacturer's instructions.

Table 1. Antibody response in serum and yolk collected from hens inoculated intranasal and intratracheal with $10^{7.0}$ ELD_{50} of A/bobwhite quail/Pennsylvania/20304/98 (H7N2) virus.[1]

DPI	Antibody source	Serology (no. positive/total tested samples)		
		AGID[2]	HI[3]	ELISA[4]
0	Serum	0/24Aa	0/24Aa	0/24Aa
	Yolk	0/1Aa	0/18Aa	0/19Aa
4	Serum	0/24Aa	0/24Aa	0/24Aa
	Yolk	0/1Aa	ND	0/20Aa
7	Serum	22/24Ab	3/24Ba	6/24Bb
	Yolk	1/5Ba	0/19Aa	0/20Aa
11	Serum	22/24Aa	19/24Aa	22/24Aa
	Yolk	4/5Aa	15/20Aa	16/20Aa
14	Serum	22/24Aa	19/24Aa	21/24Aa
	Yolk	5/5Aa	19/20Aa	20/21Aa
21	Serum	22/24Aa	20/24Aa	17/24Aa
	Yolk	4/5Aa	20/21Aa	20/21Aa
28	Serum	22/24Aa	21/24Aa	18/24Aa
	Yolk	3/5Aa	19/20Aa	16/20Aa
35	Serum	22/24Aa	21/24Aa	18/24Aa
	Yolk	4/4Aa	19/20Aa	17/20Aa
42	Serum	18/24Aa	18/24Aa	18/24Aa
	Yolk	3/5Aa	19/20Aa	18/21Aa
Total	Serum	150/216Aa	121/216Ba	120/216Ba
	Yolk	24/36Aa	111/158Ab	107/182Aa

[1]Same letter represents lack of statistic difference, and different letters represent a statistical difference. Uppercase letter represents differences between the techniques, and lowercase letter represents differences between serum and yolk samples.
[2]Agar gel immunodiffusion test.
[3]Hemagglutination-inhibition test.
[4]Enzyme-linked immunosorbent assay.

Experimental design. Twenty-four 27-week-old SPF chickens were inoculated each with $10^{7.0}$ ELD_{50} of A/Bobwhite Quail/Pennsylvania/20304/98 (H7N2) in 0.2 ml, divided 0.1 ml intranasally (choanal cleft) and 0.1 ml intratracheally. Serum and egg yolk samples were taken on 0, 4, 7, 11, 14, 21, 28, 35 and 42 days postinoculation (DPI). Five 27-week-old SPF chickens were inoculated with diluted allantoic fluid for sham controls.

Statistics. Data sets were subjected to a chi-square test to compare the three techniques by each day and compare serum samples and egg yolk for each one of the techniques tested. P values less than 0.05 were considered statistically significant. The HI titers and optical density (OD) from ELISA were compared using a Student t-test at $P < 0.05$ significance. The analysis was performed using Excel® (Microsoft, Inc., Seattle, WA).

RESULTS AND DISCUSSION

Previously detection of AIV infection by serological testing for antibodies suggested egg yolk was comparable with serum as a sample source with detection in serum at 7 DPI and egg yolk at 14 DPI (1). However, the study collected samples at 1-week intervals during the early stages of infection and did not collect samples after 21 DPI, raising questions as to the broader utility of egg yolk to replace serum as a sample source. The current study addressed the deficiencies of the earlier study by evaluating a longer infection period with more frequent sampling and compared such samples against the three primary serological techniques for detecting anti-AIV antibodies.

Serum samples and eggs from the five chickens from the sham group inoculated with sterile allantoic fluid remained negative during the entire experimental period for anti-AIV antibodies (data not shown). For the LPAIV-infected hens, when evaluating all 42 days of the experiment (Table 1), specific AIV antibodies were detected from serum by AGID in 69% of the serum samples and 66% of egg yolk samples. By HI test, 56% of the serum samples were AIV antibody positive, while 70% of the egg yolk samples were AIV antibody positive; this difference in the detection levels was statistically significant ($P = 0.0071$). The ELISA detected 55% positives for serum and 58% for egg yolk samples.

All the serum and egg yolk samples were negative for antibodies by AGID, HI, and ELISA at 0 and 4 DPI. At 7 DPI, only one sample out of five egg yolks was AGID+. Also, at 7 DPI, antibodies were detected in serum samples by all the techniques with significantly higher positivity by AGID than with HI or ELISA tests, but from 11 until 42 DPI, there was no statistical difference between the antibody detection rates by AGID, HI, and ELISA tests for serum or yolk samples on individual sampling dates. This suggests that egg yolk can be used an alternative to serum for AIV serological surveillance.

Detection of AI antibodies in the field is an important tool for a good surveillance program, and prevention and management after AI outbreaks (8), especially for LPAI, since the infected birds usually do not present with high morbidity or mortality. Besides the economic losses associated with LPAI virus infection (6), special attention is required for the subtypes H5 and H7, due to their capacity to mutate and became HPAIV (12). Antibodies detection in poultry is usually accomplished using serum samples. However such sampling requires individual bird manipulation, making the cost higher, and inducing stress in birds that could lead to a decrease in egg production.

The possibility of using egg yolk as primary sampling source for antibody detection for AIV infection would have several advantages, and it has been shown to be an effective method for detecting infection from other viruses (2,10,14), including AIV in chickens (1,13), ducks (9), and other wild birds (7). However, prior studies did not determine if the three primaty serological tests had different detection frequencies early and late in the infection period. In the current study, antibody levels were tested until 42 DPI for both serum and egg yolk. Most of

the samples were positive at similar levels, but with a slight decrease in AGID+ and HI+ serum samples at 42 DPI that were not statistically different from the number of ELISA+ serum samples. However, a previous field report using yolk samples from the 1996–1997 outbreak of H7N2 in Pennsylvania reported that the level of detection of LPAIV antibodies in egg yolk tested by AGID start to drop after 42 days, and by 77 DPI there was no detectable antibody (8).

The higher number of AGID+ compared to HI+ and ELISA+ during early stage of infection (i.e., 7 DPI) may be explained by the different classes of antibodies detected. AGID detects precipitating antibodies, mostly IgM, which predominates in the early stages of infection, while the HI and ELISA tests detect IgY antibodies that are delayed a few days after IgM in production.

The HI antibody titers were higher in egg yolk samples than in serum after 14 DPI and were statistically higher at 21 DPI, remaining significantly higher until 42 DPI (Fig. 1a). The OD measures for ELISA were significantly higher at 7 and 11 DPI for serum samples than yolk, the averages were similar at 14 DPI, and from 21 until 35 DPI, there was a switch, when the yolk samples had significantly higher ODs compared with serum. At 42 DPI the levels of antibodies for serum and egg yolk were similar (Fig. 1b). This supports previous observations that hens concentrate antibodies from the circulatory system into the egg yolk, which could make yolk a more sensitive sample late in the infection process when antibody titers are declining to low levels in the serum.

These new data suggest that LPAIV infection can be detected through AGID serological surveillance using egg yolk as early as 7 DPI, but use of HI or ELISA tests may delay detection by an additional 4 days. Furthermore, both egg yolk and serum first detected LPAIV infection on 7 DPI, although serum samples had a higher rate of anti-LPAIV antibodies (91%) than paired egg yolk samples (20%). This suggests that AGID LPAIV serological surveillance using egg yolk can detect evidence of infection as early as serum samples, but the number of samples may need to be increased to detect low infection rates. However, from 11 DPI up to 42 DPI, there was no difference in egg yolk and serum detection rates. The use of egg yolks as a sample for LPAIV antibody detection in layers has important advantages when compared with serum. Even with a delay of a few days for the majority of the samples to have detectable levels of antibodies in eggs compared with the serum, the decrease in the stress by using a less invasive method of sampling makes eggs an important and sensitive alternative to serum for assessing LPAIV infections in layer flocks.

REFERENCES

1. Beck, J. R., D. E. Swayne, S. Davison, S. Casavant, and C. Gutierrez. Validation of egg yolk antibody testing as a method to determine influenza status in white leghorn hens. Avian Dis. 47:1196–1199. 2003.

2. Brown, J., R. S. Resurrecction, T. G. Dickson, and A. Horne. The relationship of egg yolk and serum antibody. I. Infectious bursal disease virus. Avian Dis. 33:654–656. 1989.

3. Chmielewski, R., and D. E. Swayne. Avian influenza: public health and food safety concerns. Annu. Rev. Food Sci. Technol. 2:37–57. 2011.

4. Choi, K.-S., E.-K. Lee, W.-J. Jeon, M.-J. Park, Y.-N. Yoo, and J.-H. Kwon. Diagnostic utility of egg yolk for the detection of avian metapneumovirus antibodies in laying hens. Avian Dis. 54:1230–1236. 2010.

5. Da Silva, W., and D. V. Tambourgi. IgY: a promising antibody for use in immunodiagnostic and in immunotherapy. Vet. Immunol. Immunopathol. 135:173–180. 2010.

6. Davison, S., D. Galligan, T. E. Eckert, A. F. Ziegler, and R. J. Eckroade. Economic analysis of an outbreak of avian influenza, 1997–1998. J. Am. Vet. Med. Assoc. 214:1164–1167. 1999.

7. Hammouda, A., J. Pearce-Duvet, M. A. Chokri, A. Arnal, M. Gauthier-Clerck, T. Boulinier, and S. Selmi. Prevalence of influenza A antibodies in yellow-legged gull (*Larus michahellis*) eggs and adults in southern Tunisia. Vector-Borne Zoonot. 11:1583–1590. 2011.

8. Henzler, D. J., D. C. Kradel, S. Davison, A. F. Ziegler, D. Singletary, P. DeBok, H. Lu, R. Eckroade, D. Swayne, W. Lagoda, B. Schumcker, and A. Nesselrodt. Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996–98). Avian Dis. 47:1022–1036. 2003.

9. Jeong, O.-M., M.-C. Kim, H.-M. Kang, G.-W. Ha, J.-S. Oh, J.-E. Yoo, C.-H. Park, J.-S. Kwon, M.-R. Pack, H.-R. Kim, Y.-J. Kim, J.-H. Kwon, and Y.-J. Lee. Validation of egg yolk antibody based C-ELISA for avian influenza surveillance in breeder duck. Vet. Microbiol. 144:287–292. 2010.

10. Silim, A., and D. Venne. Comparison of egg-yolk and serum antibody titers to four avian viruses by enzyme-linked immunosorbent assay using paired field samples. Avian Dis. 33:643–648. 1989.

11. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. Y. M. Saif, J. R. Glisson, A. M. Fadly, L. R. McDougald, L. Nolan, and D. E. Swayne, eds. Blackwell, Ames, IA. pp. 153–184. 2008.

12. Swayne, D. E., and D. L. Suarez. Highly pathogenic avian influenza. Rev. Sci. Tech 19:463–483. 2000.

13. Trampel, D. W., E.-M. Zhou, K. Yoon, and K. J. Koehler. Detection of antibodies in serum and egg yolk following infection of chickens with an H6N2 avian influenza virus. J. Vet. Diag. Invest. 18:437–442. 2006.

14. Yeo, S. G., E. Nagy, and P. J. Krell. Indirect method for prediction of hemagglutination inhibition antibody titer to Newcastle disease virus in chickens by titration of antibodies in egg yolk. J. Vet. Diag. Invest. 15:184–187. 2003.

ACKNOWLEDGMENTS

We thank Joan Beck, Kira Moresco, and James Doster for their excellent technical assistance. This research was supported by USDA CRIS project 6612-32000-023.

Highly Pathogenic Avian Influenza Virus among Wild Birds in Mongolia

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Abstract

Mongolia combines a near absence of domestic poultry, with an abundance of migratory waterbirds, to create an ideal location to study the epidemiology of highly pathogenic avian influenza virus (HPAIV) in a purely wild bird system. Here we present the findings of active and passive surveillance for HPAIV subtype H5N1 in Mongolia from 2005–2011, together with the results of five outbreak investigations. In total eight HPAIV outbreaks were confirmed in Mongolia during this period. Of these, one was detected during active surveillance employed by this project, three by active surveillance performed by Mongolian government agencies, and four through passive surveillance. A further three outbreaks were recorded in the neighbouring Tyva Republic of Russia on a lake that bisects the international border. No HPAIV was isolated (cultured) from 7,855 environmental fecal samples (primarily from ducks), or from 2,765 live, clinically healthy birds captured during active surveillance (primarily shelducks, geese and swans), while four HPAIVs were isolated from 141 clinically ill or dead birds located through active surveillance. Two low pathogenic avian influenza viruses (LPAIV) were cultured from ill or dead birds during active surveillance, while environmental feces and live healthy birds yielded 56 and 1 LPAIV respectively. All Mongolian outbreaks occurred in 2005 and 2006 (clade 2.2), or 2009 and 2010 (clade 2.3.2.1); all years in which spring HPAIV outbreaks were reported in Tibet and/or Qinghai provinces in China. The occurrence of outbreaks in areas deficient in domestic poultry is strong evidence that wild birds can carry HPAIV over at least moderate distances. However, failure to detect further outbreaks of clade 2.2 after June 2006, and clade 2.3.2.1 after June 2010 suggests that wild birds migrating to and from Mongolia may not be competent as indefinite reservoirs of HPAIV, or that HPAIV did not reach susceptible populations during our study.

Citation: Gilbert M, Jambal L, Karesh WB, Fine A, Shiilegdamba E, et al. (2012) Highly Pathogenic Avian Influenza Virus among Wild Birds in Mongolia. PLoS ONE 7(9): e44097. doi:10.1371/journal.pone.0044097

Editor: Gavin J D. Smith, Duke-NUS Graduate Medical School, Singapore

Received February 26, 2012; **Accepted** July 30, 2012; **Published** September 11, 2012

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Funding: Support for this project was provided by the National Institute of Allergy and Infectious Disease, National Institutes of Health, Department of Health and Human Services (Contracts HHSN266200700007C, HHSN266200700009C, and HHSN266200700005C), the United States Agency for International Development (USAID), the World Bank, and the Food and Agriculture Organization of the United Nations. DOJ was supported by generous funding from the Dunemere private foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DOJ was supported by generous funding from the Dunemere private foundation. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Since its emergence in 1997 and subsequent re-emergence in 2003, highly pathogenic avian influenza virus (HPAIV) subtype H5N1 has caused the deaths of at least 357 people in 15 countries [1] and been responsible for losses of many millions of domestic poultry, negatively impacting economic growth and food security in affected countries. Prior to 2005, outbreaks in wild birds were sporadic, associated with high mortality, and thought to relate to spillover from infected domestic poultry [2–6]. This situation changed in April 2005, with an outbreak among wild migratory waterbirds at Qinghai Lake in northern China, when over 6,000 wild birds died over a period of two months [7–9].

Following events at Qinghai, there was a marked increase in outbreaks involving wild birds elsewhere in Asia, Europe and Africa. However, the extent to which wild birds contributed to the spatial expansion of HPAIV outbreaks, particularly in relation to that of the legal and illegal movement of wild and domestic fowl is difficult to resolve [10,11]. Many outbreaks involving wild birds occurred in close proximity to cases among domestic poultry [12], making it difficult to attribute the source of infection to a wild or domestic host. In such environments, it can be difficult to isolate the contribution that wild birds play in cycles of HPAIV transmission [13].

In response to the Qinghai outbreak, a wild bird surveillance system was implemented in Mongolia in 2005 to elucidate the role

of wild birds in the transmission of HPAIV H5N1. Bird species show variable susceptibility to infection with HPAIV H5N1, with experimental exposures resulting in high mortality rates among swans, geese and gulls [14,15], and variable levels of mortality or subclinical infections in ducks [15–17] Active surveillance approaches involved searching for the presence of HPAIV through screening diagnostic specimens from healthy, ill and dead birds and from the environment. In contrast, passive surveillance relied on reports of sick and dead birds received from the general public, who were requested to report waterbird mortality to provincial veterinary authorities.

Several features make Mongolia an ideal location for understanding the epidemiology of HPAIV in wild birds. Mongolia supports large populations of migratory waterfowl and shorebirds [18], including species breeding across Mongolia's extensive wetlands during the boreal summer, then departing to spend the winter in milder climates in Australasia, the Indian Subcontinent, Africa, Southeast Asia, China, Korea, and Japan [19–23]. HPAIV H5N1 is either endemic or has occurred in domestic and/or wild bird populations in all these areas except for Australasia. Other species visit Mongolia for short periods to feed while migrating to and from more northerly breeding areas. Mongolia also represents an important site for molting Anseriformes (ducks, geese and swans) that congregate during the post-breeding period when early frosts force them to vacate their Siberian breeding sites.

Mongolia presents an opportunity to study virus in wild populations in isolation from domestic poultry. It has a small domestic poultry industry with a population of fewer than 100,000 birds in 2005 [24], an overall density of <1 km^{-2} [25], with most birds raised for egg production in moderately biosecure facilities located in urban centers. In contrast, China produces approximately 5,900,000,000 domestic birds annually [26]. To date, Mongolia's modest poultry population has remained free from HPAIV outbreaks. With little backyard production, the potential for wild birds being exposed to domestic sources of virus is negligible, allowing conclusions to be drawn on the status and transmission of HPAIV in wild bird populations without the complication of local spillover from domestic hosts.

Herein we report the seven-year findings of this surveillance program, including the success of various approaches of active and passive surveillance in detecting HPAIV outbreaks, together with the results of investigations once they were detected. Isolates of low pathogenic avian influenza viruses (LPAIV) in Mongolia have been reported elsewhere [27], and so will only be summarized briefly here. The implications of the project findings are discussed both with respect to future surveillance efforts, as well as to understanding the epidemiology of HPAIV in wild bird populations.

Methods

Study Area

Lying between the latitudes of 42°N and 51°N, Mongolia is a vast, land-locked country of approximately 1.5 M km^2, that extends east-west across 2,500 km of Central Asia between Russia and China. Although a country of climatic and geographic extremes, much of the land area consists of open steppe, transitioning to taiga forest in the north and the Gobi Desert in the south and west. Wetlands and lakes of variable size fleck the landscape, with approximately 3,000 rivers stretching over 67,000 km in the north [28]. Mongolia is populated by just over three million people, over half of which depend directly or indirectly on the traditional nomadic system of livestock production, raising goats, sheep, cattle, yaks and horses [29].

Active Surveillance

Active surveillance was directed at the detection of HPAIV outbreaks (defined for the purposes of the study as presence of one or more clinically ill or dead birds from which HPAIV could be isolated), and the presence of HPAIV in clinically unaffected birds. Detection of outbreaks relied on the collection of samples from dead or ill birds located along shoreline transects; while collection of samples from live birds showing no clinical signs of illness and environmental fecal deposits were used to detect the subclinical presence of virus. The approaches used each year are summarized in Table 1. Surveys were conducted in four main regions, the 'east' (referring to Ulaanbaatar and the aimags (provinces) of Dornod, Khentii and Sukhbaatar), the 'west' (Uuvs and Khovd), the 'south-central' (Bayankhongor, Zavkhan and Gov-Altai), and the 'north-central' (Khovsgol, Bulgan and Arkhangai).

Live Bird Sampling

Live, apparently healthy birds were captured using a number of methods; none were killed for the purposes of this study. Live bird sampling focused on waterbirds particularly Anseriformes (including *Anas* ducks, shelducks, geese and swans), and Ciconiiformes (including gulls, cormorants and shorebirds). Molting Anseriformes were captured in groups, by driving birds into temporary holding pens, or individually at night with use of spotlights and nets or swan hooks. Shorebirds were caught at night using mist nets, and hand nets were used to catch cormorants and gulls. Tracheal and cloacal swabs were collected from each bird and stored individually in cryovials containing viral transport media (VTM). Oropharangeal swabs were collected instead of tracheal swabs from smaller bird species. Samples were held at 4°C up to four hours and then immersed in liquid nitrogen. Cold chain was maintained throughout delivery of samples to the laboratory. In 2006, 2007 and 2008, a duplicate set of respiratory and cloacal samples were collected and stored in cryovials containing 10% guanidine isothiocyanate solution (2007), or a solution of 1% Environ One-Stroke (2006 and 2008), for molecular analysis.

Dead/sick Bird Sampling

Shoreline transects were delineated at each site and systematically searched for sick and dead birds. Where possible they completely circumnavigated the water body, or at least exceeded 5 km on larger lakes. Birds were identified when possible to species, sex, age and general approximate time since death. Obvious signs of predation or scavenging were recorded and a photo record made of each bird. Where species could not be determined due to carcass condition, identity was recorded to the highest taxonomic level possible or simply listed as "unknown". Swab samples were collected in the same manner described for live birds. Where possible, tissues from brain, lung, spleen and pancreas were also snap frozen in VTM for virus isolation.

Fecal Sampling

Fresh fecal samples were collected where waterbirds were observed congregating or roosting. Samples were collected and preserved in the same manner as those collected from live birds. In 2005 and 2006 sampling focused on single-species flocks to enable the identification of the species being sampled. This led to a bias toward certain species that were less likely to form mixed-species groups, particularly shelducks, geese, swans, gulls and cormorants. From 2009 onward, our collection strategy changed to include species that habitually congregate in mixed-species groups (particularly focusing on ducks in the genera *Anas* and *Aythya*,

Table 1. Summary of active HPAIV surveillance approaches employed in Mongolia, from 2005–2011.

Activity	2005	2006	2007	2008	2009	2010	2011
Live bird sampling	Limited	Yes	Yes	Yes	Yes	Yes	No
Faecal sampling?	Yes	Yes	No	No	Yes	Yes	Yes
Mortality transects?	Yes	Yes	Yes	Yes	Yes	Yes	No
Number of survey sites	9	42	12	10	11	23	17
Geographic regions	NC, SC	E, W, NC, SC	NC	NC	W, NC	E, NC	E
Timing	Jul-Aug	Jul-Oct	Apr-Oct	May-Sep	Jun-Sep	May-Sep	May- Oct

Geographic regions are East (E), West (W), North-central (NC), and South-central (SC).
doi:10.1371/journal.pone.0044097.t001

which are prominent carriers of LPAIVs), sacrificing specific identity of the birds being sampled.

Passive Surveillance

Reports of sick and dead birds from herders, local veterinary, or environmental officials were relayed via the district and province veterinary offices to the State Central Veterinary Laboratory (SCVL) in Ulaanbaatar, with samples or whole birds collected by national or provincial investigation teams submitted for laboratory testing at SCVL. Samples collected from suspect cases were forwarded to the OIE Reference Laboratory at Hokkaido University for confirmation.

Outbreak Investigation

Reports of suspected outbreaks were relayed to the study team to enable follow-up outbreak investigations to take place. Standardized counts were performed at each outbreak site using recognized guidelines [30]. Point counts were supplemented with active searching to provide a more comprehensive list of species present at each site. Where feasible, attempts were made to capture and sample live birds at outbreak sites, and to collect fecal samples from areas where birds were congregating.

Diagnostics

All samples were submitted for virus isolation, except in 2006, and 2007 when inactivated duplicate samples were screened using real-time RT-PCR (rRT-PCR) [31]. Corresponding samples in VTM to those positive by rRT-PCR were then submitted for virus isolation. In 2008 all samples were screened using virus isolation of VTM samples and rRT-PCR of inactivated duplicates. Virus isolation was accomplished in 10-day old specific-pathogen-free embryonated chicken eggs using described procedures [32]. Hemagglutinating agents from virus isolation attempts were confirmed as type A influenza by antigen capture and rRT-PCR [33]. Viruses were pathotyped using chicken pathogenicity tests and sequencing of the H5 hemagglutinin proteolytic cleavage site [32,34].

Processing of samples using RT-PCR took place in several laboratories, therefore the procedures were not identical. In 2006 total RNA was extracted with a procedure optimized for cloacal swab samples. The RNA was screened for AIV by rRT-PCR using a standard protocol [33]. The rRT-PCR test was run with an internal positive control [35] to ensure that inhibitors were not causing false negative results. All rRT-PCR positive samples were processed for virus isolation in embryonated chicken eggs and were screened for H5 subtype virus with subtype specific primers and probes [36,37]. Samples from 2007 and 2008 were processed at the University of California, Davis as follows: RNA was extracted from swab samples using the MagMAX- 96 Viral Isolation Kit (Ambion Inc. Austin, TX) in accordance with the manufacturer's instructions. An rRT-PCR which targets the M gene was conducted as described by Runstadler, et al. [38,39] which was the standard test in this laboratory and run on an AB 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Amnio-allantoic fluid from eggs inoculated for virus isolation, testing positive for hemagglutination, an indicator virus presence, were processed for RNA and run on the AIV type A rRT-PCR to confirm virus presence. Regardless of the initial rRT-PCR result the subtypes of all isolates were confirmed by sequencing [27]. We assumed an individual was negative for HPAIV H5N1 if all tests conducted on samples from that individual were negative.

Ethics Statement

Bird capture and handling permits covering all four regions of Mongolia (east, west, north-central and south-central) were issued by the Mongolian Ministry of Nature, the Environment and Tourism following approval of handling and sampling protocols by the Institute of Biology at the Mongolian Academy of Sciences, with additional approval from the University of Minnesota, Institutional Animal Care and Use Committee (Protocol 1006A84613).

Results

In total, eight outbreaks of HPAIV were recorded in Mongolia during the study period (Table S5), with three more occurring within Russian territory along the shores of Uuvs Nuur, a lake that bisects the international boundary (Table S7). These eleven outbreaks occurred at just five sites (with three outbreaks each recorded at Erhel Nuur and Uuvs Nuur). An additional report of three dead whooper swans found at Zegst Nuur in Sukhbaatar province on 5 April 2011 (Ref OIE: 10500) was not considered to constitute a confirmed outbreak, as the diagnosis was based on RT-PCR and rRT-PCR for H5 only, and no virus could be isolated.

Active Surveillance

Active surveillance was conducted in all seven years at 61 locations (Figure 1, Table 1). Overall, 10,761 individual birds were sampled using active surveillance (Table 2, and Tables S1 and S3), but only one outbreak of HPAIV H5N1 was identified using the methods employed by this study, at Erhel Nuur in the north-central region on 28 July 2009, when isolates of HPAIV H5N1 were recovered from three dead juvenile Mongolian gulls *Larus mongolicus*. Although the outbreak at Erhel Nuur in 2009 was detected during active surveillance, further details are

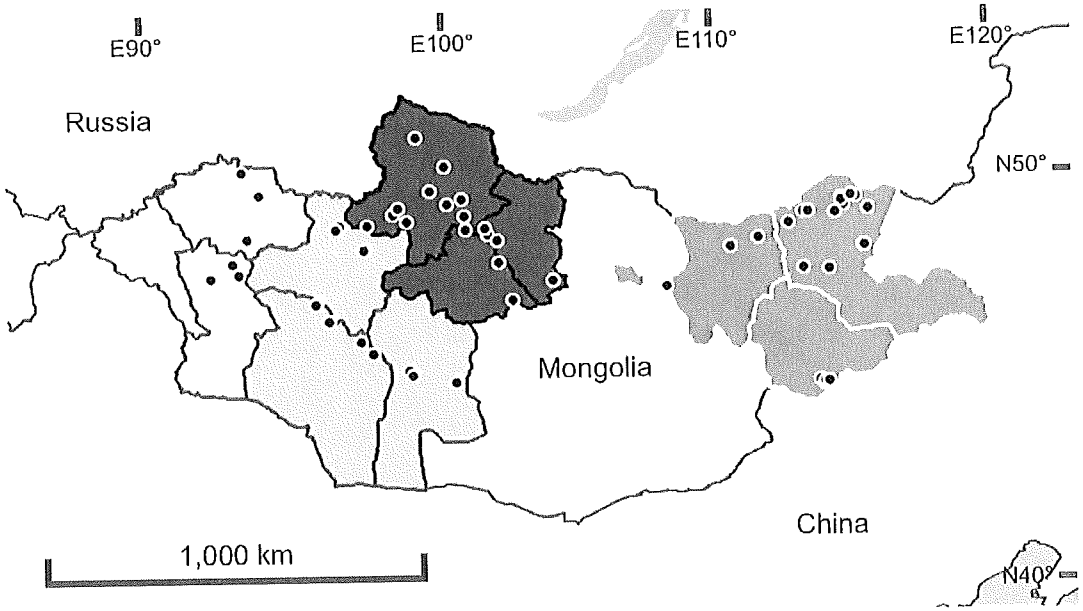

Figure 1. Map of study sites. Boundaries of study regions illustrated (white = West; light grey, white border = East; dark grey = North-central; light grey, dark border = South-central).
doi:10.1371/journal.pone.0044097.g001

provided in the section reporting the results of outbreak investigations for comparative purposes. A further three outbreaks were detected by active surveillance undertaken by Mongolian government agencies at Khunt Nuur (May 2006), Erhel Nuur (May 2006) and Doroo Tsagaan Nuur (July 2009).

A total of 56 LPAIVs were isolated from environmental fecal samples (n = 7,855), one from live clinically healthy birds (n = 2,765) and two from dead or ill birds (n = 141) sampled during active surveillance. In 2006 6.9% of samples tested positive for the matrix gene (n = 2,678), compared to 9.4% in 2007 (n = 508) and 9.4% (n = 1,377) (Table S3). Of 363 samples testing

Table 2. Summary of the number of individual birds sampled during active surveillance in Mongolia 2005–2011.

Surveillance type	Order	2005	2006	2007	2008	2009	2010	2011	Grand Total
Live bird	ANSERIFORMES	0	8	315	794	731[a]	48	0	1,896
	CICONIIFORMES	0	9	148	475	160[a]	0	0	792
	PICIFORMES	0	0	0	0	0	2	0	2
	PASSERIFORMES	0	8	0	0	0	67	0	75
Faecal	ANSERIFORMES	413	1,265	0	0	720	1,521	2,400	6,319
	GRUIFORMES		15	0	0	0	0	0	15
	CICONIIFORMES	35	1,345	0	0	140	0	0	1,520
	PASSERIFORMES	0	1	0	0	0	0	0	1
Sick/dead	ANSERIFORMES	1	10	4	1	3[b] [1]	0	0	19
	CICONIIFORMES	0	15	40	52	8[b] (3)	1	0	116
	FALCONIFORME	0	1	0	0	0	0	0	1
	UPUPIFORMES	0	0	1	0	0	0	0	1
	PASSERIFORMES	0	1	0	0	3	0	0	4
	Total	449	2,678	508	1,322	1,765	1,639	2,400	10,761

In 2008 samples were submitted for culture in embryonated eggs, and RT-PCR, the results obtained for culture are represented here.
HPAIV H5N1 viruses identified by virus isolation (VI) are indicated in parentheses, and those identified by real time reverse transcription polymerase chain reaction (PCR) are indicated in square brackets.
[a]Figures include 197 live, clinically healthy Anseriformes, and 83 Ciconiiformes sampled on Erhel Nuur between 28 July and 1 August 2009, a period that coincided with an outbreak of HPAIV H5N1 that was subsequently confirmed through laboratory analysis.
[b]Figures include one sick ruddy shelduck, and three dead juvenile Mongolian gulls at Erhel Nuur in July-August 2009 that subsequently tested positive for HPAIV H5 by rRT-PCR, and HPAIV H5N1 by virus isolation respectively.
doi:10.1371/journal.pone.0044097.t002

positive for matrix sequences only two tested positive for H5 (a fecal sample from a red crested pochard, *Rhodonessa rufina*, and a cloacal sample from a red-necked stint *Calidris ruficollis*), but no virus was isolated from the duplicates of these samples. In 2008 when all samples were tested by both RT-PCR and virus isolation, only one LPAIV was isolated, from a sample testing negative by RT-PCR for the matrix gene (from a live ruddy shelduck *Tadorna ferruginea*).

Passive Surveillance

Of the eight Mongolian outbreaks, four were detected through passive surveillance at Khunt Nuur (July 2005), Erhel Nuur (July 2005), Doitiin Tsagaan Nuur (May 2009) and Ganga Nuur (May 2010) (Table S5).

Outbreak Investigation

Five outbreak investigations were completed during this study, including Erhel Nuur (June 2005), Uuvs Nuur (June 2009), Doroo Tsagaan Nuur (August 2009), and Ganga Nuur Nature Reserve (May 2010). Although the outbreak at Erhel Nuur in 2009 was identified during active surveillance, activities performed were consistent with those at outbreak investigations and are reported here.

Population numbers, densities and species identification of live birds were obtained during each of the five outbreak investigations (Table S4). Due to the size of Uuvs Nuur, we were unable to survey birds across the entire surface area of the lake. We estimated the area surveyed at Uuvs Nuur, by assuming that all birds could be reliably identified and counted up to 2 km from the shoreline (estimated on-site using maps), and multiplying this against the length of shoreline surveyed. Counts made at all other sites represent the entire surface area of the lakes. Ganga Nuur Nature Reserve data combines surveys of its main water bodies (Ganga Nuur, Kholbo Nuur, Erdene Nuur and Huuvur Nuur).

Fifteen sick or dead birds suitable for sample collection were identified during outbreak investigations, with a further four located at Erhel Nuur in July 2009 (Table 3 and Table S2). HPAIV H5N1 virus was isolated from eight of these birds, with an additional three testing positive for HPAIV subtype H5 virus by rRT-PCR. Among birds testing positive by rRT-PCR was a dead great crested grebe *Podiceps cristatus* at Uuvs Nuur in June 2009. This bird, found 19.2 km from the border with the Russian Federation, remains the only case of HPAIV H5 on the Mongolian shores of Uuvs Nuur.

Capture of live, clinically healthy birds was only possible during the outbreak investigations at Erhel in 2005 (2 birds) and Doroo Tsagaan Nuur in 2009 (91 birds) (Table 3 and Table S2). A further 279 live birds were captured during active surveillance at Erhel Nuur in July 2009 (Table 2 and Table S1) which was later identified as an outbreak. No HPAIV H5N1 virus was isolated from any of these birds.

A total of 723 fecal samples were collected during outbreak investigations, from which no isolates of HPAIV H5N1 virus were recovered.

Discussion

Although confirmed outbreaks of HPAIV, subtype H5N1 in Mongolia occurred during four of the seven study years, our active surveillance was only successful in identifying one outbreak (Erhel Nuur, July 2009). In six of the seven years of surveillance sampling of live birds (either through capture or collection of fecal samples) concentrated on ruddy shelducks, bar-headed geese *Anser indicus* and whooper swans *Cygnus cygnus* as these species were over-represented among dead birds found at outbreaks (Tables S1, S2, S3 and S5). A total of 2,504 of these birds were sampled during active surveillance, and 503 during outbreak investigations, but no HPAIV was isolated. Duck species within the genera *Anas* and *Aythya* that may be more likely to harbor subclinical HPAIV infections [15,17] were under-represented during captures, which led to the decision to increase collections of fecal samples from these species in 2009, 2010 and 2011. A total of 4,401 samples were collected from these species during active surveillance and 292 during outbreak investigations, but also failed to yield HPAIV isolates. Taken together these findings suggest that sampling of live birds (through capture and fecal sampling) are either insensitive at detecting outbreaks, or that the species targeted were inappropriate.

Although considerably fewer samples were collected from ill and dead birds, these yielded HPAIV isolates both during active surveillance (three juvenile Mongolian gulls at Erhel Nuur in July 2009) and outbreak investigations (one whooper swan at Erhel Nuur in 2005, two ruddy shelducks and a bar-headed goose at Doroo Tsagaan Nuur in 2009, and one tundra swan *Cygnus columbianus* at Ganga Nuur in 2010). Since at least half of the confirmed Mongolian outbreaks were identified by means of passive surveillance, and sick and dead bird sampling accounted for all HPAIV isolates, passive surveillance and investigation of mortality incidents may be the most cost-effective method of identifying outbreaks of HPAIV in wild birds in Mongolia and possibly other countries. However, with 59 LPAIVs isolated during active surveillance, the wider value of this approach to understanding influenza ecology should not be discounted.

Table 3. Summary of the number of individual birds sampled during outbreak investigations in Mongolia 2005–2011.

Surveillance type	Order	2005	2006	2007	2008	2009	2010	2011	Grand Total
Live bird	ANSERIFORMES	1	0	0	0	91	0	0	92
	CICONIIFORMES	1	0	0	0	0	0	0	1
Faecal	ANSERIFORMES	412	0	0	0	141	151	0	704
	CICONIIFORMES	19	0	0	0	0	0	0	19
Sick/dead	ANSERIFORMES	4 (1)	0	0	0	4 (3)	1 (1)	0	9
	CICONIIFORMES	2	0	0	0	4 [1]	0	0	6
	Total	439	0	0	0	240	152	0	831

HPAIV H5N1 viruses identified by virus isolation (VI) are indicated in parentheses, and those identified by real time reverse transcription polymerase chain reaction (PCR) are indicated in square brackets.

doi:10.1371/journal.pone.0044097.t003

Due to the variation in strategies of laboratory analysis used during the study, it is conceivable that our ability to detect HPAIV may have been lower in some years compared to others. In particular, the pre-screening of duplicate samples using RT-PCR in 2006 and 2007 may have resulted in missing viruses among samples that were negative by RT-PCR and therefore not submitted for virus culture. Indeed, in 2008 an LPAIV was isolated from a ruddy shelduck that tested negative using RT-PCR exemplifying this possibility. However, overall RT-PCR was found to be more sensitive than virus culture in detecting viruses, with 363/4,563 (7.96%) found positive by RT-PCR compared to 79/8,406 (0.94%) by virus isolation. This is not unexpected, as unlike virus isolation, RT-PCR will detect both viable and non-viable virus.

The low density of poultry in Mongolia, coupled with the lack of any recorded outbreaks among Mongolian poultry strongly supports the hypothesis that wild birds are responsible for carrying HPAIV H5N1 to Mongolia. Furthermore, Mongolian cases only occurred in years when wild bird outbreaks were also reported in either Qinghai or Tibet during April or May (Figure 2; Table S7). The species that predominated in the Qinghai and Tibet outbreaks were similar to those that died in Mongolia (with the exception of brown-headed gull *Chroicocephalus brunnicephalus* which is uncommon in Mongolia). All isolates obtained from Mongolia in 2005 and 2006 fell within the same subclade as those obtained from Qinghai and Tibet during the same period (clade 2.2), while those obtained in 2009 and 2010 also clustered with viruses obtained from the Qinghai and Tibetan outbreaks within these years (clade 2.3.2.1). This trajectory of outbreaks is consistent with wild birds migrating northwards along the Central Asian migratory flyway during the spring, a route that is followed by birds wintering in southwestern China and the Indian subcontinent. Notably, these clades have also been isolated among domestic poultry in India (clade 2.2), Nepal (clade 2.3.2.1) and Bangladesh (both clades) [40–42]. Further isolates of clade 2.3.2.1 viruses among wild birds in Hong Kong in the winter of 2007/8 [43], in Japan during spring 2008, and again in Japan and the Korean Peninsula during the winter 2010/11 [44] suggest that this clade has also been moving along the East Asian Australasian flyway, at least to the latitude of southern China. HPAIV appears to have persisted in wild birds in the Central Asian migratory flyway for a minimum of 15 months in the case of both clade 2.2 and clade 2.3.2.1 (Tables S5, S6, S7), although the possibility of multiple reintroductions from infected domestic poultry elsewhere in the flyway cannot be discounted. However, based on the lack of any isolates of clade 2.2 from wild birds in the Central Asian flyway after June 2006, or clade 2.3.2.1 after June 2010 we hypothesize that while wild bird populations can support HPAIV infections for a limited period, they have not represented a competent reservoir in the long term. Testing this hypothesis would require that surveillance effort be increased across the flyway, with an emphasis on the reporting and investigation of dead birds.

The timing and location of HPAIV outbreaks on Mongolian waterways have shown a generally similar temporal and spatial distribution (Figure 2), with all outbreaks starting between early May and the end of July, and restricted to six locations. While the narrow spatial and temporal distribution of Mongolian outbreaks could be related to biases in surveillance locations, we do not believe this is the case. Combined with passive surveillance, which exhibits less temporal bias, Mongolian authorities have also implemented an extensive active surveillance program since 2006, targeting 97 wetland areas across 12 provinces, taking place at least twice a year.

Ecological factors are likely to play an important part in the seasonal distribution of outbreaks. Outbreaks in early May coincide with the arrival of spring migrants from southern wintering areas, including China and South Asia (mostly Anseriformes), and further south for many other species. Counts of birds obtained from April through October at Erhel Nuur in 2007 (Figure 3) illustrate how bird numbers vary during the period from spring thaw to autumn freeze. Although absolute numbers of birds are relatively small in early May, the continuation of ice coverage forces waterbirds into small areas of open water, which increases their density more than at other times of year. For instance, a total of 2,062 birds counted on Erhel Nuur on 5 May 2007 (a date equivalent to the outbreak the previous year) equates to a density of 2,222 birds km^{-2} based on a visual estimate that 95% of the lake surface was frozen. This is higher than the bird densities recorded at any other time at this site. Furthermore, in early May melting ice reduces the salinity of surface water to near zero (even on lakes that are markedly brackish for the remainder of the summer) (pers. obs.). However this effect is temporary and once all ice has melted (by mid to late May) the salinity rapidly increases and remains at a near constant until the autumn freeze. This dilution effect, coupled with low water temperatures may enhance the environmental survival of virus at this time of year [45].

The temporal delay in spring outbreaks in Uuvs Nuur (which have all occurred in early June) from those in north-central Mongolia are hard to explain. By early June spring migration is essentially over, and birds are focused on nest building and raising young. It may be that differences in the species assemblage (Table S4), or peculiarities in breeding or feeding ecology at Uuvs Nuur account for this difference (e.g. Uuvs Nuur supports large colonies of several species such as great cormorant *Phalacrocorax carbo*, Pallas's gulls *Larus ichthyaetus* and Eurasian spoonbills *Platalea leucorodia*, that are rarely found on outbreak lakes elsewhere in Mongolia), but without further information this is difficult to surmise. Although there were large numbers of birds recorded during the surveys at Uuvs Nuur in June 2009, the estimated total density (187 birds km^{-2}) was lower than recorded at all other outbreak investigations (Table S4). Outbreaks initiated in late July coincide with the highest numbers of birds on Mongolian lakes (Figure 3). For instance, counts on Erhel Nuur during the outbreak in July 2009 reached 12,278 birds, equating to a density of 662 birds km^{-2}. At this time many species, particularly ruddy shelducks, congregate in large numbers to molt, at which time they are completely flightless.

The observation that no Mongolian outbreaks have arisen in the months of August, September or October is interesting, as these months are traditionally when LPAIV subtypes reach their peak prevalence in waterfowl populations in Europe and North America [46,47]. These peaks of LPAIV subtypes are attributed to greater densities of susceptible hosts as populations are swollen by numbers of immunologically naïve juvenile birds, which congregate prior to the southward migration period. The lack of Mongolian HPAIV outbreaks detected during the late summer period suggests that the epidemiology of the virus may be quite different from natural circulation of LPAIV subtypes, which are typically less pathogenic for wild birds, although the possibility that outbreaks have been missed cannot be excluded entirely.

In many cases the numbers of sick or dead birds observed during outbreaks was unremarkable, exceeding 100 birds in only four outbreaks, with as few as a single bird involved in others. These numbers contrast with figures reported in China (Table S7), where mortalities numbering in the hundreds is more usual, with over 6,000 dead birds reported during the 2005 outbreak at

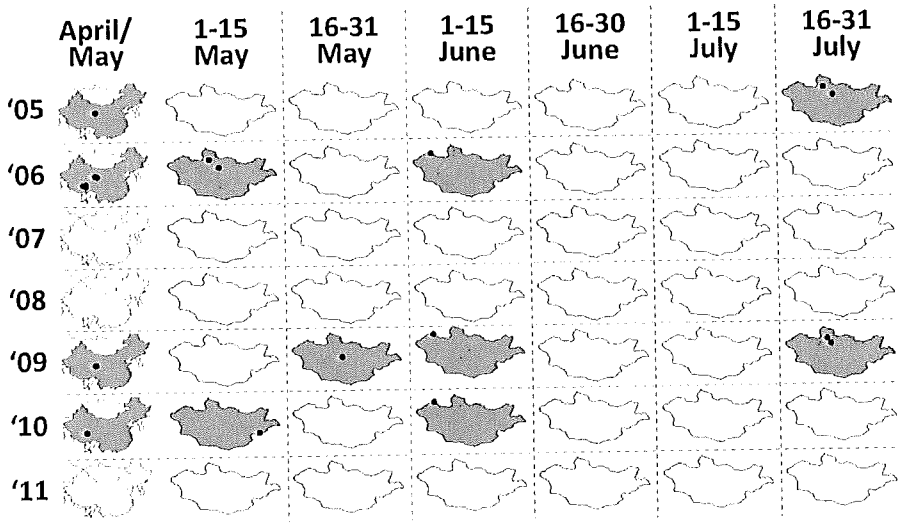

Figure 2. The spatiotemporal distribution of HPAIV outbreak onset from 2005–2011. All reported outbreaks are illustrated for the People's Republic of China during April and May (left column), and Mongolia, in two week intervals from May – July. Cells corresponding to periods during which outbreaks were first reported are indicated in grey, and locations of outbreaks are indicated in black.
doi:10.1371/journal.pone.0044097.g002

Qinghai. This disparity could be related to smaller numbers of birds on Mongolian lakes, and although total population estimates are not available in many of the Chinese outbreaks, we feel that this is unlikely. For instance, over 12,000 live birds were observed during the outbreak on Erhel Nuur in 2009, yet only three dead Mongolian gulls and a single ruddy shelduck were found despite extensive searching over several days. These birds were all flightless at the time of death, indicating that they had contracted

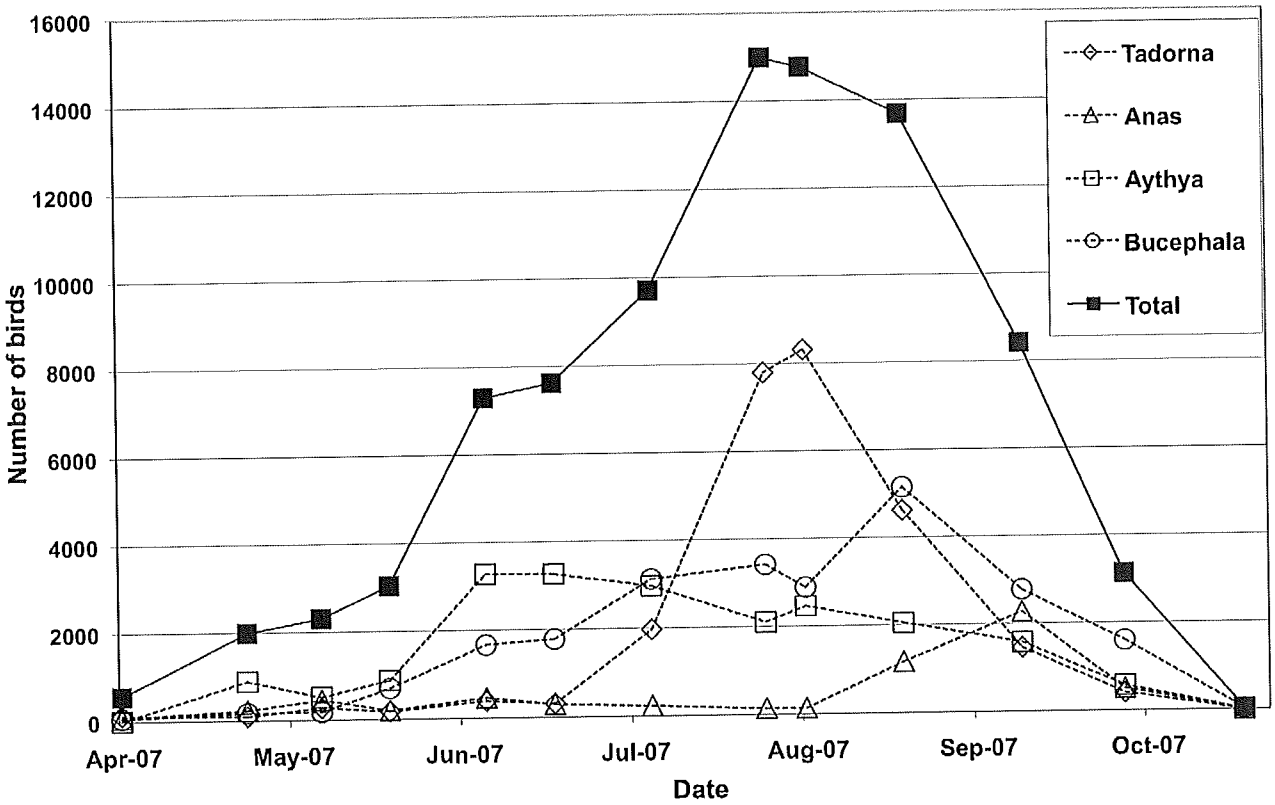

Figure 3. Monthly bird counts at Erhel Nuur from April to October 2007. Total number of birds present are indicated (solid squares), together with counts of the predominant genera *Tadorna* (open diamond), *Anas* (open triangle), *Aythya* (open square), and *Bucephala* (open circle).
doi:10.1371/journal.pone.0044097.g003

the virus locally. A review of surveillance data from Sweden and Denmark in 2006 also found that many outbreaks were associated with unspectacular levels of mortality, with 75% detected through singleton dead birds [48]. Although it is acknowledged that passive surveillance systems that investigate individual bird deaths are economically demanding, Mongolian findings indicate that not doing so will negatively influence detection sensitivity.

Although presence or abundance of a species at an outbreak site does not directly implicate it in the transmission of HPAIV, it may be instructive to look for commonalities across outbreak locations. Bird counts and species densities were assessed during five of the eleven outbreaks (Table S4) and covered all of the lakes at which outbreaks took place except Khunt Nuur. For this site, a census was made on 3 May 2007 and was considered indicative of birds present during the 4 May 2006 outbreak at that location. Only eight species were found on all of the outbreak lakes (demoiselle crane, whooper swan, ruddy shelduck, common goldeneye, common pochard, northern pintail, Mongolian gull and pied avocet). Ruddy shelduck was by far the most numerous species observed, with almost twice as many present as the next most abundant species on outbreak lakes. It has been postulated that bar-headed geese may play a role in the transmission of HPAIV across large distances in Asia [49–51], but this would not appear to be the case in all of the Mongolian outbreaks. Although bar-headed geese are common in the north-central region, they are much less common in western Mongolia, and none were observed in the vicinity of the 2009 outbreak on Uuvs Nuur, or the 2010 outbreak in Ganga Nuur Nature Reserve.

In conclusion, the occurrence of outbreaks in areas deficient in domestic poultry is strong evidence that wild birds can carry HPAIV over at least moderate distances. However, failure to detect further outbreaks of clade 2.2 after June 2006, and clade 2.3.2.1 after June 2010 suggests that wild birds migrating to and from Mongolia either are not competent as indefinite reservoirs of infection or did not reach susceptible populations during our study. For wild birds it appears that passive surveillance provides a more cost effective approach to HPAIV detection than surveillance of apparently healthy individuals.

Supporting Information

Table S1 Summary of samples collected during active surveillance and analysed by virus isolation. Includes number of individuals sampled each year through capture of live birds, collection of fecal samples and sampling of clinically sick and dead birds. Total numbers of birds from which samples were submitted for virus isolation by inoculation into embryonated chicken eggs (n) are given by species, along with numbers of isolates of LPAIVs (L) and HPAIVs (H). Samples listed in 2006 and 2007 represent duplicates of those testing positive by RT-PCR (Table S3). * Refers to a ruddy shelduck that tested negative for virus by virus isolation, but was positive for HPAIV H5N1 by RT-PCR.
(DOCX)

Table S2 Summary of samples collected during outbreak investigations. Includes number of individuals sampled through capture of live birds, collection of fecal samples and sampling of clinically sick and dead birds. Total numbers of birds from which samples were submitted for virus isolation by inoculation into embryonated chicken eggs (n) are given by species, along with numbers of isolates of LPAIVs (L) and HPAIVs (H). Refers to a great crested grebe that tested negative by virus isolation, but was positive for HPAIV H5N1 by RT-PCR.
(DOCX)

Table S3 Summary of samples collected during active surveillance and analysed by RT-PCR. Includes number of individuals sampled each year through capture of live birds, collection of fecal samples and sampling of clinically sick and dead birds. Total numbers of birds from which samples were submitted for RT-PCR (n) are given by species, along with numbers of influenza A viruses (AI) and influenza A viruses of subtype H5 (H5).
(DOCX)

Table S4 Bird counts and densities at Mongolian outbreak lakes. Densities are given in parentheses and are calculated based on the surface area of the lakes (Erhel Nuur 18.56 km^2, Khuut Nuur km^2, Doitiin Nuur km^2, Doroo Tsagaan Nuur km^2), or in the case of Uuvs Nuur in the length of shoreline surveyed (19.1 km) multiplied by an estimated maximum distance visible (2 km). Presence of species that could not be counted are indicated by the letter Y. All counts were made while outbreaks were in progress, with the exception of Khuut Nuur, where counts were made exactly one year after the May 2006 outbreak as an indication of birds present during a comparable season.
(DOCX)

Table S5 Summary of information published relating to wild outbreaks of highly pathogenic avian influenza virus in Mongolia.
(DOCX)

Table S6 Summary of information published relating to wild outbreaks of highly pathogenic avian influenza virus in China.
(DOCX)

Table S7 Summary of information published relating to wild outbreaks of highly pathogenic avian influenza virus in the Tyva Republic, Russian Federation.
(DOCX)

Acknowledgments

We would like to thank the Mongolian Ministry of Nature, Environment and Tourism, the Veterinary and Animal Breeding Agency of the Ministry of Food, Agriculture and Light Industry, the National Emergency Management Agency, the Wildlife Science and Conservation Center and the United States Embassy, Ulaanbaatar for all their good will and collaborative spirit throughout the project. Thanks also to the numerous field assistants who's hard work made this project possible, and to Sandy Shanks for editorial assistance.

Author Contributions

Conceived and designed the experiments: MG LJ WBK DOJ AF ES. Performed the experiments: CJC CYHL JSMP ES DES. Analyzed the data: MG DOJ CJC CYHL JSMP ES DES. Wrote the paper: MG DOJ LJ WBK ES. Led national influenza surveillance efforts: PD RS KG DB PB NT.

References

1. World Health Organization (2011) Archive of tables with cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. Available: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html. Accessed 2011 May 15.
2. Ellis TM, Bousfield RB, Bissett LA, Dyrting KC, Luk GSM, et al. (2004) Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol 33: 492–505.
3. Keawcharoen J, Amonsin A, Oraveerakul K, Wattanodorn S, Papravasit T, et al. (2005) Characterization of the hemagglutinin and neuraminidase genes of recent influenza virus isolates from different avian species in Thailand. ACTA Virol 49: 277–280.
4. Kwon YK, Joh SJ, Kim MC, Lee YJ, Choi JG, et al. (2005) Highly pathogenic avian influenza in magpies (Pica pica sericea) in South Korea. J Wild Dis 41: 618–623.

5. Ducatez MF, Tarnagda Z, Tahita MC, Sow A, de Landtsheer S, et al. (2007) Genetic characterization of HPAI (H5N1) viruses from poultry and wild vultures, Burkina Faso. Emerg Infect Dis 13: 611–613.
6. Alexander DJ (2007) Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Dis 51: 161–166.
7. Chen H, Smith GJD, Zhang SY, Qin K, Wang J, et al. (2005) H5N1 virus outbreak in migratory waterfowl. Nature 436: 191–192.
8. Chen HL, Li YB, Li ZJ, Shi JZ, Shinya K, et al. (2006) Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J Virol 80: 5976–5983.
9. Liu J, Xiao H, Lei F, Zhu Q, Qin K, et al. (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309: 1206.
10. Karesh WB, Cook RA, Gilbert M, Newcomb J (2007) Implications of wildlife trade on the movement of avian influenza and other infectious diseases. J Wild Dis 43: S55–S59.
11. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, et al. (2006) Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci U S A103: 19368–19373. doi: 10.1073/pnas.0609227103
12. Takekawa JY, Newman SH, Xiao X, Prosser DJ, Spragens KA, et al. (2010) Migration of Waterfowl in the East Asian Flyway and Spatial Relationship to HPAI H5N1 Outbreaks. Avian Dis 54: 466–476.
13. Gauthier-Clerc M, Lebarbenchon C, Thomas F (2007) Recent expansion of highly pathogenic avian influenza H5N1: a critical review. Ibis 149: 202–214.
14. Brown JD, Stallknecht DE, Swayne DE (2008) Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. Emerg Infect Dis 14: 136–142.
15. Brown JD, Stallknecht DE, Beck JR, Suarez DL, Swayne DE (2006) Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. Emerg Infect Dis 12: 1663–1670.
16. Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, et al. (2004) Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 78: 4892–4901. doi: 10.1128/JVI.78.9.4892-4901.2004
17. Keawcharoen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, et al. (2008) Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5NI). Emerg Infect Dis 14: 600–607.
18. Chan S, Crosby MJ, Islam MZ, Tordoff AW (2004) Important Bird Areas in Asia: Key Sites for Conservation. Cambridge, United Kingdom: BirdLife International.
19. Minton C, Jessop R, Collins P, Ewing A, Gibbs H (2005) Sighting of waders leg flagged in Victoria: report number 12. Stilt 48: 42–49.
20. Hawkes La, Balachandran S, Batbayar N, Butler PJ, Frappell PB, et al. (2011) The trans-Himalayan flights of bar-headed geese (Anser indicus). Proc Natl Acad Sci U S A 108: 9516-9519. doi: 10.1073/pnas.1017295108
21. Meyburg BU, Howey P, Bernitz Z, Pretorius R, Meyburg C (2010) The Amur Falcon (Falco amurensis) satellite tracking project. In: Suudev G, Watson R, Curti M, Yosef R, Potapov E, et al., editors. Raptors: science and conservation for present and future: The proceedings of the 6th International Conference on Asian Raptors. Ulaanbaatar, Mongolia: Asian Raptor Research and Conservation Network, Mongolian Ornithological Society, and National University of Mongolia. p. 52.
22. Newman SH, Iverson SA, Takekawa JY, Gilbert M, Prosser DJ, et al. (2009) Migration of Whooper Swans and Outbreaks of Highly Pathogenic Avian Influenza H5N1 Virus in Eastern Asia. PLoS One 4. doi: 10.1371/journal.-pone.0005729
23. Batbayar N, Takekawa JY, Newman SH, Prosser DJ, Natsagdorj T, et al. (2011) Migration strategies of Swan Geese Anser cyguoides from northeast Mongolia. Wildfowl 61: 90–109.
24. Sims L (2005) Avian Influenza in Mongolia. TCP/RAS/3007 Report to Food and Agriculture Organization of the United Nations: 1–20.
25. FAO (2007) Gridded livestock of the world 2007, G.R.W. Wint and T.P. Robinson. Rome.
26. Food and Agriculture Organization of the United Nations (2012) FAOSTAT website. Available: http://http://faostat3.fao.org/home/index.html. Accessed 2012 January 15.
27. Spackman E, Swayne DE, Gilbert M, Joly DO, Karesh WB, et al. (2009) Characterization of low pathogenicity avian influenza viruses isolated from wild birds in Mongolia 2005 through 2007. Virol J 6. doi: 10.1186/1743-422X-6-190.
28. Wingard JR, Zahler P (2006) Silent Steppe: The Illegal Wildlife Trade Crisis in Mongolia. Mongolia Discussion Papers, East Asia and Pacific Environment and Social Development Department. Washington D.C.
29. Zahler P, Joly DO, Krueger L, Osofsky SA, Shiilegdamba E (2007) Improving poverty reduction and conservation outcomes in the grassland ecosystem of Mongolia. In: Steele P, Oviedo G, McCauley D, editors. Poverty, Health and Ecosystems: experiences from Asia. Gland, Switzerland and Manila, Philippines: IUCN and Asian Development Bank. 89–96.
30. Delaney S (2005) Guidelines for participants in the International Waterbird Census (IWC). Wetlands International, Wageningen. Available: http://www.wetlands.org. Accessed 2006 April 5.
31. Spackman E, Senne D, Bulaga L, Myers T, Perdue M, et al. (2003) Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis 47: 1079–1082.
32. Swayne DE, Senne DA, Beard CW (1998) Influenza Isolation and Identification of Avian Pathogens. In: Swayne DE, editor. A Laboratory Manual for the Isolation and Identification of Avian Pathogens. American Association of Avian Pathologists, Kennett Square, Pennsylvania. 150–155.
33. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40: 3256–3260.
34. Suarez DL, Garcia M, Latimer J, Senne D, Perdue M (1999) Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. J Virol 73: 3567–3573.
35. Das A, Spackman E, Senne D, Pedersen J, Suarez DL (2006) Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. J Clin Microbiol 44: 3065–3073.
36. Spackman E, Senne D, Bulaga L, Trock S, Suarez D (2003) Development of multiplex real-time RT-PCR as a diagnostic tool for avian influenza. Avian Dis 47: 1087–1090.
37. Spackman E, Suarez D (2008) Detection and identification of the H5 hemagglutinin subtype by real-time RT-PCR. Methods Mol Biol 436: 27–33.
38. Runstadler J a, Happ GM, Slemons RD, Sheng Z-M, Gundlach N, et al. (2007) Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Arch Virol 152: 1901–1910. doi: 10.1007/s00705-007-0994-1
39. Siembieda JL, Johnson CK, Cardona C, Anchell N, Dao N, et al. (2010) Influenza A viruses in wild birds of the Pacific flyway, 2005–2008. Vector Borne Zoonotic Dis10: 793–800. doi: 10.1089/vbz.2009.0095
40. World Health Organization (2011) Updated unified nomenclature system for the highly pathogenic H5N1 avian influenza viruses. Available: http://www.who.int/influenza/gisrs_laboratory/h5n1_nomenclature/en/index.html. Accessed 2011 December 4.
41. Reid SM, Shell WM, Barboi G, Onita I, Turcitu M, et al. (2011) First reported incursion of highly pathogenic notifiable avian influenza A H5N1 viruses from clade 2.3.2 into European poultry. Transbound Emerg Dis 58: 76–78. doi: 10.1111/j.1865-1682.2010.01175.x
42. Islam MR, Haque ME, Giasuddin M, Chowdhury EH, Samad Ma, et al. (2011) New Introduction of Clade 2.3.2.1 Avian Influenza Virus (H5N1) into Bangladesh. Transbound Emerg Dis. doi: 10.1111/j.1865-1682.2011.01297.x
43. Smith GJD, Vijaykrishna D, Ellis TM, Dyrting KC, Leung YHC, et al. (2009) Characterization of Avian Influenza Viruses A (H5N1) from Wild Birds, Hong Kong, 2004–2008. Emerg Infect Dis 15: 402–407. doi: 10.3201/eid1503.081190
44. Lee D-H, Park J-K, Youn H-N, Lee Y-N, Lim T-H, et al. (2011) Surveillance and Isolation of HPAI H5N1 from Wild Mandarin Ducks (Aix galericulata). J Wild Dis 47: 994–998.
45. Stallknecht DE, Brown JD (2009) Tenacity of avian influenza viruses. Rev Sci Tech 28: 59–67.
46. Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, et al. (2007) Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathogens 3: e61.
47. Hinshaw VS, Wood JM, Webster RG, Deibel R, Turner B (1985) Circulation of influenza viruses and paramyxo- viruses in waterfowl: comparison of different migratory fly-ways in North America. Bull World Health Organ 63: 711–719.
48. Komar N, Olsen B (2008) Avian influenza virus (H5N1) mortality surveillance. Emerg Infect Dis 14: 1176–1178. doi: 10.3201/eid1407.080161
49. Prosser DJ, Takekawa JY, Newman SH, Yan B, Douglas DC, et al. (2009) Satellite-marked waterfowl reveal migratory connection between H5N1 outbreak areas in China and Mongolia. Ibis 51: 568–576.
50. Lei F, Tang S, Zhao D, Zhang X, Kou Z, et al. (2007) Characterization of {H5N1} influenza viruses isolated from migratory birds in Qinghai province of China in 2006. Avian Diseases 51: 568–572.
51. Cui P, Hou Y, Tang M, Zhang H, Zhou Y, et al. (2010) Movement patterns of Bar-headed Geese Anser indicus during breeding and post-breeding periods at Qinghai Lake, China. J Ornithol 152: 83–92. doi: 10.1007/s10336-010-0552-6.

Vaccine 30 (2012) 4964–4970

Contents lists available at SciVerse ScienceDirect

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Reduction of high pathogenicity avian influenza virus in eggs from chickens once or twice vaccinated with an oil-emulsified inactivated H5 avian influenza vaccine

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ARTICLE INFO

Article history:
Received 6 March 2012
Received in revised form 15 May 2012
Accepted 16 May 2012
Available online 29 May 2012

Keywords:
Poultry
Egg production
Avian influenza

ABSTRACT

The negative impact of high pathogenicity avian influenza virus (HPAIV) infection on egg production and deposition of virus in eggs, as well as any protective effect of vaccination, is unknown. Individually housed non-vaccinated, sham-vaccinated and inactivated H5N9 vaccinated Once or Twice adult White leghorn hens were challenged intranasally/intratracheally 3-weeks post-vaccination with H5N2 HPAIV. The non-/sham-vaccinated layers experienced 100% mortality (0% survivability) within 3–4 days post-challenge (DPC), and major changes to reproductive parameters including precipitous drops in egg production (79–0% in <5 days), production of soft and thin-shelled eggs, and deposition of virus in albumin and yolk, and on the egg shell surface of 53% of eggs. By comparison, the three H5-vaccinated groups had 83%, 100% and 100% survivability after challenge; the two H5-vaccinated Once hens that died had low pre-challenge HI titers (GMT = 16). H5-vaccinated Once or Twice groups maintained egg production after challenge (63%), but there was a mild and significant reduction in egg production as compared to pre-challenge egg production (79%). H5-vaccinated groups had reduced number of virus contaminated eggs (28%), and in most groups, reduced quantity of virus in contaminated eggs compared to non-/sham-vaccinated groups. No HPAIV-positive eggs were laid on or after 5 DPC. In conclusion, HPAIV infection had major negative impact on egg production and other reproductive parameters. H5-vaccination Once or Twice prevented declines in egg production after HPAIV challenge, reduced number of virus-infected eggs, and typically reduced the titer of virus in internal contents and on eggshell surface.

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1. Introduction

High pathogenicity avian influenza (HPAI) virus (HPAIV) infections in chickens cause multi-organ systemic disease with precipitous drops in egg production and virus replication in internal organs including the reproductive tract [1]. In the 2003 H7N7 HPAI outbreaks in the Netherlands, infected White leghorn (WL) laying hens laid pale eggs with reduced eggshell quality, and egg production dropped to near 0% in just a few days [2]. In 1999–2000 H7N1 HPAI outbreaks in Italy, egg production in both infected chickens and guinea fowl flocks dropped by 5–40% [3]. Furthermore, HPAIV has been isolated from eggs laid by experimentally and naturally infected laying chickens [4–7].

In experimental studies, vaccination against HPAIV protected young chickens against mortality and reduced respiratory and digestive tract virus shedding [8–11]. Recently, vaccination was shown to protect broiler breeder hens from mortality [12], but no data been reported on protecting egg production or virus contamination of broiler eggs or eggs for human consumption. This study was conducted to investigate how HPAIV infections affected egg production in WL hens, determine the presence and quantity of virus located on or within eggs, and determine the effect of once or twice vaccination as a mitigation strategy.

2. Materials and methods

2.1. Viruses

Virus stocks of A/chicken/Pennsylvania/1370/1983 (H5N2) (PA/83), a HPAIV, and A/turkey/Wisconsin/68 (H5N9) (Tk/WI), a LPAIV, were grown in 9 day-old specific pathogen free (SPF) embryonating chicken eggs [13].

Abbreviations: AIV, avian influenza virus; BHI, brain heart infusion media; DPC, days post-challenge; DPV, days post-vaccination; EID_{50}, mean embryo infectious doses; GMT, geometric mean titers; HA, hemagglutination; HI, hemagglutinin inhibition; HP, high pathogenicity; HPAI, high pathogenicity avian influenza; HPAIV, high pathogenicity avian influenza virus; LP, low pathogenicity; LPAIV, low pathogenicity avian influenza virus; OE, oil-in-water emulsion; SPF, specific pathogen free; WL, White leghorn.

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.vaccine.2012.05.041

2.2. Vaccines

Infectious allantoic fluid containing Tk/WI LPAIV was β-propiolactone inactivated using a 0.1% concentration and an oil-in-water emulsion (OE) vaccine was made [14]. The oil phase consisted of 36 mL mineral oil, 3 mL Span 80 (Sigma Aldrich, St. Louis, MO) and 1 mL Tween 80 (Sigma Aldrich, St. Louis, MO). Each dose of vaccine had a hemagglutination (HA) titer of 512. Sham-vaccinated groups received an OE vaccine made with sterile, non-infected SPF allantoic fluid. Each hen was vaccinated subcutaneously in the nape of the neck with 0.5 mL of sham or H5 vaccine.

2.3. Animals and housing

Reproductively active, specific-pathogen-free White leghorn hens (31–48 weeks of age) from *in house* production flocks were provided feed and water ad libitum, and 16 h of daily light. Animal care was provided under an Institutional Animal Care and Use Committee approved protocol. Experiments were carried out in an USDA-certified Biosafety Level 3-agriculture facility.

2.4. Experimental design

Hens were housed individually. Three experiments were accomplished: (1) 18 non-vaccinated hens; (2) 12 H5-vaccinated and 12 sham-vaccinated hens; and (3) 12 sham-vaccinated, 12 H5-vaccinated-Once and 11 H5-vaccinated-Twice hens. For hens receiving two H5-vaccinations, vaccinations were given 3 weeks apart. Hens were inoculated with 0.1 mL intranasally and 0.1 mL intratracheally of the HPAIV PA/83 challenge virus (10^6 EID_{50}/0.2 mL) three weeks post-vaccination.

Oropharyngeal swabs were taken on the day of death. Individual eggs were collected daily pre- and post-challenge. Each egg's entire surface area was wet swabbed before being handled and each swab was placed into a tube with 1.0 mL brain heart infusion media (BHI) (Becton, Dickinson and Company, Sparks, MD) with penicillin (2000 units/mL; Sigma Aldrich), gentamicin (200 μg/mL; Sigma Aldrich) and amphotericin B (5 μg/mL; Sigma Aldrich) and mixed thoroughly by agitation. Swab samples were stored at −70 °C until tested. Survivors at 14 DPC from all experiments were bled from a wing vein and euthanized using cervical dislocation.

2.5. Egg sampling

For yolk and albumin samples, each egg was opened and yolk and albumin separated. Albumin was mixed thoroughly and a 1.25 mL sample was removed and stored. Egg yolk was mixed thoroughly, diluted 1:2 in BHI because of high viscosity, and a 1.25 mL sample was removed and stored at −70 °C. Virus isolation was attempted from all oropharyngeal swabs as well as yolk, albumin, and shell surface swab samples. Virus isolation and titration were completed as previously described [13].

2.6. Serology

Blood samples were taken at time of vaccination, challenge and 14 DPC. Hemagglutinin inhibition (HI) assays were carried out using H5 antigen specific for the vaccine seed virus and were expressed a geometric mean titers (GMT) [13].

2.7. Virus isolation

Nine-to-11-day old SPF embryonating chicken eggs were used for virus isolation [13]. EID_{50} was determined by further titration of positive eggshell, albumin and yolk samples in 9-to-11-day old SPF embryonating chicken eggs. The minimum detectable titer was $10^{0.97}$ EID_{50}/mL for albumin and shell samples, and $10^{0.63}$/mL for yolk samples.

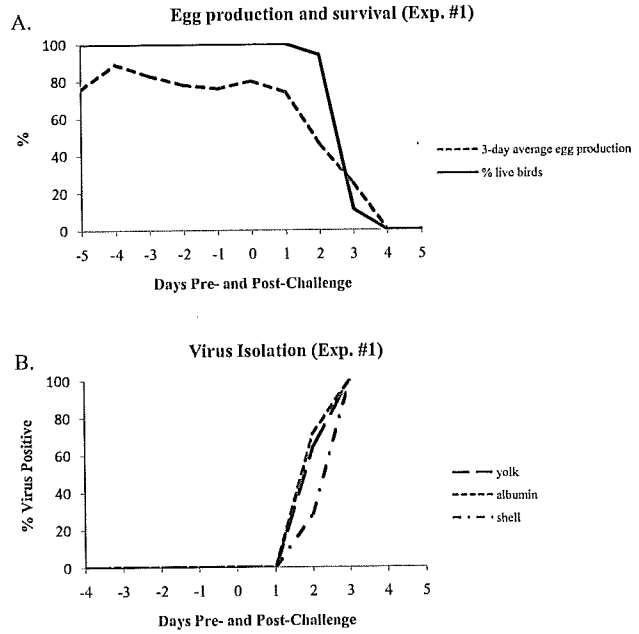

Fig. 1. Experiment 1: Egg production (three day average of percentage of eggs/hen/day); hen mortality (percentage); and virus positive eggshell surface, yolk, and albumin samples (percentage positive for virus) for hens challenged with A/chicken/Pennsylvania/1370/83 (H5N2) HPAIV.

2.8. Statistical analysis

The number of virus positive samples (total eggs, and shell, albumin and yolk samples) and number of antibody positive chickens were analyzed by chi-square test (Excel®, Microsoft Inc., Seattle, WA and Statistics Calculator, StatPac, Inc., Bloomington, MN). Virus and antibody titer data was reported as mean ± standard error of the mean (SEM) and analyzed using ANOVA and Student *t*-test (Excel®). For egg production data, data points were calculated as % eggs/hen/day on a 3 day average. To determine if individual chicken serum HI titers were predictive of preventing virus deposition in eggs laid by hens in acute infection process, regression analysis was used (Excel®). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1

All non-vaccinated chickens were negative for anti-H5 antibodies on day of challenge (Table 1). One hen died on 2 DPC, 15 hens died on 3 DPC and the remaining 2 hens died on 4 DPC (mean death time [MDT] = 3.1 days) (Fig. 1A). HPAIV was recovered from oropharyngeal swabs on the day of death for all chickens. Infection resulted in a significant decrease in egg production (Table 2) and an increase in the number of soft-shelled ($n = 10$) and thin-shelled ($n = 1$) eggs on 2 and 3 DPC ($P < 0.005$). Half of the eggs laid after challenge were virus infected (Table 3); 1 DPC = 0%, 2 DPC = 80% and 3 DPC = 100% virus positive eggs (Fig. 1B). The numbers of virus positive shell surface, albumin, and yolk samples (42–44%) were similar (Table 3).

3.2. Experiment 2

Before vaccination, all hens lacked H5 HI antibodies (Table 1). At 0 DPC (21 days post-vaccination [DPV]), sham-vaccinated hens

Table 1
HI serological response in non-vaccinated, sham-vaccinated and H5-vaccinated Once or Twice laying hens at 0 days post vaccination (DPV), 0 days post challenge (DPC) and 14 DPC. Only includes hens that laid eggs after challenge. Positives have minimum titer of 1:8.

Experiment	Group	# of hens	Time of sampling		
			0 DPV	0 DPC	14 DPC
1	Non-vaccinated	18	NA	0/18	NS
2	Sham-vaccinated	12	0/12	$0/12^{A}$ ($<8^{A}$)	NS
	H5-vaccinated Once	12	0/12	$12/12^{B}$ (23 ± 4.3^{Ba})	10/10 (1024 ± 358^{b})
3	Sham-vaccinated	12	0/12	$0/12^{A}$ ($<8^{A}$)	NS
	H5-vaccinated Once	12	0/12	$12/12^{B}$ (32 ± 5.3^{Ba})	12/12 (1024 ± 310^{Ab})
	H5-vaccinated Twice	11	0/11	$11/11^{B}$ (330 ± 101^{Ca})	11/11 (658 ± 315^{Aa})
Pooled	Non-/Sham-vaccinated	42	0/42	$0/24^{A}$ ($<8^{A}$)	NS
	H5-vaccinated Once	24	0/24	$24/24^{B}$ (27 ± 3.5^{Ba})	$22/22^{A}$ (1024 ± 310^{b})

For individual experiments 2 and 3 and pooled data, statistical significance ($P < 0.05$) for number of antibody positive (geometric mean titer of ≥8 [GMT]; Chi square) and actual titers (Student *t*-test) between sham- and H5-vaccinated groups are represented by different uppercase letters. Titer data sets (Student *t*-test) were tested for significance ($P < 0.05$) between 0 and 14 DPC; i.e. represented by different lowercase superscript letters. NA = not applicable. NS = no sample because all died by 4 DPC.

Table 2
Egg production before (−5 to 0 DPC) and after challenge (1–14 DPC) based on actual number of eggs produced and expected production of one egg per hen per day.

Exp.	Treatment group	Eggs numbers/expected production	% Egg production	*P*-value
1	Non-vaccinated hens before infection	84/108	78%	
	Non-vaccinated hens after infection	40/168	24%	<0.0001*
2	Sham-vaccinated hens before infection	55/72	76%	
	Sham-vaccinated hens after infection	23/168	14%	<0.0001*
	Once H5-vaccinated hens before infection	57/72	79%	
	Once H5-vaccinated hens after infection	105/168	63%	0.0115*
	Sham-vaccinated hens before infection	55/72	76%	
	Once H5-vaccinated hens before infection	57/72	79%	0.6885
	Sham-vaccinated hens after infection	23/168	14%	
	Once H5-vaccinated hens after infection	105/168	63%	<0.0001*
3	Sham-vaccinated hens before infection	60/72	83%	
	Sham-vaccinated hens after infection	25/168	15%	<0.0001*
	All vaccinated hens before infection	109/138	79%	
	All vaccinated hens after infection	218/336	65%	0.0026*
	Once H5-vaccinated hens before infection	55/72	76%	
	Once H5-vaccinated hens after infection	101/168	60%	0.0155*
	Twice H5-vaccinated hens before infection	54/66	82%	
	Twice H5-vaccinated hens after infection	117/168	70%	0.0016*
	Sham-vaccinated hens before infection	60/72	83%	
	Once H5-vaccinated hens before infection	55/72	76%	0.4697
	Sham-vaccinated hens before infection	60/72	83%	
	Twice H5-vaccinated hens before infection	54/66	82%	0.8145
	Sham-vaccinated hens after infection	25/168	15%	
	Once H5-vaccinated hens after infection	101/168	60%	<0.0001*
	Sham-vaccinated hens after infection	25/169	15%	
	Twice H5-vaccinated hens after infection	117/168	70%	<0.0001*
	Once H5-vaccinated hens before infection	55/72	76%	
	Twice H5-vaccinated hens before infection	54/66	82%	0.4342
	Once H5-vaccinated hens after infection	101/168	60%	
	Twice H5-vaccinated hens after infection	117/168	70%	0.5033
1	Non-vaccinated hens before infection	84/108	78%	
2	Sham-vaccinated hens before infection	55/72	76%	
3	Sham-vaccinated hens before infection	60/72	83%	0.5470
1	Non-vaccinated hens after infection	40/168	24%	
2	Sham-vaccinated hens after infection	23/168	14%	
3	Sham-vaccinated hens after infection	25/168	15%	0.0915
2	Once H5-vaccinated hens before infection	57/72	79%	
3	Once H5-vaccinated hens before infection	55/72	76%	0.6885
2	Once H5-vaccinated hens after infection	105/168	63%	
3	Once H5-vaccinated hens after infection	101/168	60%	0.6541
2	Once H5-vaccinated hens before infection	57/72	79%	
3	Once H5-vaccinated hens before infection	55/72	76%	
3	Twice H5-vaccinated hens before infection	54/66	82%	0.7357
2	Once H5-vaccinated hens after infection	105/168	63%	
3	Once H5-vaccinated hens after infection	101/168	60%	
3	Twice H5-vaccinated hens after infection	117/168	70%	0.5549
All	H5-vaccinated hens before infection	166/210	79%	
All	H5-vaccinated hens after infection	323/514	63%	<0.0001*
All	Non-/Sham-vaccinated hens before infection	199/252	79%	
All	Non-/Sham-vaccinated hens after infection	88/520	17%	<0.0001*
All	Non-/Sham-vaccinated hens before infection	199/252	79%	
All	H5-vaccinated hens before infection	166/210	79%	0.9834
All	Non-/Sham-vaccinated hens after infection	88/520	17%	
All	H5-vaccinated hens after infection	323/514	63%	<0.0001*

* Comparisons at $P < 0.05$ are significantly different.

Table 3
Number of virus positive egg, shell, albumin and yolk samples, and the average virus titer ($\log_{10}$ EID_{50}/mL) from eggs laid following challenge with HPAIV (number of positives/total eggs produced 1–4 DPC). Average titers do not include negative samples.

Exp. #	Treatment group	Virus+eggs/total eggs		Shell sample		Albumin sample		Yolk sample	
		1–4 DPC	5–14 DPC	Virus+/total eggs	Mean ± SEM (range)	Virus+/total eggs	Mean ± SEM (range)	Virus+/total eggs	Mean ± SEM (range)
1	Non-vaccinated	18/36 (50%)	–	15/36 (42%)	3.19 ± 0.34 (0.97–5.1)	16/36 (44%)	3.45 ± 0.40 (1.5–6.1)	15/36 (42%)	2.48 ± 0.34 (1.2–4.8)
2	Sham-vaccinated	14/28^{A} (50%)	–	10/28^{A} (36%)	2.59 ± 0.4^{A} (0.97–4.5)	14/28^{A} (50%)	2.69 ± 0.45^{A} (0.97–5.5)	5/28^{A} (18%)	1.68 ± 0.68^{A} (0.93–4.4)
	H5-vaccinated Once (Tk/WI)	15/39^{A} (39%)	0/66	5/39^{B} (13%)	2.41 ± 0.83^{A} (1.23–5.7)	10/39^{A} (26%)	1.51 ± 0.34^{A} (0.97–4.5)	2/39^{A} (5%)	1.79 ± 1.12^{A} (0.67 and 2.9)
3	Sham-vaccinated	15/25^{A} (60%)	–	15/25^{A} (60%)	3.06 ± 0.03^{A} (1.23–5.9)	14/25^{A} (56%)	2.83 ± 1.25^{A} (1.23–5.1)	12/25^{A} (28%)	2.00 ± 0.72^{A} (0.93–3.4)
	H5-vaccinated Once (Tk/WI)	8/33^{B} (24%)	0/86	4/33^{B} (12%)	1.30 ± 0.13^{B} (0.97–1.5)	3/33^{B} (9%)	1.15 ± 0.18^{B} (0.97–1.5)	2/33^{B} (6%)	1.90 ± 0.70*^{A} (1.2 and 2.6)
	H5-vaccinated Twice (Tk/WI)	6/31^{B} (19%)	0/68	3/31^{B} (10%)	1.06 ± 0.09^{B} (0.97–1.23)	2/31^{B} (6%)	1.10 ± 0.13*^{AB} (0.97 and 1.23)	2/31^{B} (6%)	0.82 ± 0.15*^{A} (0.67 and 0.97)
Pooled	Sham/non-vaccinated	47/89^{A} (53%)	–	40/89^{Aab} (45%)	2.99 ± 0.22^{Aa} (0.97–5.9)	44/89^{Aab} (49%)	3.01 ± 0.23^{ACa} (0.97–6.1)	32/89^{Ab} (36%)	2.17 ± 0.19^{Ab} (0.67–4.8)
	H5-vaccinated Once (Tk/WI)	23/72^{B} (32%)	0/152	9/72^{B} (13%)	1.91 ± 0.48^{Ba} (0.97–5.7)	13/72^{B} (18%)	1.42 ± 0.26^{Ba} (0.97–4.5)	4/72^{B} (6%)	1.84 ± 0.54^{Aa} (0.67–2.9)
	H5-vaccinated Twice (Tk/WI)	6/31^{B} (18%)	0/68	3/31^{B} (10%)	1.06 ± 0.09^{Ba} (0.97–1.23)	2/31^{B} (6%)	1.10 ± 0.13*^{BCa} (0.97 and 1.23)	2/31^{B} (6%)	0.82 ± 0.15*^{Aa} (0.67 and 0.97)
	H5-vaccinated All (Tk/WI)	29/103^{B} (28%)	0/220	12/103^{Ba} (12%)	1.70 ± 0.37^{Ba} (0.97–5.7)	15/103^{Ba} (15%)	1.38 ± 0.23^{Bb} (0.97–4.5)	6/103^{Ba} (6%)	1.50 ± 0.40^{Bb} (0.67–2.9)
	Total	76/192 (40%)		52/192^{a} (27%)		59/192^{ab} (31%)		38/192^{a} (20%)	

For individual experiments 2 and 3 and pooled data, statistical significance ($P < 0.05$) for number of virus positive (Chi square) and virus titers (Student *t*-test) between sham- and H5-vaccinated groups are represented by different uppercase letters. Because of low numbers of virus positive eggs in individual H5-vaccinated groups, only pooled data sets were tested for significance ($P < 0.05$) of number of virus isolates (Chi square) and virus titers (Student *t*-test) within each treatment group; i.e. represented by different lowercase superscript letters. *Only 2 virus positives were produced. In the pooled data, 88% (37 of 42) of non-/sham-vaccinated, 71% (17 or 24) H5-vaccinated Once, 45% (5 of 11) H5-vaccinated Twice, and 63% (22 of 35) of all H5-vaccinated hens laid infected eggs.

lacked H5 antibodies while H5-vaccinated hens had a low HI antibody response (GMT = 23) that was significantly lower than the HI antibody response at 14 DPC (GMT = 1024) (Table 1). All 12 sham-vaccinated birds died after HPAIV challenge (MDT = 3.4 days) (Fig. 2A) and virus was isolated from oropharyngeal swabs on the day of death. One H5-vaccinated chicken died on each of 3 and 5 DPC (Fig. 2A), each having HI antibody titers of 16 on the day of challenge and recovery of HPAIV after death.

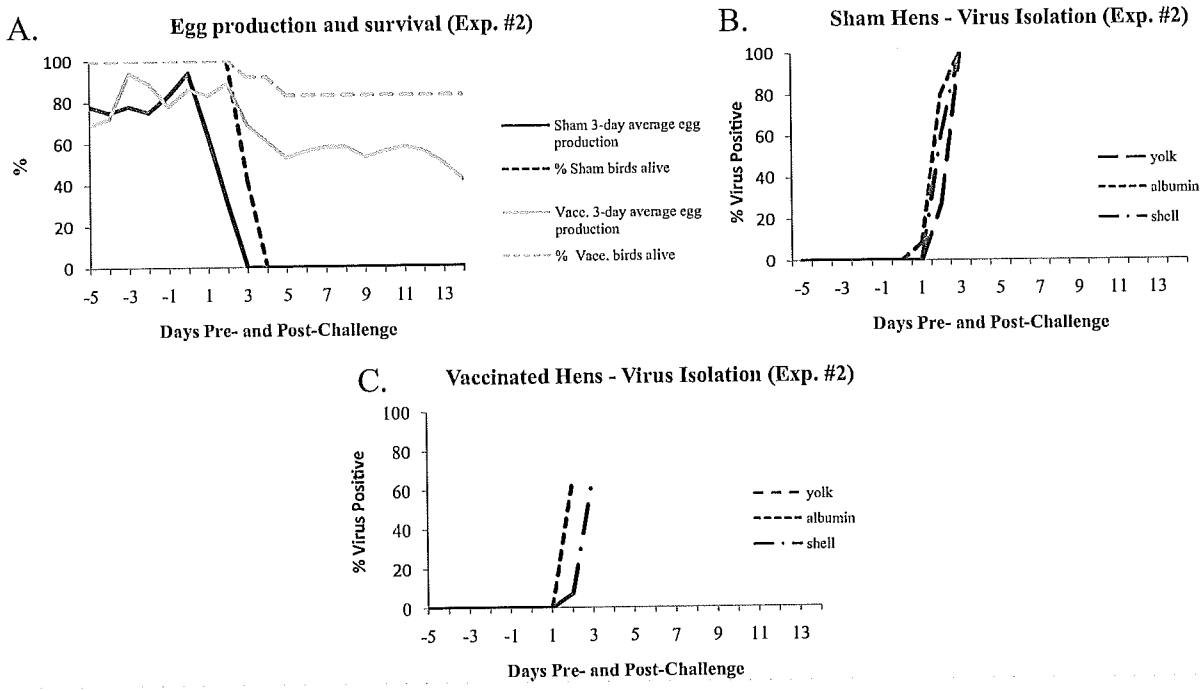

Fig. 2. Experiment 2: Egg production (three day average of percentage of eggs/hen/day); hen mortality (percentage); and virus positive eggshell surface, yolk, and albumin samples (percentage positive for virus) for sham-vaccinated and H5-vaccinated hens challenged with A/chicken/Pennsylvania/1370/83 (H5N2) HPAIV.

No significant difference in egg production was seen pre-challenge between sham-vaccinated (76%) and H5-vaccinated (79%) groups (Table 2, Fig. 2A). One soft-shelled egg was laid in the sham group on 3 DPC. HPAIV infection significantly decreased egg production in the sham-vaccinated (14%) compared to H5-vaccinated groups (63%) (Table 2). In addition, egg production pre-challenge (79%) was significantly higher compared to post challenge egg production (63%) in H5-vaccinated hens (Table 2).

The number of virus positive eggs, albumin samples and yolk samples were not significantly different between sham- and H5-vaccinated groups, but there were significantly fewer virus positive shell samples in H5-vaccinated group (Table 3, Fig. 2B and C). The average virus titers of positive shell, albumin and yolk samples were not significantly different between sham- and H5-vaccinated groups.

3.3. Experiment 3

Sham- and H5-vaccinated birds lacked H5 HI antibodies before vaccination (0 DPV) (Table 1). The sham-vaccinated chickens had no H5 HI antibodies three weeks DPV (0 DPC), but all H5-vaccinated-Once or H5-vaccinated-Twice hens had HI antibody responses, with significantly higher titers ($P < 0.0001$) in H5-vaccinated-Twice compared to H5-vaccinated-Once hens (330 versus 32, respectively) (Table 1). At 14 DPC, all survivors had higher HI antibody responses than the corresponding 0 DPC samples, but the difference in titers was only significant for the H5-vaccinated-Once hens. All sham-vaccinated hens died after HPAIV challenge (MDT = 4.0 days) and all H5-vaccinated birds survived (Fig. 3A).

H5-vaccinated-Twice hens had no differences in egg production either pre- (82%) or post-challenge (70%) while H5-vaccinated-Once hens had significantly reduced egg production between pre- and post-challenge periods (76% versus 60%, respectively) (Table 2). For pre-challenge egg production, H5-vaccinated-Once (76%), H5-vaccinated-Twice (82%), or sham-vaccinated (83%) hens had no differences in egg production (Table 3). Egg production was decreased after challenge in the sham-vaccinated group (15%) when compared to either H5-vaccinated groups post-challenge (60% and 70%) or sham-vaccinated group pre-challenge (83%) (Table 2).

H5-vaccination significantly decreased the number of virus positive eggs, and eggshell, albumin and yolk samples (Table 3, Fig. 3B–D). The virus titer of shell (H5-vaccinated-Once and H5-vaccinated-Twice) and albumin samples (H5-vaccinated-Once) were significantly less than corresponding titers for sham-vaccinated groups. The virus titers of albumin (H5-vaccinated-Twice) and yolk (H5-vaccinated-Once and H5-vaccinated-Twice) samples were less than corresponding sham-vaccinated group, but there were too few samples for further statistical analysis.

3.4. Comparisons between Experiments 1, 2 and 3

Similar data sets were tested between experiments to determine experiment-to-experiment variation. There were no significant differences in the following egg production data sets (Table 2): (1) three non-/sham-vaccinated groups before challenge, (2) three non-/sham-vaccinated groups after challenge, (3) the H5-vaccinated-Once or H5-vaccinated-Twice groups before challenge, (4) and the H5-vaccinated-Once or H5-vaccinated-Twice groups after challenge. From pooled data, H5-vaccinated and non-/sham-vaccinated groups had identical egg production before challenge (79%), but non-/sham-vaccinated groups had significantly lower egg production (17% versus 63%) after challenge (Table 2). The egg production of H5-vaccinated pooled group before (79%) and after (63%) challenge were significantly different, and for non-/sham-vaccinated groups, the egg production was significantly higher before (79%) compared to after challenge (17%) (Table 2).

There were no significant differences in the following: (1) number of virus positive eggs, eggshell or albumin samples in non-/sham-vaccinated groups; (2) number of virus positive eggs, eggshell samples or yolk samples in H5-vaccinated groups; and (3) number of or titer of positive samples between H5-vaccinated-Once or H5-vaccinated-Twice groups. Experiment 2 sham-vaccinated group had fewer virus positive yolk samples than the other two non-/sham-vaccinated groups, and experiment 3 H5-vaccinated groups had fewer virus positive albumin samples than experiment 2 H5-vaccinated group (Table 3). Using pooled data, the non-/sham-vaccinated groups had more virus positive eggs, eggshells, albumin samples and yolk samples than the H5-vaccinated-Once or H5-vaccinated-Twice groups, and the average titers were significantly higher in eggs from non-/sham-vaccinated group (Table 3).

The pooled non-/sham-vaccinated hens had lower HI titers at 0 DPC (GMT < 8) than H5-vaccinated-Once hens (GMT = 27) (Table 1). For H5-vaccinated-Once hens, survivors at 14 DPC had higher HI titers (GMT = 1024) compared to 0 DPC (GMT = 27) (Table 1). When classifying hens into two serological titer groups, hens with HI results in titer groups 1 (GMT = 4, 8 or 16) had a significantly higher proportion of eggs with virus on the surface ($P = 0.021$) or virus in internal contents ($P = 0.0007$) than eggs laid by hens in titer group 2 (GMT $\geq$ 32). However, regression failed to show an association between serum HI titers and virus titers recovered from eggs during the acute infection process (data not shown).

4. Discussion

The current study demonstrated that non-vaccinated and sham-vaccinated layers when challenged with PA/83 H5N2 HPAIV had 100% mortality within 3–4 DPC, and major changes to reproductive parameters including precipitous drops in egg production (0% in <5 days), production of soft and thin-shelled eggs, and recovery of virus from albumin, yolk and eggshell surface. HPAIV was recovered from 53% of the eggs laid between 1 and 4 DPI with average virus titers of $10^{2.17\pm0.19}$ (range = $10^{0.93-4.8}$), $10^{3.01\pm0.23}$ (range = $10^{0.97-6.1}$) and $10^{2.99\pm0.22}$ (range = $10^{0.97-5.9}$) EID_{50}/mL for yolk, albumin and eggshell surface, respectively. In 1983–84 naturally occurring H5N2 HPAIV cases in the USA, seven clinically-affected WL and broiler breeder flocks had recovery of virus from 31% of eggs collected during the acute stage of clinical disease; i.e. 1–18 days after appearance of clinical signs [4]. HPAIV was isolated from albumin, yolk or eggshell surface [4]. In an experimental study using 10^6 EID_{50} PA/83, WL hens had 92% mortality (MDT = 6.1 days), total cessation of egg production by 4 DPC, production of thin-shelled and soft-shelled eggs, and production of infected eggs on 3 and 4 DPC with maximum titer of $10^{4.9}$ EID_{50}/mL [5,15]. In another experiment using 10^4 EID_{50} of a similar H5N2 HPAIV in hens, 8% of the eggs were infected and the virus positive eggs were laid only on the last day of life [6]. The clinical features, drops in egg production, production of abnormal eggs, and virus presence on and in eggs from our non-/sham-vaccinated groups was similar to previous natural and experimental HPAIV infections of WL hens validates our model system for assessing the impact of vaccination on ameliorating HPAIV infection in reproductively active chickens.

In the current study, H5-vaccinated-Once or H5-vaccinated-Twice laying chickens were protected against H5N2 HPAIV challenge, but the extent of protection varied with the specific parameter measured. Examining pooled data, H5-vaccinated groups had lower mortality rates, better egg production, and reduced number of and virus quantity in contaminated eggs as compared to non-/sham-vaccinated groups. Historically,

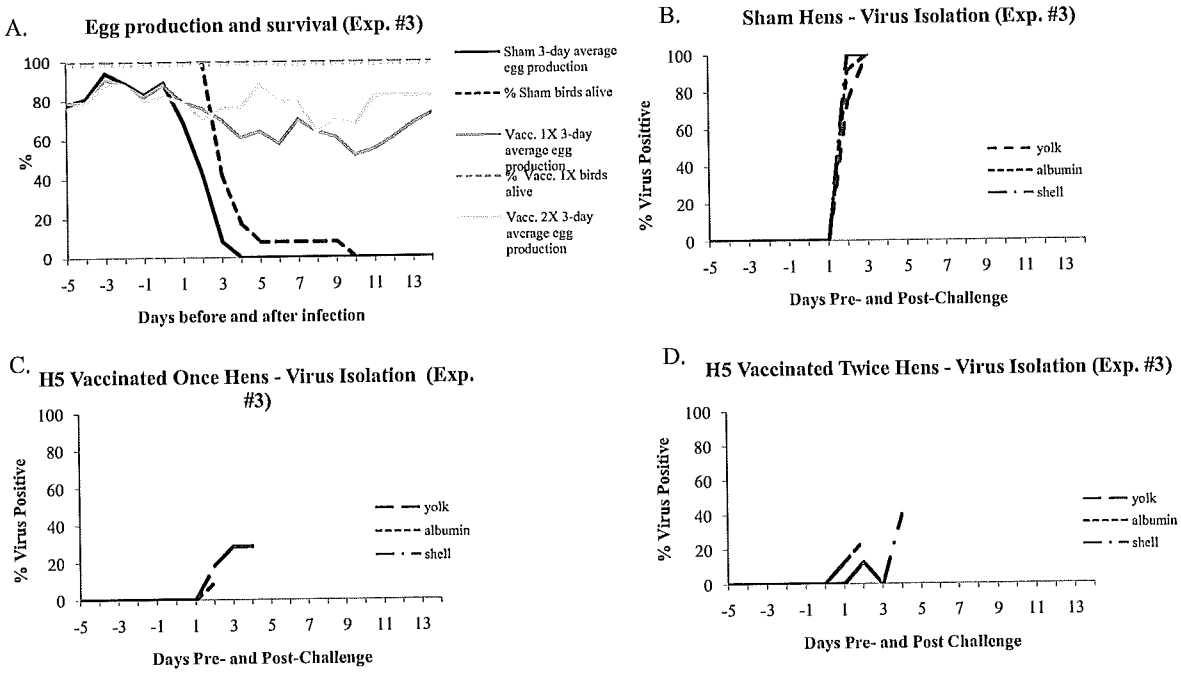

Fig. 3. Experiment 3: Egg production (three day average of percentage of eggs/hen/day); hen mortality (percentage); and virus positive eggshell surface, yolk, and albumin samples (percentage positive for virus) for sham-vaccinated and H5-vaccinated-Once and Twice hens challenged with A/chicken/Pennsylvania/1370/83 (H5N2) HPAIV.

prevention of mortality has been the most frequent measure of protection reported in HPAIV challenge studies [16]. In our study, H5-vaccinated groups had only 6% mortality compared to the 100% mortality in non-/sham-vaccinated groups. The two H5-vaccinated-Once hens that died had low pre-challenge HI titers of 16. However, similar low HI titers were not a consistent negative predictor of mortality as seven hens with titers of 8 or 16 survived virulent challenge. Previously, HI titers ≥ 8 was associated with protection from mortality in H5-vaccinated young chickens when challenged with antigenically related H5 HPAIV, but some vaccinated chickens that lacked HI antibodies were also protected from virulent challenge [14,17]. Another study suggested that HI values were not predictive of protection; however, much of the data was generated using antigenic variant challenge viruses that arose after usage of vaccine in the field for several years generating field viruses resistant to classic H5 vaccines [12].

H5-vaccinated-Once or H5-vaccinated-Twice groups did maintain egg production as compared to the complete cessation of egg production in non-/sham-vaccinated groups by 4 DPC. H5-vaccinated hens had a significant decrease in number of virus positive eggs (28%) compared to non-/sham-vaccinated hens (53%) and, the mean infectious titer of HPAIV-positive eggs was lower in eggs laid by H5-vaccinated groups ($10^{1.70}$, $10^{1.38}$ and $10^{1.50}$ EID_{50}/mL) compared to non-/sham-vaccinated groups ($10^{2.99}$, $10^{3.01}$ and $10^{2.17}$ EID_{50}/mL) for eggshell surface, albumin and yolk, respectively. Specifically, the differences in titers for albumen and yolk in all three treatment groups were not statistically significant in experiment 2. One previous experiment had higher rates of virus recovery from eggs (62.5%, 36.4% and 23.1% of eggs) laid by H5-vaccinated broiler breeder hens infected with H5N1 HPAIV than we observed (39%, 24% and 19% of eggs) [12]. The mean virus titer in the infected broiler breeder eggs were higher for eggshell ($10^{6.8}$, $10^{5.1}$, and $10^{4.5}$ EID_{50}/mL), but similar for albumin (10^{3} and $10^{2.4}$ EID_{50}/mL) and yolk ($10^{2.7}$ EID_{50}/mL) samples compared to samples in our study when converting the genome equivalent copies to EID_{50} using standard infectious virus curves [12,18] (D.E. Swayne, unpublished data); i.e. approximately 58 GEC are equivalent to 1 EID_{50}. The similarity of titers in infected eggs from H5-vaccinated broiler breeder hens and WL hens in the current study, suggests commonality in systemic immunity and protection between these two different chicken types. However, the higher eggshell titers from broiler breeder eggs suggests either greater fecal contamination as eggs pass through the cloaca, the terminal canal shared by both digestive and reproductive tracts, or greater replication in epithelium of the oviduct, specifically in the shell-gland and/or vagina [19]. Previously, H5N2 HPAIV quantity in feces from infected hens varied between 10^{7-9} EID_{50}/g of feces, and resulted in eggshell contamination during laying process [15,6].

Although most of the parameters indicated that H5-vaccinated-Once and H5-vaccinated-Twice hens had equal protection from HPAIV challenge, serological data suggested that protection, measured by lack of an amnestic response (≥ 3 fold rise in titer) post challenge, was better in H5-vaccinated-Twice compared to H5-vaccinated-Once hens; i.e. amnestic response was seen in all H5-vaccinated-Once hens but in only 27% of H5-vaccinated-Twice hens (unpublished data). The initial HI serological titers in H5-vaccinated-Once hens were low (GMT = 27), but booster vaccination increased the HI titers (GMT = 330). However, even at these high titers, direct upper respiratory challenge only reduced and did not prevent infection of the reproductive tracts as evident by isolation of virus from 28% of eggs laid by H5-vaccinated hens. However, no HPAIV was recovered from eggs laid by H5-vaccinated hens after 4 DPC.

A recent case report of H5N1 HPAI on an Egyptian thrice vaccinated WL farm exhibited 20% decrease in egg production with abnormal eggs, and 27% mortality over 12 days, resulted from infection by a mutant field virus that escaped vaccinal-induced immunity [20]. Interestingly, the two chicken flocks with low HI antibody titers (GMT = 3.7 and 9.2) had HPAI, but the two flocks with high HI titers (GMT = 97 and 128) were unaffected. In our study, WL hens with HI titers ≤ 16 had a significantly higher frequency (76% virus recovery; $P < 0.0001$) of virus recovery from shell surface or

internal contents than eggs from chickens with HI titers ≥ 32 (29% virus recovery). However, having HI titers ≥ 32, including as high as 512, did not prevent infection and deposition of virus in eggs. Natural exposure may occur with a lower quantity of virus, thus preventing population infection as occurred in chicken in the two houses with high HI titers [20]. However, antigenic variants of H5N1 HPAIV have arisen in Indonesia, Egypt, China and Vietnam that have infected and caused significant mortality in flocks of broiler breeder and WL chickens with high HI titers and high population immunity [10]. These antigenic variants killed well-immunized chickens in experimental studies using classic H5 vaccines in chickens and included deposition of HPAIV in eggs laid by clinically-unaffected vaccinated chickens [10,12,21]. Analysis of HI titers as predictor of protection may need assessment of sera against both HI antigen from the vaccine and circulating field strains.

Acknowledgements

This research was supported by Cooperative Research Information System (CRIS) Project No. 6612-32000-048. The authors thank Kira Moresco and James Doster for excellent technical assistance.

References

[1] Kuiken T, Van Den Brand J, van RD, Pantin-Jackwood M, Swayne DE. Comparative pathology of select agent influenza a virus infections. Vet Pathol 2010;47:893–914.
[2] Elbers ARW, Koch G, Bouma A. Performance of clinical signs in poultry for the detection of outbreaks during the avian influenza A (H7N7) epidemic in The Netherlands in 2003. Avian Pathol 2005;34:181–7.
[3] Zanella A, Dall'Ara P, Martino PA. Avian influenza epidemic in Italy due to serovar H7N1. Avian Dis 2001;45:257–61.
[4] Cappucci Jr DT, Johnson DC, Brugh M, Smith TM, Jackson CF, Pearson JE, et al. Isolation of avian influenza virus (subtype H5N2) from chicken eggs during a natural outbreak. Avian Dis 1985;29:1195–200.
[5] Swayne DE, Beck JR. Heat inactivation of avian influenza and Newcastle disease viruses in egg products. Avian Pathol 2004;33:512–8.
[6] Bean WJ, Kawaoka Y, Wood JM, Pearson JE, Webster RG. Characterization of virulent and avirulent A/Chicken/Pennsylvania/83 influenza viruses: potential role of defective interfering RNAs in nature. J Virol 1985;54:151–60.
[7] Moses HE, Brandley CA, Jones EE. The isolation and identification of fowl plague virus. Am J Vet Res 1948;9:314–28.
[8] Eggert D, Thomas C, Spackman E, Pritchard N, Rojo F, Bublot M, et al. Characterization and efficacy determination of commercially available Central American H5N2 avian influenza vaccines for poultry. Vaccine 2010;28:4609–15.
[9] Bublot M, Pritchard N, Cruz J, Mickle TR, Selleck P, Swayne DE. Efficacy of a fowlpox-vectored avian influenza H5 vaccine against Asian H5N1 highly pathogenic avian influenza virus challenge. Avian Dis 2007;51: 498–500.
[10] Swayne DE, Kapczynski DR. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunol Rev 2008;225:314–31.
[11] Beato MS, Toffan A, De Nardi R, Cristalli A, Terregino C, Cattoli G, et al. A conventional, inactivated oil emulsion vaccine suppresses shedding and prevents viral meat colonisation in commercial (Pekin) ducks challenged with HPAI H5N1. Vaccine 2007;25:4064–72.
[12] Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM. Multiple dose vaccination with heterologous H5N2 vaccine: immune response and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens. Vaccine 2011;29:6219–25.
[13] Swayne DE, Senne DA, Suarez DL. Avian influenza. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR, editors. Isolation and identification of avian pathogens. Jacksonville, FL: American Association of Avian Pathologist; 2008. p. 128–34.
[14] Swayne DE, Beck JR, Garcia M, Stone HD. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathol 1999;28:245–55.
[15] Beard CW, Brugh M, Johnson DC. Laboratory studies with the Pennsylvania avian influenza viruses (H5N2). Proc US Anim Health Assoc 1984;88:462–73.
[16] Swayne DE, Kapczynski DR. Vaccines, vaccination and immunology for avian influenza viruses in poultry. In: Swayne DE, editor. Avian influenza. Ames, IA: Blackwell Publishing; 2008. p. 407–51.
[17] Kumar M, Chu HJ, Rodenberg J, Krauss S, Webster RG. Association of serologic and protective responses of avian influenza vaccines in chickens. Avian Dis 2007;51:481–3.
[18] Fereidouni SR, Starick E, Beer M, Wilking H, Kalthoff D, Grund C, et al. Highly pathogenic avian influenza virus infection of mallards with homo- and heterosubtypic immunity induced by low pathogenic avian influenza viruses. PLoS One 2012;4:e6706.
[19] Capua I, Mutinelli F. A color atlas and text on avian influenza. Bologna: Papi Editore; 2001. p. 1–287.
[20] Kilany WH, Arafa A, Erfan AM, Ahmed MS, Nawar AA, Selim AA, et al. Isolation of highly pathogenic avian influenza H5N1 from table eggs after vaccinal break in commercial layer flock. Avian Dis 2010;54:1115–9.
[21] Grund C, Abdelwhab E, Arafa AS, Ziller M, Hassan MK, Aly MM, et al. Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. Vaccine 2011;29:5567–73.

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The role of vaccines and vaccination in high pathogenicity avian influenza control and eradication

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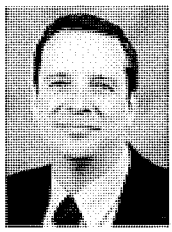

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"The eradication of H5N1 high pathogenicity avian influenza has been more complex than the other 29 high pathogenicity avian influenza epizootics because it has involved multiple countries, affected more diverse types of poultry production systems, infected wild birds and had a major public health element."

Thirty epizootics of high pathogenicity avian influenza (HPAI) or fowl plague have occurred in poultry and other birds in the world since influenza virus was discovered to be the etiological agent of the disease in 1955 [1]. The largest epizootic of the past 50 years was the H5N1 panzootic, which began in the Guangdong province of southern China in 1996 and has since spread to affect 63 countries, resulting in the death or culling of over 250 million poultry and wild birds. Twenty four epizootics were eradicated using a four-component stamping-out strategy that included education, biosecurity, rapid diagnostics and surveillance, and elimination of infected birds. However, the inability of the stamping-out strategy to eradicate H5N2 HPAI in Mexico and H7N3 HPAI in Pakistan during 1994–1995 challenged the dogma of immediate eradication being achievable under all national political structures, poultry production systems, economic conditions and HPAI outbreak situations using traditional eradication methods [2]. Therefore, Mexico and Pakistan initiated the use of a fifth control component, increasing resistance of poultry to HPAI viruses by vaccination, which had been used previously to control low pathogenicity avian influenza (AI) in the USA and Italy [3–6]. More recently, vaccines for HPAI control have been utilized in the Democratic People's Republic of Korea against H7N7 HPAI (2005) and in 13 of the 63 countries affected with H5N1 HPAI (1996 to present) [2]. Although the H5N1 HPAI panzootic continues, the H5N2 Mexican, H7N3 Pakistani and H7N7 Korean HPAI epizootics have ended. The recent Chinese Taipei (H5N2; 2012) and South African (H5N2; 2011) epizootics are nearing eradication using stamping-out programs only.

The ability to eradicate HPAI is influenced by the competency of the governmental veterinary medical authority, the density of poultry within the country and the level of governmental participation [7,8]. The eradication of H5N1 HPAI has been more complex than the other 29 HPAI epizootics because it has involved multiple countries, affected more diverse types of poultry production systems, infected wild birds and had a major public health element. The latter aspect has involved 607 hospitalized cases with 358 deaths, and the H5N1 HPAI virus has the potential to become a future pandemic virus [101]. This public health aspect resulted in mobilization of a global effort to eradicate H5N1 HPAI from poultry, but the veterinary infrastructure and finances have not been adequate to conduct an effective stamping-out campaign in all countries [8]. Therefore, vaccination was administered in order to decrease bird susceptibility to infection,

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KEYWORDS: avian influenza • birds • H5N1 • poultry • vaccination

 10.1586/ERV.12.60 © 2012 Expert Reviews Ltd ISSN 1476-0584

reduce the number of birds infected and their subsequent deaths, and decrease the amount of HPAI virus shed into the environment, thereby reducing transmission to both birds and humans. This new strategy has prevented disease and death in poultry and allowed maintenance of rural livelihoods and food security within affected countries [9,10]. Such a strategy has bought time to restructure poultry production systems and veterinary services to work towards a long-term goal of H5N1 HPAI eradication. Vaccines have been primarily used in some developing and transition countries, but with less usage in developed countries.

Between 2002 and 2010, 113.98 billion doses of AI vaccine were used to protect an at-risk national poultry population of 131 billion (41.9% coverage rate) in 15 countries from HPAI and were comprised of 95.5% inactivated vaccines administered by injecting individual birds and 4.5% live recombinant vectored vaccines administered by mass application – that is, spray [2]. The majority were used in four countries affected by enzootic H5N1 HPAI and were applied in nationwide campaigns to protect poultry in commercial, semicommercial and village production systems. China, the world's largest poultry producer and consumer, has used the most H5 vaccine (103.72 billion doses; 90.99%), followed by Egypt (5 billion doses; 4.65%), Indonesia (2.6 billion doses; 2.32%) and Vietnam (1.6 billion doses; 1.43%). Vaccine usage began after H5N1 HPAI became enzootic in poultry populations. The remaining ten countries or regions had minor usage of vaccine, accounting for less than 0.7% of the total, and the vaccine was used in targeted vaccination programs to high-risk poultry either as a preventative measure, or as a management tool during eradication. The percentage of vaccine usage in least developed, developing/transition and developed countries is as follows: Russia (0.37%), Pakistan (0.12%), Hong Kong Special Administrative Region (0.08%), Kazakhstan (0.03%), Côte d'Ivoire (<0.01%), Democratic People's Republic of Korea (<0.01%; H7N7 HPAI only), France (<0.01%), Israel (<0.01%), Mongolia (<0.01%), The Netherlands (<0.01%) and Sudan (<0.01%).

> "...since 2006, some field viruses in H5N1 enzootic countries have not been protected by one or more H5 vaccines."

Vaccine for the H5N1 panzootic was initially administered in Hong Kong (2002), followed by Indonesia (2003) and Mainland China (2004). Initially, poor vaccine quality and inadequate manufacturing capacity were major limitations to implementing effective field vaccination programs, but vaccines have improved in quality and efficacy over past decade, with manufacturing capacity exceeding demand by the end of 2006. Currently, reserve vaccine manufacturing capacity is available, primarily in China. The available vaccines have utilized seed strains shown in multiple experimental studies to be efficacious against H5N1 field viruses [11,12]. However, since 2006, some field viruses in H5N1 enzootic countries have not been protected by one or more H5 vaccines [12–14]. This need for changes in vaccine seed strains has been met by development, licensing, manufacturing and field application of vaccines based on reverse genetic (rg) seed strains containing the hemagglutinin gene of relevant field viruses that have been altered to be low virulence. China has lead this effort with commercialization of vaccines with four different rg vaccine seed strains, and all vaccine manufactured in China since late 2006 have utilized rg seed strains [15]. In the majority of situations, the vaccine seed strains have matched the circulating field viruses and provided good protection. However, vaccination has not been a panacea for eradication of H5N1 HPAI, not because of vaccine seed strain failures, but because of inability to obtain population immunity in susceptible poultry species [16,17]. The principle issue has been logistical – that is, inability to organize and vaccinate all at-risk poultry populations, especially in the numerous small farm and village poultry, which predominate in developing countries. For example, in two mass campaigns to vaccinate village and small farm poultry in Egypt and Indonesia, only 20–40% were vaccinated. However, even in a developed country, The Netherlands, vaccination was difficult with only 0.07–0.27% of hobby poultry being vaccinated [2,18,19].

> "...vaccination has not been a panacea for eradication of H5N1 high pathogenicity avian influenza, not because of vaccine seed strain failures, but because of inability to obtain population immunity in susceptible poultry species."

Vaccine usage rates have varied in individual countries with the highest being almost 100% in Hong Kong after full implementation of vaccination in 2004 [2]. Hong Kong achieved poultry population immunity to H5N1 HPAI, which is emphasized by having only one outbreak of H5N1 (one farm) since 2004, an antigenic variant clade 2.3.4 H5N1 HPAI virus that overcame vaccinal immunity [102]. Unfortunately, China, Egypt, Vietnam and Indonesia have not consistently attained the 60–80% vaccine coverage rate necessary to achieve national population immunity. For each year, the individual country vaccination coverage rates ranged from 11.1 to 83.3%, depending on population estimates of village and small farm poultry. These production sectors are logistically the most difficult to implement vaccination programs. For example for 2006–2010, the average vaccine coverage rate in Egypt was 69.9% based on World Organization for Animal Health production numbers, but only 27.8–48.6% when based on higher small farm and village poultry estimates of the Egyptian Ministry of Agriculture [2]. By contrast, the ten nonenzootic countries/regions used targeted vaccination strategies, which translated into lower national vaccine coverage rates (<15%), but these programs have been more effective and have resulted in elimination of vaccine usage or greatly reduced usage. Although these coverage rates fall short of the 60–80% vaccine coverage rate to have national population immunity, their targeted approach to one or more subsets of poultry populations, based on risk assessment and reliable surveillance data, achieved immunity in the at-risk population:

- Eradication in France, The Netherlands, Cote d'Ivoire, Israel and Sudan followed by elimination of vaccination;
- Eradication in Hong Kong (2009) with vaccination continuing as an extra preventative measure;

- Continued preventative vaccination in Kazakhstan and Mongolia but without HPAI in poultry;
- No HPAI cases in Russia and Pakistan since 2008 with proposed cessation of vaccination.

Over the next 5 years, the highest priority needs for improving vaccines and vaccination in H5N1 HPAI enzootic control programs include:

- Restructuring and modernization of veterinary services in order to develop, implement, monitor and revise poultry vaccination programs, as well as restructuring poultry production systems to improve movement control, enhance biosecurity and better educate farmers on HPAI control and eradication;
- Improving national HPAI surveillance programs to determine geographic locations, poultry species and production sectors where the virus resides and use such data to develop targeted vaccination programs to achieve 60–80% coverage in at-risk poultry populations and provide continual feedback to modify the program as local conditions change;
- Restructuring funding for H5N1 HPAI control so that sufficient funds are available to veterinary medicine and agricultural production systems to develop effective control and eradication programs that will prevent human infection and eliminate the virus, since domestic poultry are the reservoir of this zoonotic virus, and effective control must be initiated at the virus source to eliminate the public health threat;
- Improving vaccination rates among domestic ducks, which are a large asymptomatic reservoir of H5N1 HPAI virus in Asia, by linking control of an economically significant disease such as duck virus enteritis (DVE) with H5N1 HPAI through the potential use of recombinant live DVE vaccine with H5 gene insert, thus providing protection from both DVE and H5 HPAI in a single vaccine;
- Developing new vaccine vector platforms for cost-effective vaccination of short-lived meat chickens that do not require catching and handling of individual birds (e.g., through using spray or *in ovo* application), and provide protection after a single vaccination. Recombinant Newcastle disease viruses (NDV) with H5 hemagglutinin gene insert (rNDV-AIV-H5) can be applied by spray application, but rNDV-AIV-H5 has had limited field use as a vaccine because of inhibition of a protective primary immune response by maternal or field-derived antibodies to the NDV vector, but rNDV-AIV-H5 is effective in a prime-boost two-dose vaccination program. The newly developed recombinant herpesvirus of turkeys can be applied *in ovo* or at 1 day of age in the hatchery and provide protection in meat chickens throughout their short production life using only one vaccination, but this vaccine needs extensive testing in the field to determine its utility. Development of other spray-administered viruses could provide additional vectors for delivering H5 genes if maternal antibodies and/or field-induced antibodies are lacking against the vectors within the poultry population;
- Continuing development of antigenically relevant, rg AI seed strains for use in inactivated vaccines as field viruses drift antigenically away from current AI vaccine seed strains, and these seed strains may need to be region specific as the H5N1 viruses became geographically isolated and evolve into different genetic subclades or antigenic subgroups;
- Developing a more time-responsive vaccine licensing and registration process by the national veterinary biological authorities for replaceable 'vaccine cassettes', which would allow vaccine seed strains to be updated more quickly.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

References

1 Swayne DE. Impact of vaccines and vaccination on global control of avian influenza. *Avian Dis.* 56, (2012) (In Press).

2 Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev. Sci. Tech.* 30(3), 839–870 (2011).

3 Villarreal C. Avian influenza in Mexico. *Rev. Sci. Tech.* 28(1), 261–265 (2009).

4 Halvorson DA. The control of H5 or H7 mildly pathogenic avian influenza: a role for inactivated vaccine. *Avian Pathol.* 31(1), 5–12 (2002).

5 Capua I, Marangon S. The use of vaccination as an option for the control of avian influenza. *Avian Pathol.* 32(4), 335–343 (2003).

6 Naeem K, Siddique N. Use of strategic vaccination for the control of avian influenza in Pakistan. *Dev. Biol. (Basel)* 124, 145–150 (2006).

7 Pavade G, Awada L, Hamilton K, Swayne DE. Analysis of economic indicators, poultry density and performance of veterinary services for control of high pathogenicity avian influenza in poultry. *OIE Rev. Sci. Tech.* 30(3), 661–671 (2011).

8 Sims LD. Lessons learned from Asian H5N1 outbreak control. *Avian Dis.* 51(Suppl. 1), 174–181 (2007).

9 Sims LD. Experience in control of avian influenza in Asia. *Dev. Biol. (Basel)* 130, 39–43 (2007).

10 Lubroth J, Morzaria S, Thiermann A. Global strategy for highly pathogenic avian influenza: progressive control and eradication, and postoutbreak recovery. In: *Avian Influenza.* Swayne DE (Ed.). Wiley-Blackwell Publishing, IA, USA, 561–585 (2008).

11 van den Berg T, Lambrecht B, Marche S, Steensels M, Van BS, Bublot M. Influenza vaccines and vaccination strategies in birds. *Comp. Immunol. Microbiol. Infect. Dis.* 31(2–3), 121–165 (2008).

12 Swayne DE, Kapczynski D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. *Immunol. Rev.* 225, 314–331 (2008).

13 Cattoli G, Milani A, Temperton N *et al.* Antigenic drift in H5N1 avian influenza virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. *J. Virol.* 85(17), 8718–8724 (2011).

14 Grund C, Abdelwhab el-SM, Arafa AS *et al.* Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. *Vaccine* 29(33), 5567–5573 (2011).

15 Chen H. Avian influenza vaccination: the experience in China. *Rev. Sci. Tech.* 28(1), 267–274 (2009).

16 Peyre M, Samaha H, Makonnen YJ *et al.* Avian influenza vaccination in Egypt: limitations of the current strategy. *J. Mol. Genet. Med.* 3(2), 198–204 (2009).

17 Peyre M, Fusheng G, Desvaux S, Roger F. Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience. *Epidemiol. Infect.* 137(1), 1–21 (2009).

18 Mariner J. Final Report: Operational Research in Indonesia for More Effective Control of Highly Pathogenic Avian Influenza. International Livestock Research Institute, Nairobi, Kenya, 1–154 (2011).

19 Government of Egypt. *Integrated National Plan for Avian and Human Influenza. Animal Health and Livelihood Sustainability Strategy.* Ministry of Agriculture and Land Reclamation, Cairo, Egypt, 1–27 (2010).

Websites

101 WHO. Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003–2012. www.who.int/influenza/human_animal_interface/EN_GIP_20120706CumulativeNumberH5N1cases.pdf

102 Highly pathogenic avian influenza, Hong Kong (SAR-PRC). http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=7609

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Evaluation of different embryonating bird eggs and cell cultures for isolation efficiency of avian *Influenza A Virus* and Avian paramyxovirus serotype 1 from real-time reverse transcription polymerase chain reaction –positive wild bird surveillance samples

Kira A. Moresco, David E. Stallknecht and David E. Swayne

J VET Diagn Invest 2012 24: 563
DOI: 10.1177/1040638712440991

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Evaluation of different embryonating bird eggs and cell cultures for isolation efficiency of avian *Influenza A Virus* and Avian paramyxovirus serotype 1 from real-time reverse transcription polymerase chain reaction–positive wild bird surveillance samples

Kira A. Moresco, David E. Stallknecht, David E. Swayne[1]

Abstract. Virus isolation rates for *Influenza A Virus* (FLUAV) and Avian paramyxovirus serotype 1 (APMV-1) from wild bird surveillance samples are lower than molecular detection rates for the specific viral genomes. The current study was conducted to examine the possibility of increased virus isolation rates from real-time reverse transcription polymerase chain reaction (real-time RT-PCR) using alternative virus isolation substrates such as embryonating duck eggs (EDEs), embryonating turkey eggs (ETEs), Madin–Darby canine kidney (MDCK) cell cultures, and African green monkey kidney (Vero) cell cultures. Rectal swabs of birds in the orders *Anseriformes* and *Charadriiformes* were tested by real-time RT-PCR for the presence of FLUAV and APMV-1 genomes, and virus isolation (VI) was attempted on all real-time RT-PCR–positive samples. Samples with threshold cycle (Ct) ≤37 had VI rates for FLUAV of 62.5%, 50%, 43.8%, 31.5%, and 31.5% in embryonating chicken eggs (ECEs), ETEs, EDEs, MDCK cells, and Vero cells, respectively. A higher isolation rate was seen with ECEs compared to either cell culture method, but similar isolation rates were identified between the different embryonating avian eggs. Virus isolation rates for APMV-1 on samples with real-time RT-PCR Ct ≤37 were 75%, 100%, 100%, 0%, and 37.5% in ECEs, ETEs, EDEs, MDCK cells, and Vero cells, respectively. Significantly higher VI rates were seen with ECEs as compared to either cell culture method for all real-time RT-PCR–positive samples. Because of the limited availability and high cost of ETEs and EDEs, the data support the continuing usage of ECEs for primary isolation of both FLUAV and APMV-1 from real-time RT-PCR–positive wild bird surveillance samples.

Key words: Avian embryo; avian influenza; real-time polymerase chain reaction.

Wild birds are the reservoir for *Influenza A Virus* (FLUAV; family Orthomyxoviridae, genus *Influenzavirus A*) and Avian paramyxoviruses (APMV; family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Avulavirus*). Influenza A virus can be categorized as 1 of 16 different hemagglutination subtypes (H1–H16) in combination with 1 of 9 different neuraminidase subtypes (N1–N9).[14] Avian paramyxovirus can be categorized as 1 of 9 serotypes, of which serotype 1 is the most common (APMV-1), with *Newcastle disease virus* strains being the virulent form of this serotype (i.e., intracerebral pathogenicity index of ≥0.7).[1–3] At least 2 primary wild aquatic bird reservoirs for low pathogenicity avian influenza (LPAI) viruses exist in nature: birds in the order *Anseriformes*, which are composed of ducks, geese, and swans, and birds in the order *Charadriiformes*, which are composed of gulls, terns, and waders.[4,12] Virus isolates of APMV-1 have frequently been obtained from migratory waterfowl and aquatic birds, but infections have been identified in at least 241 species of birds.[3] *Influenza A virus* and APMV-1 have been transmitted from wild birds to domestic poultry, causing various outcomes ranging from asymptomatic infections to mild or even severe disease. The most frequently infected poultry species with LPAI viruses from wild birds are domestic ducks. Turkeys are the most frequently infected poultry species in the order *Galliformes*, and chickens have a lower frequency, based on population

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numbers, of infection with wild bird LPAI viruses. This information raises the question of whether embryonating duck or turkey eggs might be more efficient at isolating LPAI viruses from wild bird samples.

The standard method for virus isolation (VI) of FLUAV and APMV-1 is inoculation of sample material into the chorioallantoic sac (CAS) of embryonating chicken eggs (ECEs), but these viruses have been isolated using multiple types of cell cultures including the commonly used Madin–Darby canine kidney (MDCK) and African green monkey kidney (Vero) cell cultures. Influenza A virus and APMV-1 both replicate in ECEs and hemagglutinate avian and mammalian erythrocytes.[2,13,14] Identification of both viruses can be accomplished with standard hemagglutination inhibition (HI) assays (Carbrey EA, Beard CW, Cooper RJ, et al.: 1974, Hemagglutination and hemagglutination-inhibition tests with Newcastle disease virus: microtiter technique. *In:* Proceedings of the 17th annual meeting of the American Association of Veterinary Laboratory Diagnosticians, pp. 1–6, Roanoke, Virginia), antigen detection, or molecular-based assays such as real-time reverse transcription polymerase chain reaction (real-time RT-PCR).[5,9,10,15] Real-time RT-PCR is commonly used to screen wild and domestic bird samples to reduce the number of samples entered into the more labor- and resource-intensive VI methods. However, the most efficient method for VI is unknown. The purpose of the current study was to determine if 4 additional VI methods were more efficient than standard ECEs for isolating FLUAV and APMV-1 from real-time RT-PCR–positive (real-time RT-PCR+) wild bird surveillance samples. Virus isolation was performed on all real-time RT-PCR+ samples using ECEs, embryonating duck eggs (EDEs), embryonating turkey eggs (ETEs), and MDCK and Vero cell cultures.

Cloacal swabs were collected from 197 wild aquatic birds in Minnesota, North Dakota, New Jersey, and Delaware from 2003 to 2008; 50.8% ($n = 100$) were obtained from the order *Charadriiformes* (ruddy turnstone, *Arenaria interpres*) and 49.2% ($n = 97$) from *Anseriformes* consisting of the mallard (*Anas platyrhynchos*, $n = 58$), blue-winged teal (*Anas discors*, $n = 28$), green-winged teal (*Anas carolinensis*, $n = 1$), wood duck (*Aix sponsa*, $n = 4$), gadwall (*Anas strepera*, $n = 1$), ring-necked duck (*Aythya collaris*, $n = 1$), and ruddy duck (*Oxyura jamaicensis*, $n = 1$). Samples were stored in 2.0 ml of brain heart infusion media and frozen at –80°C until processed.

Wild bird samples were tested using real-time RT-PCR directed to the FLUAV matrix gene and a duplex real-time RT-PCR directed to APMV-1 matrix and polymerase genes. Real-time RT-PCR was performed as previously described[5,9,10,15] using 100 µl of original sample material. Real-time RT-PCR+ samples for FLUAV, APMV-1, and mixed infections were inoculated into 10-day-old specific pathogen–free (SPF) ECEs, 12-day-old SPF ETEs, and 12-day-old FLUAV antibody–negative EDEs. One hundred microliters of sample material was injected into the CAS of 3 eggs using standard methods, and the embryos were incubated for 4 days at 37°C.[14] Amnioallantoic fluid (AAF) from all embryos was harvested for hemagglutination (HA) testing using 0.5% chicken erythrocytes (Carbrey EA, et al.: 1974, Hemagglutination and hemagglutination-inhibition tests with Newcastle disease virus). Amnioallantoic fluid from dead embryos was harvested separately; AAF from live embryos was harvested and pooled. If HA negative, 200 µl of AAF was reinoculated into CAS for a second isolation attempt by the previously described method.[14] For the third VI attempt, 200 µl of AAF was inoculated into the yolk sac of 6-day-old ECEs and 8-day-old ETEs and EDEs and allowed to incubate for 4 days at 37°C. The yolk sac membrane and embryo were collected and forcefully passed through a syringe. Contents were centrifuged at 200 × *g* for 10 min; supernatant was collected and tested for HA activity.[6,16]

Real-time RT-PCR+ samples for FLUAV and APMV-1 were inoculated into MDCK and Vero cell cultures for 3 isolation attempts. Cells were seeded into 96-well cell culture plates with 1× Dulbecco modified Eagle medium (DMEM), 5% fetal bovine serum, and antibiotic and antimycotic solution (10,000 U/ml of penicillin G, 10,000 µg/ml of streptomycin, 25 µg/ml of amphotericin B) and allowed to monolayer overnight at 37°C and 5% CO_2. The monolayers were washed twice with sterile phosphate buffered saline and inoculated with 20 µl of original sample into each of 3 wells and allowed to incubate for 45 min at 37°C. After incubation, 150 µl of 1× DMEM supplemented with antibiotic/antimycotic solution was added. Infected cells were allowed to incubate for 4 days. Cells were frozen at –80°C and thawed. Fifty microliters of supernatant was harvested for HA testing (Carbrey EA, et al.: 1974, Hemagglutination and hemagglutination-inhibition tests with Newcastle disease virus), and an additional 20 µl of supernatant was used as inoculum for the second VI attempt. This method was repeated for a total of 3 VI attempts. Any sample with positive HA results was grown to a volume sufficient for differential diagnostic testing.

Samples with positive HA results were identified as FLUAV using a commercial antigen-capture enzyme-linked immunosorbent assay[a] or APMV by standard HI assay (Carbrey EA, et al.: 1974, Hemagglutination and hemagglutination-inhibition tests with Newcastle disease virus). The APMV serotype was determined using reference antigen/antiserum combinations for APMV serotypes 1–4, 6, and 7. Statistical comparisons were made using statistical software.[b] Data were organized into 2 × 2 contingency tables and analyzed for significant differences in VI using the Fisher exact test; statistical significance was set to $P < 0.05$. The results for different VI methods were compared to each other for all real-time RT-PCR+ samples and real-time RT-PCR+ samples with threshold cycle (Ct) ≤37.

There were 23.4% ($n = 46$) of samples that were real-time RT-PCR+ for the FLUAV matrix gene; 54.3% ($n = 25$) were

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Table 1. Virus isolation results for *Influenza A virus* (FLUAV) and Avian paramyxovirus serotype 1 (APMV-1) in embryonating chicken eggs (ECEs), embryonating turkey eggs (ETEs), embryonating duck eggs (EDEs), Madin–Darby canine kidney (MDCK) cells, and African green monkey kidney (Vero) cells.*

	FLUAV VI results per passage				APMV-1 VI results per passage			
	Passage 1 (CAS)	Passage 2 (CAS)	Passage 3 (YS)	Total	Passage 1 (CAS)	Passage 2 (CAS)	Passage 3 (YS)	Total
ECE	7	2	1	10	7	2	0	9
ETE	6	2	0	8	8	2	1	11
EDE	6	2	0	8	8	4	0	12
MDCK	5	–	–	5	–	–	–	–
Vero	4	1	–	5	4	1	–	5

* One hundred ninety-seven cloacal swabs from wild aquatic birds were screened by real-time reverse transcription polymerase chain reaction assays, and the FLUAV-positive ($n = 46$) and APMV-I–positive ($n = 17$) samples were subjected to virus isolation (VI) attempts. CAS = chorioallantoic sac; YS = yolk sac; – = negative result.

obtained from *Anseriformes* and 45.6% ($n = 21$) obtained from *Charadriiformes*. The Ct values ranged from 29.39 to 43.32. The FLUAV VI results for ECEs, ETEs, EDEs, and MDCK and Vero cell cultures are reported in Table 1. The majority of the VI occurred in the real-time RT-PCR samples with Ct ≤37 ($n = 16$). With real-time RT-PCR samples with Ct ≤37, ECEs, ETEs, EDEs, and MDCK and Vero cells had VI rates of 62.5% ($n = 10$), 50% ($n = 8$), 43.8% ($n = 7$), 31.5% ($n = 5$), and 31.5% ($n = 5$), respectively. For Ct >37 ($n = 30$), ECEs, ETEs, and MDCK and Vero cells had a VI rate of 0.0% ($n = 0$), and EDEs had a VI rate of 3% ($n = 1$). The ECEs supported the growth of 3 FLUAV isolates not recovered using ETEs and EDEs. The ETEs supported the growth of 1 FLUAV isolate not recovered using ECEs or EDEs, and EDEs supported the growth of 1 FLUAV isolate not recovered using ECEs and ETEs. Overall, for the 5 VI methods, there was no Ct value below which there was 100% predictable isolation of FLUAV (i.e., low Ct value always correlating with positive VI). The ECEs had higher VI rates than the MDCK and Vero cell cultures for all real-time RT-PCR+ samples as well as those samples with Ct values ≤37 (Table 2) that were close to statistical significance, possibly due to small virus-positive rates. No significant differences in isolation rates were detected in the pairwise comparisons between the other VI methods.

For the duplex APMV-1 real-time RT-PCR, 8.6% ($n = 17$) were positive for class I and II APMV-1; 47.2% ($n = 8$) were from *Anseriformes*, and the remaining 52.9% ($n = 9$) were from *Charadriiformes*. The Ct values ranged from 28.19 to 39.85. The APMV VI results for ECEs, ETEs, EDEs, and MDCK and Vero cell cultures are reported in Table 1. The majority of the VIs occurred from real-time RT-PCR samples with Ct values ≤37 ($n = 8$, 47%). The APMV-1 isolation rates for ECEs, ETEs, EDEs, and MDCK and Vero cells were 75% ($n = 6$), 100% ($n = 8$), 100% ($n = 8$), 0%, and 37.5% ($n = 3$), respectively. For Ct values >37 ($n = 9$), ECEs and ETEs both had a VI rate of 33% ($n = 3$), EDEs had a VI rate of 44% ($n = 4$), and Vero cells had a VI rate of 22.2% ($n = 2$). The ECEs supported the growth of 1 APMV-1 isolate not recovered using ETEs or EDEs. The ETEs supported the growth of 3 APMV-1 isolates not recovered using ECEs or EDEs. The EDEs supported the growth of 1 APMV-1 isolate not isolated in ECEs or ETEs. Overall, for the 5 VI methods, there was no Ct value below which there was predictable isolation of an APMV-1 isolate. For all real-time RT-PCR+ samples, significantly higher rates of APMV-1 isolation were seen with ECEs, ETEs, and EDEs as compared to either cell culture method (Table 3), but no significant differences in VI rates were observed in pairwise comparisons between embryonating avian eggs. Vero cell cultures had significantly higher VI rates than MDCK cell cultures, as no APMV-1 was isolated by this latter method for all sample sets that were real-time RT-PCR+. For samples with Ct values ≤37, significantly higher VI rates were seen with ECEs, ETEs, and EDEs compared to MDCK cell cultures and near significant (ECE) or significant (ETE and EDE) against Vero cell cultures.

Table 2. Statistical test results (P values) for embryonating chicken eggs (ECEs), embryonating turkey eggs (ETEs), embryonating duck eggs (EDEs), Madin–Darby canine kidney (MDCK) cells, and African green monkey kidney (Vero) cells for isolation of *Influenza A Virus* from all real-time reverse transcription polymerase chain reaction (RT-PCR)–positive (lower left numbers) or real-time RT-PCR–positive samples with threshold cycle values ≤37 (upper right numbers).

	ECE	ETE	EDE	MDCK	Vero
ECE	1.00	0.36	0.24	0.08	0.08
ETE	0.40	1.00	0.50	0.24	0.24
EDE	0.40	0.60	1.00	0.36	0.36
MDCK	0.13	0.28	0.28	1.00	1.00
Vero	0.13	0.28	0.28	1.00	1.00

In poultry, FLUAV infections are most frequent in domestic ducks, which are the same species as wild mallards (i.e., *A. platyrhynchos*), followed by domestic turkeys and

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Table 3. Statistical test results (P values) for embryonating chicken eggs (ECEs), embryonating turkey eggs (ETEs), embryonating duck eggs (EDEs), Madin–Darby canine kidney (MDCK) cells, and African green monkey kidney (Vero) cells for isolation of Avian paramyxovirus serotype 1 from all real-time reverse transcription polymerase chain reaction (RT-PCR)–positive (lower left numbers) or real-time RT-PCR–positive samples with threshold cycle values ≤37 (upper right numbers).

	ECE	ETE	EDE	MDCK	Vero
ECE	1.00	0.23	0.23	0.00*	0.16
ETE	0.50	1.00	1.00	<0.01*	0.01*
EDE	0.50	1.00	1.00	<0.01*	0.01*
MDCK	<0.01*	<0.01*	<0.01*	1.00	0.02*
Vero	0.04*	0.02*	0.02*	0.12	1.00

* Significantly different ($P < 0.05$).

least frequent in chickens when compared to the relative populations of global production for each species. Because the VI of wild bird surveillance samples in ECEs consistently yields fewer viable virus isolates than are detected by real-time RT-PCR, the possibility of increasing the rate of VI using ETEs and EDEs, and 2 common cell culture methods, was investigated.

The real-time RT-PCR assays directed to the FLUAV matrix gene, and the APMV matrix and polymerase genes, are commonly used as prescreen tools to reduce the number of samples selected to continue into laborious and time-consuming VI methods.[11] Real-time RT-PCR assays detected FLUAV matrix gene in 23.4% (n = 46) of samples, from which ECEs produced viable isolates in 21.7% (n = 10), and ETEs and EDEs produced isolates in 17.4% (n = 8), but samples with high Ct values (>37) rarely yielded virus isolates. When restricting VI attempts to Ct values ≤37, the success rate rose to 62.5%, 50%, and 43.8% for ECEs, ETEs, and EDEs, respectively. Real-time RT-PCR detected APMV-1 matrix and polymerase genes in 8.5% (n = 17) of samples, from which ECEs produced viable isolates in 52.9% (n = 9), ETEs in 58.8% (n = 11), and EDEs in 70.5% (n = 12) of real-time RT-PCR+ samples. When restricting VI attempts to Ct ≤37, the success rate rose to 75% for ECEs and 100% for both ETEs and EDEs, respectively. Other studies report similar findings related to VI rate and Ct values. A 2008 report described that, when Ct values >37 were used as the cutoff for VI attempts, only 3.5% of virus isolates would have been missed.[8] A previous report noted increased VI from samples with Ct values of 33 ± 2 as well as an occasional VI-positive result in samples with Ct values of 36.[7,9] Focusing VI attempts to samples with lower Ct values would save time and reduce resources for the isolation of viruses, especially for FLUAV. The low isolation rate for samples with >37 Ct values could be from the presence of nonviable viruses, resulting from the failure to maintain a cold chain from the collection site to the laboratory, improper maintenance of the temperature, or multiple freeze-thaw cycles in processing the material in the laboratory. For APMV-1, the isolation rate was higher than for FLUAV, possibly because of a lower Ct range for APMV-1 compared to FLUAV.

There were no significant differences in the numbers of FLUAV isolated when utilizing ECEs, EDEs, or ETEs on all real-time RT-PCR+ samples or restricting VI attempts to only samples with real-time RT-PCR+ Ct values of ≤37, although ECEs yielded 3 additional isolates not recovered with ETEs or EDEs. Similarly, VI results for APMV-1 show there were no significant differences in isolation frequency when utilizing ECEs, EDEs, and ETEs, irrespective of comparing all real-time RT-PCR+ samples or limiting to just samples with Ct values ≤37. The ETEs yielded 3 isolates not recovered with ECEs or EDEs. In addition, ETEs and EDEs yielded APMV-1 isolates on all samples with Ct values ≤37. The ETEs and EDEs may be reasonable alternatives for VI attempts when ECEs fail to isolate APMV-1 on samples with real-time RT-PCR Ct values ≤37.

Overall, there was reduced VI efficiency for samples with Ct values >37. While there was no definite predictive VI outcome associated with a specific range of Ct values, the range of real-time RT-PCR Ct values should be evaluated for each study before entering into labor- and resource-intensive VI studies. The expectation of increased VI efficiency of FLUAV and APMV-1 from wild bird surveillance samples with use of ETEs and EDEs is generally impractical, except maybe as a second passage for APMV-1 real-time RT-PCR+ samples that were VI negative with ECEs. However, the VI efficiency of ECEs was similar or higher than VI efficiency using ETEs and EDEs for FLUAV in surveillance samples used in the current study.

The ECEs had significantly or near significantly higher rates of VI than either MDCK or Vero cell cultures for FLUAV or APMV-1 real-time RT-PCR+ samples. Similarly, ECEs produced higher FLUAV VI rates from wild bird cloacal swabs than produced by MDCK.[6] While VI using cell culture is a lower cost method,[6] the use of MDCK or Vero cell culture provides fewer FLUAV isolates than ECEs, and such cell culture methods would be disadvantageous unless used in a high throughput system with the goal of maximizing the number of samples for VI attempts. The ECEs remain the preferred method of FLUAV and APMV-1 VI from real-time RT-PCR+ wild bird surveillance samples.

Acknowledgements

This project was completed as partial requirement by the senior author for a Master of Science degree. Maple Leaf Farms is thanked for generously providing the high quality embryonating duck embryos. Dr. Patti Miller is thanked for APMV antigen and antiserum. Joan Beck and Tim Olivier are thanked for providing technical assistance.

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Sources and manufacturers

a. BinaxNow®, Binax Inc., Scarborough, ME.
b. GraphPad Prisma 5.0A, GraphPad Software, La Jolla, CA.

Declaration of conflicting interests

The author(s) declared that they had no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Research funding provided by ARS CRIS project no. 6612-32000-048-00D.

References

1. Alexander DJ: 1995, The epidemiology and control of avian influenza and Newcastle disease. J Comp Pathol 112:105–126.
2. Alexander DJ: 2008, Newcastle disease virus and other avian paramyxoviruses. *In*: A laboratory manual for the isolation and identification of avian pathogens, ed. Swayne DE, Glisson JR, Jackwood MW, et al., 4th ed., pp. 156–163. American Association of Avian Pathologists, Jacksonville, FL.
3. Alexander DJ, Senne DA: 2008, Newcastle disease, other avian paramyxoviruses, and pheumovirus infections. *In*: Diseases of poultry, ed. Saif YM, Fadly AM, Glisson JR, et al., 12th ed., pp. 75–115. Blackwell, Ames, IA.
4. Hanson BA, Luttrell MP, Goekjian VH, et al.: 2008, Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? J Wild Dis 44:351–361.
5. Kim LM, Suarez DL, Afonso CL: 2008, Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. J Vet Diagn Invest 20:414–425.
6. Lira J, Moresco KA, Stallknecht DE, et al.: 2010, Single and combination diagnostic test efficiency and cost analysis for detection and isolation of avian influenza virus from wild bird cloacal swabs. Avian Dis 54(1 Suppl):606–612.
7. Munster VJ, Baas C, Lexmond P, et al.: 2009, Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. J Clin Microbiol 47:666–673.
8. Slomka MJ, Pavlidis T, Banks J, et al.: 2007, Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. Avian Dis 55 (1 Suppl):373–377.
9. Spackman E, Senne DA, Bulaga LL, et al.: 2003, Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis 47(3 Suppl):1079–1082.
10. Spackman E, Senne DA, Myers TJ, et al.: 2002, Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40:3256–3260.
11. Spackman E, Suarez DL, Senne DA: 2008, Avian influenza diagnostic and surveillance methods *In*: Avian influenza, ed. Swayne DE, pp. 299–308. Blackwell, Ames, IA.
12. Stallknecht DE, Shane SM: 1988, Host range of avian influenza virus in free-living birds. Vet Res Commun 12: 125–141.
13. Swayne DE, Halvorson D: 2008, Influenza. *In*: Diseases of poultry, ed. Saif YM, Fadly AM, Glisson JR, et al., 12th ed., pp. 152–184. Blackwell, Ames, IA.
14. Swayne DE, Senne DA, Beard CW: 2008, Avian influenza. *In*: A laboratory manual for the isolation and identification of avian pathogens, ed. Swayne DE, Glisson JR, Jackwood MW, et al., 5th ed., pp. 128–134. American Association of Avian Pathologists, Jacksonville, FL.
15. Wise MG, Suarez DL, Seal BS, et al.: 2004, Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J Clin Microbiol 42:329–338.
16. Woolcock PR, McFarland MD, Lai S, Chin RP: 2001, Enhanced recovery of avian influenza virus isolates by a combination of chicken embryo inoculation methods. Avian Dis 45:1030–1035.

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Journal of Wildlife Diseases, 48(2), 2012, pp. 322–334
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AVIAN INFLUENZA VIRUS INFECTION DYNAMICS IN SHOREBIRD HOSTS

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ABSTRACT: To gain insight into avian influenza virus (AIV) transmission, exposure, and maintenance patterns in shorebirds at Delaware Bay during spring migration, we examined temporal AIV prevalence trends in four Charadriiformes species with the use of serial cross-sectional data from 2000 through 2008 and generalized linear and additive models. Prevalence of AIV in Ruddy Turnstones (*Arenaria interpres morinella*) increased after arrival, peaked in mid-late May, and decreased prior to departure. Antibody prevalence also increased over this period; together, these results suggested local infection and recovery prior to departure. Red Knots (*Calidris canutus rufa*), Sanderlings (*Calidris alba*), and Laughing Gulls (*Leucophaeus atricilla*) were rarely infected, but dynamic changes in antibody prevalence differed among species. In Red Knots, declining antibody prevalence over the stopover period suggested AIV exposure prior to arrival at Delaware Bay with limited infection at this site. Antibody prevalence was consistently high in Laughing Gulls and low in Sanderlings. Both viral prevalence and antibody prevalence in Sanderlings varied directly with those in turnstones, suggesting virus spillover to Sanderlings. Results indicate that, although hundreds of thousands of birds concentrate at Delaware Bay during spring, dynamics of AIV infection differ among species, perhaps due to differences in susceptibility, potential for contact with AIV at this site, or prior exposure. Additionally, Ruddy Turnstones possibly act as a local AIV amplifying host rather than a reservoir.

Key words: AIV, avian influenza virus, Charadriiformes, Delaware Bay, disease ecology, infection dynamics, Ruddy Turnstone (*Arenaria interpres morinella*), shorebird.

INTRODUCTION

Although Anseriformes (swans, geese, and ducks) and Charadriiformes (gulls and shorebirds) are the natural reservoirs of avian influenza viruses (AIV), our current understanding of AIV epidemiology in these reservoirs is limited by the patchy nature of surveillance across space, time, and species (Stallknecht and Brown, 2007). Among ducks, AIV prevalence typically peaks in late summer and autumn as birds, particularly naïve juveniles, aggregate on premigratory staging grounds (Olsen et al., 2006). Some species of Charadriiformes, such as gulls (family Laridae) and shorebirds (families Charadriidae and Scolopacidae), also are important in global AIV epidemiology, and certain subtypes are associated with and are particularly well adapted to gull species (Hinshaw et al., 1983; Yamnikova et al., 2003; Fouchier et al., 2005). Although transmission among ducks occurs primarily through fecally contaminated water (Hinshaw et al., 1979), transmission and maintenance among Charadriiformes species is poorly understood.

Initial studies conducted at Delaware Bay on the Atlantic coast of North America reported that AIV prevalence was 2.4–20% among sampled shorebirds and gulls in spring compared to 3.5–8% in autumn and 0% in winter and summer

(Kawaoka et al., 1988). Estimated mean springtime prevalence among shorebirds and gulls was 6.3% across 21 yr (Krauss et al., 2010). Both studies recognized one shorebird species, the Ruddy Turnstone (*Arenaria interpres morinella*), as disproportionately infected, and Hanson et al. (2008) reported that mean springtime prevalence was significantly higher among Ruddy Turnstones (11%) than among 10 other species (0.5%). Despite this consistent seasonal pattern of infection among shorebirds at Delaware Bay, AIV have not been detected more than occasionally in shorebirds at other times and locations, particularly in the western hemisphere (e.g., Escudero et al., 2008; Iverson et al., 2008; Winker et al., 2008; Ghersi et al., 2009).

An estimated >1 million shorebirds use Delaware Bay each spring (Clark et al., 1993), where, by feasting on eggs of horseshoe crabs (*Limulus polyphemus*) on spawning beaches, they rapidly gain up to 70% of their arrival body masses to fuel long-distance flights to breeding grounds in the Arctic (Robinson et al., 2003). Each year, shorebirds begin arriving in early May following migrations from wintering and stopover areas in South America and the Caribbean (Myers et al., 1990; Morrison and Harrington, 1992) and depart for breeding grounds during late May–early June (Robinson et al., 2003). The stopover period is approximately 5 wk, although individual birds can remain for shorter periods (Gillings et al., 2009). From 12% to 80% of the North American populations of six species use Delaware Bay in spring, including Red Knots (*Calidris canutus rufa*), Ruddy Turnstones, Sanderlings (*Calidris alba*), Semipalmated Sandpipers (*Calidris pusilla*), Dunlins (*Calidris alpina*), and Short-billed Dowitchers (*Limnodromus griseus*) (reviewed by US Fish and Wildlife Service, 2003). Additionally, adjacent salt marshes in New Jersey support breeding colonies of Laughing Gulls (*Leucophaeus atricilla*), Herring Gulls (*Larus argentatus*), and Great Black-backed Gulls (*Larus marinus*), and non-breeding Ring-billed Gulls (*Larus delawarensis*) that also feed on horseshoe crab eggs alongside shorebirds (Burger et al., 2007).

Delaware Bay is adjacent to the Delmarva Peninsula, an important poultry-producing region. Because transmission of AIV from wild birds to poultry occasionally occurs with appropriate contact (Spackman, 2009), understanding the scale, scope, and timing of AIV epidemics in nearby wild bird populations is important to address this risk.

Our objectives were to describe temporal patterns of AIV prevalence and antibody prevalence within three shorebird species (Ruddy Turnstones, Red Knots, and Sanderlings) and one gull species (Laughing Gulls) during the spring migratory stopover at Delaware Bay, and compare patterns between species. Such information provided insight into possible AIV sources, transmission, exposure, and maintenance patterns at this location, as well as potential exposures outside of the Delaware Bay stopover.

MATERIALS AND METHODS

Field and laboratory methods

Fieldwork was conducted during 17–24 May 2006, 10 May–3 June 2007, and 7 May–4 June 2008 at Delaware Bay (39°N, 75°W). Shorebirds and gulls were captured with cannon nets as part of long-term population studies. Following banding and measurement, swab samples for virus isolation were collected, stored, processed, and tested in embryonated chicken eggs as previously described (Hanson et al., 2008). Cloacal swabs were collected from all birds; in addition, oropharyngeal swabs were collected from Laughing Gulls in 2008 and analyzed separately (none were positive). Fresh Laughing Gull feces were swabbed in limited cases and only from within the borders of monospecific breeding colonies. Presence of AIV was confirmed by hemagglutination (Swayne et al., 1998) and reverse transcriptase polymerase chain reaction (RT-PCR) for matrix gene (Spackman and Suarez, 2008) on allantoic fluid. All isolates were low pathogenic (LPAI) viruses (unpublished data).

During 2007–2008, blood samples were collected by jugular venipuncture from a

random subset of swabbed birds. Collected volume ranged from <0.5% to 1% of a bird's body mass in grams. Samples were kept on ice in the field and sera were stored at −20 C until testing. Antibodies against AIV nucleoprotein (NP) were tested with a commercial blocking enzyme-linked immunoassay (bELISA; FlockChek AI MultiS-Screen Antibody Test Kit, IDEXX Laboratories, Westbrook, Maine, USA). Samples yielding S/N ratios of <0.5 were considered positive, per manufacturer's recommendations.

Morphometric data for individual sampled birds were obtained from the Shorebird Resighting Database (http://www.bandedbirds.org). Research was conducted under University of Georgia Animal Care and Use Committee approval and state and federal scientific collection permits.

Statistical analyses

Computations and statistical analyses were performed in JMP version 8 (SAS Institute Inc., Cary, North Carolina, USA). Virus isolation data from four species (Ruddy Turnstone, Red Knot, Sanderling, and Laughing Gull) were evaluated for temporal trends within stopover seasons. Data from 2006–2008 were pooled with results from 2000–2005 originally reported in Hanson et al. (2008). For each species, data from all 9 yr (4 yr for Laughing Gulls; 2005–2008) were used to estimate the average trend, if any. Additionally, the sampling date range spanned the majority of the stopover season in 2002, 2007, and 2008 (25, 25, and 29 days, respectively) and allowed evaluation of each of these years separately. Within a logit-link generalized linear model (GLM) framework, changes in prevalence were modeled as a linear, quadratic, or three-node knotted spline (Hastie and Tibshirani, 1990) function of date, to obtain maximum model fit. Because shorebirds are expected to gain mass over the stopover period, the above analyses were repeated with masses of sampled birds (available 2006–2008). Laughing Gulls were not weighed at the time of sampling and were excluded from these analyses. In some cases several models adequately described the prevalence trend; however, we present the single model that minimized Akaike's Information Criterion (AIC_c) (Burnham and Anderson, 2002).

We also evaluated trends in antibody prevalence over time and over mass gain in each species with the use of GLM. Because the bELISA imperfectly detects recent AIV infection in wild bird species (test sensitivity=0.754, specificity=1; Brown et al., 2009), antibody prevalence calculations were adjusted according to Rogan and Gladen (1978):

$$P=\frac{P^T+Sp-1}{Se+Sp-1},$$

where P=true antibody prevalence, P^T=test antibody prevalence, Sp=test specificity, and Se=test sensitivity. Thus, the adjusted antibody prevalence for each day or 10-g mass class (weighted by n) was used rather than raw antibody status of individual birds. Serology data were pooled by shorebird species because temporal patterns did not differ between years (year effect with day as covariate, for Ruddy Turnstones: $\chi^2=1.2$, df=1, $P=0.27$; Red Knots: $\chi^2=0.79$, df=1, $P=0.37$; Sanderlings: $\chi^2=0.02$, df=1, $P=0.88$), but were examined for 2007–2008 separately in Laughing Gulls because the pattern varied between years ($\chi^2=6.8$, df=1, $P=0.009$). For Ruddy Turnstones, an estimate of the minimum proportion of birds that were exposed to AIV (i.e., seroconverted) during the stopover period was determined by subtracting the estimated antibody prevalence upon arrival at Delaware Bay from that upon departure.

Nonparametric Kendall's τ_b correlation was used to assess the relationship of AIV prevalence on a given year and day between species pairs. To reduce measurement bias, we included data only when $n\geq5$ simultaneously for both species. We further used logistic regression to examine the relationship between isolating any AIV in a species (i.e., AIV presence/absence) and prevalence in Ruddy Turnstones on that date. Here, only data when $n\geq30$ were used.

RESULTS

Prevalence of AIV infection (Table 1) and AIV antibodies (Table 2) varied by species; both infection and antibody prevalence were highest in Ruddy Turnstones and lowest in Sanderlings. Red Knots and Laughing Gulls both exhibited high antibody prevalence but low infection prevalence. Annual AIV prevalence in Ruddy Turnstones was 10–20 times greater than in Red Knots and Sanderlings and 5–6 times greater than in Laughing Gulls.

AIV dynamics in Ruddy Turnstones

Prevalence of AIV varied with time similarly for 2000–2008 pooled data (three-node knotted spline fit: $\chi^2=20.0$,

TABLE 1. Springtime stopover site avian influenza virus prevalence among four species of Charadriiformes at Delaware Bay, 2000–2008.

	Ruddy Turnstone		Red Knot		Sanderling		Laughing Gull	
Year	*n* (positive)	Prevalence (%) (95% confidence interval [CI])	*n* (positive)	Prevalence (%) (95% CI)	*n* (positive)	Prevalence (%) (95% CI)	*n* (positive)	Prevalence (%) (95% CI)
2002	736 (75)	10 (8.2–13)	364 (2)	0.5 (0.2–2.0)	248 (2)	0.8 (0.2–2.9)	n/a[a]	n/a[a]
2007	415 (19)	4.6 (3.0–7.0)	242 (1)	0.4 (0.1–2.3)	191 (0)	0.0 (0.0–2.0)	48 (0)	0.0 (0.0–7.4)
2008	582 (97)	17 (14–20)	277 (2)	0.7 (0.2–3.0)	225 (1)	0.4 (0.1–2.5)	149 (4)[b]	2.7 (1.0–6.7)
2000–2008	3,660 (431)	12 (12–13)	2,438 (21)	0.9 (0.6–1.3)	1,269 (15)	1.2 (0.7–1.9)	332 (6)[a]	1.8 (0.8–3.9)[a]

[a] Laughing Gulls were sampled during 2005–2008 only.

[b] Two cloacal swabs and two fecal swabs positive.

df=2, $P<0.0001$) and in each individual year (2002: $\chi^2=12.9$, df=2, $P=0.002$; 2007: $\chi^2=6.7$, df=2, $P=0.036$; 2008: $\chi^2=14.0$, df=2, $P=0.0009$; Fig. 1). Although peak prevalence varied among years, peaks occurred within 2 calendar days of each other (Table 3). In all years, prevalences among birds sampled during periods of arrival and departure (<15 May or >May 30; Robinson et al., 2003) were lower than among birds sampled during 15–30 May (2002: 4.7% vs. 12%, $\chi^2=9.0$, df=1, $P=0.003$; 2007: 1.2% vs. 6.9%, $\chi^2=10.3$, df=1, $P=0.001$; 2008: 11% vs. 20%, $\chi^2=11.5$, df=1, $P=0.0007$).

Estimated AIV prevalence also varied over mass, both for 2006–2008 pooled data (quadratic fit, $\chi^2=28.4$, df=2, $P<0.0001$) and in individual years (2007: quadratic fit, $\chi^2=10.2$, df=2, $P=0.006$; 2008: three-node knotted spline fit, $\chi^2=12.0$, df=2, $P=0.003$; Fig. 2). In both 2007 and 2008, prevalence was lower among birds with masses suggesting recent arrival or impending departure (≤99 or ≥157g; Robinson et al., 2003) than among birds with midrange masses (2007: 0.8% vs. 6.3%, $\chi^2=8.3$, df=1, $P=0.004$; 2008: 12% vs. 20%, $\chi^2=3.8$, df=1, $P=0.050$). Mass associated with peak prevalence varied by only 2 g in different years (Table 3).

Adjusted antibody prevalence increased over the stopover season ($\chi^2=116.0$, df=1, $P<0.0001$; Fig. 3a) from <40% positive on 10 May to >95% positive by 25 May. Adjusted antibody prevalence was lower among birds sampled on or before 17 May, corresponding to the rapid prevalence increase (Fig. 1), than among birds sampled later (51% vs. 100%; $\chi^2=143.3$, df=1, $P<0.0001$). Adjusted antibody prevalence also increased with mass ($\chi^2=100.7$, df=1, $P<0.0001$). Antibody prevalence among birds weighing ≤99 g was significantly lower than among heavier

TABLE 2. Springtime stopover site avian influenza virus antibody prevalence among four species of Charadriiformes at Delaware Bay, 2007–2008.

Species	Year	*n* (positive)	Calculated antibody prevalence (%)	Adjusted antibody prevalence (%)[a] (95% CI)
Ruddy Turnstone	2007–2008	380 (243)	64	85 (78–91)
Red Knot	2007–2008	211 (125)	59	79 (70–87)
Sanderling	2007–2008	153 (4)	2.6	3.4 (0.1–6.8)
Laughing Gull	2007	31 (23)	74	98 (78–100)
	2008	52 (25)	48	64 (46–82)

[a] Adjusted for test sensitivity=0.754 (Brown et al., 2009), according to Rogan and Gladen (1978).

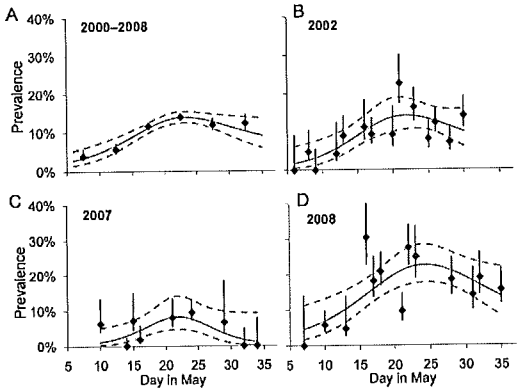

FIGURE 1. Avian influenza virus prevalence in Ruddy Turnstones over the course of the spring stopover at Delaware Bay, (A) for 2000–2008 pooled data, and for each year sufficient serial data were available: (B) 2002, (C) 2007, and (D) 2008. Lines of fit (95% confidence interval) and prevalence (±SE) for each day (B, C, and D) or 5-day time span (A) when $n \geq 5$ are shown. All lines of fit are significant at $\alpha=0.05$.

birds (41% vs. 91%; $\chi^2=86.6$, df=1, $P<0.0001$), and antibody prevalence among birds weighing ≥157 g was higher than among lighter birds (100% vs. 82%; $\chi^2=8.7$, df=1, $P=0.003$).

Evidence and estimates of seroconversion: Individual Ruddy Turnstones that were tested both by VI and bELISA ($n=363$) were divided into four categories based on results of the two tests. Both the mean sample date and mean mass varied across categories (one-way analysis of variance [ANOVA]; date: $F=22.8$, df=3, $P<0.0001$; mass: $F=21.0$, df=3, $P<0.0001$) and increased in the order: VI negative and bELISA negative, VI positive and bELISA negative, VI positive and bELISA positive, VI negative and bELISA positive (Fig. 4). Migrants typically arrive at Delaware Bay asynchronously during late April–mid-May, but depart en masse over a few days in late May–early June (Clark et al., 1993; Gillings et al., 2009). Assuming that arriving birds have similar masses regardless of arrival date (Gillings et al., 2009), we compared the expected antibody prevalence among Ruddy Turnstones at the mean arrival mass (96 g; Niles et al. unpublished data, cited in US Fish and Wildlife Service, 2003) to the estimated antibody prevalence on the mean departure date (1 June; unpublished data), or 48% and 99%, respectively. Thus, approximately 51% of the Ruddy Turnstone population seroconverted during the stopover.

AIV dynamics in Red Knots

AIV prevalence in Red Knots remained low (<2%) over the stopover period during each year and was not associated with mass (2006–2008 pooled data; $\chi^2=2.9$, df=1, $P=0.090$; data not shown). In contrast to Ruddy Turnstones, adjusted antibody prevalence in Red Knots decreased significantly over time ($\chi^2=15.0$, df=1, $P=0.001$; Fig. 3b). Antibody prevalence varied with 10-g mass class in a

TABLE 3. Estimated peak avian influenza virus prevalence by day and body mass in Ruddy Turnstones, 2000–2008, Delaware Bay. See text for details of model fit.

	By day		By mass[a]	
Year	Peak prevalence (%) (95% confidence interval [CI])	Date of peak prevalence	Peak prevalence (%) (95% CI)	Mass at peak prevalence (g)
2002	14 (11–19)	22 May	–	–
2007	8.2 (4.7–14)	22 May	9.3 (5.3–16)	133
2008	23 (18–28)	24 May	24 (18–30)	132
2000–2008	13 (11–14)	24 May	–	–
2006–2008	–	–	19 (16–22)	134

[a] Mass data were not available prior to 2006.

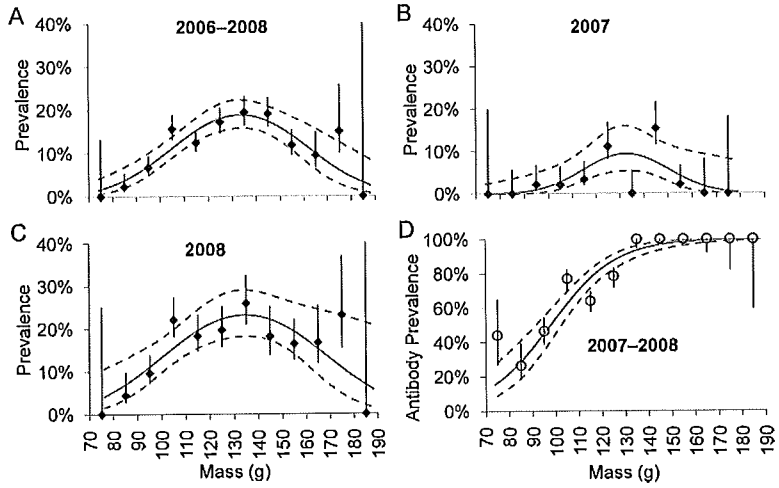

FIGURE 2. Avian influenza virus prevalence during (A) 2006–2008, (B) 2007, and (C) 2008, and (D) antibody prevalence during 2007–2008 in Ruddy Turnstones by 10-g mass classes, Delaware Bay. Lines of fit and 95% confidence interval are shown (all fits are significant at $\alpha=0.01$). Also shown are prevalences and adjusted antibody prevalences (±SE) positioned at the midpoint of each class (e.g., adjusted antibody prevalence among birds 90–99 g is plotted at 95 g).

quadratic manner ($\chi^2=6.5$, df=2, $P=0.039$), and was higher among midweight birds than among birds with either near-arrival (≤114 g) or near-departure (≥176 g) masses (82% vs. 70%; $\chi^2=4.2$, df=1, $P=0.041$).

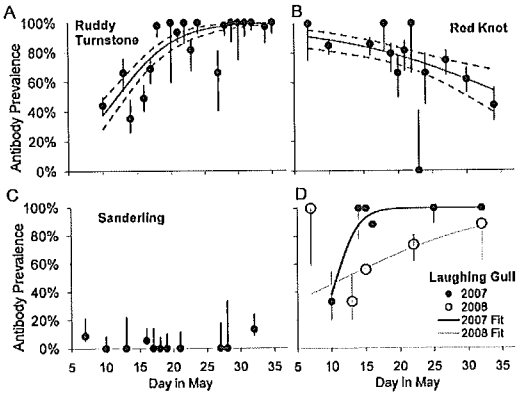

FIGURE 3. Estimated AIV antibody prevalence (95% CI) over the stopover season at Delaware Bay in (A) Ruddy Turnstones, (B) Red Knots, (C) Sanderlings, and (D) Laughing Gulls. Also shown are adjusted antibody prevalences (±SE) on each sample date (data from both years combined for Ruddy Turnstones, Red Knots, and Sanderlings). All fits are significant at $\alpha=0.001$.

AIV dynamics in Sanderlings

Viral prevalence increased with day during 2000–2008 ($\chi^2=4.5$, df=1, $P=0.034$) but not within any individual year (all $P>0.05$; data not shown). All Sanderling AIV isolations occurred during 17–30 May (prevalence=1.5%); this prevalence was significantly higher than the 0% prevalence during the arrival period ($\chi^2=6.2$, df=2, $P=0.013$) but not the departure period ($P>0.05$). Prevalence did not vary with mass, either for pooled 2006–2008 data or during 2008 alone (all $P>0.05$). However, a significant trend was observed when birds weighing >80 g (representing <5% of tested birds) were excluded ($\chi^2=4.8$, df=1, $P=0.028$; Fig. 5a). Prevalences among arrival- (≤53 g), intermediate- (54–90 g), and departure-weight (≥91 g) birds were 1.3%, 1.6% , and 0%, respectively, and were not statistically different ($\chi^2=0.31$, df=2, $P=0.86$).

Adjusted antibody prevalence did not vary over time ($\chi^2=1.3$, df=1, $P=0.26$; Fig. 3c) but increased with mass ($\chi^2=4.0$, df=1, $P=0.045$; Fig. 5b). Adjusted antibody prevalence did not differ among

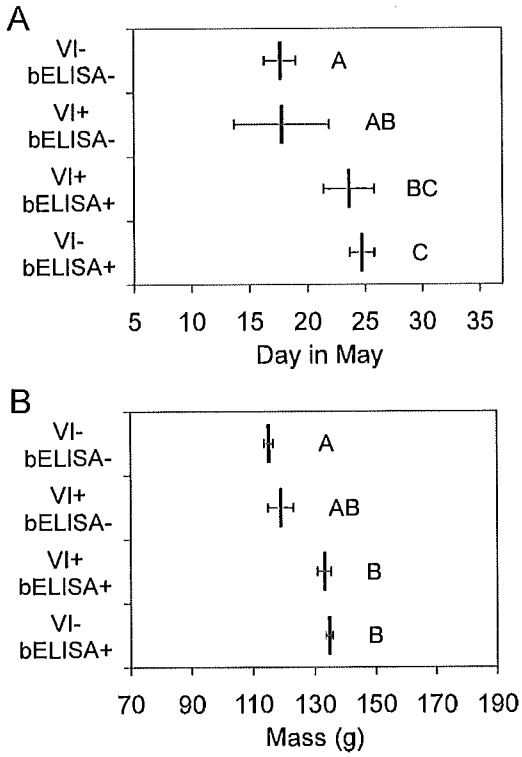

FIGURE 4. Mean (±SE) (A) sample date and (B) mass of Ruddy Turnstones testing positive (+) or negative (−) on virus isolation (VI) and serology (bELISA). Within each panel, the mean sample date or mass among birds belonging to categories not connected by the same letter are significantly different (Tukey–Kramer post hoc tests; $\alpha=0.05$).

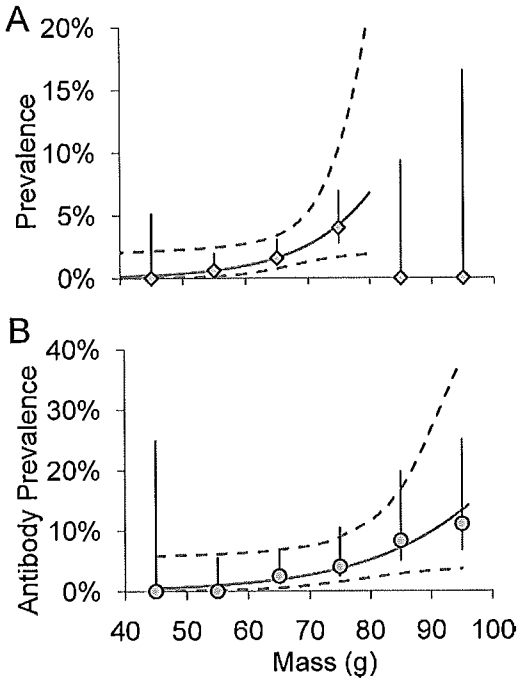

FIGURE 5. Avian influenza virus (A) prevalence (2006–2008) and (B) antibody prevalence by 10-g mass class (2007–2008) in Sanderlings. Lines of fit and 95% confidence interval are shown (all fits are significant at $\alpha=0.05$); see text for details.

arrival-, midrange, and departure-weight birds (0%, 3.2%, and 12%, respectively; $\chi^2=1.7$, df=2, $P=0.43$). However, sample sizes were $n=11$ and $n=13$ in arrival- and departure-weight groups, respectively.

AIV dynamics in Laughing Gulls

Infection prevalence did not vary temporally within a stopover season during 2005–2008 or the individual years 2007 or 2008 (all $P>0.05$; data not shown). Adjusted daily antibody prevalences increased with time and were higher in 2007 than in 2008 (Fig. 3d); a significant year×day interaction was present (whole model: $\chi^2=19.4$, df=3, $P=0.0002$; day effect: $\chi^2=12.1$, df=1, $P=0.0005$; year effect: $\chi^2=11.4$, df=1, $P=0.0008$; year×day effect: $\chi^2=4.6$, df=1, $P=0.032$).

AIV prevalence correlations between species

Prevalence among Sanderlings was positively correlated with prevalence among Ruddy Turnstones captured on the same day ($n=26$ catches, Kendall's $\tau_b=0.34$, $P=0.033$). Daily prevalences among Red Knots and Laughing Gulls were not correlated with prevalence among Ruddy Turnstones ($n=35$, $\tau_b=0.16$, $P=0.23$; and $n=10$, $\tau_b=-0.061$, $P=0.82$, respectively), nor were prevalences correlated among Sanderlings, Red Knots, and Laughing Gulls (all $P>0.05$). Additionally, the probability of detecting AIV among a sample of ≥30 Sanderlings was positively associated with AIV prevalence among Ruddy Turnstones on that day (logistic regression: $\chi^2=6.28$, df=1, $P=0.012$) and was likely (≥50% probability) when Ruddy Turnstone

prevalence exceeded 18.6%. There was no association between prevalence among Ruddy Turnstones and AIV presence among Red Knots ($\chi^2<0.01$, df=1, $P=0.98$) or Laughing Gulls ($\chi^2=0.01$, df=1, $P=0.94$) on a given date.

DISCUSSION

Ruddy Turnstones

There are few reports of AIV in Ruddy Turnstones at times and locations outside the Delaware Bay spring migratory stopover period. The consistent temporal pattern of infection and seroconversion (between and within years) at this site suggests an annual and localized epidemic; reported AIV isolations from Ruddy Turnstones at Delaware Bay each May since 1985 (Krauss et al., 2004; Hanson et al., 2008; Krauss et al., 2010) clearly demonstrate this predictable event.

All plots of AIV prevalence over time and mass gain had similar patterns of near-zero prevalence during early May when turnstones are at arrival weights, followed by abrupt increases in prevalence on approximately 16–17 May as the season progressed and birds gained weight. Antibody prevalence also increased during each stopover season; together these measures indicate that most exposures and infections occurred after arrival at Delaware Bay. The AIV infection duration is unknown in shorebirds, but the narrowness of the epidemic curve suggests short periods of shedding similar to the 2–8 days reported in wild Mallards (Latorre-Margalef et al., 2009). Infections decreased after 24 May, possibly due to an increase in population immunity. In 2007, the entire epidemic lasted less than 25 days before a complete fadeout, but in 2008 it lasted longer. We did not sample after 4 June because the small remaining number of birds were more dispersed and difficult to capture, but these data would have been useful to accurately determine the epidemic's span.

Similarly, patterns of antibody prevalence indicated population seroconversion in the wake of an epidemic. Generally, individual birds were virus- and antibody-negative at the beginning of the stopover, became infected, seroconverted within a few days of infection, and then recovered from infection but retained circulating antibodies. This pattern is expected with an acute infectious disease (Nunn and Altizer, 2006). We estimate that at least half of birds seroconverted during the stopover; this figure could include both previously unexposed birds and birds that had been previously exposed (at Delaware Bay or elsewhere) but whose antibodies had fallen below detectable levels (i.e., they were re-exposed at Delaware Bay). Because virtually all turnstones were antibody positive just prior to departure, the population proportion exposed at the stopover is likely higher than the above estimate.

Although the conditions that enhance AIV transmission in Ruddy Turnstones at Delaware Bay each spring are currently unknown, the high population density (up to 67 birds/m^2; Gillings et al., 2007) of this species at Delaware Bay is an obvious and unique situation; such densities are not encountered during other times in their annual cycle (Nettleship, 2000). The sudden aggregation of susceptible Ruddy Turnstones at Delaware Bay could provide the population threshold (Lloyd-Smith et al., 2005) needed to initiate and sustain annual AIV epidemics (Krauss et al., 2010). Up to 80% of the *A. interpres morinella* population migrates through Delaware Bay each year (Morrison et al., 2001), and aerial counts have exceeded 100,000 turnstones on a single day (Clark et al., 1993). During the breeding, fall migration, and wintering periods Ruddy Turnstones are typically much more dispersed, often seen individually or in small flocks ($n<50$; Nettleship, 2000). Thus, potential bird-to-bird transmission of AIV could be interrupted when they do not regularly encounter a large number of conspecifics.

A majority of turnstones did not have detectable antibodies against AIV upon

arrival at Delaware Bay. Though the duration for which anti-NP antibodies can be detected following initial exposure is unknown, it seems reasonable that the majority of Ruddy Turnstones had not been recently exposed to AIV, and indeed might never have been exposed (e.g., young adult birds migrating through Delaware Bay for the first time) or were last exposed during the preceding spring. Further, previous population exposure might have involved different AIV subtypes to which detected antibodies were not protective or only partially protective.

In addition to population density and immunity, increased potential virus contact at Delaware Bay due to the local density of other AIV reservoir species also should be considered. The largest Laughing Gull breeding colony on the Atlantic coast and smaller colonies of Herring Gulls, Great Black-backed Gulls, egrets, herons, and other waterbirds are located in close proximity on the Cape May Peninsula in New Jersey, as are migrant and resident waterfowl. Laughing and Herring Gulls number tens of thousands of breeding pairs (Pierotti and Good, 1994; Burger, 1996) and often feed alongside shorebirds on the beaches (Burger et al., 2007). Gulls are recognized AIV reservoirs and transmission might be associated with breeding behavior (Velarde et al., 2010). The high prevalence of AIV antibodies detected in gulls in the present study supports their possible involvement. Infection data in gulls, waterfowl, and other resident waterbirds prior to shorebird arrival would help determine if epidemiologic links exist between species groups.

Two additional unique and poorly understood factors that might enhance AIV transmission or susceptibility at this site are environmental conditions that allow effective exposure to and transmission of AIV and increased susceptibility related to the physiologic changes associated with long-distance migration and rapid weight gain. Migration and refueling at stopovers are physiologically stressful activities, and stress hormones such as corticosterone that could be important to shorebird stopover physiology (Piersma et al., 2000; Mizrahi et al., 2001) might also be immunosuppressive.

Because the majority of Ruddy Turnstones are infected with and recover from AIV during the spring stopover, and from Delaware Bay disperse onto the breeding grounds and remain dispersed during fall migration and winter, it is possible that AIV infection does not persist year-round in this species. This is supported by the low antibody prevalence we observed on arrival. Rather than being an AIV reservoir, which implies endemicity, it is possible that Ruddy Turnstones are a local amplifying host under conditions of high density, low flock immunity, and increased exposure to AIV. Similar conditions might exist at other locations involving Ruddy Turnstones or another permissive species; in October 2007, AIV were detected in Ruddy Turnstones in Peru (Ghersi et al., 2009). Although shorebirds have been implicated in rare transhemispheric movement of AIV gene segments (Krauss et al., 2007), a recent analysis suggests that AIV are more likely to be carried long distances by gulls and that shorebirds, such as Ruddy Turnstones, are probably local secondary hosts (Pearce et al., 2010).

Other species

Of the three syntopic species studied, only Sanderlings exhibited dynamical changes in AIV prevalence over the stopover period. Prevalence increased with day (≤30 May), and with mass (≤80 g), and was positively correlated with prevalence in Ruddy Turnstones. Sanderlings appear relatively resistant to AIV infection (perhaps due to decreased susceptibility or limited contact), given low prevalence and antibody prevalence at Delaware Bay, and zero prevalence at other times and locations (Hlinak et al., 2006; Munster et al., 2007; Winker et al., 2007; Escudero et al., 2008; Hanson et al.,

2008; Iverson et al., 2008; Ghersi et al., 2009). Because infections in Sanderlings are sporadic and likely depend on the infected proportion of syntopic birds, they are probably due to spillover events from Ruddy Turnstones acting as amplifying hosts (Fenton and Pedersen, 2005).

The high antibody prevalence in Red Knots, which declined over the season, was an unexpected finding. Red Knots are not often found infected at Delaware Bay or the limited number of other locations where they have been sampled (D'Amico et al., 2007; Hanson et al., 2008). Although the duration of antibody detectability is unknown, this pattern suggests that Red Knots were exposed to AIV recently prior to arrival at Delaware Bay, perhaps on their wintering grounds or at another stopover during northward migration. Red Knots often winter in large flocks at Tierra del Fuego in Argentina and Chile and congregate at several stopovers prior to Delaware Bay (Harrington, 2001); therefore, opportunity could exist for AIV spread within localized populations throughout much of the year. Although AIV has not been detected on wintering grounds, available information is limited. More information regarding when and where Red Knots are exposed or infected would be helpful to understand what role, if any, LPAI infection has had on recent population declines (Baker et al., 2004; Niles et al., 2009).

High antibody prevalence could also result from long-lasting immunity following prior, or repeated, exposure(s) at Delaware Bay (Buehler et al., 2010). However, antibody prevalence declines rather than remaining high over the stopover duration, suggesting that Red Knots are not re-exposed to AIV while at Delaware Bay. This pattern is difficult to understand because knots and turnstones feed together on the Bay beaches. Perhaps subtle differences in host species ecology support transmission of virus among turnstones but not to knots. For example, Red Knots primarily consume horseshoe crab eggs available at the surface, but Ruddy Turnstones regularly dig several centimeters deep to reach buried egg masses (Tsipoura and Burger, 1999). If fecally excreted virus becomes rapidly unavailable on the beach surface (e.g., through UV irradiation or percolation into the sand due to wave action) but remains infectious when adsorbed to subsurface sand particles (Jin et al., 1997) or horseshoe crab eggs, then Red Knots might encounter viable AIV less often than Ruddy Turnstones. Sanderlings and gulls often raid turnstone excavations, whereas Red Knots rarely do (Burger et al., 2007; Vahl et al., 2007), perhaps helping to explain why Sanderlings and gulls are infected more often. Other differences in host ecology such as roost environments could also contribute to differential transmission. Additionally, we did not examine whether Red Knots shed AIV from the respiratory rather than the intestinal tract.

Laughing Gulls exhibited significant but variable increases in antibody prevalence over the stopover period; sample sizes were relatively small. Given that a majority of Ruddy Turnstones were exposed to AIV over the stopover season and that Laughing Gulls and turnstones share multiple habitats in the Delaware Bay area (data not shown), it is not surprising that many Laughing Gulls also were exposed. To date, Laughing Gulls have not been extensively sampled at Delaware Bay or elsewhere (reviewed by Bogomolni et al., 2008) and infection data are limited in this study. More systematic sampling of Laughing Gulls including time periods before shorebird arrival and after their departure would help to characterize AIV dynamics in this species further.

CONCLUSIONS

This study is the first to detail AIV infection dynamics over the entire course of an epidemic within Charadriiformes populations. The observed dynamical

changes in prevalence and antibody prevalence allowed insight into when and where these four species were infected with or exposed to AIV. Although Ruddy Turnstones are seemingly the most important species in AIV epidemics at Delaware Bay, the detailed dynamic changes reported here indicate that they largely become infected, seroconvert, and recover locally. The source of viruses for these annual epidemics has not been identified, but AIV could arrive with a few infected Ruddy Turnstones (or other shorebirds) or be acquired from local sources such as gulls or waterfowl. Because infection apparently is a local phenomenon, Ruddy Turnstones might primarily act as a local virus amplifier, potentially generating reassortant viruses derived from many distant sources (Pearce et al., 2010). Other species, such as Red Knots, might be exposed at other (unidentified) sites, but further research is needed to define their possible roles in AIV epidemiology.

ACKNOWLEDGMENTS

This research was funded through Specific Cooperative Agreement 58-6612-2-0220 between the Southeast Poultry Research Laboratory, Agricultural Research Service, Department of Agriculture (USDA-ARS) and the Southeastern Cooperative Wildlife Disease Study, and by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health and Human Services, under contract HHSN266200700007C. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. Banding data and morphometric measurements are the property of the Natural Heritage & Endangered Species Program, Division of Fish and Wildlife, Delaware Department of Natural Resources and Environmental Control, and the Nongame and Endangered Species Program, Division of Fish and Wildlife, New Jersey Department of Environmental Protection; we thank these agencies for granting access to these data. We are grateful to many people who provided field and laboratory support, particularly E. Casey, L. Coffee, M. Cole, J. Cumbee, D. Downs, S. Gibbs, W. Hamrick, S. Keeler, G. Martin, S. McGraw, C. McKinnon, J. Murdock, R. Paulson, J. Smith, and B. Wilcox.

LITERATURE CITED

Baker, A. J., P. M. Gonzalez, T. Piersma, L. J. Niles, I. D. S. do Nascimento, P. W. Atkinson, N. A. Clark, C. D. T. Minton, M. K. Peck, and G. Aarts. 2004. Rapid population decline in Red Knots: Fitness consequences of decreased refueling rates and late arrival in Delaware Bay. Proceedings of the Royal Society B: Biological Sciences 271: 875–882.

Bogomolni, A. L., R. J. Gast, J. C. Ellis, M. Dennett, K. R. Pugliares, B. J. Lentell, and M. J. Moore. 2008. Victims or vectors: A survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. Diseases of Aquatic Organisms 81: 13–38.

Brown, J. D., D. E. Stallknecht, R. D. Berghaus, M. P. Luttrell, K. Velek, W. Kistler, T. Costa, M. J. Yabsley, and D. Swayne. 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clinical and Vaccine Immunology 16: 824–829.

Buehler, D. M., B. I. Tieleman, and T. Piersma. 2010. How do migratory species stay healthy over the annual cycle? A conceptual model for immune function and for resistance to disease. Integrative and Comparative Biology 50: 346–357.

Burger, J 1996. Laughing Gull (*Larus atricilla*). *In* The birds of North America online, A. Poole (ed.). Cornell Lab of Ornithology, Ithaca, New York, Species Account no. 225, http://bna.birds.cornell.edu/bna/species/225. Accessed January 2011.

———, S. A. Carlucci, C. W. Jeitner, and L. Niles. 2007. Habitat choice, disturbance, and management of foraging shorebirds and gulls at a migratory stopover. Journal of Coastal Research 23: 1159–1166.

Burnham, K. P., and D. R. Anderson. 2002. Model Selection and multimodel inference: A practical information-theoretic approach. 2nd Edition. Springer, New York, New York, 488 pp.

Clark, K. E., L. J. Niles, and J. Burger. 1993. Abundance and distribution of migrant shorebirds in Delaware Bay. Condor 95: 694–705.

D'Amico, V. L., M. Bertellotti, A. J. Baker, and L. A. Diaz. 2007. Exposure of Red Knots (*Calidris canutus rufa*) to select avian pathogens; Patagonia, Argentina. Journal of Wildlife Diseases 43: 794–797.

Escudero, G., V. J. Munster, M. Bertellotti, and P. Edelaar. 2008. Perpetuation of avian influenza in the Americas: Examining the role of shorebirds in Patagonia. Auk 125: 494–495.

Fenton, A., and A. B. Pedersen. 2005. Community epidemiology framework for classifying disease threats. Emerging Infectious Diseases 11: 1815–1821.

FOUCHIER, R. A. M., V. MUNSTER, A. WALLENSTEN, T. M. BESTEBROER, S. HERFST, D. SMITH, G. F. RIMMELZWAAN, B. OLSEN, AND A. D. M. E. OSTERHAUS. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from Black-headed Gulls. Journal of Virology 79: 2814–2822.

GHERSI, B. M., D. L. BLAZES, E. ICOCHEA, R. I. GONZALEZ, T. KOCHEL, Y. TINOCO, M. M. SOVERO, S. LINDSTROM, B. SHU, A. KLIMOV, A. E. GONZALEZ, AND J. M. MONTGOMERY. 2009. Avian influenza in wild birds, central coast of Peru. Emerging Infectious Diseases 15: 935–938.

GILLINGS, S., P. W. ATKINSON, S. L. BARDSLEY, N. A. CLARK, S. E. LOVE, R. A. ROBINSON, R. A. STILLMAN, AND R. G. WEBER. 2007. Shorebird predation of horseshoe crab eggs in Delaware Bay: Species contrasts and availability constraints. Journal of Animal Ecology 76: 503–514.

———, ———, A. J. BAKER, K. A. BENNETT, N. A. CLARK, K. B. COLE, P. M. GONZALEZ, K. S. KALASZ, C. D. T. MINTON, L. J. NILES, R. C. PORTER, I. DE LIMA SERRANO, H. P. SITTERS, J. L. WOODS, AND T. L. SHAFFER. 2009. Staging behavior in Red Knot (*Calidris canutus*) in Delaware Bay: Implications for monitoring mass and population size. Auk 126: 54–63.

HANSON, B. A., M. P. LUTTRELL, V. H. GOEKJIAN, L. NILES, D. E. SWAYNE, D. A. SENNE, AND D. E. STALLKNECHT. 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? Journal of Wildlife Diseases 44: 351–361.

HARRINGTON, B. A 2001. Red Knot (*Calidris canutus*). *In* The birds of North America online, A. Poole (ed.). Cornell Lab of Ornithology, Ithaca, New York, Species Account no. 563, http://bna.birds.cornell.edu/bna/species/563. Accessed January 2011.

HASTIE, T. J., AND R. J. TIBSHIRANI. 1990. Generalized additive models. Chapman and Hall, London, UK, 352 pp.

HINSHAW, V. S., R. G. WEBSTER, AND B. TURNER. 1979. Water-borne transmission of influenza A viruses. Intervirology 11: 66–68.

———, G. M. AIR, G. C. SCHILD, AND R. W. NEWMAN. 1983. Characterization of a novel hemagglutinin subtype (H13) of influenza A viruses from gulls. Bulletin of the World Health Organization 61: 677–679.

HLINAK, A., R. U. MUHLE, O. WERNER, A. GLOBIG, E. STARICK, H. SCHIRRMEIER, B. HOFFMANN, A. ENGELHARDT, D. HUBNER, F. J. CONRATHS, D. WALLSCHLAGER, H. KRUCKENBERG, AND T. MULLER. 2006. A virological survey in migrating waders and other waterfowl in one of the most important resting sites of Germany. Journal of Veterinary Medicine Series B, Infectious Diseases and Veterinary Public Health 53: 105–110.

IVERSON, S. A., J. Y. TAKEKAWA, S. SCHWARZBACH, C. J. CARDONA, N. WARNOCK, M. A. BISHOP, G. A. SCHIRATO, S. PAROULEK, J. T. ACKERMAN, H. IP, AND W. M. BOYCE. 2008. Low prevalence of avian influenza virus in shorebirds on the Pacific coast of North America. Waterbirds 31: 602–610.

JIN, Y., M. V. YATES, S. S. THOMPSON, AND W. A. JURY. 1997. Sorption of viruses during flow through saturated sand columns. Environmental Science & Technology 31: 548–555.

KAWAOKA, Y., T. M. CHAMBERS, W. L. SLADEN, AND R. G. WEBSTER. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163: 247–250.

KRAUSS, S., D. WALKER, S. P. PRYOR, L. NILES, C. H. LI, V. S. HINSHAW, AND R. G. WEBSTER. 2004. Influenza A viruses of migrating wild aquatic birds in North America. Vector-Borne and Zoonotic Diseases 4: 177–189.

———, C. A. OBERT, J. FRANKS, D. WALKER, K. JONES, P. SEILER, L. NILES, S. P. PRYOR, J. C. OBENAUER, C. W. NAEVE, L. WIDJAJA, R. J. WEBBY, AND R. G. WEBSTER. 2007. Influenza in migratory birds and evidence of limited intercontinental virus exchange. PLoS Pathogens 3: 1684–1693.

———, D. E. STALLKNECHT, N. J. NEGOVETICH, L. J. NILES, R. J. WEBBY, AND R. G. WEBSTER. 2010. Coincident Ruddy Turnstone migration and horseshoe crab spawning creates an ecological "hot spot" for influenza viruses. Proceedings of the Royal Society B: Biological Sciences 277: 3373–3379.

LATORRE-MARGALEF, N., G. GUNNARSSON, V. J. MUNSTER, R. A. M. FOUCHIER, A. D. M. E. OSTERHAUS, J. ELMBERG, B. OLSEN, A. WALLENSTEN, P. D. HAEMIG, T. FRANSSON, L. BRUDIN, AND J. WALDENSTRÖM. 2009. Effects of influenza A virus infection on migrating Mallard ducks. Proceedings of the Royal Society B: Biological Sciences 276: 1029–1036.

LLOYD-SMITH, J. O., P. C. CROSS, C. J. BRIGGS, M. DAUGHERTY, W. M. GETZ, J. LATTO, M. S. SANCHEZ, A. B. SMITH, AND A. SWEI. 2005. Should we expect population thresholds for wildlife disease? Trends in Ecology & Evolution 20: 511–519.

MIZRAHI, D. S., R. L. HOLBERTON, AND S. A. GAUTHREAUX. 2001. Patterns of corticosterone secretion in migrating Semipalmated Sandpipers at a major spring stopover site. Auk 118: 79–91.

MORRISON, R. I. G., AND B. A. HARRINGTON. 1992. The migration system of the Red Knot *Calidris canutus rufa* in the New World. Wader Study Group Bulletin 64 (Supplement): 71–84.

———, R. E. GILL, B. A. HARRINGTON, S. SKAGEN, G. W. PAGE, C. L. GRATTO-TREVOR, AND S. M. HAIG. 2001. Estimates of shorebird populations in North America. Canadian Wildlife Service Occasional Paper 104: 64.

MUNSTER, V. J., C. BAAS, P. LEXMOND, J. WALDENSTROM, A. WALLENSTEN, T. FRANSSON, G. F. RIMMELZWAAN, W. E. P. BEYER, M. SCHUTTEN, B. OLSEN, A. OSTERHAUS, AND R. A. M. FOUCHIER. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathogens 3: 630–638.

MYERS, J. P., M. SALLABERRY, E. ORTIZ, G. CASTRO, L. M. GORDON, J. L. MARON, C. T. SCHICK, E. TABILO, P. ANTAS, AND T. BELOW. 1990. Migration routes of New World Sanderlings (*Calidris alba*). Auk 107: 172–180.

NETTLESHIP, D. N 2000. Ruddy Turnstone (*Arenaria interpres*). *In* The birds of North America online, A. Poole (ed.). Cornell Lab of Ornithology, Ithaca, New York, Species Account no. 537, http://bna.birds.cornell.edu/bna/species/537. Accessed January 2011.

NILES, L. J., J. BART, H. P. SITTERS, A. D. DEY, K. E. CLARK, P. W. ATKINSON, A. J. BAKER, K. A. BENNETT, K. S. KALASZ, N. A. CLARK, J. CLARK, S. GILLINGS, A. S. GATES, P. M. GONZALEZ, D. E. HERNANDEZ, C. D. T. MINTON, R. I. G. MORRISON, R. R. PORTER, R. KEN ROSS, AND C. R. VEITCH. 2009. Effects of horseshoe crab harvest in Delaware Bay on Red Knots: Are harvest restrictions working? Bioscience 59: 153–164.

NUNN, C. L., AND S. ALTIZER. 2006. Host–parasite dynamics and epidemiological principles. *In* Infectious diseases in primates: Behavior, ecology, and evolution, C. L. Nunn and S. Altizer (eds.). Oxford University Press, New York, New York, pp. 98–133.

OLSEN, B., V. J. MUNSTER, A. WALLENSTEN, J. WALDENSTROM, A. OSTERHAUS, AND R. A. M. FOUCHIER. 2006. Global patterns of influenza A virus in wild birds. Science 312: 384–388.

PEARCE, J. M., A. M. RAMEY, H. S. IP, AND R. E. GILL. 2010. Limited evidence of trans-hemispheric movement of avian influenza viruses among contemporary North American shorebird isolates. Virus Research 148: 44–50.

PIEROTTI, R. J., AND T. P. GOOD. 1994. Herring Gull (*Larus argentatus*). *In* The birds of North America online, A. Poole (ed.). Cornell Lab of Ornithology, Ithaca, New York, Species Account no. 124, http://bna.birds.cornell.edu/bna/species/124. Accessed January 2011.

PIERSMA, T., J. RENEERKENS, AND M. RAMENOFSKY. 2000. Baseline corticosterone peaks in shorebirds with maximal energy stores for migration: A general preparatory mechanism for rapid behavioral and metabolic transitions? General and Comparative Endocrinology 120: 118–126.

ROBINSON, R. A., P. W. ATKINSON, AND N. A. CLARK. 2003. Arrival and weight gain of Red Knot *Calidris canutus*, Ruddy Turnstone *Arenaria interpres* and Sanderling *Calidris alba* staging in Delaware Bay in spring. British Trust for Ornithology Research Report 307: 51.

ROGAN, W. J., AND B. GLADEN. 1978. Estimating prevalence from the results of a screening test. American Journal of Epidemiology 107: 71–76.

SPACKMAN, E 2009. The ecology of avian influenza virus in wild birds: What does this mean for poultry? Poultry Science 88: 847–850.

———, AND D. SUAREZ. 2008. Type A influenza virus detection and quantitation by real-time RT-PCR. Methods in Molecular Biology 436: 19–26.

STALLKNECHT, D. E., AND J. D. BROWN. 2007. Wild birds and the epidemiology of avian influenza. Journal of Wildlife Diseases 43: S15–S20.

SWAYNE, D. E., D. A. SENNE, AND C. W. BEARD. 1998. Avian influenza. *In* A laboratory manual for the isolation and identification of avian pathogens, D. E. Swayne (ed.). American Association of Avian Pathologists, Kennett Square, Pennsylvania, pp. 150–155.

TSIPOURA, N., AND J. BURGER. 1999. Shorebird diet during spring migration stopover on Delaware Bay. Condor 101: 635–644.

US FISH AND WILDLIFE SERVICE. 2003. Delaware Bay shorebird-horseshoe crab assessment report and peer review. US Fish and Wildlife Service Migratory Bird Publication R9-03/02, Arlington, Virginia, 99 pp.

VAHL, W. K., J. VAN DER MEER, K. MEIJER, T. PIERSMA, AND F. J. WEISSING. 2007. Interference competition, the spatial distribution of food and free-living foragers. Animal Behaviour 74: 1493–1503.

VELARDE, R., S. E. CALVIN, D. OJKIC, I. K. BARKER, AND É. NAGY. 2010. Avian influenza virus H13 circulating in Ring-billed Gulls (*Larus delawarensis*) in southern Ontario, Canada. Avian Diseases 54: 411–419.

WINKER, K., K. G. MCCRACKEN, D. D. GIBSON, C. L. PRUETT, R. MEIER, F. HUETTMANN, M. WEGE, I. V. KULIKOVA, Y. N. ZHURAVLEV, M. L. PERDUE, E. SPACKMAN, D. L. SUAREZ, AND D. E. SWAYNE. 2007. Movements of birds and avian influenza from Asia into Alaska. Emerging Infectious Diseases 13: 547–552.

———, E. SPACKMAN, AND D. E. SWAYNE. 2008. Rarity of influenza A virus in spring shorebirds, southern Alaska. Emerging Infectious Diseases 14: 1314–1316.

YAMNIKOVA, S. S., A. S. GAMBARYAN, A. B. TUZIKOV, N. V. BOVIN, M. N. MATROSOVICH, I. T. FEDYAKINA, A. A. GRINEV, V. M. BLINOV, D. K. LVOV, D. L. SUAREZ, AND D. E. SWAYNE. 2003. Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae. Avian Diseases 47: 1164–1168.

Submitted for publication 8 May 2011.
Accepted 30 September 2011.

AVIAN DISEASES 56:818–828, 2012

Symposium Keynote Address—

Impact of Vaccines and Vaccination on Global Control of Avian Influenza

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Received 11 April 2012; Accepted 12 June 2012; Published ahead of print 13 June 2012

SUMMARY. There are 30 recorded epizootics of H5 or H7 high pathogenicity avian influenza (HPAI) from 1959 to early 2012. The largest of these epizootics, affecting more birds and countries than the other 29 epizootics combined, has been the H5N1 HPAI, which began in Guangdong China in 1996, and has killed or resulted in culling of over 250 million poultry and/or wild birds in 63 countries. Most countries have used stamping-out programs in poultry to eradicate H5N1 HPAI. However, 15 affected countries have utilized vaccination as a part of the control strategy. Greater than 113 billion doses were used from 2002 to 2010. Five countries have utilized nationwide routine vaccination programs, which account for 99% of vaccine used: 1) China (90.9%), 2) Egypt (4.6%), 3) Indonesia (2.3%), 4) Vietnam (1.4%), and 5) Hong Kong Special Administrative Region (<0.01%). Mongolia, Kazakhstan, France, The Netherlands, Cote d'Ivoire, Sudan, North Korea, Israel, Russia, and Pakistan used <1% of the avian influenza (AI) vaccine, and the AI vaccine was targeted to either preventive or emergency vaccination programs. Inactivated AI vaccines have accounted for 95.5% of vaccine used, and live recombinant virus vaccines have accounted for 4.5% of vaccine used. The latter are primarily recombinant Newcastle disease vectored vaccine with H5 influenza gene insert. China, Indonesia, Egypt, and Vietnam implemented vaccination after H5N1 HPAI became enzootic in domestic poultry. Bangladesh and eastern India have enzootic H5N1 HPAI and have not used vaccination in their control programs. Clinical disease and mortality have been prevented in chickens, human cases have been reduced, and rural livelihoods and food security have been maintained by using vaccines during HPAI outbreaks. However, field outbreaks have occurred in vaccinating countries, primarily because of inadequate coverage in the target species, but vaccine failures have occurred following antigenic drift in field viruses within China, Egypt, Indonesia, Hong Kong, and Vietnam. The primary strategy for HPAI and H5/H7 low pathogenicity notifiable avian influenza control will continue to be immediate eradication using a four-component strategy: 1) education, 2) biosecurity, 3) rapid diagnostics and surveillance, and 4) elimination of infected poultry. Under some circumstances, vaccination can be added as an additional tool within a wider control strategy when immediate eradication is not feasible, which will maintain livelihoods and food security, and control clinical disease until a primary strategy can be developed and implemented to achieve eradication.

RESUMEN. *Estudio recapitulativo por invitación*— Impacto de las vacunas y la vacunación para el control global de la influenza aviar. Se han presentado 30 epizootias de la influenza aviar de alta patogenicidad H5 o H7 de 1959 a principios del año 2012. La mayor de estas epizootias, que ha afectado más aves y países en comparación con las otras 29 epizootias combinadas, ha sido la influenza aviar altamente patógena H5N1, que comenzó en Guangdong, China, en 1996 y que causó la muerte o la eliminación de más de 250 millones de aves de corral y/o aves silvestres en 63 países. La mayoría de los países han utilizado los programas de sacrificio sanitario en aves de corral para erradicar la influenza aviar altamente patógena H5N1. Sin embargo, 15 países afectados han utilizado la vacunación como parte de la estrategia de control. Más de 113 millones de dosis fueron utilizadas desde 2002 al 2010. Cinco países han utilizado programas rutinarios de vacunación en todo el país, que representan el 99% de las vacunas utilizadas: 1) China (90.9%), 2) Egipto (4.6%), 3) Indonesia (2.3%), 4) Vietnam (1.4%), y 5) Hong Kong, Región Administrativa Especial (<0.01%). Mongolia, Kazajstán, Francia, Holanda, Costa de Marfil, Sudán, Corea del Norte, Israel, Rusia y Pakistán han utilizado menos del uno porciento de la vacuna contra influenza aviar y la vacunación fue dirigida a programas de vacunación preventiva o de emergencia. Las vacunas inactivadas contra la influenza aviar han representado el 95.5% de las vacunas utilizadas y las vacunas con virus vivos recombinantes han representado el 4.5% de las vacunas utilizadas. Estas últimas son principalmente la vacuna recombinante que utiliza al virus de la enfermedad de Newcastle como vector con un inserto del gene H5 del virus de la influenza aviar. China, Indonesia, Egipto y Vietnam implementaron la vacunación después que la influenza aviar de alta patogenicidad H5N1 se convirtió en enzoótica en aves domésticas. Bangladesh y la India oriental tienen influenza aviar de alta patogenicidad H5N1 enzoótica y no han utilizado la vacunación en sus programas de control. La enfermedad clínica y la mortalidad se han prevenido en los pollos, los casos en humanos se han reducido, y los medios de subsistencia rurales y la seguridad alimentaria se han mantenido mediante el uso de vacunas durante los brotes de influenza aviar altamente patógena. Sin embargo, brotes de campo se han producido en los países donde se ha llevado a cabo la vacunación, sobre todo debido a la insuficiente cobertura de las especies objetivo, se han producido fracasos en la vacunación después de cambios por derivación antigénica de los virus de campo en China, Egipto, Indonesia, Hong Kong y Vietnam. La estrategia principal de control para la influenza aviar de alta patogenicidad y de baja patogenicidad H5/H7 de declaración obligatoria continúa siendo la erradicación inmediata utilizando una estrategia de cuatro componentes: 1) educación, 2) bioseguridad, 3) el diagnóstico rápido y vigilancia, y 4) la eliminación de aves infectadas. En algunas circunstancias, la vacunación se puede agregar como una herramienta adicional dentro de una estrategia más amplia de control cuando la erradicación inmediata no es factible, la que mantendrá los medios de subsistencia y la seguridad alimentaria, y controlará a la enfermedad clínica hasta que una estrategia primaria pueden ser desarrollada e instrumentada para lograr la erradicación.

Key words: avian influenza, highly pathogenic avian influenza, vaccination, vaccine

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Abbreviations: AGDP = agricultural gross domestic product; AI = avian influenza; FAO = Food and Agriculture Organization; GDP = gross domestic product; GNI = gross national income; HDI = human development index; HP = high pathogenicity; HPAI = high pathogenicity avian influenza; LP = low pathogenicity; LPNAI = low pathogenicity notifiable avian influenza; OECD = Organization for Economic Cooperation and Development; OIE = World Organization for Animal Health; OFFLU = OIE and FAO animal influenza network; PVS = OIE Performance of Veterinary Services evaluation

HIGH PATHOGENICITY AVIAN INFLUENZA EPIZOOTICS AND CONTROL

Since identification of influenza A virus as the cause of fowl plague or high pathogenicity avian influenza (HPAI) in 1955, 30 epizootics of HPAI in birds have occurred around the world (Table 1) (30). Traditional control strategies for HPAI have relied upon four basic categories of components: 1) education (including behavioral change communications), 2) biosecurity (including modifications to the ways in which poultry are reared and sold, movement management, and cleaning and disinfection), 3) diagnostics and surveillance, and 4) elimination of infected poultry, usually through culling or depopulation (30). In 1995, a fifth category was added, decreasing host susceptibility, with the field implementation of vaccines and vaccination in Mexican and Pakistani control programs. In the future, increasing host resistance through genetics may replace or at least augment vaccination as a means to decrease host susceptibility.

Of the 30 HPAI epizootics, 28 have involved single countries, and two have involved multiple countries (1996–present, H5N1 in 63 countries of Asia, Europe, and Africa; and 2003, H7N7 in The Netherlands, Belgium, and Germany) (30). The number of birds affected ranges from 1300 to over 250 million (Fig. 1). This number is quite small considering over 58 billion poultry are raised per year in the world. Most of the epizootics were short, spanning <1 yr, and most were addressed using a comprehensive control program involving stamping-out, which led to eradication (Fig. 2). However, four epizootics used vaccines and vaccination as a part of the control strategies: 1) Mexico, H5N1, 1994–95 (32); 2) Pakistan, H7N3, 1995–2004 (16); 3) Asia/Africa/Europe, H5N1, 1996–continuing (24,29); and 4) North Korea, H7N7, 2005 (17). These four epizootics used vaccination as an added control component because either traditional stamping-out programs did not eradicate the disease, or resources were not available or were inadequate to accomplish eradication. In these situations, vaccination was used as a tool to reduce infection pressure while maintaining rural livelihoods and food security, controlling the clinical disease, and allowing development of infrastructure that could lead to eradication.

There is no "one control strategy" for HPAI eradication (30). The H5N1 HPAI panzootic that emerged in 1996 in China is unique because of the 16 yr of reported outbreaks, 9 yr of multicountry control experience (2003–12), multiple introductions into some countries with multiple eradications, and wild bird involvement. There have been several quantifiable successes, including eradication in 57 countries, reduction in number of outbreaks in poultry, reduction in the time to eradication, reduction in rate of human cases in countries with adequate surveillance, and the refinement of the role of vaccines and vaccination as tools in a comprehensive control program. In addition, we must ask ourselves, "What did the 57 countries that eradicated H5N1 HPAI do that was 'right'?" Their control strategies had sufficient application of each of the four components (education, biosecurity, diagnostic and surveillance, and elimination of infected poultry) to achieve eradication. If the other countries have not achieved eradication using the same four components, then what will the addition of vaccines and vaccination to the control strategy do? In experimental studies, vaccines and vaccination have been shown to increase resistance to virus infection, reduce virus replication in respiratory and gastrointestinal tracts, and prevent illness and death in poultry. In the field, these aspects will reduce environmental contamination by the virus (12,18), reduce transmission to birds, as has been demonstrated experimentally (4,13), reduce human exposure and infections (18,29), and maintain livelihood and food security of rural poor (18). However, vaccines and vaccination cannot eradicate HPAI; i.e., eradication can only be achieved through a comprehensive strategy coordinating vaccines and vaccination with the other four control components.

There have been some reports of AI vaccine "failures" in the field with cases of HPAI in four countries with enzootic infection that vaccinate, and isolation of H5N1 and/or identification of disease clinically consistent with HPAI in vaccinated flocks (14,27,31). Statements about the cause of these vaccine "failures" in HPAI field control are short on facts and long on speculations. What is the true situation in the field? What can we learn by digging down deep into individual countries? OFFLU, the joint OIE and FAO animal influenza network of experts, conducted an evaluation of global avian influenza control strategies for poultry, especially AI vaccine and vaccination components, during 2010–11 (21,29). The evaluation of HPAI control programs was divided into three levels: 1) overall economic condition of the countries, along with functionality of their veterinary services and poultry density, 2) international comparisons of HPAI control programs, and 3) detailed examination of the application of the HPAI control program within individual countries.

General economic, agricultural, and veterinary services impact of control. The initial study compared HPAI control against a country's national poultry density, the performance of veterinary services, and economic indicators. Data on outbreak duration, eradication time, mortality rate, and culling rate were collected (19,20). These data were compared to poultry density, economic indicators (gross domestic product [GDP], agricultural GDP [AGDP], % AGDP, GDP per capita, gross national income [GNI], human development index [HDI], and membership in the Organisation for Economic Cooperation and Development [OECD]), and the functionality of national veterinary services based on OIE Performance of Veterinary Services (PVS) evaluation of critical competencies. Countries were grouped as least, developing/transition, and developed for analysis based on United Nations classification.

Poultry density of least-developed countries showed significant positive correlation with the HPAI outbreak data; i.e., as poultry density at the national level is increased, HPAI outbreaks are longer with higher mortality and culling rates (21). This suggests that high-density poultry farms in least-developed countries may be a risk factor for easy transmission of virus and prolonged HPAI outbreaks because biosecurity practice levels are low and surveillance systems are weak. However, in developing/transition and developed countries, no correlation existed at the national level between poultry density and eradication data, suggesting that increased

Table 1. List of the 30 epizootics of HPAI in domestic poultry and wild birds between 1959 and 2012 (30).

Year	Country	Subtype	Species
1959	Scotland	H5N1	Chickens (*Gallus gallus domesticus*)
1961	South Africa	H5N3	Common terns (*Sterna hirundo*)
1963	England	H7N3	Turkeys (*Meleagridis gallopavo*)
1966	Canada	H5N9	Turkeys
1975	Australia	H7N7	Chickens and ducks (*Anas platyrhyncos*)
1979	Germany	H7N7	Chickens and geese (*Anser anser domesticus*)
1979	England	H7N7	Turkeys
1983–84	USA	H5N2	Chickens, turkeys, partridges (*Alectoris chukar*), guinea fowl (*Numida meleagris*), and miscellaneous poultry
1983	Ireland	H5N8	Turkeys, chickens, ducks
1985	Australia	H7N7	Chickens
1991	England	H5N1	Turkeys
1992	Australia	H7N3	Chickens, ducks
1994	Australia	H7N3	Chickens
1994–95	Mexico	H5N2	Chickens
1995 & 2004	Pakistan	H7N3	Chickens
1996–cont.	Asia, Africa, Europe	H5N1	Various poultry and wild birds
1997	Australia	H7N4	Chickens, emus (*Dromaius novaehollandiae*)
1997	Italy	H5N2	Various poultry
1999–2000	Italy	H7N1	Chickens, turkeys, guinea fowl, quail, ducks, pheasants, ostriches (*Struthio camelus*)
2002	Chile	H7N3	Chickens, turkeys
2003	Netherlands, Belgium, Germany	H7N7	Chickens and various other poultry
2004	USA	H5N2	Chickens and various other poultry
2004	Canada	H7N3	Chickens
2004 & 2006[A]	South Africa	H5N2	Ostriches and various other poultry
2005	Peoples' Republic of Korea	H7N7	Chicken
2007	Canada	H7N3	Chickens
2008	England	H7N7	Chickens
2009	Spain	H7N7	Chickens
2011–cont.	South Africa	H5N2	Ostriches
2012–cont.	Chinese Taipei	H5N2	Chickens

[A]Additional studies are needed to determine if these are two distinct lineages for the hemagglutinin genes of these H5N2 viruses. In this table, they are grouped together as one lineage of virus.

poultry density is offset by improved biosecurity practices to reduce transmission. However, with intracountry analysis by region, province, or county, high poultry density even in developed countries may prolong outbreaks (26). There was no significant association between HPAI outbreak data and the economic indicators of GDP, AGDP, % AGDP, GDP/capita, GNI, and HDI (21). However, OECD membership was associated with shorter and significantly fewer HPAI outbreaks, quicker eradication times, lower reported mortality rates (compared to at-risk susceptible poultry), and higher culling rates than non-OECD member countries. OECD members not only have high-income economies, but they also require governmental transparency and good governance.

The role of national, provincial, and local veterinary services in control of animal diseases is critical in implementing policy. OIE has developed and implemented the PVS tool, which evaluates the function of critical competencies of a country's veterinary services. For this study, core competencies were evaluated against HPAI outbreak data for staffing of veterinarians and paraveterinarians; professional competencies of veterinarians; continuing education; emergency funding; veterinary laboratory diagnosis; epidemiologic surveillance; availability of veterinary medicines and biologic agents; transparency; and disease prevention, control, and eradication measures. A negative correlation was identified between HPAI control data and the following factors (i.e., HPAI control was worse if individual competency scores were low)(21):

1) Low competency score for staffing of veterinarians and other professionals correlated with longer eradication time, higher mortality rate, higher culling rate, and larger number of outbreaks.
2) Lower competency score for staffing of veterinary paraprofessionals correlated with higher mortality rate.
3) Lower competency score for professional competencies of veterinarians correlated with higher mortality rate.
4) Lower competency score for continuing education correlated with higher mortality rate.
5) Lower competency score for emergency funding correlated with longer eradication time.
6) Lower competency score for epidemiologic surveillance correlated with longer HPAI eradication time.
7) Lower competency score for veterinary medicines and veterinary biologic agents correlated with higher culling rate and greater number of outbreaks.
8) Lower competency score for transparency correlated with higher culling rate and greater number of outbreaks.
9) Lower competency score for disease prevention, control, and eradication measures correlated with longer eradication time, higher culling rate, and greater number of outbreaks.

In general, these data emphasize that lower critical competencies of veterinary services are associated with poorer AI outbreak control, as is evident by greater numbers of HPAI outbreaks, the longer

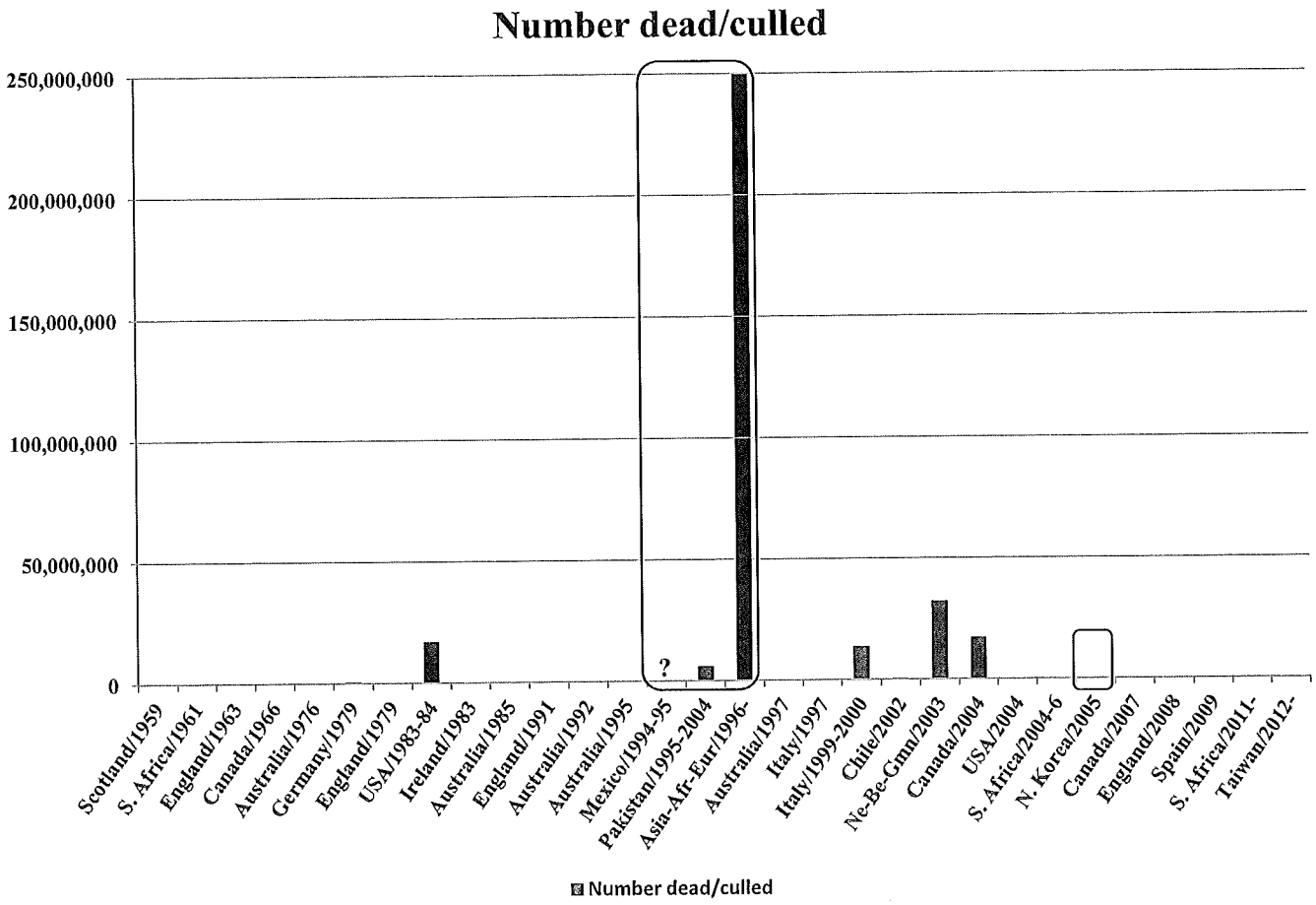

Fig. 1. Number of birds that died or were culled for HPAI in the 30 reported epizootics from 1959 to early 2012. Lack of visible bar indicates <1 million birds.

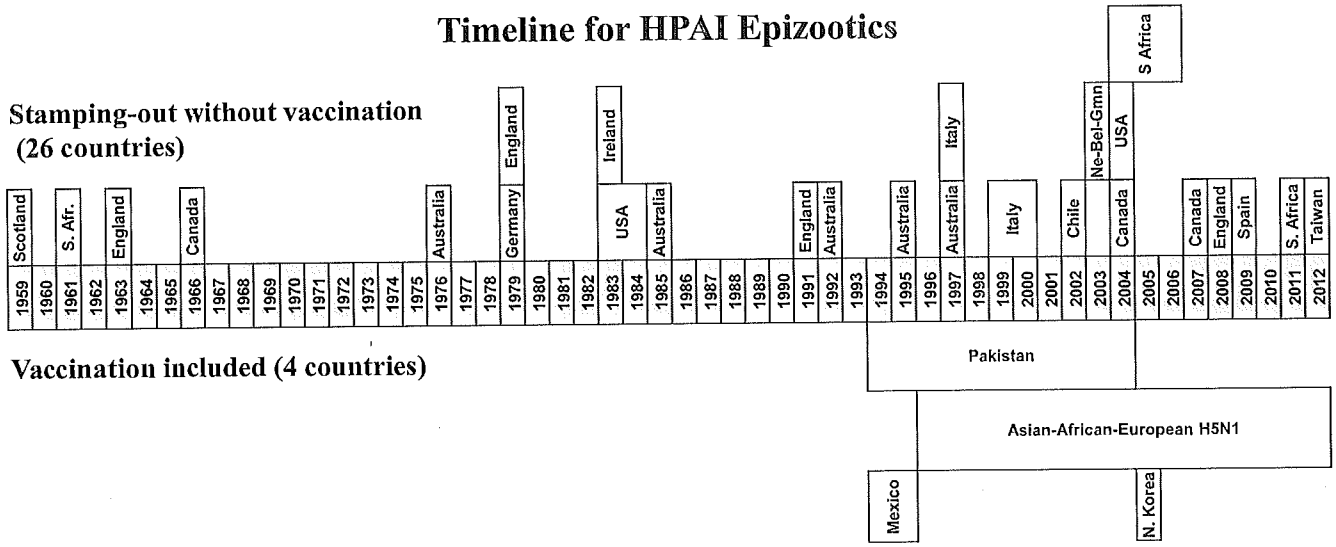

Fig. 2. Time line for the 30 reported HPAI epizootics from 1959 to early 2012.

duration of outbreaks, and higher mortality rates. Interestingly, the link between competencies of veterinary services, including emergency funding, and control of HPAI corroborates previous analyses emphasizing that adequate finances and good governance, as espoused in OECD membership, are essential for functionality in animal disease control.

International comparisons of HPAI control programs. An assessment of the specific components in AI control programs for countries that reported HPAI and/or H5/H7 low pathogenicity notifiable avian influenza (LPNAI) outbreaks during 2002–10 was accomplished (29). Sixty-nine of 80 countries completed and returned a questionnaire (86%) on their national HPAI and H5/H7 LPNAI control programs. Responding countries were from six continents: Asia ($n = 24$), Africa ($n = 10$), North America ($n = 5$), South America ($n = 1$), Europe ($n = 28$), and Australia/Oceania ($n = 1$). Of the 69 countries, 27 countries (39%) had HPAI outbreaks in poultry, 11 countries (16%) had HPAI outbreaks in wild birds, 26 countries (38%) had HPAI outbreaks in both poultry and wild birds, and five countries (7%) had H5/H7 LPNAI outbreaks in poultry. In addition, 14 (20%) of the 69 countries had both HPAI and H5/H7 LPNAI outbreaks in poultry. All 69 countries had national HPAI and H5/H7 LPNAI control programs. These programs had many common components, including: quarantine and additional movement restrictions or controls, tracing of poultry in the outbreak area, enhanced biosecurity measures, farmer and public education and awareness about the disease, monitoring, rapid diagnostics, stamping-out of positive cases, disinfection of facilities and equipment, decontamination and disposal of infectious materials, and compensation. Some countries had more detailed and tailored plans that also included additional components, such as crisis management framework, high-throughput rapid diagnostic testing, early processing of at-risk noninfected poultry, emergency vaccination, pen-side testing as a screening tool, and zoning of movement restrictions and surveillance. The practice of eliminating infected poultry varied with country. For HPAI, some countries practiced culling only on the infected premises, while with others, culling also included dangerous contacts and contiguous premises. With some countries, a zonal approach was used of culling all poultry within 0.5, 1, or 3 km of the infected premises. With AI control programs in some countries, alternatives for elimination of poultry infected with H5/H7 LPNAI were used, including marketing of recovered birds, but most elected to cull infected poultry. The quantitative implementation of each component varied with individual country, resulting in variations in the speed of eradication.

Fifty-eight percent of countries specified a vaccination option for HPAI, while only 39% had vaccination as an option in H5/H7 LPNAI control strategies. Vaccination can be used in three ways: as a preventive tool when risk of introduction is high, or as an emergency action after an outbreak begins, or as a routine measure when infection is enzootic within the country. However, for an emergency vaccination program to be effective, a vaccine bank, field trials, and a vaccination implementation plan in the field need to be in place before the emergency occurs. Fifty-eight percent of the countries had written plans with specific vaccine and vaccination use criteria, but only 14% had completed AI vaccine and vaccination simulation exercises or worked out the logistics of implementing a vaccination program. Field trials for vaccine efficacy had been conducted by 25% of the countries for H5, and 7% of the countries for H7 vaccines. Thirty percent of countries had used vaccines for HPAI control; 16% of countries used it in poultry, only 10% used it in collections of captive birds, and 4% used it in both poultry and bird collections. For H5/H7 LPNAI control, 12% of countries used vaccines. Non-H5/H7 LPAI vaccines were used in 17% of countries, where H9N2 was the most common and was used in 10 countries. However, some vaccines have been used to control H1 and H3 swine influenza viruses in turkeys in Canada and the United States, H6 AI viruses in poultry in Germany, South Africa, and the United States, and H2 and H4 AI viruses in poultry in the United States (3,29).

Vaccine banks have been an important part of preparation for emergency vaccination programs. Ten countries had vaccine banks containing H5 vaccines, and three countries had both H5 and H7 vaccines in their banks. The quantity of vaccines ranged from 0.5 to 55 million doses per subtype, but most countries had ≤3.5 million doses per subtype. The vaccines were acquired in 2006 ($n = 4$), 2007 ($n = 1$), 2008 ($n = 1$), 2009 ($n = 1$), and 2010 ($n = 3$), or were not specified. Expiration dates of vaccines ranged from 1 to 4 yr. As vaccines expired, countries have identified two main options for the future: rotating stocks from commercial vaccine manufacturers or opting out of vaccine banks because of perceived decrease in risk for HPAI or H5/H7 LPNAI introduction. Most countries have concluded that maintaining government-held vaccine banks has too high a cost-benefit ratio.

The H5/H7 AI vaccines have been used in six different types of programs: 1) zoo and captive-held nonpoultry, 2) single poultry farms, 3) ring vaccination of poultry around AI outbreaks, 4) targeted vaccination of high-risk poultry, 5) targeted vaccination of specific sectors of poultry, and 6) routine vaccination of poultry. First, a large program funded by the European Union conducted vaccination on zoo birds and captive-held nonpoultry in 13 countries. In addition, seven other countries had vaccination programs for such bird populations (29). Second, single poultry farms conducted vaccination programs in poultry. Examples include the 2003–04 vaccination of layers in a single company of Connecticut (USA) layers for H7N2 LPNAI and the 2006 vaccination of ostriches on a single Israeli farm for H5N1 HPAI (29). Third, ring vaccination has been practiced in poultry around H7N3 outbreak zones in Pakistan since 2004 (29). Fourth, vaccination was targeted for high-risk poultry such as outdoor ducks in France during 2006 and free-range layers in the Netherlands from 2006 to 2008, but such vaccinations were a small percentage of susceptible poultry (29). Fifth, vaccination was focused to specific sectors of poultry, such as turkeys and capons in northern Italy from 2003 to 2005 for H7N3 and/or H5N2 LPNAI (5,15,29). Sixth, routine vaccination of all poultry within a country either as a campaign or age-dependent strategy as has occurred in China, Egypt, Vietnam, and Indonesia against H5N1 HPAI since 2002 (29). In addition, Mexico also has continued a routine vaccination program for H5N2 LPNAI in chickens since 1995 (32,33).

From the survey, the top five reasons cited for not using H5/H7 vaccines were absence of notifiable AI in the country, no immediate risk for notifiable AI outbreaks, successful stamping-out campaigns, the lack of adequate resources for vaccination, and the high cost of vaccines. By comparison, the reasons for using vaccines, as indicated by the survey, by countries that are using, have used, or may use H5/H7 vaccines include stamping-out measures were not adequate in large outbreaks, to control localized infection "persistent" in some population of poultry species (i.e., domestic ducks), to protect expensive breeds and birds, the presence of enzootic disease, and availability and adequacy of vaccination resources.

A common question concerning vaccination is whether vaccination created H5N1 HPAI enzootic status? This is best answered by examining individual countries. For Bangladesh and eastern India, H5N1 HPAI has become enzootic without AI vaccination. In Egypt, 383 outbreaks occurred before vaccination was initiated, and 573

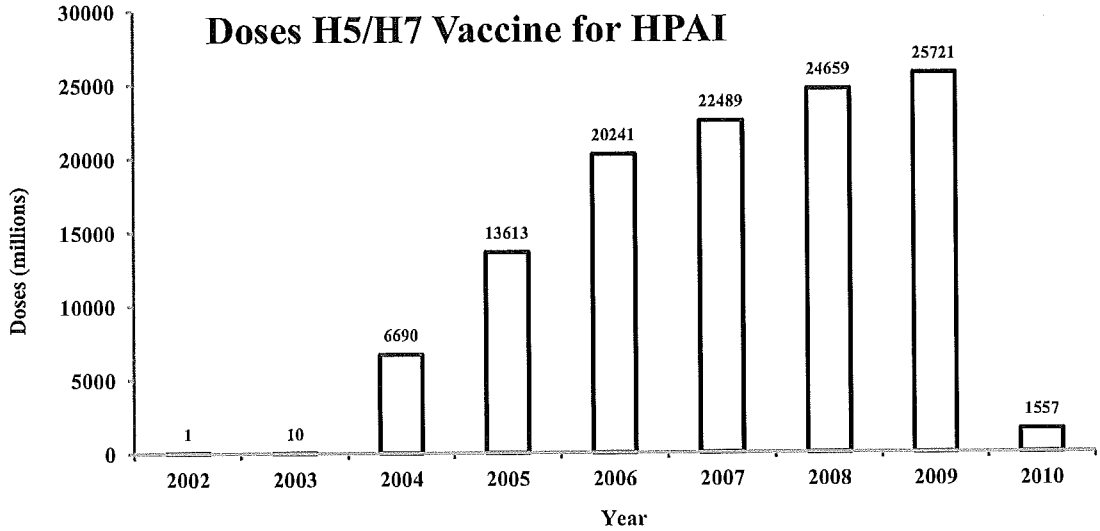

Fig. 3. Summary of the doses of vaccine used in poultry for HPAI by year from 2002 to 2010.

outbreaks occurred before 1% of the poultry population had been vaccinated (6,11). In China, the first H5N1 cases were reported in 1996 in published literature, but no official outbreaks were reported to OIE until 2004, when China received OIE membership. During the first two months of 2004, 48 outbreaks occurred in 16 provinces throughout the whole country (i.e., H5N1 HPAI cases in the northern, southern, western, and eastern provinces of China), while vaccination for mainland China did not begin until after the 2004 outbreaks (8,9,19). For Indonesia, vaccination began mid-2004, but the first cases of H5N1 HPAI occurred in July 2003, with 312 outbreaks and 10.9 million deaths by June 2004 (19,23). Vaccination in Vietnam did not start until October 2005, but by the end of 2004, 24% of communes and 60% of towns had cases, with 17% of the poultry population being affected (19,25). These data suggest that H5N1 HPAI was enzootic before vaccination was effectively applied (29).

Vaccine usage for HPAI has varied by year, with initial implementation in 2002 in Hong Kong (Fig. 3) (29). Usage increased in 2003 with full implementation of vaccination in Hong Kong. Usage jumped in 2004 with initiation of vaccination in China and Indonesia, but the demand in China alone was greater than the manufacturing capacity, and manufacturing was only expanded sufficiently to meet the demand in 2006. The total usage from 2002 to 2010 was over 113 billion doses in 15 countries, with China leading the total vaccine usage (>103 billion doses; 90.99%; Figs. 4, 5). This is followed by 5 billion doses in Egypt, 2.6 billion in Indonesia, and 1.6 billion in Vietnam, which account for 4.65%,

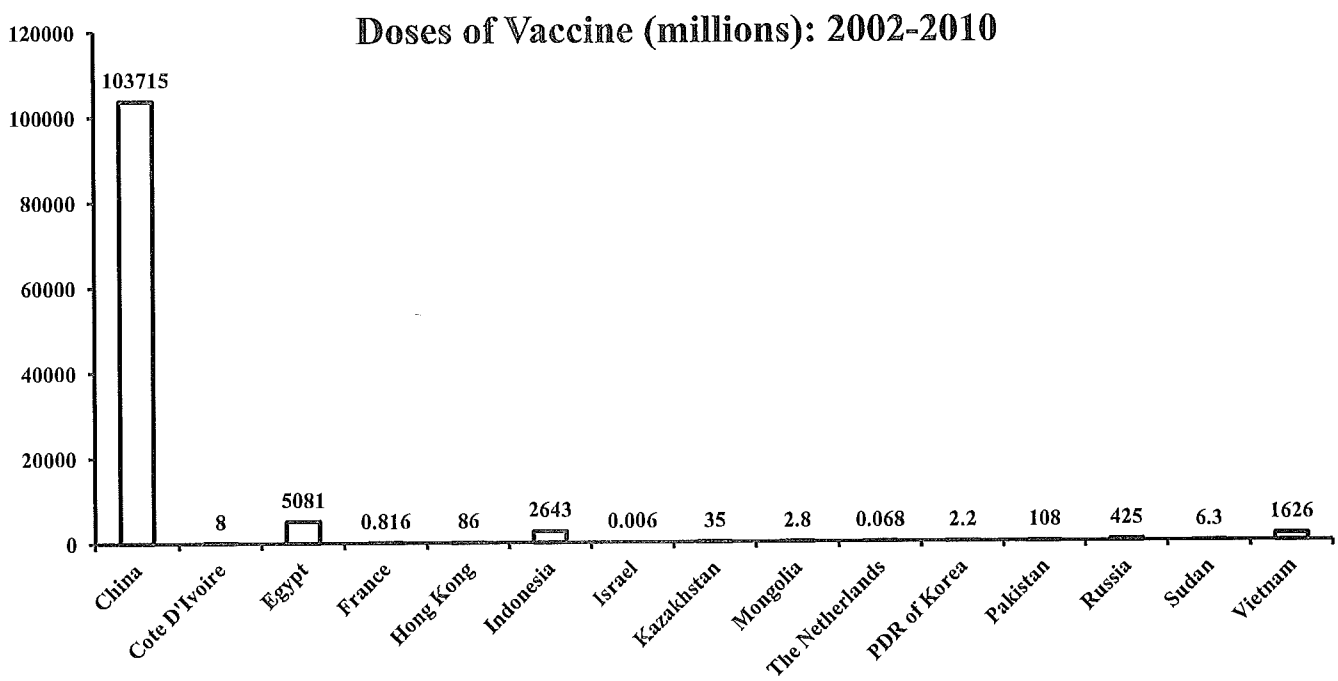

Fig. 4. Summary of H5 vaccine doses used in poultry by the 15 countries vaccinating poultry against HPAI from 2002 to 2010.

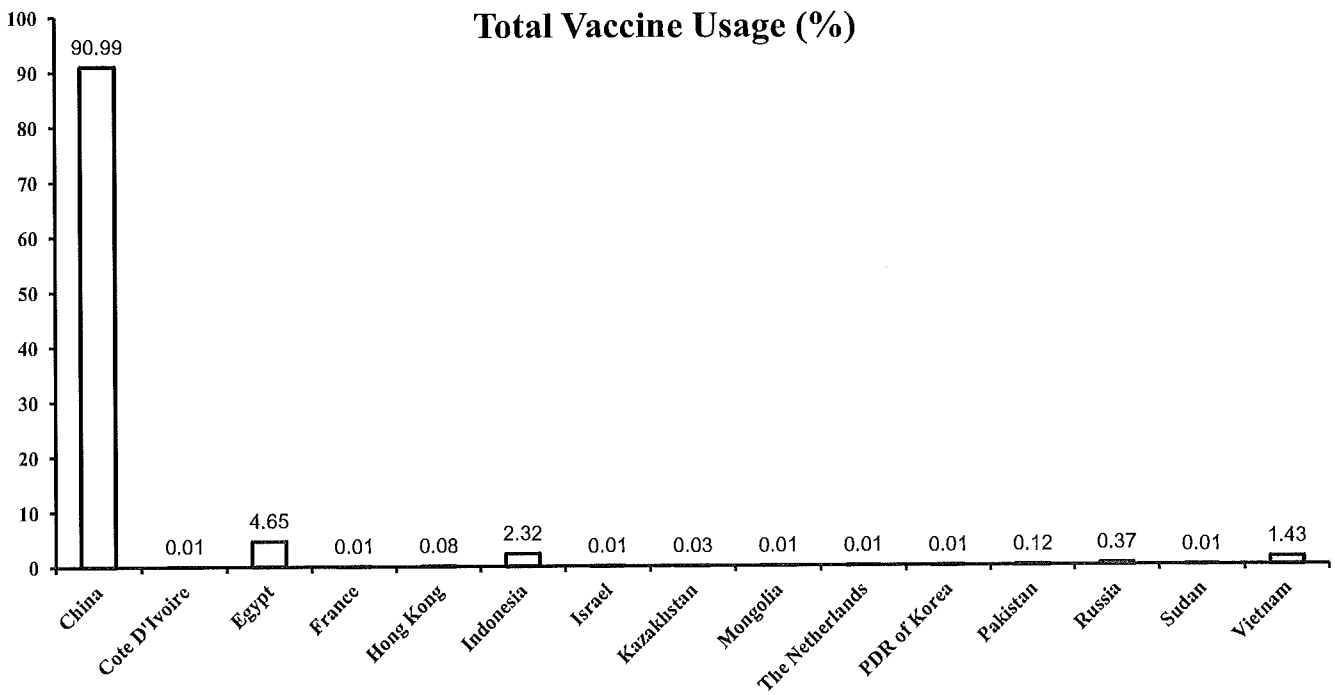

Fig. 5. Percentage of total vaccine doses used in poultry by the 15 countries vaccinating poultry against HPAI from 2002 to 2010.

2.32%, and 1.43% of vaccine used against HPAI, respectively. Inactivated AI vaccines have accounted for 95.5%, and live recombinant virus vaccines have accounted for 4.5% of vaccine used. The latter was primarily recombinant Newcastle disease vectored vaccine with H5 influenza gene insert. These 113 billion doses were used in an at-risk national poultry population of 131 billion (41.9% coverage rate), compared to global poultry production of 520 billion (10.9% coverage rate). However, this high vaccine usage was associated with the very high poultry production in China, compared to a much lower production in Egypt, Indonesia, and Vietnam, and the even smaller usage in the other 11 countries/regions (Fig. 6). However, the usage rates varied in individual countries (Fig. 7), with a high of 86.2% for Hong Kong for all years, but when fully implemented in 2004, the program was 100% in Hong Kong, the only location to achieve a level of effective population immunity. In other countries, the vaccination programs reached a lower percentage of the poultry population because of the difficult logistics of implementing vaccination in sector 3 and 4 poultry. In addition, estimates of sector 3 and 4 poultry may be too low. For example, Egypt had a calculated 69.9% coverage rate based on official OIE production numbers, but with estimates from the Egyptian Ministry of Agriculture for a greater poultry population in sector 3 and 4 production systems, the vaccination coverage dropped to between 27.8% and 48.6% (29). The coverage rate for poultry in China, Mongolia, and Vietnam was approximately 50% (Fig. 7), but with the other 10 countries, coverage was less than 15%.

The type of poultry covered varied with vaccination program in each country. For 2004–09, Egypt concentrated most heavily on meat and breeder chickens (78.2% of 1.9 billion birds) and layers (31.6% of 91 million hens), and had much lower coverage in turkeys (10.4% of 8.1 million birds), ducks (15% of 60 million birds), and geese (1.4% of 40 million birds; Fig. 8). In contrast, Vietnam's program obtained a higher vaccination rate in meat and breeder ducks (90% of 322 million population) and lower vaccination rate in meat and breeder chickens (50% of 1.3 billion population). Finally, Pakistan concentrated on higher vaccination rates among long-lived broiler and layer breeders (25.8% of 54 million population) and layers (2.8% of 344 million population), but did not vaccinate short-lived meat chickens (0% of 2.7 billion population). Usage of vaccine for control of H5/H7 LPNAI accounted for <10.1 billion doses, which corresponds to 8.1% of H5/H7 vaccine as compared to HPAI vaccine usage.

Detailed intracountry application of vaccination in a HPAI control program. Countries with highly functional, coordinated, adequately funded national, provincial, and local veterinary services had the greatest success with implementing and monitoring HPAI vaccination programs. These countries generally had predeveloped logistic plans for vaccination and had exercised vaccination plans as table-top or field exercises. Vaccines were available commercially or from a vaccine bank within the country, which indicated the vaccines had completed the licensing and registration process or the country had an emergency authority to use the vaccines. Generally, stockpiles of vaccine were available in the country through either existing manufacturing capacity or importation from countries that manufactured such vaccines. The time period when the vaccination program was initiated had an impact on success, such that shorter times between diagnosis of the index case and vaccination implementation impacted time to eradication. For vaccination to be effective, there must be population immunity within the control zone or compartment. This was easier to achieve with implementation of vaccination in industrial production sectors 1 and 2 than in semicommercial and village sectors 3 and 4 of least-developed and developing/transition countries. To be effective, population immunity must be a minimum of 60%, but optimal immunity requires >80% immunity in the susceptible at-risk poultry population. Single vaccination has been attempted for achieving protection in meat chickens, but such programs have not been effective in producing immunity in the field (22), and experimental studies have also questioned the utility of single-dose vaccine except under very defined conditions (10). To

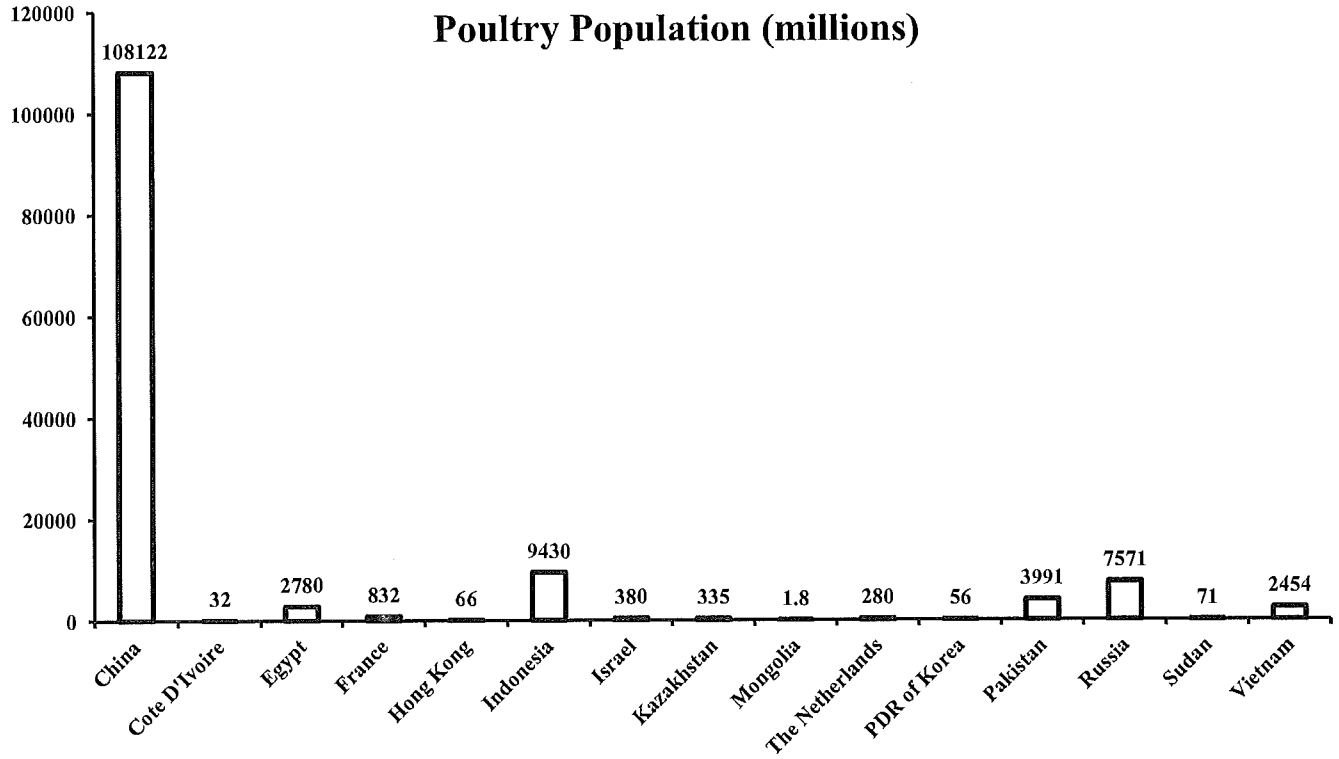

Fig. 6. Poultry production populations for 15 H5 vaccinating countries for 2002–10.

achieve high population immunity economic incentives are required for farmers to vaccinate, and such vaccination must control a real disease and not a theoretical disease. For example, it has been difficult to convince farmers in least-developed and developing/transitional countries to vaccinate for the good of public health or to prevent a "pandemic" such as in domestic ducks, which have minimal disease to no disease in the field when infected with H5N1 HPAI virus. Vaccination compliance has dropped in Vietnam for sector 3 duck farms because the farmers do not see the benefit in livelihood and food security terms. The major reason for vaccine and vaccination "failure" in the field has been the inability to achieve population immunity in 80% of the susceptible population as the result of low vaccine

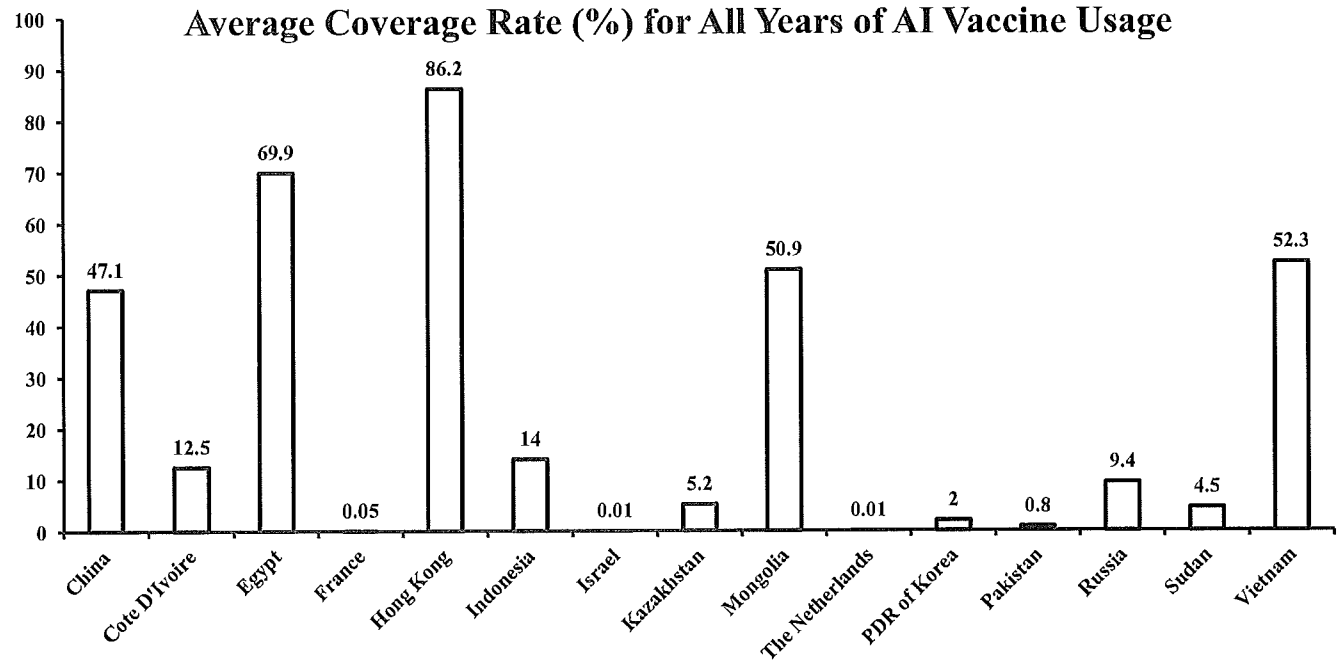

Fig. 7. Average national vaccine coverage rate for 15 countries vaccinating against HPAI for 2002–10.

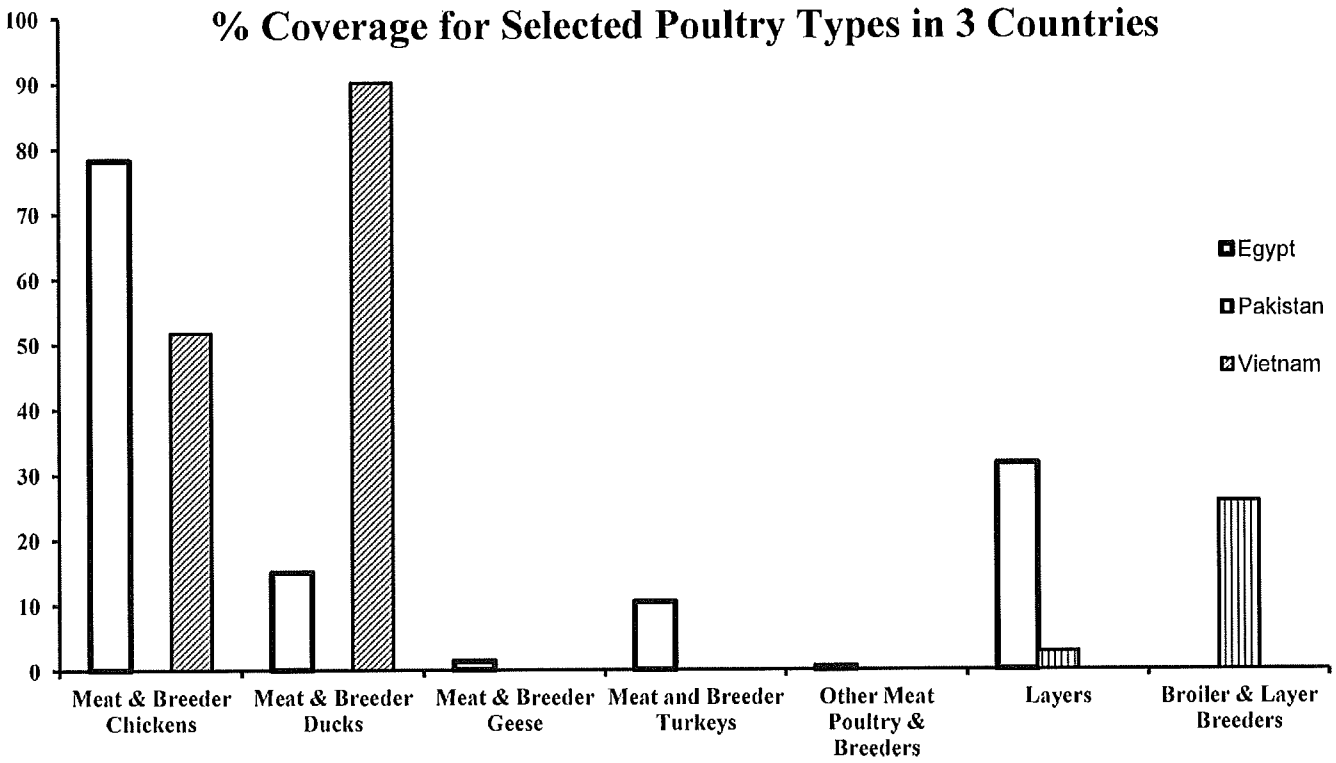

Fig. 8. Vaccine coverage rate for different types of poultry in Egypt, Vietnam, and Pakistan for 2002–10.

administration or coverage rate. In Egypt, for sectors 3 and 4, only 36% of the poultry are vaccinated, but in commercial sectors 1 and 2, the vaccination coverage is 50%–60% (22).

Recently, sporadic failures of vaccines and vaccination have been the result of continued usage of antigenically inappropriate vaccine seed strain after H5N1 HPAI viruses had circulated in the field for multiple years and antigenically drifted away from vaccine seed strains. This necessitates conducting *ongoing* surveillance of field strains and conducting challenge studies on a biannual basis using epidemiologically relevant field strains. This may require use of more than one seed strain or bivalent vaccines in countries with long-term vaccine use.

Field viruses have appeared in Egypt, China, Vietnam, Hong Kong, and Indonesia that are resistant to H5 vaccine seed strains, but not against H7 vaccine seed strains used in Pakistan (1,28). In Egypt, which has vaccinated poultry since early 2006, several field viruses have been identified that are resistant to classic seed strains Mexico/94 and RE-1 vaccine seed strains (2,7,14,28). China has also experienced reduced effectiveness of H5 seed strains, necessitating changes of vaccine seed strains as resistance occurred in the field. They have used various vaccine seed strains since 2004: A/Turkey/England/1973 (H5N2) LPNAI virus from 2004 to 2006; RE-1, a reverse genetic (rg) A/goose/Guangdong/1/1996 (H5N1), clade 0 lineage virus produced from 2004 to 2008; RE-4, an rgA/chicken/Shanxi/2006 (H5N1), clade 7 lineage virus produced for 2006–07; RE-5, a rgA/duck/Anhui/1/2006 (H5N1), clade 2.3.4 lineage virus produced from 2008; and RE-6, a rgA/duck/Guangdong/S1322/2010 (H5N1), clade 2.3.2 lineage virus with production beginning in 2012. In 2011, Vietnam identified several 2.3.2.1 strains resistant to immunity from RE-1 and RE-5 vaccines, and they proposed usage of a new RE-6 starting in 2012. Hong Kong experienced an H5N1 HPAI outbreak in a well-vaccinated flock during 2008. The isolated virus was clade 2.3.4 and was resistant to protection from the Mexican/94 vaccine seed strain. Indonesia began experiencing outbreaks of clinical disease consistent with HPAI in well-vaccinated flocks during 2007. Historically, the classic H5 vaccines are antigenically similar, but H5N1 strains were isolated from flocks with "vaccine breaks" that overcame protection from Mexico/94, RE-1, and other H5 vaccine seed strains. H5 vaccine-resistant strains have not been reported from Pakistan, Russia, or the other countries that have used a more targeted vaccination program (29). Additional details of HPAI vaccination programs are presented elsewhere (29).

Vaccination protocols should be developed and tailored to fit the poultry species and type of bird, the production sector, and the immune status of the population. Movement controls on poultry populations are necessary to prevent dispersion of infected poultry to live poultry markets from any production sector, i.e., sectors 1, 2, 3, and 4.

Future Directions in AI vaccines and vaccination. Over the next 5 yr, specific changes will be needed to further improve vaccines and vaccination of poultry in controlling HPAI:

1) age- and species-specific targeted vaccination based on surveillance, and applied to both clinically affected and asymptomatically infected species instead of attempts to apply vaccines through timed campaigns in all poultry within a country;
2) improved vaccines that can be applied by mass application methods such as aerosol, *in ovo*, feed, etc., and improvement of application methods for broader population coverage;
3) specific protocols to achieve a two-vaccine-dose regime at an early age, such as a priming vaccination at 1–10 days of age and a boost vaccination between 10 and 30 days of age;
4) Marek's disease herpesvirus vectored vaccine with H5 gene insert for single-dose protection for short-lived broilers, or priming with other recombinant vaccines (recombinant fowlpox virus or Newcastle disease vector with H5 gene insert) and boost with inactivated H5 AI vaccine;

5) recombinant vaccine for ducks and geese, such as duck virus enteritis (DVE) vaccine virus with AI HA gene that will protect from an economic disease (i.e., DVE) but also reduce infection by H5N1 HPAI virus; and
6) development of more realistic AI vaccine exit strategies based on risk models in current and future production systems, and not based on imposed time lines without scientific merit.

SUMMARY

Countries with high poultry density that are least economically developed have had the most difficulty in controlling/eradicating HPAI, emphasizing need to improve biosecurity and other parts of HPAI control programs. An effective HPAI control program requires good governance, transparency, competent veterinary services, and economic support. The primary strategy for HPAI and H5/H7 LPNAI control will continue to be immediate eradication by a four-component strategy: education, biosecurity, rapid diagnostics and surveillance, and elimination of infected poultry. Vaccination can be a second tier component or "tool" when immediate eradication is not feasible, to maintain livelihoods and food security, and to control clinical disease until a primary strategy can be developed and implemented to achieve eradication.

ADDENDUM

After submission of the manuscript, an outbreak of H7N3 HPAI began in layer chickens in Mexico during June 2012. A stamping-out program has been initiated and vaccination of layers in the outbreak area has begun. This increases the number of HPAI epizootics since 1959 to 31.

REFERENCES

1. Abbas, M. A., E. Spackman, R. Fouchier, D. Smith, Z. Ahmed, N. Siddique, L. Sarmento, K. Naeem, E. T. McKinley, A. Hameed, S. Rehmani, and D. E. Swayne. H7 avian influenza virus vaccines protect chickens against challenge with antigenically diverse isolates. Vaccine 29:7424–7429. 2011.

2. Abdelwhab, E. M., C. Grund, M. M. Aly, M. Beer, T. C. Harder, and H. M. Hafez. Multiple dose vaccination with heterologous H5N2 vaccine: immune response and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens. Vaccine 29:6219–6225. 2011.

3. Abolnik, C., E. Cornelius, S. P. R. Bisschop, M. Romito, and D. Verwoerd. Phylogenetic analyses of genes from South African LPAI viruses isolated in 2004 from wild aquatic birds suggests introduction by Eurasian migrants. Dev. Biol. (Basel) 124:189–199. 2006.

4. Bouma, A., A. T. Muljono, A. Jatikusumah, A. Nell, S. Mudjiartiningsih, I. Dharmayanti, Siregar E. Sawitri, I. Claassen, G. Koch, and J. A. Stegeman. Field trial for assessment of avian influenza vaccination effectiveness in Indonesia. Rev. Sci. Tech. Off. Int. Epiz. 27:633–642. 2008.

5. Capua, I., A. Schmitz, V. Jestin, G. Koch, and S. Marangon. Vaccination as a tool to combat introductions of notifiable avian influenza viruses in Europe, 2000 to 2006. Rev. Sci. Tech. Off. Int. Epiz. 28:245–259. 2009.

6. Castellan, D. FAO mission in support of avian influenza prevention and control in Egypt. FAO, Rome. pp 1–30. 2006.

7. Cattoli, G., A. Milani, N. Temperton, B. Zecchin, A. Buratin, E. Molesti, M. M. Aly, A. Arafa, and I. Capua. Antigenic drift in H5N1 avian influenza virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. J. Virol. 85:8718–8724. 2011.

8. Chen, H. Avian influenza vaccination: the experience in China. OIE Rev. Sci. Tech. 28:267–274. 2009.

9. Chen, H., and Z. Bu. Development and application of avian influenza vaccines in China. Curr. Top. Microbiol. Immunol. 333:153–162. 2009.

10. Eggert, D., and D. E. Swayne. Single vaccination provides limited protection to ducks and geese against H5N1 high pathogenicity avian influenza virus. Avian Dis. 54:1224–1229. 2010.

11. Freiji, M. Lessons learnt from avian influenza H5N1 epidemic in Egypt. In: Conference on Co-ordinated Response Strategy to Avian Influenza, 29 May 2006. International Financial Corporation, World Bank Group, Cairo, Egypt, presentation. 2006.

12. Gilbert, M., X. Xiao, D. U. Pfeiffer, M. Epprecht, S. Boles, C. Czarnecki, P. Chaitaweesub, W. Kalpravidh, P. Q. Minh, M. J. Otte, V. Martin, and J. Slingenbergh. Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia. Proc. Natl. Acad. Sci. U. S. A. 105:4769–4774. 2008.

13. Goot, J. A., G. Koch, M. C. Jong, and M. v. Boven. Transmission dynamics of low- and high-pathogenicity A/Chicken/Pennsylvania/83 avian influenza viruses. Avian Dis. 47(3):939–941. 2003.

14. Kilany, W. H., A. Arafa, A. M. Erfan, M. S. Ahmed, A. A. Nawar, A. A. Selim, S. G. Khoulosy, M. K. Hassan, M. M. Aly, H. M. Hafez, and E. M. Abdelwhab. Isolation of highly pathogenic avian influenza H5N1 from table eggs after vaccinal break in commercial layer flock. Avian Dis. 54:1115–1119. 2010.

15. Marangon, S., I. Capua, G. Pozza, and U. Santucci. Field experiences in the control of avian influenza outbreaks in densely populated poultry areas. Dev. Biol. (Basel) 119:155–164. 2004.

16. Naeem, K., and N. Siddique. Use of strategic vaccination for the control of avian influenza in Pakistan. Dev. Biol. (Basel) 124:145–150. 2006

17. [OIE] World Organization for Animal Health. Avian influenza in Korea (Democratic People's Republic of) [Internet]. OIE Disease Information 18(14) [cited 2005 May 15]. Available from: http://www.oie.int/eng/info/hebdo/AIS_76.HTM#Sec42005

18. OIE. Avian influenza vaccination: OIE information document and Verona recommendations [Internet]. [cited 2011 Mar 23] Available from: http://www.oie.int/eng/info_ev/Other%20Files/A_Guidelines%20on%20AI%20vaccination.pdf 2007

19. OIE. Animal health data (prior to 2005): Handistats II [Internet]. [cited 2011 Jul 27]. Available from: http://web.oie.int/hs2/report.asp?lang=en 2011

20. OIE. World animal health information system [Internet] [cited 2011 May 19]. Available from: http://web.oie.int/wahis/public.php?page=home 2011

21. Pavade, G., L. Awada, K. Hamilton, and D. E. Swayne. Analysis of economic indicators, poultry density and performance of veterinary services for control of high pathogenicity avian influenza in poultry. OIE Rev. Sci. Tech. 30:661–671. 2011.

22. Peyre, M., H. Samaha, Y. B. Makonnen, A. Saad, A. Abd-Elnabi, S. Galal, T. Ettel, G. Dauphin, J. Lubroth, F. Roger, and J. Domenech. Avian influenza vaccination in Egypt: limitations of the current strategy. J. Mol. Genet. Med. 3:198–204. 2009.

23. Sawitri, S., E. Darminto, J. Weaver, and A. Bouma. The vaccination programme in Indonesia. Dev. Biol. (Basel) 130:151–158. 2007.

24. Sims, L. D. Experience in control of avian influenza in Asia. Dev. Biol. (Basel) 130:39–43. 2007.

25. Sims, L. D., and I. H. Brown. Multi-continential epidemic of H5N1 high pathogenicity avian influenza (1996–2007). In: Avian influenza. D. E. Swayne, ed. Blackwell, Ames, IA. pp. 251–286. 2008.

26. Stegeman, A., A. Bouma, A. R. Elbers, M. C. de Jong, G. Nodelijk, F. de Klerk, G. Koch, and M. van Boven. Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003: course of the epidemic and effectiveness of control measures. J. Infect. Dis. 190:2088–2095. 2004.

27. Swayne, D. E., and D. R. Kapczynski. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunol. Rev. 225:314–331. 2008.

28. Swayne, D. E., and D. R. Kapczynski. Vaccines, vaccination and immunology for avian influenza viruses in poultry. In: Avian influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA. pp. 407–451. 2008.

29. Swayne, D. E., G. Pavade, K. Hamilton, B. Vallat, and K. Miyagishima. Assessment of national strategies for control of high pathogenicity avian influenza and low pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. OIE Rev. Sci. Tech. 30:839–870. 2011.

30. Swayne, D. E., D. L. Suarez, and L. D. Sims. Influenza. In: Diseases of poultry, 13th ed. D. E. Swayne, J. R. Glisson, L. R. McDougald, V. Nair, L. K. Nolan, and D. L. Suarez, eds. Wiley-Blackwell, Ames, IA, In press. 2013.

31. Takano, R., C. A. Nidom, M. Kiso, Y. Muramoto, S. Yamada, Y. Sakai-Tagawa, C. Macken, and Y. Kawaoka. Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003–2007. Virology 390:13–21. 2009.

32. Villarreal, C. Experience in control of avian influenza in the Americas. Devel. Biol. 130:53–60. 2007

33. Villarreal, C. L. Control and eradication strategies of avian influenza in Mexico. Dev. Biol. (Basel) 124:125–126. 2006

ACKNOWLEDGMENTS

I thank the following for providing data and insight: Keith Hamilton, Gounalan Pavade, Bernard Vallat, K. Miyagishima, Lena Awada, and staff of World Organisation for Animal Health (Paris and regional offices); Gwenaelle Dauphin, Mia Kim, Filip Claes, Juan Lubroth, and staff at FAO, Animal Production and Health Division (Rome and field); chief veterinary officers and staff of 69 participating countries; Les Sims; Marisa Peyre; David Suarez, Erica Spackman, Mary Pantin-Jackwood, and Joan Beck of the Southeast Poultry Research Laboratory, USDA/ARS; Peter Daniels and Frank Wong of the Australian Animal Health Laboratory; Ron Fouchier of Erasmus University; and Derek Smith of Cambridge University.

Virus Research 167 (2012) 297–301

Contents lists available at SciVerse ScienceDirect

Virus Research

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Biochemical characterization of the small hydrophobic protein of avian metapneumovirus

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ARTICLE INFO

Article history:
Received 24 March 2012
Received in revised form 13 May 2012
Accepted 16 May 2012
Available online 29 May 2012

Keywords:
RNA viruses
Paramyxoviruses
Avian metapneumovirus
SH protein
Glycosylation
Homodimer

ABSTRACT

Avian metapneumovirus (AMPV) is a paramyxovirus that has three membrane proteins (G, F, and SH). Among them, the SH protein is a small type II integral membrane protein that is incorporated into virions and is only present in certain paramyxoviruses. In the present study, we show that the AMPV SH protein is modified by N-linked glycans and can be released into the extracellular environment. Furthermore, we demonstrate that glycosylated AMPV SH proteins form homodimers through cysteine-mediated disulfide bonds, which has not been reported previously for SH proteins of paramyxoviruses.

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1. Introduction

Avian metapneumovirus (AMPV), previously known as turkey rhinotracheitis virus or avian pneumovirus, is a paramyxovirus that causes acute respiratory disease in turkeys and has recently emerged as a major economic problem for the world-wide poultry industry (Branigan et al., 2006; Catelli et al., 2010; Easton et al., 2004; Govindarajan et al., 2006; Naylor et al., 2007; Njenga et al., 2003; Toquin et al., 2006; Velayudhan et al., 2005). Based on its genome and biological characteristics, AMPV and its human counterpart human metapneumovirus (HMPV) are assigned to the genus Metapneumovirus within the subfamily Pneumovirinae. For AMPV, there exist four subgroups, namely, A, B, C, and D (Easton et al., 2004; Velayudhan et al., 2008). Interestingly, sequence analysis has revealed that AMPV subgroup C (AMPV-C) is more closely related to HMPV than to any of the other AMPV subgroups (Govindarajan and Samal, 2005; Toquin et al., 2003; van den Hoogen et al., 2001, 2002). Due to its closely related to HMPV, AMPV-C has been the subject of extensive investigations.

In addition to G (attachment) and F (fusion), AMPV-C encodes third membrane protein that is a small hydrophobic (SH) protein. The SH protein is only present in certain paramyxoviruses, which include mumps, PIV5, human and animal RSV, and metapneumovirus (Collins and Crowe, 2007; Collins and Graham, 2008; Easton et al., 2004; He et al., 2001). Using AMPV-C as a model system, our previous study demonstrated that the SH protein is an integral membrane protein with $N_{in}C_{out}$ orientation located in both the plasma membrane as well as within intracellular compartments (Deng et al., 2011). Furthermore, we showed that AMPV-C SH protein localizes in the endoplasmic reticulum (ER), Golgi, and cell surface, and is transported through ER-Golgi secretory pathway (Deng et al., 2011). In the present study, we report that the SH protein of AMPV-C is modified by N-linked glycans and can be released into the cell culture media. Furthermore, we reveal that glycosylated AMPV SH proteins form homodimers through cysteine-mediated disulfide bonds, which has not been reported previously.

2. Materials and methods

2.1. Plasmids, cells, and antibodies

AMPV subgroup C SH expression plasmid pSH^{HA} was described in our previous study (Deng et al., 2011). This plasmid

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http://dx.doi.org/10.1016/j.virusres.2012.05.013

expression vector expresses SH protein with an HA epitope tag at the C-terminus. COS-1 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. Monoclonal antibody against the HA epitope was purchased from Sigma–Aldrich. Secondary antibody goat anti-mouse IgG-HRP was purchased from Abcam.

2.2. Western blot assay

COS-1 cells grown in 60-nm plates were transfected with 5.0 μg of pSH^{HA}. Transfection was performed using TransIT-LT1 (Mirus) following the procedure outlined by the manufacturer. For the preparation of supernatant-derived materials, the culture media was harvested 24 h post-transfection and pelleted by centrifugation at 2000 × g for 10 min. The clarified supernatants were passed through a 0.22 μm filter before being layered onto a 20% sucrose cushion (PBS) and centrifuged at 100,000 × g for 2 h at 4 °C in a SW41 rotor (Beckman Coulter). The re-suspension media of the pellets followed by low-speed centrifugation was subject to SDS–PAGE and Western blot analysis as supernatant-derived materials. For the preparation of cell lysates, at 24 h post-transfection, cells were harvested and lysed in lysis buffer (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100) in the presence of 1x protease inhibitor cocktail (Pierce, Rockford, IL). The cell lysates were prepared by removing nuclei by centrifugation at 2000 × g at 4 °C for 10 min. Pellets and cell lysates were separated on a 12% SDS–PAGE and transferred to a nitrocellulose membrane (Pierce), then blocked in a PBS buffer containing 0.5% Tween-20 and 5% dry milk powder. SH fusion proteins were detected by a standard Western blotting procedure coupled with the ECL system (Pierce) using anti-HA monoclonal antibody and goat anti-mouse IgG-HRP. Protein bands in western-blots were analyzed by ImageJ software (http://rsbweb.nih.gov/ij/) to quantify the percentage of SH^{HA} proteins present in supernatants relative to total SH^{HA} proteins present in both cell lysates and supernatants. This parameter was used to determine the SH protein secretion efficiency.

For detection of SH homodimer formation, the same SDS–PAGE/Western blot protocol was used except for that SDS–PAGE samples containing SH^{HA} proteins were prepared in the absence (non-reduced condition) or presence of 5% β-mercaptoethanol (reduced condition) before their separation in the gels.

2.3. Glycosidase digestion experiment

Digestion of SH^{HA} protein was carried out with various glycosidases (New England Biolabs): Peptide N-glycosidase F (PNGase F), O-glycanase, and sialidase, at 37 °C for 12 h according to a published protocol (Liu et al., 2007). SH^{HA} proteins were immunoprecipitated from crude cellular lysates with anti-HA monoclonal antibody, and then eluted from protein G plus agarose beads (Pierce) by boiling them for 5 min in commercial denaturing buffer provided with deglycosidase enzymes (New England Biolabs). Enzymes (equivalent to 2000 units) and corresponding reaction buffers were added to SH^{HA} proteins according to protocol provided by manufacturer. Digested samples were finally subjected to western blot analysis by probing with anti-HA monoclonal antibody.

3. Results and discussion

3.1. Intracellular expression profile of the SH protein

To gain insight into biochemical properties of the SH protein, we determined its expression profile. Western blot analysis of both intracellular and extracellular SH proteins in transfected cells was used.

As shown in Fig. 1A, the SH^{HA} fusion protein expressed inside of the cells primarily exhibited four forms. The faint band that migrated at 30 kDa matches the predicted size of the SH protein and likely represents the full-length unglycosylated fusion protein ($SH^{HA}0$). More high prominent molecular weight protein bands in the 43–55 kDa range were also seen, and were likely due to various levels of protein glycosylation as reported for other SH proteins (Collins et al., 1990; Fuentes et al., 2007; Hiebert et al., 1988; Olmsted and Collins, 1989; Rixon et al., 2004). The three most predominant bands of approximately 43, 48, and 52 kDa within the smear were termed as $SH^{HA}1$, $SH^{HA}2$, and $SH^{HA}3$, respectively. SH proteins were also found in the cell culture media at approximately 16% of the amount in the cell lysate (Fig. 1B). This observation suggested that the SH protein is capable of release into the extracellular environment, which agree with our previous study in that we showed that the SH protein is transported through ER-Golgi secretory pathway (Deng et al., 2011).

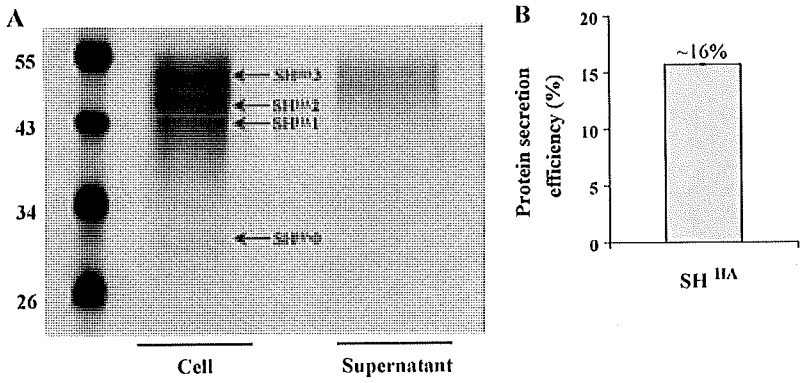

Fig. 1. Intracellular expression profile of the SH protein. (A) SH protein expression profile. COS-1 cells were transfected with pSH^{HA} plasmid. At 24 h posttransfection, cell culture media and cell lysates were analyzed via Western blot with anti-HA monoclonal antibody. (B) SH protein secretion efficiency in transfected COS-1 cells. COS-1 cells were transfected with pSH^{HA} plasmid and 24 h post-transfection samples from the supernatant were analyzed by western blot as compared to whole cell lysates. Approximately 10% of original cell extract and corresponding cell supernatant were used in the Western blot. Protein bands in western blots were scanned and quantified from three repeats by ImageJ software (http://rsbweb.nih.gov/ij/) to determine the percentage of SH^{HA} proteins present in supernatants relative to total SH^{HA} proteins present in both cell lysates and supernatants, as an indicator of SH protein secretion efficiency. Numbers on the left indicate the migration positions of molecular mass markers (in kilodaltons). $SH^{HA}0$ indicates an unmodified full-length SH fusion protein, while $SH^{HA}1/SH^{HA}2/SH^{HA}3$ represents three major glycosylated species of SH fusion proteins with different molecular weights.

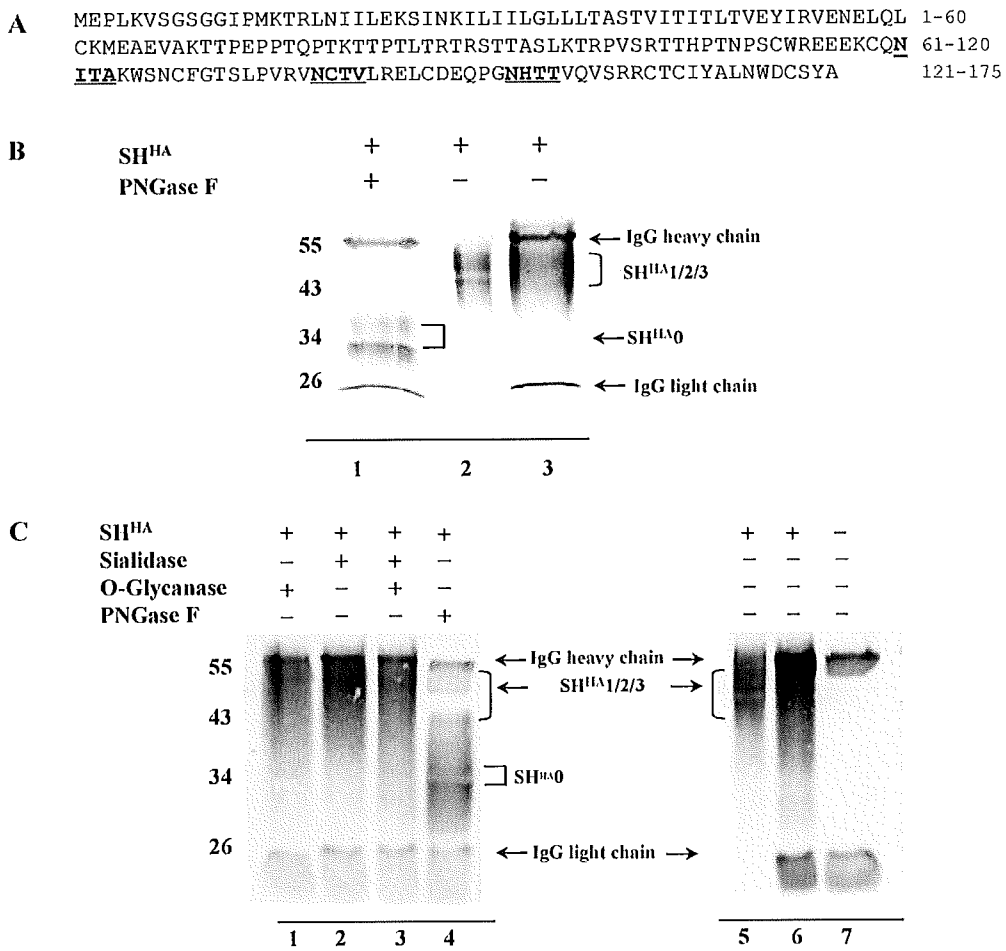

Fig. 2. N-linked glycosylations of the SH protein. (A) Predicted amino acid sequence of AMPV-C SH protein (Yunus et al., 2003). Three potential N-linked glycosylation sites are underlined and bold-typed. (B) Deglycosylation of SH protein with PNGase F. Cellular lysates expressing SH^{HA} protein were immunoprepcipitated with anti-HA monoclonal antibody. Immunoprepcipitates were treated (lane 1) or not (lane 3), as indicated, with PNGase F, and analyzed by western blot. Pre-immunoprepcipitation sample of SH^{HA} protein was analyzed in parallel (lane 2). (C) Enzymatic deglycosylation of SH protein. Cellular lysates prepared from COS-1 cells transfected with pSH^{HA} plasmid or empty vector alone (mock) were immunoprepcipitated with anti-HA monoclonal antibody. Equal portion of immunoprepcipitates was treated with indicated enzymes, respectively, and analyzed by western blot. Lane 1, O-glycanse-treated sample; lane 2, sialidase-treated sample; lane 3, O-glycanse + sialidase-treated sample; lane 4, PNGase F-treated sample; lane 5, untreated pre-immunoprecipitation sample; lane 6, untreated immunoprecipitation sample; lane 7, untreated immunoprecipitation mock sample. Numbers on the left indicate the migration positions of molecular mass markers (in kilodaltons). The migration of IgG molecules (heavy and light chains) and SH^{HA} proteins in both deglycosylated ($SH^{HA}0$) and glycosylated ($SH^{HA}1/2/3$) forms are indicated on the right (B) and on the middle of gel (C), respectively.

3.2. N-linked glycosylations of the SH protein

AMPV-C SH protein contains three potential N-linked glycosylation sites (Fig. 2A) and their locations are highly conserved among different AMPV-C virus strains (data not shown), suggesting their functional significance. To determine whether the SH protein is modified by the addition of N-linked glycans, immunoprecipitated SH proteins were incubated in the absence (Fig. 2B, lane 3) or presence of recombinant peptide N-glycosidase F (PNGase F, an enzyme removing N-linked glycans) (Fig. 2B, lane 1) and analyzed by western blot with anti-HA monoclonal antibody. Cell lysates expressing SH^{HA} prior to immunoprecipitation served as an input control (Fig. 2B, lane 2). PNGase F digestion converted SH^{HA} from a smear of glycosylated form ($SH^{HA}1/2/3$, lane 3 in Fig. 2B) to a deglycosylated form ($SH^{HA}0$, lane 1 in Fig. 2B). This conversion provides evidence that SH is modified by N-linked glycosylations. The reason why deglycosylated SH^{HA} protein ($SH^{HA}0$) runs as a doublet is not clear but could be due to other modifications such as phosphorylation. Phosphorylation of viral glycoproteins in varicella-zoster virus and herpes simplex virus type 1 has been previously established (Grose et al., 1989; Miriagou et al., 2000; Montalvo and Grose, 1986). Alternatively, it could be possible that the truncated form of non-glycosylated SH may arise from translational initiation at the second methionine of the protein, 13 residues downstream of the first one. Two extra bands; ~25 kDa band corresponding to the IgG light chain, and ~55 kDa band corresponding to the IgG heavy chain, were also detected (Fig. 2B, lanes 1 and 3). Presence of these two bands was further demonstrated in western blot analysis of immunoprecipitated complexes from mock transfections (Fig. 2C, lane 7).

To rule out the possibility that SH protein undergoes O-linked glycosylation, we digested immunoprecipitated SH^{HA} proteins with O-glycanase and sialidase either alone or in combination and assayed these samples plus controls using western-blot. Treatment of SH^{HA} with these enzymes had no apparent effect on SH^{HA} protein's mobility in western-blot (Fig. 2C, lanes 1–3) compared to PNGase F-treated positive control (Fig. 2C, lane 4). The data indicate that SH is resistant to O-glycanase digestion and thus is not O-glycosylated. Previous studies of small membrane hydrophobic proteins showed that SH of both human and bovine respiratory syncytial viruses and NB of human influenza virus are modified by polylactosaminoglycan (Anderson et al., 1992; Williams and Lamb,

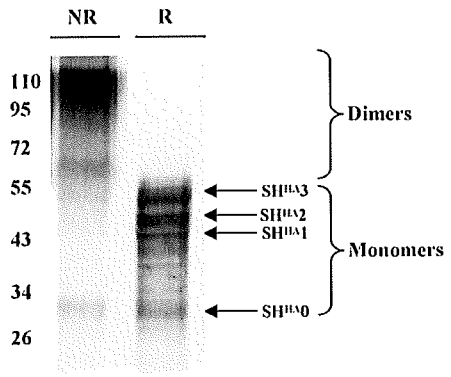

Fig. 3. Dimerization of the SH protein. COS-1 cells were transfected with pSH^{HA} plasmid and then boiled in SDS loading buffer in the absence (lane 1, non-reduced condition) or presence of 5% β-mercaptoethanol (lane 2, reduced condition). Proteins were then separated by SDS–PAGE electrophoresis and western blot was conducted using anti-HA antibody. Numbers on the left indicate the migration positions of molecular mass markers (in kilodaltons). Note that the lane 1 (NR) and lane 2 (R) data were obtained in the same experiment, and have been placed together arbitrarily according to their migration positions relative to molecular mass markers in the gel.

1988). It will be interesting to address whether AMPV SH protein can also undergo modification of polylactosaminoglycan, in addition to N-linked glycosylation observed here.

3.3. Dimerization of the SH protein

Using bimolecular fluorescence complementation assay, we previously showed that AMPV-C SH is capable of interacting with itself inside cells to form a homodimer (Deng et al., 2011). Considering that the predicted SH ectodomain contains 9 cysteine residues (Fig. 2A), we speculate that cysteine-mediated disulfide bond may play a role in SH dimer formation. To test this hypothesis (Fig. 3) a traditional biochemical experiment was performed in which SH^{HA} protein lysates were treated under non-reducing and reducing conditions, separately. The resulting samples were then analyzed by SDS–PAGE and western blot. We observed that the bands in non-reducing condition were shifted to positions of approximately twice the size of the bands observed under reducing conditions, suggesting dimer formation likely through cysteine disulfide bond linkage (Fig. 3). Thus, these findings support the concept that the SH protein forms disulfide-linked dimers. A portion of the monomeric SH^{HA}0 protein was still present under non-reducing condition but apparently all of the larger forms migrate as large forms (Fig. 3, NR lane). This demonstrated that the unglycosylated form is not disulfide bonded in a dimer.

3.4. Conclusion and discussion

Our experiment generated a number of new observations with respect to biochemical properties of the AMPV SH protein. Significantly, we found that cysteine disulfide bond linkage plays an important role in SH homodimer formation. Interestingly, disulfide linked homodimers was not observed in the unglycosylated form of the AMPV SH protein. This result suggests a functional relationship between glycosylation and cysteine-mediated dimer formation in the AMPV SH protein. This phenomenon has not been observed before so it will be interesting to investigate whether SH proteins of other viruses possess this biochemical property.

Through deglycosylation study we demonstrated that the AMPV SH protein is modified by N-linked glycans, which is similar to other SH proteins reported previously (Collins et al., 1990; Fuentes et al., 2007; Hiebert et al., 1988; Olmsted and Collins, 1989; Rixon et al., 2004). However, SH protein secretion has not been observed in other paramyxoviruses, it is worth noting that secretion of viral glycoproteins in the paraxomovirus family has been demonstrated in the G (attachment) protein of HRSV (Hendricks et al., 1988). This phenomenon is implicated as an immune evasion mechanism utilized by HRSV (Hendricks et al., 1988). The biological relevance of SH protein secretion demonstrated in our study is not known. However, it could be envisioned that SH may have a role, possibly by acting as a "decoy", in the modulation of the host immune response, which will be a subject of future study.

Taken together, our study has demonstrated some important biochemical properties of metapneumovirus SH protein and represents the first step toward defining the role of SH protein in metapneumovirus replication and pathogenesis.

Acknowledgments

We thank Elizabeth Kolb for editing the manuscript and Xiuqin Xia for outstanding technical assistance. This work was supported by the SD AES (3AH203 to FL), NIH 5K02AI076125-02 award to FL, and USDA, ARS CRIS project 6612-32000-056-00D (to QY).

References

Anderson, K., King, A.M., Lerch, R.A., Wertz, G.W., 1992. Polylactosaminoglycan modification of the respiratory syncytial virus small hydrophobic (SH) protein: a conserved feature among human and bovine respiratory syncytial viruses. Virology 191 (1), 417–430.

Branigan, P.J., Day, N.D., Liu, C., Gutshall, L.L., Melero, J.A., Sarisky, R.T., Del Vecchio, A.M., 2006. The cytoplasmic domain of the F protein of human respiratory syncytial virus is not required for cell fusion. Journal of General Virology 87 (Pt 2), 395–398.

Catelli, E., Lupini, C., Cecchinato, M., Ricchizzi, E., Brown, P., Naylor, C.J., 2010. Field avian metapneumovirus evolution avoiding vaccine induced immunity. Vaccine 28 (4), 916–921.

Collins, P.L., Crowe, J.E.J., 2007. Respiratory syncytial virus and metapneumovirus. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Field Virology. , 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 1601–1646.

Collins, P.L., Graham, B.S., 2008. Viral and host factors in human respiratory syncytial virus pathogenesis. Journal of Virology 82 (5), 2040–2055.

Collins, P.L., Olmsted, R.A., Johnson, P.R., 1990. The small hydrophobic protein of human respiratory syncytial virus: comparison between antigenic subgroups A and B. Journal of General Virology 71 (Pt 7), 1571–1576.

Deng, Q., Weng, Y., Lu, W., Demers, A., Song, M., Wang, D., Yu, Q., Li, F., 2011. Topology and cellular localization of the small hydrophobic protein of avian metapneumovirus. Virus Research 160 (1–2), 102–107.

Easton, A.J., Domachowske, J.B., Rosenberg, H.F., 2004. Animal pneumoviruses: molecular genetics and pathogenesis. Clinical Microbiology Reviews 17 (2), 390–412.

Fuentes, S., Tran, K.C., Luthra, P., Teng, M.N., He, B., 2007. Function of the respiratory syncytial virus small hydrophobic protein. Journal of Virology 81 (15), 8361–8366.

Govindarajan, D., Buchholz, U.J., Samal, S.K., 2006. Recovery of avian metapneumovirus subgroup C from cDNA: cross-recognition of avian and human metapneumovirus support proteins. Journal of Virology 80 (12), 5790–5797.

Govindarajan, D., Samal, S.K., 2005. Analysis of the complete genome sequence of avian metapneumovirus subgroup C indicates that it possesses the longest genome among metapneumoviruses. Virus Genes 30 (3), 331–333.

Grose, C., Jackson, W., Traugh, J.A., 1989. Phosphorylation of varicella-zoster virus glycoprotein gpI by mammalian casein kinase II and casein kinase I. Journal of Virology 63 (9), 3912–3918.

He, B., Lin, G.Y., Durbin, J.E., Durbin, R.K., Lamb, R.A., 2001. The SH integral membrane protein of the paramyxovirus simian virus 5 is required to block apoptosis in MDBK cells. Journal of Virology 75 (9), 4068–4079.

Hendricks, D.A., McIntosh, K., Patterson, J.L., 1988. Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. Journal of Virology 62 (7), 2228–2233.

Hiebert, S.W., Richardson, C.D., Lamb, R.A., 1988. Cell surface expression and orientation in membranes of the 44-amino-acid SH protein of simian virus 5. Journal of Virology 62 (7), 2347–2357.

Liu, L., Bastien, N., Li, Y., 2007. Intracellular processing, glycosylation, and cell surface expression of human metapneumovirus attachment glycoprotein. Journal of Virology 81 (24), 13435–13443.

Miriagou, V., Stevanato, L., Manservigi, R., Mavromara, P., 2000. The C-terminal cytoplasmic tail of herpes simplex virus type 1 gE protein is phosphorylated in vivo and in vitro by cellular enzymes in the absence of other viral proteins. Journal of General Virology 81 (Pt 4), 1027–1031.

Montalvo, E.A., Grose, C., 1986. Varicella zoster virus glycoprotein gpI is selectively phosphorylated by a virus-induced protein kinase. Proceedings of the National Academy of Sciences of the United States of America 83 (23), 8967–8971.
Naylor, C.J., Ling, R., Edworthy, N., Savage, C.E., Easton, A.J., 2007. Avian metapneumovirus SH gene end and G protein mutations influence the level of protection of live-vaccine candidates. Journal of General Virology 88 (Pt 6), 1767–1775.
Njenga, M.K., Lwamba, H.M., Seal, B.S., 2003. Metapneumoviruses in birds and humans. Virus Research 91 (2), 163–169.
Olmsted, R.A., Collins, P.L., 1989. The 1A protein of respiratory syncytial virus is an integral membrane protein present as multiple, structurally distinct species. Journal of Virology 63 (5), 2019–2029.
Rixon, H.W., Brown, G., Aitken, J., McDonald, T., Graham, S., Sugrue, R.J., 2004. The small hydrophobic (SH) protein accumulates within lipid-raft structures of the Golgi complex during respiratory syncytial virus infection. Journal of General Virology 85 (Pt 5), 1153–1165.
Toquin, D., de Boisseson, C., Beven, V., Senne, D.A., Eterradossi, N., 2003. Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. Journal of General Virology 84 (Pt 8), 2169–2178.
Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C., Eterradossi, N., 2006. European and American subgroup C isolates of avian metapneumovirus belong to different genetic lineages. Virus Genes 32 (1), 97–103.
van den Hoogen, B.G., Bestebroer, T.M., Osterhaus, A.D., Fouchier, R.A., 2002. Analysis of the genomic sequence of a human metapneumovirus. Virology 295 (1), 119–132.
van den Hoogen, B.G., de Jong, J.C., Groen, J., Kuiken, T., de Groot, R., Fouchier, R.A., Osterhaus, A.D., 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nature Medicine 7 (6), 719–724.
Velayudhan, B.T., McComb, B., Bennett, R.S., Lopes, V.C., Shaw, D., Halvorson, D.A., Nagaraja, K.V., 2005. Emergence of a virulent type C avian metapneumovirus in turkeys in Minnesota. Avian Diseases 49 (4), 520–526.
Velayudhan, B.T., Noll, S.L., Thachil, A.J., Halvorson, D.A., Shaw, D.P., Goyal, S.M., Nagaraja, K.V., 2008. Comparative pathogenicity of early and recent isolates of avian metapneumovirus subtype C in turkeys. Canadian Journal of Veterinary Research 72 (4), 371–375.
Williams, M.A., Lamb, R.A., 1988. Polylactosaminoglycan modification of a small integral membrane glycoprotein, influenza B virus NB. Molecular and Cellular Biology 8 (3), 1186–1196.
Yunus, A.S., Govindarajan, D., Huang, Z., Samal, S.K., 2003. Deduced amino acid sequence of the small hydrophobic protein of US avian pneumovirus has greater identity with that of human metapneumovirus than those of non-US avian pneumoviruses. Virus Research 93 (1), 91–97.

Li et al. Virology Journal 2012, 9:227
http://www.virologyj.com/content/9/1/227

VIROLOGY JOURNAL

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Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies

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Abstract

Background: Many viruses have evolved multiple strategies to prevent super infection of host cells by more than one virion. This phenomenon, known as super infection exclusion, may play an important role on virus evolution because it can affect the frequency of reassortment and/or recombination. Newcastle disease virus (NDV), a negative sense single-stranded RNA virus, is characterized by its continuous evolutionary dynamics and by a low frequency of recombination events. However, the mechanisms that contribute to the low recombination rates on NDV are still not completely understood.

Methods: In this study we assessed the ability of two NDV strains (LaSota and B1) to super infect host cells *in vitro*. We generated a recombinant NDV strain LaSota expressing the red fluorescent protein (RFP) and used it in co-infection assays with a related NDV strain B1 expressing the green fluorescent protein (GFP). DF-1 cells were inoculated with both viruses at the same time or at different intervals between primary infection and super infection.

Results: When both viruses were inoculated at the same time point, a 27% co-infection rate was observed, whereas when they were inoculated at different time points the super infection rates decreased to levels as low as 1.4%.

Conclusions: These results indicate that although different NDV strains can co-infect host cells *in vitro*, the super infection rates are low, specially as the time between the primary infection and super infection increases. These results confirm the occurrence of super infection exclusion between different strains of NDV.

Keywords: NDV, Co-infection, Super infection, Interference

Introduction

Newcastle disease (ND) is one of the most important diseases of poultry, causing significant economic losses to poultry producers around the world [1]. The etiologic agents of ND is Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1), an enveloped, non-segmented, single-stranded, negative sense RNA ([–]ssRNA) virus of the genus *Avulavirus* family *Paramyxoviridae* [1]. The NDV genome is approximately 15.2 Kb in length and contains six genes encoding for the nucleoprotein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L). At least one additional protein (V) is produced through editing of the phosphoprotein messenger RNA (mRNA) [1,2].

NDV is known for its continuous evolutionary dynamics, with different isolates undergoing simultaneous evolutionary changes around the world [2,3]. This evolutionary dynamic is likely resultant from the lack of proofreading activity of the viral RNA dependent RNA polymerase [4,5]. While mutations introduced during virus replication seem to play a major role on NDV evolution, recombination has only been rarely reported [3,6-9]. In general, the low frequency of recombination in [–]ssRNA viruses reflect specific aspects of their life cycle, including their genome

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organization and the mechanisms involved in transcription and replication [10]. Other aspects the infection cycle and virus-host interactions may also affect the recombination rates in RNA viruses [10]. For NDV, however, the strategies/mechanisms that contribute to the low frequency of recombination are not completely understood.

Many enveloped viruses have evolved mechanisms to prevent infection of host cells by more than one virion [11-13]. This phenomenon, known as super infection exclusion, likely affects the frequency of recombination within a viral population because co-infection of a cell by related viruses is required for the exchange of genetic material. In this study we assessed the ability of two NDV strains (LaSota and B1) to super infect host cells *in vitro*. We generated a recombinant NDV strain LaSota, encoding the red fluorescent protein (RFP; rLS-RFP) and used it in co-infection assays with a related strain of NDV (B1) expressing the green fluorescent protein (GFP; rB1-GFP).

Methods

Cells and viruses

HEp-2 (ATCC, CCL-23), Vero (ATCC, CCL-81) and DF-1 (ATCC, CRL-12203) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics and antimycotic (100U/ml Penicillin, 100 μg/ml Streptomycin, 0.25 μg/ml Amphotericin B, Thermo Scientific), and maintained at 37°C with 5% CO_2. DF-1 cells were supplemented with 10% allantoic fluid (AF) (from specific pathogen free [SPF] embryonated chicken eggs) during all virus infections. The NDV strain LaSota was obtained from ATCC and the recombinant NDV strain B1 expressing the green fluorescence protein (rB1-GFP) was kindly provided by Dr. Peter Palese, Department of Microbiology, Mount Sinai University [14]. All viruses were propagated in 9-day-old SPF embryonated chicken eggs and the virus titres were determined by limiting dilution, calculated according to the Spearman and Karber's method, and expressed as Log_{10} tissue culture infectious dose 50 ($TCID_{50}$)/ml. The modified vaccinia virus Ankara/T7 (MVA/T7) was used in the infection/transfection experiments to rescue the recombinant virus NDV-LS/RFP.

Generation of the recombinant NDV-LaSota containing the RFP gene

The recombinant NDV LaSota expressing the red fluorescent protein (RFP) (rLS-RFP) was generated from the backbone of the vector pLS/aMVP-C G [15]. The open reading frame of the RFP was inserted between the fusion (F) and hemagglutinin-neuraminidase (HN) genes as an extra transcriptional unit (Figure 1). Initially, an intermediate plasmid containing the aMPV-C G gene and its flanking regions (including the NDV gene start [GS] and gene end [GE] sequences) was generated by PCR amplification (primers: LSFF-5'-TCAGATGAGAGCCACTACAAAAA TGTGAGC-3' and LS6682R-5'ACTCAAGGGCCACTTG CT-3') and cloning of the PCR product into the TOPO TA® vector (Invitrogen). The aMPV-C G protein coding sequence was replaced with the open reading frame (ORF) of the RFP gene using the In-Fusion® PCR Cloning kit (Clontech) and two sets of primers (including LS-NI-up-5'-CTTGCACCTGGAGGGCGCCAAC-3' and LS-NI-down-5'-CGTACACAGATGAGGAACGAAGGTTTCCCT-3' to linearize the vector, and NI-RFP-F-5'CCCTCCAGGTG CAAGATGGCCTCCTCCGAGGAC-3' and NI-RFP-R-5'-CCTCATCTGTGTACGCTACAGGAACAGGTGGTG-3'

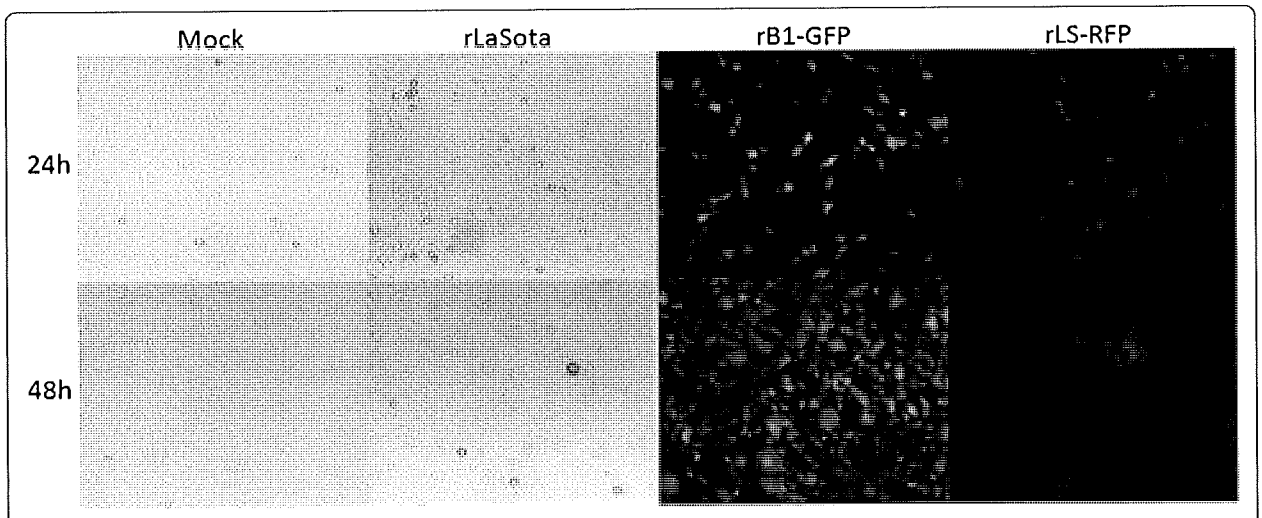

Figure 1 Cytopathic effect and expression or RFP and GFP in rLS-RFP- and rB1-GFP-infected DF-1 cells. Cells were inoculated with each virus at a MOI of 0.01 and examined under a fluorescence microscope 24 and 48 h pi.

to amplify the RFP ORF from the pCMV-DsRed-Express plasmid, Clontech). The coding sequences of the RFP and its flanking regions were PCR amplified (primers: LSFF and LS6682R, described above) and cloned into the vector containing the full-length LaSota genome using the InFusion PCR cloning kit and specific primers (LS-vec-F-up-5'-GTGGCTCTCATCTGATCTAGAGTATTATTC-3' and LS-vec-6682-down-5'-5'AAGTGGCCCTTGAGTCTCCGT TGGCATTGTTA-3'). The resultant plasmid, designated pLS-RFP, was amplified in Stbl2 competent cells (Invitrogen) and purified using a QIAprep Spin Miniprep kit (Qiagen).

Rescue of the rLS-RFP was performed in HEp-2 cells according to standard protocols [15]. HEp-2 cells were seeded on a six-well plate (1×10^6 cells/well), infected with MVA/T7 at a multiplicity of infection (MOI) of 3, and one hour later transfected with the plasmid pLS-RFP (2 μg), and the helper plasmids pTM-NP (1 μg), pTM-P (0.5 μg), and pTM-L (0.1 μg) using Lipofectamine 2000™ (Invitrogen). Seventy-two hours post-infection/transfection cells were harvested and subjected to three freeze and thaw cycles. Approximately 300 μl of the cell lysate were inoculated into the allantoic cavity of 9-day-old SPF embryonated chicken eggs for amplification of the recombinant virus. Rescue of rLS-RFP was confirmed by hemagglutination (HA) and fluorescence assays.

Biological characterization of rLS-RFP

The pathogenicity of the rLS-RFP virus was assessed by standard mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests [16]. To compare the cytopathic effect (CPE) induced by rLS-RFP, the parental NDV-LaSota and the rB1-GFP, DF-1 cells were infected with each virus (MOI=0.01) and evaluated under a light and a fluorescence microscope at 24 and 48 hours post infection (pi). Growth characteristics of rLS-RFP, NDV-LaSota, and rB1-GFP were assessed in DF-1 cells. Cells were infected with rLS-RFP, NDV-LaSota, or rB1-GFP (MOI=0.01), harvested at 0, 12, 24, 36 and 48h pi and subjected to three freeze and thaw cycles. Viral titres were determined by limiting dilution, calculated according to the Spearman and Karber's method, and expressed as Log_{10} $TCID_{50}$/ml.

Super infection assays

The ability of rLS-RFP and rB1-GFP to super infect host cells was assessed in a chicken fibroblast cell line (DF-1). Initially, rLS-RFP and rB1-GFP were mixed (MOI=3 of each virus), and inoculated in DF-1 cells. After 1h of adsorption at 37°C the virus inoculum was removed, cells were washed 1X with DMEM, and complete culture media was added to the cells. At 12h pi cells were stained with HCS Nuclear Mask Blue (Invitrogen) and examined under a fluorescence microscope (Nikon, Eclipse T*i*, Melville, NY). Pictures were taken at 20X magnification and the numbers of red-, green-, or yellow-fluorescent cells (red + green) were determined using the ImageJ (version 1.46) software (NIH, http://rsbweb.nih.gov/ij/index.html). The numbers of cells infected with rLS-RFP, and rB1-GFP, or co-infected with both viruses (yellow-fluorescent cells) were expressed as percentage of infected cells. At least three fields were randomly selected to calculate the mean infectivity and super infection percentages.

To assess the effect of the time of super infection in the ability of rLS-RFP and rB1-GFP to co-infect host cells, DF-1 cells were culture in 12-well plates and inoculated with both viruses at different intervals between the primary infection and the super infection. Cells were inoculated with rLS-RFP and rB1-GFP (MOI=3) and super infected at 1 and 3h pi with rB1-GFP or rLS-RFP (MOI=3), respectively. In a second experiment, cells were inoculated with rLS-RFP or rB1-GFP (MOI=1) and super infected at 12, and 24h pi with rB1-GFP or rLS-RFP (MOI=3), respectively. At 24h post super infection cells were stained with HCS Nuclear Mask Blue (Invitrogen), and examined under a fluorescence microscope (Nikon, Eclipse T*i*, Melville, NY). The numbers of red-, green-, and yellow-fluorescent cells were determined as described above. In addition, Vero cells were inoculated with rLS-RFP or rB1-GFP (MOI=3) and super infected at 3h pi with rB1-GFP or rLS-RFP (MOI=3), respectively. At 24h post super infection cells were examined under a fluorescence microscope (Nikon, Eclipse T*i*, Melville, NY). Statistical analysis was performed with the two-tailed paired Student's *t*-test, and p values < 0.05 were considered statistically significant.

Results

Construction and characterization of recombinant NDV LaSota expressing the red fluorescent protein (rLS-RFP)

The recombinant NDV LaSota encoding the red fluorescent protein (RFP) was constructed using reverse genetic approaches. The cDNA clone pLS/aMPV-C G [15], containing the complete genome sequences of NDV strain LaSota and the coding sequences of the G protein of avian metapneumovirus type C, was used as backbone to generate the rLS-RFP virus. The open reading frame of the aMPV G protein (1,758 nt) was replaced by the coding sequence of the RFP (678 nt) using the In-Fusion cloning strategy. The rLS-RFP was rescued in HEp-2 cells and propagated in 9-days-old embryonated chicken eggs. DNA sequencing of regions flanking the inserted gene confirmed the integrity of NDV LaSota and of RFP sequences in the recombinant virus (data not shown).

To determine whether the resultant recombinant virus expresses the RFP, DF-1 cells were infected and examined

under a fluorescence microscope. Expression of RFP was observed in cells infected with the rLS-RFP at 24h and 48h post-infection (pi), demonstrating efficient expression of RFP by the recombinant virus (Figure 1). The rB1-GFP virus was used as a positive control for the expression of GFP (Figure 1).

Biological characterization of the rLS-RFP virus

The pathogenicity and replication kinetics of rLS-RFP were assessed *in vivo* and *in vitro*, respectively. The intracerebral pathogenicity index (ICPI) and the mean death time (MDT) of rLS-RFP were determined using standard protocols. Insertion of RFP into the NDV-LaSota backbone resulted in a slight attenuation of the recombinant virus, as evidenced by a decreased intracerebral pathogenicity index (ICPI; 0.0) in day-old chicks and a longer mean death time (MDT; 127 h) in embryonated chicken eggs inoculated with rLS-RFP, when compared to those inoculated with the parental virus NDV strain LaSota (Table 1). The rB1-GFP presented a MDT >150 h. No differences in the cytopathic effect (CPE) induced by the parental and recombinant viruses (rLS-RFP and rB1-GFP) were observed in DF-1-infected cells (Figure 1). Additionally, no significant differences in the replication kinetics and viral yields were detected between NDV-LaSota, rLS-RFP, and rB1-GFP in embryonated chicken eggs- or in DF-1-infected cells (Table 1; Figure 2), indicating that insertion of RFP in the F-HN intergenic region did not significantly affect replication of the recombinant virus.

Super infection with rLS-RFP and rB1-GFP results in low rates of co-infection *in vitro*

The ability of two NDV strains (LaSota and B1) to super infect host cell was assessed *in vitro*. DF-1 cells were co-infected with rLS-RFP (MOI=3) and rB1-GFP (MOI = 3) and the expression of RFP and GFP was examined by fluorescence microscopy. Red- and green-fluorescent cells were observed as early as 12 h pi, confirming that the cells were infected with rLS-RFP or rB1-GFP, respectively (Figure 3A). The co-infection rates were ~27% as evidenced by the presence of yellow-fluorescent cells at 24 h pi (Figure 3A, panel a, and d).

The effect of the time of infection on the ability of NDV LaSota and B1 to super infect host cells was also investigated. DF-1 cells were infected with rLS-RFP or rB1-GFP and super infected with rB1-GFP or rLS-RFP at different intervals post-primary infection, respectively (1, 3, 12 and 24 h pi). As in the co-infection assay described above, super infection at intervals of 1, 3, 12 and 24 h resulted in low rates of co-infection, confirming the occurrence of viral interference or super infection exclusion among these viral strains [17] (Figure 4A, B, and C; Figure 5A,B and C). Notably, the co-infection rates significantly decreased as the time between primary infection and super infection increased (Figure 4A,B and C; Figure 5A,B and C). To rule out the possibility that the super infection exclusion observed in DF-1 cells was mediated by a host antiviral response induced by the primary infection, we performed the super infection assays in Vero cells which are defective in interferon production [18]. Similar to the results observed in DF-1 cells, infection with one NDV strain (rLS-RFP or rB1-GFP) interfered with the super infection by the other, as evidenced by low rates of yellow-fluorescent cells at 24 h pi (Additional file 1: Figure S1). These results demonstrate the occurrence of super infection exclusion between two different strains of NDV in DF-1 and Vero cells.

Table 1 Biological characterization of the rLS-RFP

Virus	MDT	EID_{50}	$TCID_{50}$	ICPI
NDV LaSota	110hs	3.20×10^{9}	2.37×10^{8}	0.15
rLS-RFP	127hs	3.60×10^{8}	1.78×10^{8}	0
rB1-GFP	>150hs	1.26×10^{8}	1.78×10^{7}	NT[a]

[a]Not tested.

Discussion

In the present study we assessed the ability of two NDV strains (LaSota and B1) to co-infect host cells *in vitro*. Results here revealed that NDV strains LaSota and B1 are able to co-infect one host cell simultaneously. However, the super infection rates are low and they decrease as the time between the primary infection and super infection increases. These findings suggest the occurrence of super infection exclusion during NDV infection.

Super infection exclusion is a phenomenon by which an established virus infection blocks co-infection by a

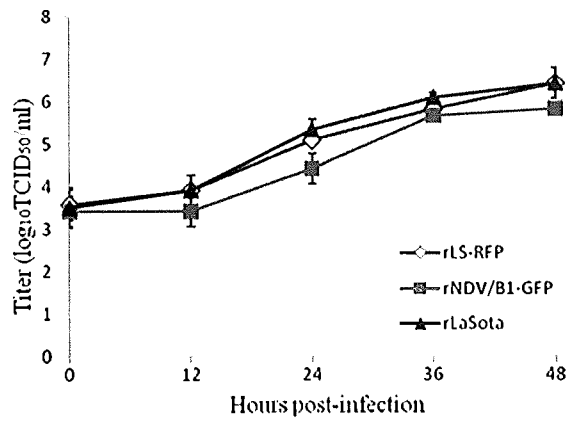

Figure 2 Replication kineticts of NDV-LaSota, rLS-RFP, and rB1-GFP. DF-1 cells were infected with rLS-RF, rB1-GFP or LaSota strain (MOI=0.01), harvested at the indicated time points and the virus titres determined by limiting dilution and expressed as Log_{10} $TCID_{50}$/ml. Results shown represent the mean titre calculated from two independent experiments.

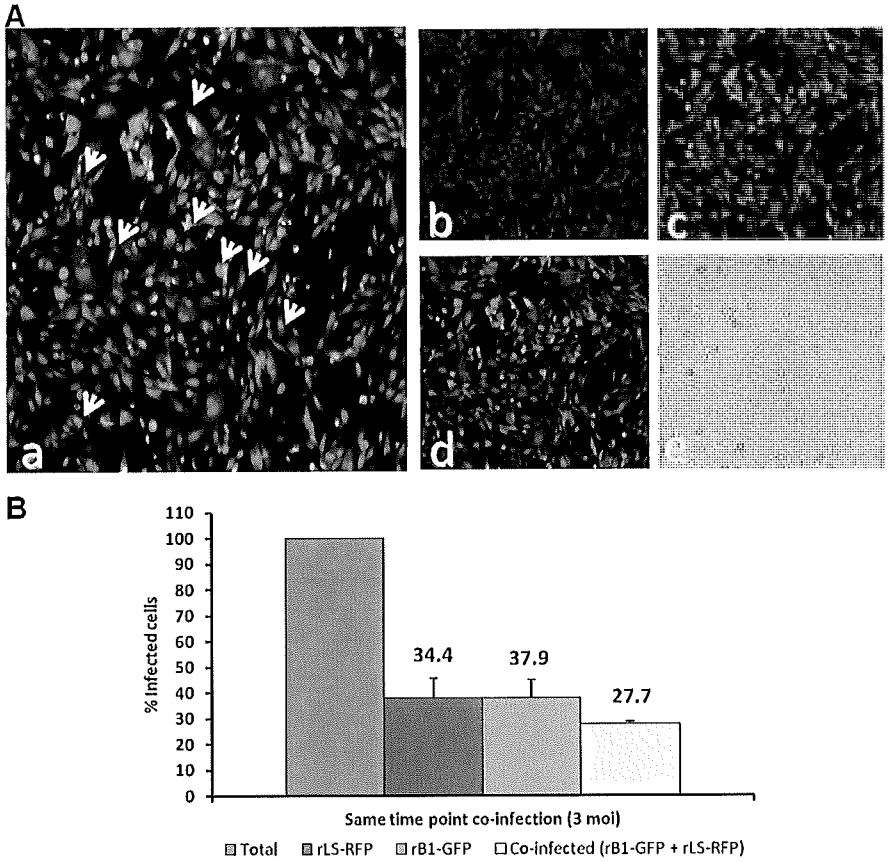

Figure 3 Co-infection of DF-1 with rLS-RFP and rB1-GFP. (**A**) Monolayers of DF-1 cells were infected with rLS-RFP and rB1-GFP (MOI=3) at the same time point. At 24h post infection, cells were stained with HCS NuclearMask Blue and examined under a fluorescence microscope. **a.** merged green, red and blue channels; **b.** red channel; **c.** green channel, **d.** merged green and red channels; **e.** bright field. (**B**) Percentage of infected cells. Cells were infected as described in A. Three fields were randomly selected and used to determine the numbers of red-, green- and yellow-fluorescent cells using the ImageJ software. Results shown represent mean percentages of three fields. The percentages of cells infected with each virus (rLS-RFP of rB1-GFP) and the percentage of cells co-infected are expressed as a relative percentage of the total number of infected cells.

homologous super infecting virus [11]. Here we have shown that primary infection of DF-1 cells with either rLS-RFP or rB1-GFP decreases the co-infection rates by the heterologous strain. Interestingly, the co-infection rates decreased as the time between the primary infection and the super infection increased, corroborating with findings from early studies [17]. Super infection exclusion can occur at three different steps of the virus life cycle, including attachment, entry and/or intracellular replication [11-13]. It has been shown that for the paramyxovirus human parainfluenza type 3 the neuraminidase activity of the HN protein is responsible for blocking super infection by a heterologous strain [13]. Whether NDV HN exerts a similar function in blocking super infection or whether the virus has evolved additional mechanisms to prevent super infection remains to be determined.

Although the biological significance of super infection exclusion for NDV and other paramyxoviruses is unknown, this phenomenon can contribute to the low frequency of recombination observed for these viruses. The limited number of cells that are co-infected during NDV infection may negatively affect recombination rates. As shown here, super infection rates were low even when a high multiplicity of infection (MOI=3 for each virus) was used (Figure 3A and B), and they significantly decreased as the time interval between each infecting virus increased (Figure 4A,B, and C; Figure 5A,B and C). Results from the super infection assays on Vero cells (Additional file 1: Figure S1A and B), known to be interferon defective [18], indicate that the low co-infection rates observed in DF1 cells are due to super infection exclusion between NDV strains LaSota and B1 and not due to an antiviral response of the host cells generated against the primary infection. It is important to note that in addition to specific viral mechanisms that may contribute to super infection exclusion (i.e. neuraminidase activity of the HN protein), the

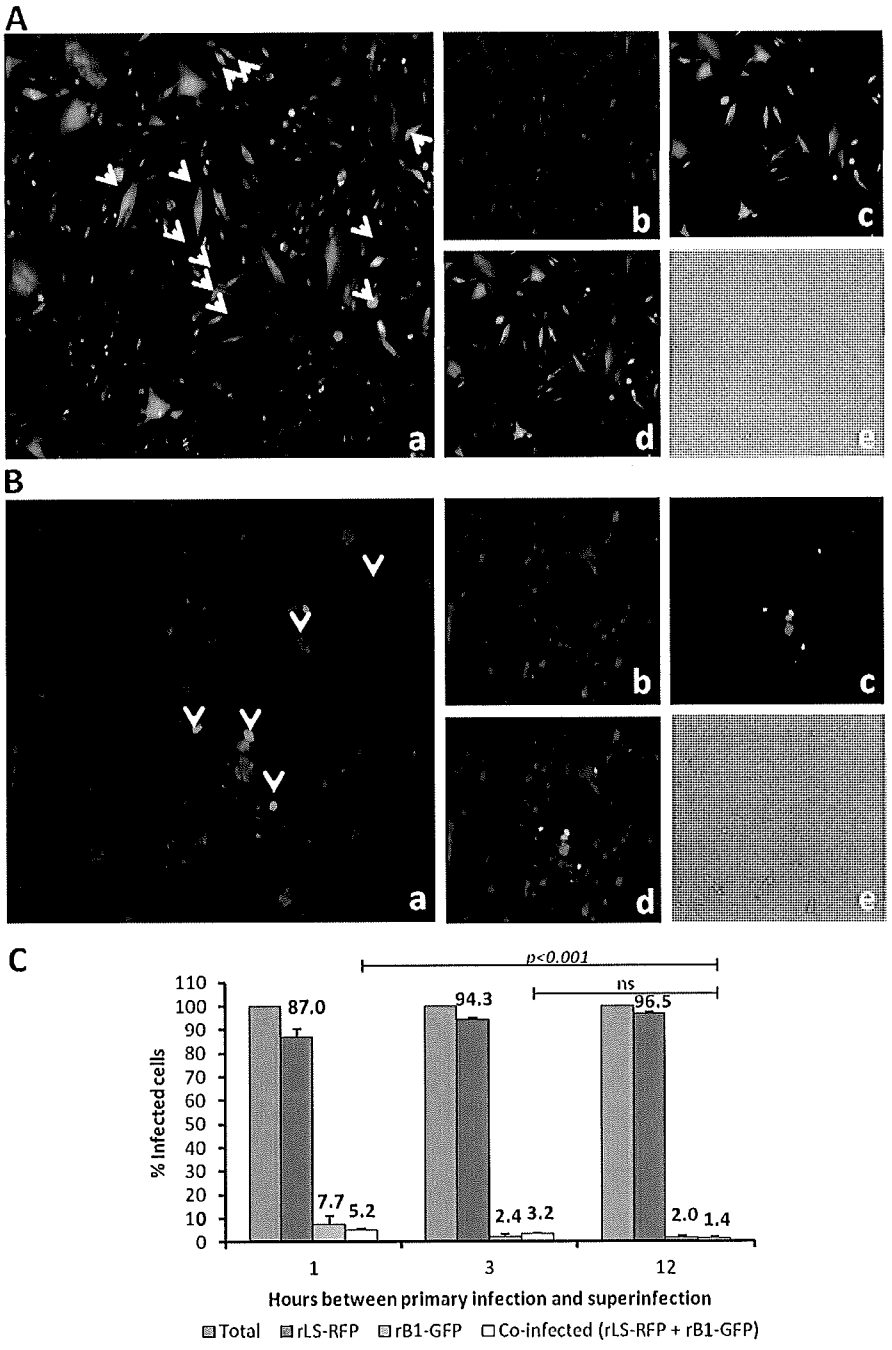

Figure 4 Co-infection of DF-1 cells with rLS-RFP and rB1-GFP. (**A**) DF-1 cells infected with rLS-RFP (MOI=3) and super infected with rB1-GFP (MOI=3) at 1 h post primary infection. At 24h post infection, cells were stained with HCS NuclearMask Blue and examined under a fluorescence microscope. **a**. merged green, red and blue channels; **b**. red channel; **c**. green channel, **d**. merged green and red channels; **e**. bright field. (**B**) DF-1 cells infected with rLS-RFP and super infected with rLS-RFP at 12 h post primary infection. **a**. merged green, red and blue channels; **b**. red channel; **c**. green channel, **d**. merged green and red channels; **e**. bright field. (**C**) Percentage of infected cells. Cells were infected as described in A and B. Three fields for each indicated time point were randomly selected and used to determine the numbers of red-, green- and yellow-fluorescent cells using the ImageJ software. Results shown represent mean percentages of three fields. The percentages of cells infected with each virus (rLS-RFP of rB1-GFP) and the percentage of cells co-infected are expressed as a relative percentage of the total number of infected cells.

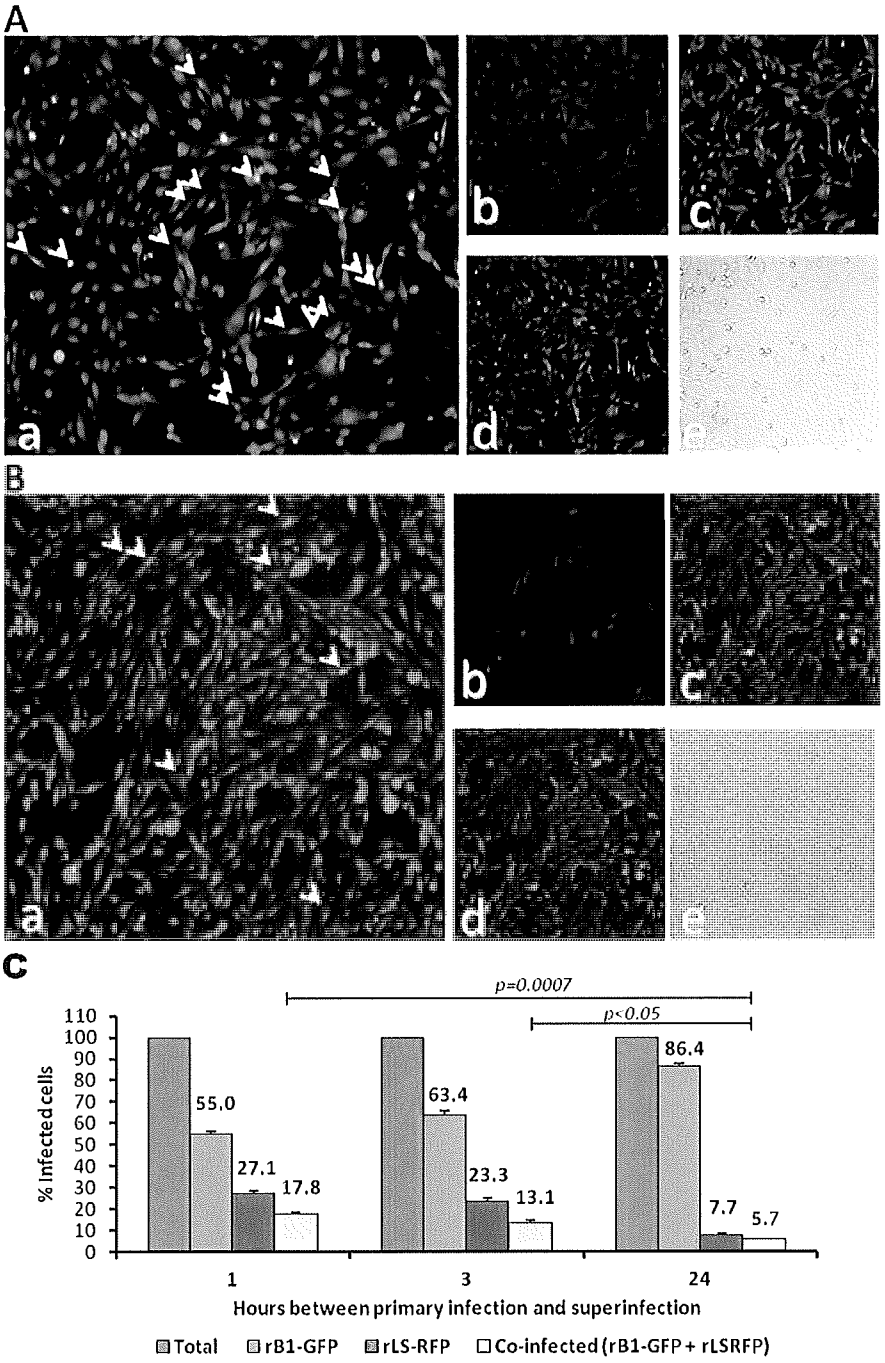

Figure 5 Co-infection of DF-1 cells with rB1-GFP and rLS-RFP. (A) DF-1 cells infected with rB1-GFP (MOI= 3) and super infected with rLS-RFP (MOI=3) at 1 h pi. At 24h post infection, cells were stained with HCS NuclearMask Blue and examined under a fluorescence microscope. **a**. merged green, red and blue channels; **b**. red channel; **c**. green channel, **d**. merged green and red channels; **e**. bright field. **(B)** DF-1 cells infected with rB1-GFP and super infected with rLS-RFP at 12 h pi. At 24h post infection, cells were stained with HCS NuclearMask Blue and examined under a fluorescence microscope. **a**. merged green, red and blue channels; **b**. red channel; **c**. green channel, **d**. merged green and red channels; **e**. bright field. **(C)** Percentage of infected cells. Cells were infected as described in A and B. Three fields for each indicated time point were randomly selected and used to determine the numbers of green-, red- and yellow-fluorescent cells using the ImageJ software. Results shown represent mean percentages of three fields. The percentages of cells infected with each virus (rB1-GFP or rLS-RFP) and the percentage of cells co-infected are expressed as a relative percentage of the total number of infected cells.

co-infection rates are likely affected by other factors *in vivo,* including strain-specific cell and tissue tropism, the time interval between infection with different strains and the presence of predisposing immunity.

In the future, it will be interesting to use the recombinant virus generated here (rLS-RFP) to determine the mechanism responsible for super infection exclusion in NDV and to assess the potential for recombination among different NDV strains. Complete genome sequencing following super infection experiments will allow us to determine if recombination can indeed occur in NDV and how frequent it may be.

Conclusions

In summary, the present study describes the characterization of a recombinant NDV strain encoding a red fluorescent protein that was used in co-infection studies. We have shown that NDV strains LaSota and B1 are able to co-infect host cells *in vitro,* however the co-infection rates are low and they decrease as the time between primary infection and super infection increases.

Additional file

Additional file 1: Figure S1. Co-infection of Vero cells with rB1-GFP and rLS-RFP. (A) Vero cells were infected with rLS-RFP (MOI=3) and super infected with rB1-GFP (MOI=3) at 3 h post primary infection. At 24h post infection, cells were examined under a fluorescence microscope. a. red channel; b. green channel; c. merged green, and red channels. (B) Vero cells were infected with rB1-GFP and super infected with rLS-RFP at 12 h post primary infection. At 24h post infection, cells were examined under a fluorescence microscope. a. green channel; b. red channel; c. merged green and red channels.

Abbreviations

ND: Newcastle disease; NDV: Newcastle disease virus; APMV-1: Avian paramyxovirus type 1; [–] ssRNA: Non-segmented single-stranded, negative sense RNA; NP: Nucleoprotein; P: Phosphoprotein; M: Matrix protein; F: Fusion protein; HN: Hemagglutinin-neuraminidase; L: RNA-dependent RNA polymerase; mRNA: Messenger RNA; RFP: Red fluorescent protein; ICPI: Intracerebral pathogenicity index; MDT: Mean death time; CPE: Cytopathic effect; TCID50: Tissue culture infectious dose 50; DMEM: Dulbecco's modified eagle medium; FBS: Fetal bovine serum; AF: Allantoic fluid; SPF: Specific pathogen free; MVA/T7: Modified vaccinia virus Ankara/T7; GS: Gene start; GE: Gene end; ORF: Open reading frame; HA: Hemagglutination; MOI: Multiplicity of infection.

Competing interests

The authors declare no competing interest.

Authors' contributions

JL constructed the recombinant virus and performed the co-infection studies. HH contributed for recombinant virus construction. QY contributed and coordinated the recombinant virus construction. DGD conceived the co-infection studies. DL participated on experimental design. PJM coordinated the research. All authors have read and approved the final manuscript.

Acknowledgments

We thank Tim Olivier and Xiuqin Xia for technical assistance, the SEPRL sequencing facility personnel for nucleotide sequencing, and Dr. Peter Palese (Department of Microbiology, Mount Sinai School of Medicine) for providing the recombinant virus rB1-GFP used in this study. J. Li and H. Hu were sponsored by a scholarship from China Scholarship Council. This work was supported by USDA funding, CRIS no. 6612-32000-064-00D.

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Received: 10 May 2012 Accepted: 26 September 2012
Published: 3 October 2012

References

1. Alexander DJ, Senne DA: Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In *Diseases of Poultry*. 12th edition. Edited by Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE. Ames: Iowa State University Press; 2008:75–116.
2. Miller PJ, Decanini EL, Afonso CL: Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infect Genet Evol* 2010, 10:26–35.
3. Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL: Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect Genet Evol* 2012, 12:1770–1779.
4. Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S: Rapid evolution of RNA genomes. *Science* 1982, 215:1577–1585.
5. Chare ER, Gould EA, Holmes EC: Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. *J Gen Virol* 2003, 84:2691–2703.
6. Song Q, Cao Y, Li Q, Gu M, Zhong L, Hu S, Wan H, Liu X: Artificial recombination may influence the evolutionary analysis of Newcastle disease virus. *J Virol* 2011, 85:10409–10414.
7. Qin Z, Sun L, Ma B, Cui Z, Zhu Y, Kitamura Y, Liu W: F gene recombination between genotype II and VII Newcastle disease virus. *Virus Res* 2008, 131:299–303.
8. Han GZ, He CQ, Ding NZ, Ma LY: Identification of a natural multi-recombinant of Newcastle disease virus. *Virology* 2008, 371:54–60.
9. Zhang R, Wang X, Su J, Zhao J, Zhang G: Isolation and analysis of two naturally-occurring multi-recombination Newcastle disease viruses in China. *Virus Res* 2010, 151:45–53.
10. Simon-Loriere E, Holmes EC: Why do RNA viruses recombine? *Nat Rev Microbiol* 2011, 9:617–626.
11. Walters KA, Joyce MA, Addison WR, Fischer KP, Tyrrell DL: Super infection exclusion in duck hepatitis B virus infection is mediated by the large surface antigen. *J Virol* 2004, 78:7925–7937.
12. Lee YM, Tscherne DM, Yun SI, Frolov I, Rice CM: Dual mechanisms of pestiviral super infection exclusion at entry and RNA replication. *J Virol* 2005, 79:3231–3242.
13. Horga MA, Gusella GL, Greengard O, Poltoratskaia N, Porotto M, Moscona A: Mechanism of interference mediated by human parainfluenza virus type 3 infection. *J Virol* 2000, 74:11792–11799.
14. Park MS, Shaw ML, Munoz-Jordan J, Cros JF, Nakaya T, Bouvier N, Palese P, Garcia-Sastre A, Basler CF: Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol* 2003, 77:1501–1511.
15. Hu H, Roth JP, Estevez CN, Zsak L, Liu B, Yu Q: Generation and evaluation of a recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus subgroup C as a bivalent vaccine in turkeys. *Vaccine* 2011, 29:8624–8633.
16. Alexander DJ: Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech* 2000, 19:443–462.
17. Bratt MA, Rubin H: Specific interference among strains of Newcastle disease virus. I. Demonstration and measurement of the interference. *Virology* 1967, 33:598–608.
18. Emeny JM, Morgan MJ: Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. *J Gen Virol* 1979, 43:247–252.

doi:10.1186/1743-422X-9-227
Cite this article as: Li *et al.*: Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies. *Virology Journal* 2012 9:227.

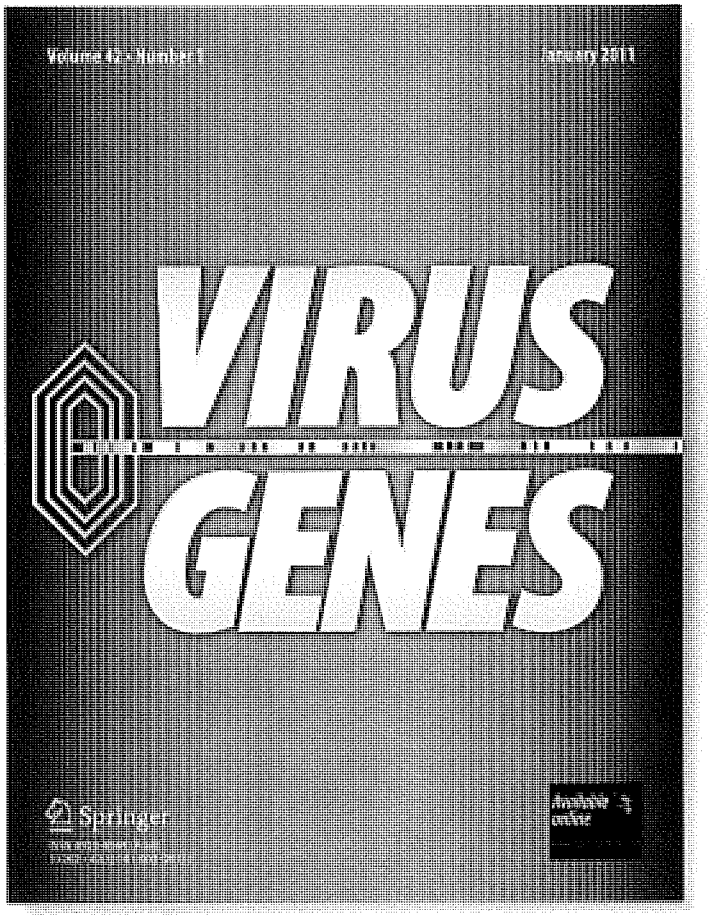
Characterization of H5N1 highly pathogenic avian influenza viruses isolated from poultry in Pakistan 2006–2008

Jamie L. Wasilenko, Mary Pantin-Jackwood, Taseer Ahmed Khan, Ayaz Ahmed, Shafqat F. Rehmani, Nazir Ahmed Lone, David E. Swayne, et al.

Virus Genes

ISSN 0920-8569
Volume 44
Number 2

Virus Genes (2012) 44:247-252
DOI 10.1007/s11262-011-0689-2



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Characterization of H5N1 highly pathogenic avian influenza viruses isolated from poultry in Pakistan 2006–2008

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Received: 22 August 2011 / Accepted: 31 October 2011 / Published online: 15 November 2011
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Abstract Nine avian influenza viruses (AIV), H5N1 subtype, were isolated from dead poultry in the Karachi region of Pakistan from 2006 to 2008. The intravenous pathogenicity indices and HA protein cleavage sites of all nine viruses were consistent with highly pathogenic AIV. Based on phylogenetic analysis of the HA genes, these isolates belong to clade 2.2 and both the HA and NA are closely related to each other (nucleotide identities above 99.0%) and to other Middle Eastern H5N1 AIV isolates (nucleotide identities above 98.0%). The phylogenetic data suggest that the virus in both epornitics of H5N1 HPAIV in commercial poultry in the Karachi region of Pakistan between 2006 and 2008 were from a very closely related source, however, there is inadequate epidemiological data to determine what the reservoir was for the virus between the 2006 and 2007 outbreaks other than that there was a single introduction into the region.

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Keywords Avian influenza · High pathogenicity · H5N1 · Pakistan · Hemagglutinin · Neuraminidase

Introduction

Avian influenza virus (AIV) was first reported in Pakistan in 1995 when an H7N3 high pathogenicity avian influenza virus (HPAIV) infected commercial poultry [1, 2]. Since then there have been sporadic outbreaks of H7N3 HPAIV and low pathogenicity (LP) AIV and H9N2 LPAIV resulting in the loss of large numbers of poultry [2–4]. The H7N3 and H9N2 outbreaks have been controlled primarily by vaccination and biosecurity. In February 2006, the first H5N1 HPAIV outbreaks occurred in the northwest Frontier region of Pakistan in commercial poultry farms and later in 2006 extended to the capital region of Islamabad [5]. In early 2007, the H5N1 HPAIV was detected in wild birds [5], and later that same year the first human cases of H5N1 HPAIV infection in Pakistan were reported [6]. Cases of H5N1 were not reported in commercial poultry in the southern region of Pakistan (Karachi/Sindh region) until March of 2007, although a 2006 AIV isolate (A/chicken/Pakistan/NARC-228/2006) was later identified to be an H5N1 HPAIV. Similar to the other outbreaks in Pakistan, localized vaccination and stamping out procedures were employed to try to contain the virus and were temporarily successful until additional outbreaks were reported in the Karachi region in January of 2008 [5]. Pakistan declared itself free of H5N1 HPAI in September of 2008 with no further H5N1 outbreaks reported to date. In this brief report, we characterize several H5N1 isolates from Pakistan and discuss the epidemiological implications of these isolates.

Table 1 Percent nucleotide identity (HA top, NA bottom), intravenous pathogenicity index, and GenBank accession numbers for the H5N1 HPAIVs from Pakistan included in this study

Isolate name	NARC-228/06	SPVC-5/07	SPVC-8/07	SPVC-7/07	SPVC-1/08	SPVC-7/08	SPVC-8/08	SPVC-15/08	SPVC-20/08	IVPI	GenBank accession number	
											HA	NA
A/chicken/Pakistan/NARC-228/06	100	99.2	99.6	99.4	99.8	99.8	99.6	99.6	99.4	2.8	CY068896	CY068897
A/chicken/Pakistan/SPVC-5/07	99.3	100	99.4	98.9	99.4	99.4	99.1	99.2	99.6	2.5	CY068904	CY068905
A/chicken/Pakistan/SPVC-7/07	99.7	99.6	100	99.4	99.8	99.8	99.6	99.6	99.5	2.7	CY068906	CY068907
A/chicken/Pakistan/SPVC-8/07	99.4	99.0	99.4	100	99.5	99.5	99.3	99.3	99.1	2.6	CY068910	CY068911
A/chicken/Pakistan/SPVC-1/08	99.5	99.1	99.5	99.8	100	99.9	99.7	99.7	99.5	2.9	CY068900	CY068901
A/chicken/Pakistan/SPVC-7/08	99.6	99.5	99.9	99.3	99.3	100	99.7	99.7	99.5	2.7	CY068908	CY068909
A/chicken/Pakistan/SPVC-8/08	99.7	99.6	100	99.4	99.5	99.9	100	99.8	99.2	2.9	CY068912	CY068913
A/chicken/Pakistan/SPVC-15/08	99.7	99.6	100	99.4	99.5	99.9	100	100	99.3	2.8	CY068898	CY068899
A/chicken/Pakistan/SPVC-20/08	99.3	100	99.6	99.0	99.1	99.5	99.6	99.6	100	2.5	CY068902	CY068903

IVPI Intravenous pathogenicity index

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Materials and methods

Viruses

Nine H5N1 HPAIV isolates (Table 1) were selected for genetic (full HA and NA gene sequencing) and pathogenicity testing to add to epidemiological and biological information about the H5N1 AIVs in Pakistan. All nine isolates were obtained from oral swabs or tracheal tissue from dead chickens suspected to be infected with AIV at commercial poultry operations in the Karachi region of Pakistan. Virus was isolated and propagated in embryonating chicken eggs as per standard methods [7].

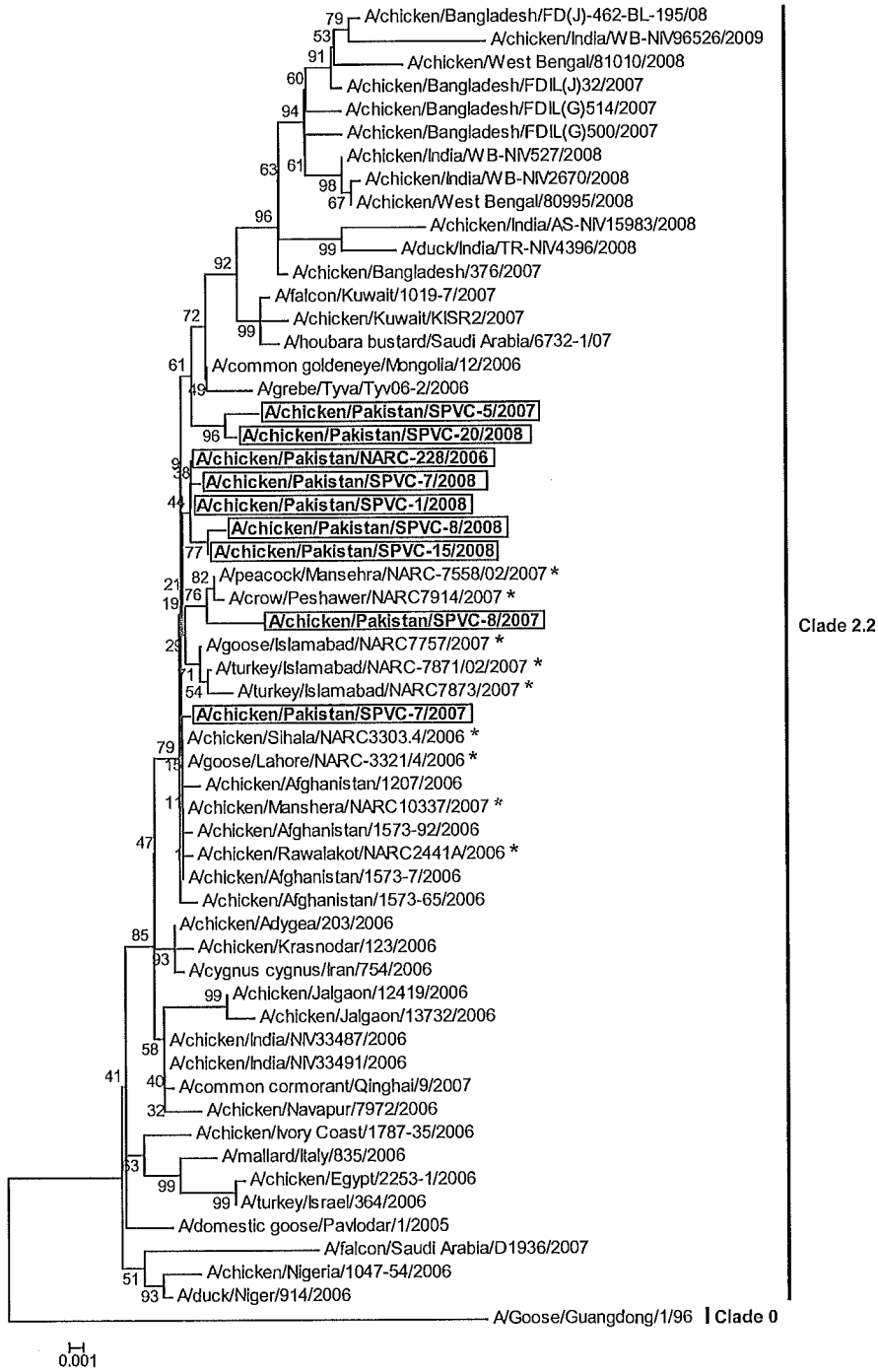

Fig. 1 Phylogenetic analysis of the coding region of the **a** HA and **b** NA genes from Pakistani H5N1 HPAIV viruses. The tree was constructed with MEGA 4.0 using the neighbor-joining method with 1,000 bootstrap replicates. Evolutionary distances were computed using the Kimura 2-parameter method and are listed as units of the number of base substitutions per site. The trees were rooted to the A/goose/Guangdong/1/96 sequence and the nine Pakistani virus isolates referred to in this study are indicated in *bold* and other viruses from Pakistan are labeled with an *asterisk* (*)

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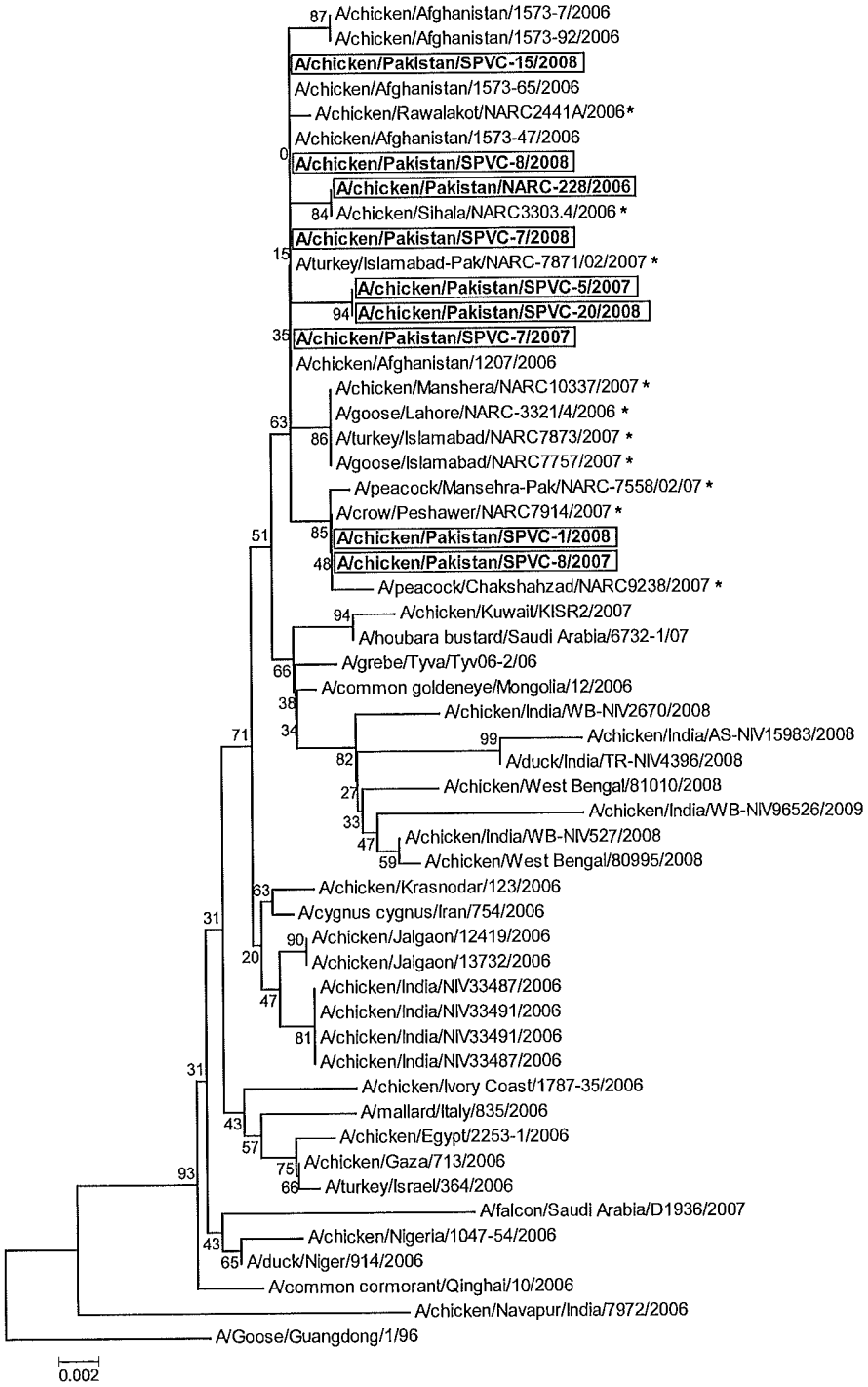
Fig. 1 continued



Pathogenicity testing

Pathogenicity tests for the intravenous pathogenicity index (IVPI) was conducted in accordance with the procedures of the World Organization for Animal Health (OIE) [5] at the Sindh Poultry Vaccine Center, Karachi, Pakistan. In brief, eight specific pathogen-free chickens were inoculated intravenously with each AIV isolate which had been propagated in embryonated chickens eggs. The chickens were observed for 10 days for morbidity and mortality which was used to calculate the IVPI.

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Sequencing

The full HA and NA genes were sequenced directly at Southeast Poultry Research Laboratory in Athens, GA, USA, as described previously [8].

Sequence analysis

Phylogenetic analysis was conducted by aligning the sequences with the CLUSTAL W algorithm and trees were constructed with Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [9] using the neighbor-joining method with 1,000 bootstrap replicates and evolutionary distances were computed using the Kimura 2-parameter method [10] and are listed as units of the number of base substitutions per site. Additional H5 and N1 genes were selected to include in the trees to best demonstrate the most closely related isolated and the relationship of the nine Pakistani viruses to other H5 and N1 genes within their clade and more globally, respectively. The trees were rooted to the A/goose/Guangdong/1/1996 sequence (Fig. 1).

Results

Pathogenesis studies

The IVPI for the nine isolates ranged from 2.5 to 2.9 (Table 1), which is consistent with HPAIV according to the OIE standards where isolates with a score of >1.2 are classified as HPAIV based on the OIE standard test [5].

HA and NA sequences

All nine virus isolates had the same HA proteolytic cleavage site: RRRKKR*G, which is consistent with H5 HPAIV [5, 11] and the in vivo studies. The HA gene sequences of all nine isolates assorted into clade 2.2. Among the HA genes of the nine viruses, there was 99.0–99.8% nucleotide (nt) and 98.8–99.6% amino acid (aa) identity. The NA genes of these nine viruses had 99.0–100% nt and 98.9–100% aa identity and all had a stalk deletion from residues 49 to 68. Both the HA and NA of all nine viruses were most closely related to H5N1 HPAIVs from Pakistan and Afghanistan collected during the same time frame (2006–2008) with nt identities above 98.0%.

Discussion

Characterization of the H5N1 HPAIVs from the Karachi region of Pakistan is crucial for understanding the epidemiology and biology of this virus lineage in the region and globally. Since AIV is a rapidly mutating virus and the animal health labs in Pakistan largely rely on conventional RT-PCR for the detection and identification of AIV, maintaining an up-to-date database of AIV sequence information is critical to assuring that diagnostic tests are reliable. Also, although the results of the pathogenicity tests are expected, it demonstrates that the H5N1 HPAIV has maintained its high virulence for chickens.

Isolates from the Middle East and Russia were closely related to these isolates indicating that all these clade 2.2 isolates were introduced into these regions by likely a common mechanism during 2006 when the H5N1 HPAIV spread from Southeast Asia. Consistent with this is that the NA gene from these isolates had the same NA stalk deletion reported with the viruses from the outbreak at Qinghai lake in 2005 and 2006 [12]. These outbreaks were notable as a benchmark for the spread of the H5N1 virus out of Asia in 2006 because of the infection of migratory wild birds at Qinghai Lake [13]. One epidemiological question for the virus in Pakistan is how it was maintained for 6 months while there were no reported detections in commercial poultry. One possibility is wild birds or non-commercial domestic birds harbored the virus. Successful control of H5N1 HPAIV in Pakistan was primarily accomplished by a combination of vaccination and good biosecurity in commercial poultry. Although surveillance for HPAIV in poultry in Pakistan continues and new isolates will be characterized as they are collected, presently H5N1 HPAIV appears to have been eradicated from the country and has not been isolated since 2008.

Acknowledgments The authors gratefully acknowledge Joan Beck, Joyce Bennett and Melissa Scott at Southeast Poultry Research Laboratory for technical assistance. Funding was provided by the United States Department of State Biosecurity Engagement Program through a Memorandum of Agreement with the United States Department of Agriculture, Agricultural Research Service.

References

1. K. Naeem, The Avian Influenza H7N3 Outbreak in South Central Asia. 4th international symposium on avian influenza, Athens, GA, 1997, pp. 31–35
2. K. Naeem, M. Hussain, Vet. Rec. **137**, 439 (1995)
3. K. Naeem, N. Siddique, M. Ayaz, M.A. Jalalee, Avian Dis. **51**, 189–193 (2007)
4. K. Naeem, A. Ullah, R.J. Manvell, D.J. Alexander, Vet. Rec. **145**, 560 (1999)
5. OIE, Avian influenza: home page. World Organization for Animal Health, 2008
6. WHO, Human cases of avian influenza A (H5N1) in north-west frontier province, Pakistan, October–November 2007. Wkly Epidemiol Rec, Geneva, 2008, pp. 359–364
7. D.E. Swayne, D. Senne, D.L. Suarez, in *Isolation and Identification of Avian Pathogens*, ed. by L. Dufour-Zavala, D.E. Swayne, J. Glisson, J. Pearson, W. Reed, M. Jackwood, P.

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Woolcock (American Association of Avian Pathologists, Jacksonville, 2008), pp. 128–134
8. D.L. Suarez, M. Garcia, J. Latimer, D. Senne, M. Perdue, J. Virol. **73**, 3567–3573 (1999)
9. K. Tamura, J. Dudley, M. Nei, S. Kumar, Mol. Biol. Evol. **24**, 1596–1599 (2007)
10. M. Kimura, J. Mol. Evol. **16**, 111–120 (1980)
11. D.A. Senne, B. Panigrahy, Y. Kawaoka, J.E. Pearson, J. Suss, M. Lipkind, H. Kida, R.G. Webster, Avian Dis. **40**, 425–437 (1996)
12. H. Chen, Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, Y. Kawaoka, J. Virol. **80**, 5976–5983 (2006)
13. G. Wang, D. Zhan, L. Li, F. Lei, B. Liu, D. Liu, H. Xiao, Y. Feng, J. Li, B. Yang, Z. Yin, X. Song, X. Zhu, Y. Cong, J. Pu, J. Wang, J. Liu, G.F. Gao, Q. Zhu, J. Gen. Virol. **89**, 697–702 (2008)

AVIAN DISEASES 56:479–487, 2012

Differences in Pathogenicity, Response to Vaccination, and Innate Immune Responses in Different Types of Ducks Infected with a Virulent H5N1 Highly Pathogenic Avian Influenza Virus from Vietnam

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Received 6 December 2011; Accepted 7 March 2012; Published ahead of print 7 March 2012

SUMMARY. In a previous study, we found clear differences in pathogenicity and response to vaccination against H5N1 highly pathogenic avian influenza (HPAI; HA clade 2.3.4) between Pekin (*Anas platyrhynchos* var. *domestica*) and Muscovy (*Cairina moschata*) ducks vaccinated using a commercial inactivated vaccine (Re-1). The objective of the present study was to further investigate the pathogenicity of H5N1 HPAI viruses in different species of ducks by examining clinical signs and innate immune responses to infection with a different strain of H5N1 HPAI virus (HA clade 1) in two domestic ducks, Pekin and Muscovy, and one wild-type duck, mallard (*Anas platyrhynchos*). Protection conferred by vaccination using the Re-1 vaccine against infection with this virus was also compared between Pekin and Muscovy ducks. Differences in pathogenicity were observed among the virus-infected ducks, as the Muscovy ducks died 2 days earlier than did the Pekin and mallard ducks, and they presented more-severe neurologic signs. Conversely, the Pekin and mallard ducks had significantly higher body temperatures at 2 days postinfection (dpi) than did the Muscovy ducks, indicating possible differences in innate immune responses. However, similar expression of innate immune-related genes was found in the spleens of virus-infected ducks at this time point. In all three duck species, there was up-regulation of IFN-α, IFN-γ, IL-6, CCL19, RIG-I, and MHC class I and down-regulation of MHC class II, but variable expression of IL-18 and TLR7. As in our previous study, vaccinated Muscovy ducks showed less protection against virus infection than did Pekin ducks, as evidenced by the higher mortality and higher number of Muscovy ducks shedding virus when compared to Pekin ducks. In conclusion, infection with an H5N1 HPAI virus produced a systemic infection with high mortality in all three duck species; however, the disease was more severe in Muscovy ducks, which also had a poor response to vaccination. The differences in response to virus infection could not be explained by differences in the innate immune responses between the different types of ducks when examined at 2 days dpi, and earlier time points need to be evaluated.

RESUMEN. Diferencias en la patogenicidad, la respuesta a la vacunación, y la respuesta inmune innata en diferentes tipos de patos infectados con un virus de influenza aviar de alta patogenicidad virulento H5N1 en Vietnam.

En un estudio anterior, se encontraron claras diferencias en la patogenicidad y en la respuesta a la vacunación contra un virus de la influenza aviar altamente patógena H5N1 (clado de hemoaglutinina 2.3.4) entre patos Pekín (*Anas platyrhynchos* var *domestica*) y patos reales (*Cairina moschata*), que fueron vacunados con una vacuna comercial inactivada (Re-1). El objetivo del presente estudio fue investigar más a fondo la patogenicidad del virus de influenza aviar H5N1 en diferentes especies de patos mediante el examen de los signos clínicos y la respuesta inmune innata a la infección con una cepa diferente del virus de la influenza aviar altamente patógena H5N1 (subtipo HA 1) en dos patos domésticos, Pekín y pato real, y un pato silvestre, y mallard (*Anas platyrhynchos*). La protección conferida por la vacunación con la vacuna Re-1 contra la infección con este virus también fue comparada entre Pekín y los patos reales. Se observaron diferencias en la patogenicidad entre los patos infectados por el virus, como con los patos reales que murieron dos días antes en comparación con los patos Pekín y mallard, y presentaron signos neurológicos más severos. Por el contrario, los patos Pekín y reales mostraron temperaturas corporales significativamente superiores en los dos días posteriores a la infección en comparación con los patos reales, indicando las posibles diferencias en la respuesta inmune innata. Sin embargo, una expresión similar de los genes relacionados con las respuestas inmunes innatas se encontraron en los bazos de patos infectados por virus en este punto del tiempo. En las tres especies de patos, se observó regulación positiva de IFN-α, IFN-γ, IL-6, CCL19, RIG-I y MHC de clase I y regulación negativa de MHC de clase II, y expresión variable de IL-18 y TLR7. Al igual que en nuestro estudio anterior, los patos reales vacunados mostraron una menor protección contra la infección por el virus en comparación con los patos Pekín como lo demuestra la mayor mortalidad y el mayor número de patos reales eliminando el virus en comparación con los patos Pekín. En conclusión, la infección con un virus H5N1 de la influenza aviar altamente patógena produce una infección sistémica con una elevada mortalidad en las tres especies de patos, sin embargo, la enfermedad fue más grave en los patos reales, los cuales también mostraron una pobre respuesta a la vacunación. Las diferencias en la respuesta a la infección por el virus no pueden ser explicadas por diferencias en las respuestas inmunitarias innatas entre los diferentes tipos de patos cuando se examinaron a los dos días después de la infección, y puntos mas tempranos deben ser evaluados.

Key words: highly pathogenic avian influenza, H5N1 type, ducks, pathogenicity, innate immunity, vaccination

Abbreviations: AI = avian influenza; BHI = brain heart infusion; CCL19 = chemokine ligand 19; dpi = days postinfection; ECE = embryonating chicken eggs; EID_{50} = 50% egg infective dose; HEPA = high efficiency particulate air; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; IFN-α = interferon alpha; IFN-γ = interferon gamma; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; IL-18 = interleukin 18; MDT = mean death time; MHC-I = major histocompatibility complex class I; MHC-II = major histocompatibility complex class II; MIP3b = macrophage inflammatory protein 3 beta; PG = prostaglandin; qRRT-PCR = quantitative real-time reverse transcriptase–PCR; RIG-I = retinoic acid-inducible gene 1; SPF = specific-pathogen-free; TLR7 = toll-like receptor 7

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The natural host species and reservoir for avian influenza (AI) viruses are wild aquatic birds, especially those of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, terns, and auks) (51,55,60). Normally, ducks naturally or experimentally infected with H5 and H7 highly pathogenic AI (HPAI) viruses develop only subclinical to mild disease (2,14,41,46). However, the pathobiology of HPAI viruses has changed, as many Asian lineage H5N1 HPAI viruses can replicate systemically and produce neurologic dysfunction and death in ducks. H5N1 HPAI viruses are currently 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. The pathogenicity of H5N1 HPAI viruses in ducks can vary depending on the virus and on the age and species of the ducks tested (6,21,40,42,44,57). Asymptomatic infected migratory ducks are suspected of contributing to the spread of H5N1 HPAI viruses westward from Qinghai Lake, China in 2005 to Europe, Africa, India, and the Middle East (11,25,26). On the other hand, domestic ducks have a higher likelihood of perpetuating H5N1 HPAI viruses. Free-range, as well as backyard domestic ducks, have been associated with the spread of H5N1 HPAI viruses (19,22,26,48). Because of this, reducing the risk of H5N1 HPAI virus infection in ducks is important for controlling the continuing circulation and spread of H5N1 HPAI viruses (12,23,28,54).

Vaccination has proven effective in protecting ducks against H5N1 HPAI and is being used in several countries to help control the disease (47). However, vaccinated ducks may still shed virus into the environment, which may result in possible transmission to other hosts and contribute to an endemic situation (10). Several studies have been conducted to determine vaccine efficacy in ducks against HPAI lethal challenge (5,7,16,27,37,43,52,59,61), and differences in response to vaccination have been reported between domestic duck species (52,53). Differences in virus pathogenicity among duck species have also been described (21,25,31). In a recent study, mallard and Muscovy ducks infected with different H5N1 HPAI viruses (HA clades 1, 2.3.2, and 2.3.4) showed clear differences in response to infection, with the Muscovy ducks presenting a higher mortality than did mallards, regardless of the virus given (44). Distinctions between species are not necessarily due to domestic versus wild duck species. A study comparing morbidity, mortality, and viral shedding in five species of wild North American ducks with two different H5N1 HPAI viruses found that only one of the five duck species became sick or died with either virus (8).

Host immune responses most likely play a role in the differences observed in pathogenicity and response to vaccination, but little is known about the immune response in ducks to AI virus infection. Some studies have addressed individual innate immune gene expression in duck-origin cells infected with AI viruses by the use of reverse-transcriptase–PCR (RT-PCR) assays (1,24,29,49), but studies exploring host gene expression in ducks infected with AI viruses have been very limited (4,18,45). One of these immune related genes, retinoic acid-inducible gene 1 (RIG-I), is present in ducks and plays a critical role in the innate immune response to influenza (4,34). Up-regulation of RIG-I in ducks after infection with H5N1 HPAI viruses has been previously reported (4). There is also little information available about fever in birds, although there is evidence that suggests that functional characteristics of avian fever are similar to those found in mammals (20). Prostaglandins (PGs), as in mammalian fevers, seem to play a role in the generation of fever in birds (3,20,33). A role for cytokines, including interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), has also been suggested in the signaling pathways that lead to PG synthesis (33). In a recent study investigating the role for pro-inflammatory cytokines in duck fever, it was concluded that interleukins IL-1β and IL-6 might mediate fever by instigating an accelerated synthesis of brain-derived PG. IL-6 was also suggested to serve as one of the terminal mediators of the avian febrile response (35).

In our previous study, we compared the response to vaccination with a commercially available H5N1 inactivated vaccine, Re-1, between Pekin (*Anas platyrhynchos* var. *domestica*) and Muscovy (*Cairina moschata*) ducks, and found that Muscovy ducks developed lower viral antibody titers induced by vaccination than did Pekin ducks, and they presented with higher morbidity and mortality after challenge with an HA clade 2.3.4 H5N1 HPAI virus from Vietnam (9). When comparing the response to virus infection in nonvaccinated ducks, differences were also observed, with infected Muscovy ducks presenting a more-severe disease than did Pekin ducks. Up-regulation of interferon alpha (IFN-α), RIG-I, IL-6, and major histocompatibility complex class II (MHC-II), and down regulation of MHC-II, was found in both types of ducks with significant differences in expression of RIG-I, IL-6, and MHC-II genes (9).

In order to better understand the differences observed in pathogenicity between different species of ducks, in this study we compared the outcomes of infection with another H5N1 HPAI virus (HA clade 1) in Pekin, Muscovy, and mallards ducks. Innate immune responses were also examined by determining the expression of several immune related genes including IFN-α, interferon gamma (IFN-γ), interleukin 18 (IL-18), IL-6, toll-like receptor 7 (TLR7), chemokine ligand 19 (CCL19), RIG-I, major histocompatibility complex class I (MHC-I), and MHC-II. To confirm our previous findings, we also studied the response of Pekin and Muscovy ducks to vaccination against avian influenza, using the same previously used vaccine, and determined protection against challenge with the HA clade 1 H5N1 HPAI virus.

MATERIALS AND METHODS

Virus and vaccine. The H5N1 HPAI virus A/Dk/NauGiang/NCVD07-12/07, belonging to HA clade 1, was obtained from the National Center for Veterinary Diagnosis, Hanoi, Vietnam. The virus was propagated in embryonating chicken eggs (ECE), and virus 50% egg infective dose (EID_{50}) was determined as previously described (38). Allantoic fluid was diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to obtain an inoculum with 10^5 EID_{50} per 0.1 ml per bird. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI medium. The commercial inactivated Re-1 vaccine (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China) (58) was used to vaccinate the ducks. This vaccine was produced by reverse genetics and derived its HA and NA genes from A/Goose/Guangdong/96; the virus was attenuated by removing the multiple basic amino acids at the HA cleavage site. The six internal genes of this recombinant virus were derived from the high-growth A/Puerto Rico/8/34 (PR8) virus. All experiments using H5N1 HPAI viruses, including work with animals, were performed in biosecurity level-3 enhanced facilities at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service, United States Department of Agriculture (USDA); all personnel were required to wear a powered air-protection respirator with a high efficiency particulate air (HEPA)-filtered air supply (3M™, St. Paul, MN).

Duck experiment. Pekin ducks, Muscovy ducks, and mallard ducks (*Anas platyrhynchos*) were obtained at day of age from commercial farms. Serum samples were collected from 15 ducks of each species to ensure that the birds were serologically negative for AI virus as determined by an ELISA test using IDEXX FlockChek AI MultiS-Screen (Idexx Laboratories, Westbrook, ME). Ducks were cared for and housed in accordance with protocols approved by SEPRL's Institutional Animal Care and Use Committee.

Table 1. Morbidity and mortality. Pekin, Muscovy, and mallard ducks were challenged intranasally at 30 days of age with 10^5 EID_{50} of A/Dk/NauGiang/NCVD07-12/07 (HA clade 1) H5N1 HPAI virus. A group of Pekin and Muscovy ducks was also vaccinated at 14 days of age with inactivated Re-1 AI vaccine.

Groups	No. of ducks	No. of ducks euthanatized at 2 dpi	Body temperatures at 2 dpi (F)[A]	No. of ducks with neurologic signs/total (day of onset)	Mortality/total (MDT)
Pekin, nonchallenged controls	5	5	107.2 ± 0.18[a]	—	—
Pekin, challenged	8	5	108.9 ± 0.26[b]	3/3 (3 dpi)	3/3 (4.5)
Pekin, vaccinated, challenged	10	0	107.3 ± 0.32[a]	1/10 (5 dpi)	1/10 (6)
Muscovy, nonchallenged controls	10	5	106.3 ± 0.20[a]	—	—
Muscovy, challenged	11	5	106.5 ± 0.62[a]	6/6 (1 dpi)	6/6 (2.3)
Muscovy, vaccinated, challenged	10	0	107.4 ± 0.28[a]	4/10 (3 dpi)	4/10 (3.5)
Mallard, nonchallenged controls	5	5	106.8 ± 0.14[a]	—	—
Mallard, challenged	10	5	109.9 ± 0.26[b]	5/5 (3–4 dpi)	5/5 (5)

[A]Mean ± SD. Groups with different lowercase letters are significantly different ($P < 0.05$).

At 15 days of age, 10 Pekin and 10 Muscovy ducks were vaccinated subcutaneously in the nape of the neck with 0.5 ml of the Re-1 vaccine (Table 1) following manufacturer's recommendations. At 30 days of age, these vaccinated ducks and eight unvaccinated Pekin, 11 Muscovy, and 10 mallard ducks were inoculated intranasally via the choanal slit with $10^{5.0}$ EID_{50} of the H5N1 HPAI virus in 0.1 ml (Table 1). Five Pekin, five Muscovy, and five mallard ducks received 0.1 ml of the sham inoculum (nonchallenged controls). Blood samples for serology were also collected at this time from the vaccinated ducks. Ducks were housed in self-contained isolation units that were ventilated under negative pressure with HEPA-filtered air, maintained under continuous lighting, and given *ad libitum* access to feed and water. Ducks were observed daily for clinical signs of disease. Body temperatures were taken from all ducks at 2 days postinfection (dpi) using a rectal digital thermometer. Oropharyngeal and cloacal swabs were collected at 3 and 5 dpi from the vaccinated ducks to determine viral shedding. Five ducks from the nonchallenged groups and five from the virus-challenged groups were euthanatized at 2 dpi with 0.5 ml sodium pentobarbital (5 gr/ml) per bird, gross lesions recorded, and tissues collected separately from each duck for virus isolation. Portions of the brain, lung, spleen, skeletal muscle, and heart from 3 ducks per group were collected in BHI with antibiotics, stored frozen at −70 C, and titers of infectious virus subsequently determined. Trachea, lung, heart, brain, adrenal gland, duodenum, pancreas, liver, kidney, spleen, bursa, thymus, thyroid, and thigh tissue from 3 ducks per group were also collected from necropsied ducks. Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Duplicate sections were stained by immunohistochemistry methods to determine influenza viral antigen distribution in individual tissues (38). Additional spleen sections were collected from five ducks per group and frozen immediately at −80 C for subsequent use in cytokine detection by quantitative real-time reverse transcriptase-PCR (qRRT-PCR). During the experiment, moribund ducks, in pain, with severe neurologic signs or that were unable to reach food or water were also euthanatized. At 12 dpi all surviving ducks were bled and euthanatized.

Serology. Hemagglutination inhibition (HI) assays were performed with serum collected from vaccinated ducks immediately before virus challenge and 12 days after challenge to quantify antibody response to vaccination, as previously described (43). Beta-propiolactone-inactivated antigen (Ag) made from either a virus with a similar HA as the recombinant vaccine virus (Goose/Hong Kong/ 437-6/99) or with the challenge virus (A/Dk/VN/88/07) was used in the test. HI titers were reported as $\log_2$ titers, with 3 $\log_2$ titers being the minimum positive titer.

Virus titers in tissues. Titers of infectious virus from tissues collected at 2 dpi were determined as previously described (39). In brief, tissues were normalized by weight and homogenized in BHI medium, and clarified homogenates were titrated for virus infectivity in eggs. Tissues were homogenized to a 10% (wt/vol) final concentration. Tenfold dilutions of the 10% homogenates (100 µl) were inoculated into 10-day-old ECEs and virus titers as $\log_{10}$ EID_{50}/gram of tissue were calculated. The minimal detectable titer was $10^{1.97}$ EID_{50}/g.

Determination of virus shedding. Oropharyngeal and cloacal swab samples collected from vaccinated ducks were suspended in 2 ml sterile BHI and frozen at −70 C until RNA extraction. RNA was extracted using a previously described combination of Trizol LS reagent (Invitrogen, Inc. Carlsbad, CA) and the MagMax AI/ND RNA isolation kit (Ambion, Inc., Austin, TX) (15). qRRT-PCR targeting the influenza M gene (50) 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. A standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge virus, and results were reported as EID_{50}/ml equivalents (43). The calculated qRRT-PCR detection limit was 10^2 EID_{50}/ml per reaction.

qRRT-PCR for immune related genes. Differences in expression of innate immune-related genes was determined in spleen samples collected from five nonchallenged and five virus-challenged ducks normalized by weight and homogenized in BHI medium. The spleen was chosen because it is an immune organ in birds which is also affected by systemic infection with H5N1 HPAI viruses, as previously demonstrated (9,21,42). Total RNA was extracted from spleen homogenates using Trizol following the manufacturer's instructions (Invitrogen), followed by purification with the Qiagen RNeasy® Midiprep Kit (Qiagen). The RNA concentrations of all samples were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). One-hundred micrograms of RNA from each sample were additionally treated with a on-column DNase digestion (RNase-free DNase Set; Qiagen) for 15 min at room temperature. DNase digest efficacy was determined in random samples by PCR amplification using MHC-II primers (9). RNA integrity numbers were measured with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and were between 8 and 10. The relative quantitation in gene expression following infection was performed using several sets of primers (Table 2). β-actin was used as the endogenous control. MHC-I and MHC-II, interleukin 18 (IL-18), and CCL19, also known as macrophage inflammatory protein 3 beta (MIP3b), primers were based on published sequences and designed using DNASTAR Lasergene primer select software (Madison, WI). Primers for IFN-α, IFN-γ, IL-1β, IL-6, TLR7, and RIG-I have been previously published (1,4) (Table 2). Primer pair specificity among duck species was determined by dissociation curves. Primers for IL-1β could not be optimized to avoid primer dimers in circumstances when target presence was low and, therefore, was only used for qualitative comparison. All reactions were run in triplicate with the ABI Prism® 7500 (Applied Biosystems, Carlsbad, CA) using Power SYBR® Green RNA-to-C™ 1-Step Kit (Applied Biosystems). qRRT-PCR conditions and cycling parameters were the same for all primer pairs used and were as follows: 50 ng RNA per reaction on cycle at 50 C for 30 min, 1 cycle at 95 C for 15 min, 40 cycles of 95 C for 15 sec, and 58 C for 32 sec, followed by dissociation curve determination of the products with 1 cycle at 95 C for 15 sec, 55 C for 20 sec, 99 C for 15 sec, and 60 C for 15 sec. In order to confirm correct target amplification, PCR products

Table 2. Primers sequences used in real-time RT-PCR.

Target	Forward primer	Reverse primer	Size (bp)	Source reference
IFN-α	gggccccgcaaccctt	ctgtaggtgtggttctggaggaa	76	(8)
IFN-γ	aaataccctttccaatgactacaagaa	agccttgcgttggattttca	90	Adams (pers. comm.)
IL-1β	tcatcttctaccgcctggac	cttgtaggtggcgatgttga	151	Adams (pers. comm.)
IL-6	ttcgacgaggagaaatgctt	ccttatcgtcgttgccagat	150	(1)
TLR7	cctttcccagagagcattca	tcaagaaatatcaagataatcacatca	154	(1)
MHC-I	cccactccctgcgctatttctaca	cctccccatccacataccccacac	90	(8)
MHC-II	tggatgaagaacgggcaggag	ggatgaagggcaggtaggagaact	103	(8)
CCL19	ctgcctgaggacgagcgagaa	tgatgaacacgttggcagggattt	104	AY682098
IL-18	ctgcctctattttgctgatgacga	gaaggaagcgatggaggatttt	74	DQ522948
RIG-I	gtgtatggaggaaaaccctatttctt	ggagggtcatacctgttgtttgat	92	(3)
β-actin	cccccgtgctgtgttcccatctatcg	gggtgctcctcaggggctactctcac	228	(45)

were cloned into pCR-TOPO2.1 sub-cloning vectors (Invitrogen) and sequenced using M13 forward and M13 reverse.

Calculations and statistical analysis. A one-way ANOVA with Tukey's post-test was used to analyze the ducks' body temperatures and virus titers in tissues using Prism v.5.01 software (GraphPad Software Inc., San Diego, CA). For statistical purposes, any tissue sample in which virus was not isolated was given a numeric value of $10^{1.9}$ EID_{50}/g. Statistical significance was set at $P < 0.05$. Cytokine gene expression fold change was determined by the $2^{-\Delta\Delta Ct}$ method (30) using β-actin as the endogenous reference gene to normalize the level of target gene expression. A one-way ANOVA with Tukey's multiple comparison tests (GraphPad Software Inc.) was used to determine the statistical significance between fold change values of the three duck species. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical signs and mortality. All nonvaccinated ducks challenged with the H5N1 HPAI virus presented neurologic signs and died in less than 5 days (Table 1); however, Muscovy ducks showed earlier and more-severe neurologic signs and had a lower mean death time (MDT) than did the Pekin and mallard ducks. Neurologic signs included whole body tremors, uncontrollable shaking, marked loss of balance, tilted head, seizures, loss of vision, and paralysis. Ducks from these groups were also depressed and had anorexia and watery, greenish diarrhea. Nine of 10 vaccinated Pekin ducks and six of 10 vaccinated Muscovy ducks survived the virus challenge, but all presented mild clinical signs consisting of conjunctivitis and anorexia, and three ducks from each group had fever at 2 dpi (>108.0 C). Four of 10 vaccinated Muscovy ducks and one of 10 vaccinated Pekin ducks had mild to severe neurologic signs and died. By 12 dpi, all surviving ducks, except one Muscovy duck, had recuperated. The surviving Muscovy duck had mild torticolis. The nonvaccinated Pekin and mallard ducks challenged with the H5N1 HPAI virus had significantly higher body temperatures than the Muscovy ducks (Table 1). The body temperatures of the vaccinated Pekin and Muscovy ducks challenged with virus were not significantly different from the nonchallenged controls and the virus-challenged Muscovy ducks.

Hemagglutination inhibition titers and virus shedding in vaccinated Pekin and Muscovy ducks. Results are presented in Table 3. Mean titers are given for only the positive ducks. Five of 10 vaccinated Pekin ducks had positive prechallenge HI titers when using the vaccine-like virus antigen in the HI assay; however, only one of 10 was positive when using the challenge virus. Two of 10 Muscovy ducks were seropositive when using the vaccine-like virus in the test, and the titers were lower than the titers in Pekin ducks. No titers were detected when using the challenge virus. This is a common occurrence when HI tests are done using a virus different than what the ducks were initially exposed to; in this case, the vaccine. Moderate to high postchallenge HI titers were observed in the vaccinated ducks when using the vaccine-like virus (>7 $\log_2$) or the challenge virus (>3 $\log_2$) in the test, with Muscovy ducks showing higher titers than the Pekin ducks.

There were clear differences between Pekin and Muscovy ducks in regard to the number of ducks shedding virus after challenge and in the duration of virus shedding (Table 3). Only one or two of 10 Pekin ducks shed virus at 3 and 5 dpi. Most Muscovy ducks shed viruses from the oropharyngeal route at both 3 and 5 dpi, and half shed virus through the cloacal route at 3 dpi.

Virus titers in tissues collected from Pekin, Muscovy, and mallard ducks challenged with the H5N1 HPAI virus. Moderate to high virus titers ($\log_{10}$ 5.1–7.8 EID_{50}) were present in the lung, brain, heart, spleen, and muscle of virus-challenged ducks (Fig. 1). There were significantly higher virus titers in the hearts of the Muscovy ducks than in the Pekin and mallard ducks, but no significant difference in titers was found between the other tissues, although the titers were higher in the Muscovy duck tissues

Table 3. Hemagglutination inhibition titers and virus shedding. Pekin and Muscovy ducks were vaccinated with an inactivated commercial vaccine at 15 days of age and challenged intranasally at 30 days of age with an H5N1 HPAI virus. Serum samples were taken at the day of challenge and 12 days postchallenge. HI titers were obtained using a virus similar to the vaccine or the challenge virus as antigen in the test. Swab samples were taken from all birds remaining at 3 and 5 dpi. Mean titers are given for only the positive values.

	HI titers no. of positive ducks/total ducks (mean titers ± SEM)				Virus shedding no. of positive ducks/total ducks (mean titers ± SEM)[A]			
					3 dpi		5 dpi	
Groups	Prechallenge (vaccine Ag)	Prechallenge (challenge Ag)	Postchallenge (vaccine Ag)	Postchallenge (challenge Ag)	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
Pekin	5/10 (2.8 ± 0.49)	1/10 (0.4)	9/9 (7.3 ± 0.42)	9/9 (3.8 ± 0.53)	2/10 (2.08 ± 0.35)	0/10	1/10 (3.0)	1/10 (3.1)
Muscovy	2/10 (1.1 ± 0.43)	0/10	6/6 (9.16 ± 0.31)	6/6 (5.5 ± 0.50)	6/8 (3.5 ± 0.37)	4/8 (3 ± 0.71)	6/7 (2.7 ± 0.14)	1/7 (3.2)

[A]$\log_{10}$ EID_{50} equivalents were determined using qRRT-PCR specific for type A avian influenza matrix gene. Numbers in parentheses are averages of viral titers shed from birds in each group.

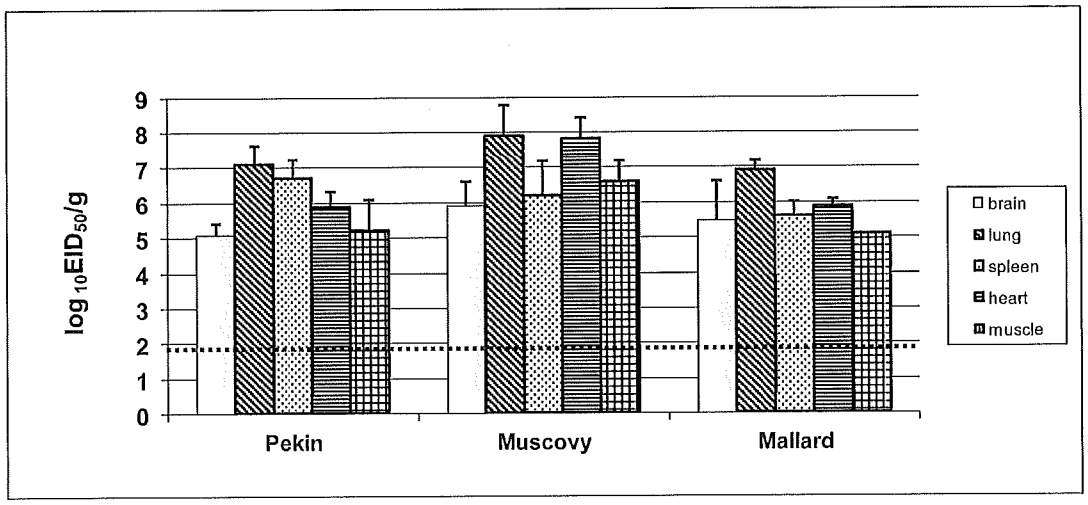

Fig. 1. Virus titers in tissues collected from ducks infected with the A/Dk/NauGiang/NCVD07-12/07 H5N1 HPAI virus. Tissues were collected at 2 dpi from three ducks. Values are the mean ± standard deviation. The threshold of detection was $10^{1.97}$ EID_{50}/g. There was a significant difference in virus titers in the heart of the Muscovy *versus* the Pekin and mallard ducks. No significant difference was found between the other tissues.

when compared to the Pekin and mallards titers, except for in the spleen.

Gross and microscopic lesions and viral antigen distribution in tissues. The virus-challenged Pekin, Muscovy, and mallard ducks presented characteristic lesions of infection with H5N1 HPAI viruses including dehydration, empty intestines, splenomegaly, thymic atrophy, dilated and flaccid hearts with increased pericardial fluid, and congested malacic brains (40). The presence of microscopic lesions and virus antigen in tissues was studied in order to determine differences in virus tissue tropism in the three types of ducks. Microscopic lesions were widespread in tissues from all ducks examined and have been previously described for virulent H5N1 HPAI viruses isolated in Vietnam (42). Viral antigen staining was present in multiple tissues of all ducks infected with the virus, indicating systemic infection (Table 4). 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. In lymphoid organs, viral antigen was only 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 Kupfer cells in the liver, smooth muscle of the ventriculus, autonomic ganglia of the enteric tract, and feather epidermal cells. Virus infection in Muscovy ducks resulted in more widespread viral antigen staining in tissues compared to Pekin and mallard ducks (Table 4). Virus staining was found in the enteric tract, kidney, and skeletal muscle of the Muscovy ducks but not in the Pekin and mallard ducks.

Innate immune-related host genes expression. RNA extracted from spleens was used to investigate differences in the innate immune response among the three duck species by examining gene expression patterns of various cytokines and other innate immune-response genes (Fig. 2). Although there were differences in the sequences of the cytokines examined between the different duck species, the amplified products were >98% similar. A similar trend of up- or down-regulation of innate immune genes was observed in spleens when examined at 2 dpi (Fig. 2). All three duck species showed up-regulation of IFN-α, IFN-γ, IL-6, RIG-I, CCL19, and MHC-I. Anti-viral type I IFN-α gene expression was significantly higher in Pekin and mallard ducks when compared to Muscovy ducks. IFN-γ gene expression was up-regulated in all three duck

Table 4. Distribution of viral antigen in tissues collected from ducks intranasally inoculated with the A/Dk/NauGiang/NCVD07-12/07 H5N1 HPAI virus. Tissues were collected from three ducks at 2 days postchallenge.

Tissues	Pekin	Muscovy	Mallard
Trachea	+/++/++[A]	++/+/+++	+/++/++
Lung	+/+/+++	++/++/+++	+/+/+++
Heart	++/++/++	++/+++/+++	++/++/++
Brain	+++/++/++	+++/++/++	+++/++/++
Adrenal gland	++/+/++	+++/+++/++	++/+/++
Duodenum	−/−/−	+/+/+	−/−/−
Pancreas	+/+/+++	++/++/+++	+/+/+++
Liver	+/++/++	++/+++/+++	+/++/++
Kidney	−/−/−	−/+/+	−/−/−
Spleen	+/++/+	++/++/++	+/++/+
Bursa	++/++/+	+/++/++	++/++/+
Thymus	+++/+++/+++	++/+++/+++	+++/+++/+++
Skeletal muscle	+/+/−	−/+/+	+/+/−

[A]Duck 1/duck 2/duck 3; (−) no virus antigen staining; (+) infrequent; (++) common; (+++) widespread.

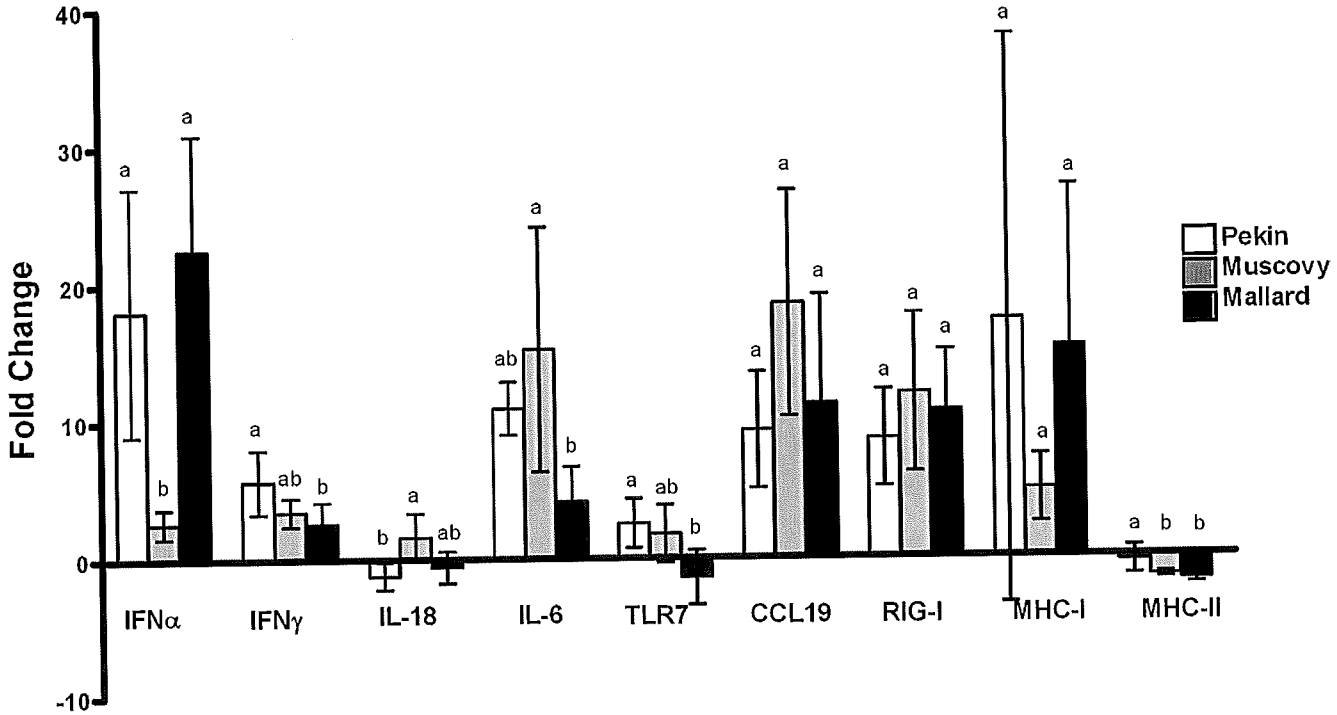

Fig. 2. Changes in expression of IFN-α, IFN-γ, IL-18, IL-6, TLR7, CCL19, RIG-I, MHC-I, and MHC-II genes in spleens of Pekin, Muscovy, and mallard ducks infected with A/Dk/NauGiang/NCVD07-12/07 H5N1 HPAI virus at 2 dpi. Changes were quantified by qRRT-PCR and expressed as fold change in the birds infected compared with noninfected birds ($n = 5$). Bars represent standard deviation of the mean. Different lowercase letters indicate significant differences of means ($P < 0.05$).

species with a significant difference in expression between Pekin and mallard ducks. Expression of IL-18 gene, a signal cytokine which stimulates the production of IFN-γ, was also increased (less than 2-fold) in Muscovy ducks and was statistically different from Pekin ducks, in which it was decreased. A significant difference in expression of the pro-inflammatory cytokine IL-6 gene was found between Muscovy and mallard ducks, increasing 15-fold in Muscovy ducks, while induction in mallard and Pekin was 11- and 4-fold, respectively. Pro-inflammatory signal mediator TLR7 expression in Pekin ducks was statistically different compared to mallard ducks, increasing in expression by 2-fold in Pekin and Muscovy ducks but down-regulated 1.4-fold in mallards. Chemokine CCL19 gene expression was up-regulated more than 9-fold in all three types of ducks with no significant difference in expression between types of ducks. RIG-I, an innate immunity response gene in ducks (4), was also up-regulated 8- to 11-fold in all three duck species, but there was no statistically significant difference in expression between ducks. MHC-I molecule expression increased in all three duck types, but expression was very variable and not statistically different. Expression of the MHC-II molecule was minimally decreased in all three duck types. IL-1β gene expression (qualitative comparison) decreased in all three duck species (data not shown).

DISCUSSION

Until 2002, H5N1 HPAI viruses caused only mild or no clinical disease in ducks. Since then, many H5N1 HPAI viruses have shown to be pathogenic in ducks (40). Nevertheless, the pathogenicity of H5N1 HPAI viruses in ducks varies depending on viral strain, the age of infected ducks and, as this and other studies demonstrates, the duck species. Based on the results of this study, there is a clear difference in response to H5N1 HPAI virus infection between the domestic Muscovy ducks when compared to the domestic Pekin and the closely related wild mallard ducks. Muscovy ducks belong to a different avian species than the Pekin and mallard ducks and seem to be more closely related to geese than ducks. This could explain the differences observed in pathogenicity and response to vaccination among these different types of ducks.

All three types of ducks presented 100% mortality after challenged with the Vietnam H5N1 HPAI HA clade 1 virus used. Similar results were obtained in Pekin ducks inoculated with other virulent viruses from Vietnam (9,37,42,43,63). The high mortality observed in the mallard ducks was not expected, based on previous research showing that mallards were less susceptible to disease produced by H5N1 HPAI viruses than were other duck species (8,17,25,36,44). The Muscovy ducks MDT was half that of the Pekin or mallard ducks, and they showed earlier and more severe neurologic signs, similar to what Phoung *et al.* (44) reported when comparing Muscovy and mallard ducks. Interestingly, Muscovy ducks did not mount as strong a feverish response to virus infection as the Pekin and the mallard ducks did, which was also previously found when comparing infections between Muscovy and Pekin ducks infected with another H5N1 HPAI virus (9). Furthermore, a larger number of vaccinated, challenged Muscovy ducks showed neurologic signs and died earlier than the Pekin ducks. In addition, 75% of the vaccinated Muscovy ducks shed virus, mostly via the oropharyngeal route, versus only 20% of the vaccinated Pekin ducks, and a larger number of Muscovy ducks overall (85%) shed virus until 5 dpi. Together, these results indicate Muscovy ducks are more susceptible to H5N1 HPAI infection than are Pekin and mallard ducks.

Virus distribution in tissues from the nonvaccinated, virus-challenged ducks examined at 2 dpi indicated some differences between the different types of ducks. Muscovy ducks had viral

antigen present in the enteric tract, kidney, and skeletal muscle that was not seen in Pekin and mallard ducks. In addition, Muscovy ducks had widespread viral staining in the heart, adrenal glands, and liver when compared to the lesser virus staining observed in tissues from Pekin and mallard ducks. However, no statistical difference in virus titers was found in the brain, lung, spleen, and muscle in the three types of ducks. This corroborates the severe systemic infection of this particular H5N1 HPAI virus in ducks. The significantly higher virus titers found in the hearts of the Muscovy ducks might explain the earlier deaths observed in this species. Mortality in domestic ducks has been associated with heart failure resulting from increased H5N1 HPAI viral cardiotropism (56,62). The immune system of the Pekin and mallard ducks could also have more-efficiently controlled virus replication and spread, thus prolonging the time of death of the ducks.

Avian influenza virus infection in naive hosts triggers a cascade of host defenses that are responsible for control and clearance of the virus and include innate and subsequent adaptive immune responses. Differences in the innate immune response between duck species is supported by the findings in this study, as virus-challenged Pekin and mallard ducks survived longer than the Muscovy ducks and presented higher body temperatures after virus infection. Body temperatures of Pekin ducks were also higher than in Muscovy ducks after infection with another H5N1 HPAI virus (9). Vaccinated Pekin ducks also mounted a better humoral immune response to vaccination, which conferred them better protection against the subsequent challenge with the H5N1 HPAI virus. This was shown by more vaccinated Pekin ducks presenting detectable antibodies prechallenge, as compared to Muscovy ducks, and less Pekin ducks shedding virus after challenge. Antibody titers after virus challenge where higher in the Muscovy ducks as a consequence of more-severe infection because of the lower levels of protection. This demonstrates that the humoral response also differs between Pekin and Muscovy ducks and supports our previous findings (9).

Differences in innate immune responses could have provided Pekin and mallard ducks an immunologic advantage in response to AI virus infection when compared to the Muscovy ducks. To examine the innate immune response to infection, expression of several innate immune-related genes was examined in spleens collected at the peak of virus infection at 2 dpi. Relative quantitative RT-PCR showed up-regulation of most innate immunity associated genes, with no significant differences observed between the three types of ducks with most cytokines, a consequence of the great variability in gene expression between the ducks in each group. Despite being more closely related and responding similarly to virus infection, no obvious gene expression pattern emerged that was shared by the domestic Pekin and mallard ducks but was different from that found in the domestic Muscovy ducks. In this study, we were unable to design primers for IL-2 that would work well for all three species, or quantitative primers for IL-1β, but could determine a decreased expression of IL-1β in all three species (data not shown). IL-6 up-regulation was previously shown to occur in both Pekin and Muscovy ducks after infection with another H5N1 HPAI virus, with IL-6 levels significantly higher in the Muscovy ducks (9). Similarly in this study, IL-6 gene expression in the Muscovy ducks was higher than in Pekin and mallard ducks, but the difference was not statistically significant between the Pekin and the Muscovy ducks as previously found. As in mammals, IL-6 has been associated with fever induction in ducks (35). Based on the higher body temperatures found in the virus-infected Pekin and mallard ducks, higher levels of IL-6 were expected when compared to the Muscovy ducks. It is not clear if this is a true difference in innate immune responses between the ducks or a consequence of the timing when the samples were taken. Ducks were sampled at 2 dpi, 2 days before any of the remaining Pekin and Muscovy ducks died. However, at this time point, four Muscovy ducks were found dead and the remaining two ducks died the day after. Consequently, the five Muscovy ducks sampled at 2 dpi, although still alive, could have been in different declining states, which might account for the lower body temperatures observed in these ducks. IFN-α and IFN-γ expression was up-regulated in all ducks, similar to what has been reported *in vivo* and *in vitro* in duck cells infected with other H5N1 HPAI viruses (1,24,29,45). IL-18, which stimulates the production of IFN-γ and thus facilitates Th1 immune responses (13), was not up-regulated in Pekin or mallard ducks but was in the Muscovy ducks. CCL19 is associated with modulation of inflammatory responses, so it is not surprising that it was up-regulated in all three duck species. Similar up-regulation of RIG-I was observed in all three types of ducks.

RIG-I, a cytoplasmic 5′-triphosphate RNA sensor found in ducks but not in chickens, initiates expression of other antiviral genes (4,32,64). RIG-I expression in 6-wk-old mallard ducks after infection with a H5N1 virus was greatly induced, but expression levels varied depending on the time after infection at which they were measured (4). We also found RIG-I up-regulation in Pekin and Muscovy ducks infected with another virulent H5N1 HPAI virus (9), but expression was higher in Pekin ducks, indicating that the virus strain might also affect cytokine gene expression. The expression of the TLR7 gene, a receptor implicated in the immune response to influenza virus, was up-regulated in Pekin and Muscovy ducks but down-regulated in the mallards. Finally, an interesting trend of up-regulated MHC-I occurring in tandem with down-regulated MHC-II was observed in this study and has been previously reported (1,9,45). The variability in immune-related gene expression among the ducks precludes us from making conclusions about differences in innate immune responses between the types of ducks studied. For this study, we chose to examine the innate immune response at the peak of virus infection, but a more-detailed time-course study would be required to show differences in responses to infection between the ducks. Differences in response are linked to the time and the magnitude of the innate immune response, with resistant animals typically producing an earlier induced innate immune response than do the more-susceptible animals.

In conclusion, different types of ducks respond differently to H5N1 HPAI virus infection and vaccination. Our findings clearly demonstrate a lower protection conferred by vaccination in Muscovy ducks to H5N1 HPAI virus infection when compared to Pekin ducks. This, and the earlier morbidity and mortality after infection, leads us to believe that in Muscovy ducks the innate immune response and, consequently, the adaptive immune response is less efficient in controlling virus infection. More studies examining the expression of more innate immune-related genes and at earlier time points should help determine this. The results from this study provide insight into the nature of immune responses in ducks to infection with H5N1 HPAI viruses.

REFERENCES

1. Adams, S. C., Z. Xing, J. Li, and C. J. Cardona. Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Mol. Immunol. 46:1744–1749. 2009.

2. Alexander, D. J., G. Parsons, and R. J. Manvell. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5

subtype for chickens, turkeys, ducks and quail. Avian Pathol. 15:647–662. 1986.

3. Baert, K., L. Duchateau, S. De Boever, M. Cherlet, and P. De Backer. Antipyretic effect of oral sodium salicylate after an intravenous E. coli LPS injection in broiler chickens. Br. Poult. Sci. 46:137–143. 2005.

4. Barber, M. R., J. R. Aldridge Jr., R. G. Webster, and K. E. Magor. Association of RIG-I with innate immunity of ducks to influenza. Proc. Natl. Acad. Sci. U. S. A. 107:5913–5918. 2010.

5. Beato, M. S., A. Toffan, R. De Nardi, A. Cristalli, C. Terregino, G. Cattoli, and I. Capua. A conventional, inactivated oil emulsion vaccine suppresses shedding and prevents viral meat colonisation in commercial (Pekin) ducks challenged with HPAI H5N1. Vaccine 25:4064–4072. 2007.

6. Bingham, J., D. J. Green, S. Lowther, J. Klippel, S. Burggraaf, D. E. Anderson, H. Wibawa, D. M. Hoa, N. T. Long, P. P. Vu, D. J. Middleton, and P. W. Daniels. Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. Avian Pathol. 38:267–278. 2009.

7. Boltz, D. A., B. Douangngeun, S. Sinthasak, P. Phommachanh, P. Midouangchanh, D. Walker, R. Keating, A. M. Khalenkov, M. Kumar, and R. G. Webster. Field assessment of an H5N1 inactivated vaccine in chickens and ducks in Lao PDR. Arch. Virol. 154:939–944. 2009.

8. Brown, J. D., D. E. Stallknecht, J. R. Beck, D. L. Suarez, and D. E. Swayne. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. Emerg. Infect. Dis. 12:1663–1670. 2006.

9. Cagle, C., T. L. To, T. Nguyen, J. Wasilenko, S. C. Adams, C. J. Cardona, E. Spackman, D. L. Suarez, and M. Pantin-Jackwood. Pekin and Muscovy ducks respond differently to vaccination against H5N1 highly pathogenic avian influenza (HPAI) virus. Vaccine 29:6549–6557. 2011.

10. Capua, I., and D. J. Alexander. Avian influenza vaccines and vaccination in birds. Vaccine 26(Suppl. 4):D70–73. 2008.

11. Cattoli, G., I. Monne, A. Fusaro, T. M. Joannis, L. H. Lombin, M. M. Aly, A. S. Arafa, K. M. Sturm-Ramirez, E. Couacy-Hymann, J. A. Awuni, K. B. Batawui, K. A. Awoume, G. L. Aplogan, A. Sow, A. C. Ngangnou, I. M. El Nasri Hamza, D. Gamatie, G. Dauphin, J. M. Domenech, and I. Capua. Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. PLoS One 4:e4842. 2009.

12. Chen, H., G. Deng, Z. Li, G. Tian, Y. Li, P. Jiao, L. Zhang, Z. Liu, R. G. Webster, and K. Yu. The evolution of H5N1 influenza viruses in ducks in southern China. Proc. Natl. Acad. Sci. U. S. A. 101:10452–10457. 2004.

13. Chen, H. Y., B. A. Cui, P. A. Xia, X. S. Li, G. Z. Hu, M. F. Yang, H. Y. Zhang, X. B. Wang, S. F. Cao, L. X. Zhang, X. T. Kang, and K. Tu. Cloning, in vitro expression and bioactivity of duck interleukin-18. Vet. Immunol. Immunopathol. 123:205–214. 2008.

14. Cooley, A. J., H. Van Campen, M. S. Philpott, B. C. Easterday, and V. S. Hinshaw. Pathological lesions in the lungs of ducks with influenza A viruses. Vet. Pathol. 26:1–5. 1989.

15. Das, A., E. Spackman, M. J. Pantin-Jackwood, and D. L. Suarez. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of avian influenza virus by RT-PCR. J. Vet. Diagn. Invest. 21:771–778. 2009.

16. Eggert, D., and D. E. Swayne. Single vaccination provides limited protection to ducks and geese against H5N1 high pathogenicity avian influenza virus. Avian Dis. 54:1224–1229.

17. Fereidouni, S. R., E. Starick, M. Beer, H. Wilking, D. Kalthoff, C. Grund, R. Hauslaigner, A. Breithaupt, E. Lange, and T. C. Harder. Highly pathogenic avian influenza virus infection of mallards with homo- and heterosubtypic immunity induced by low pathogenic avian influenza viruses. PLoS One 4:e6706. 2009.

18. Fleming-Canepa, X., C. Brusnyk, J. R. Aldridge, K. L. Ross, D. Moon, D. Wang, J. Xia, M. R. Barber, R. G. Webster, and K. E. Magor. Expression of duck CCL19 and CCL21 and CCR7 receptor in lymphoid and influenza-infected tissues. Mol. Immunol. 48:1950–1957.

19. Gilbert, M., P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Kalpravidh, H. Wagner, and J. Slingenbergh. Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerg. Infect. Dis. 12:227–234. 2006.

20. Gray, D. A., S. K. Maloney, and P. R. Kamerman. Lipopolysaccharide-induced fever in Pekin ducks is mediated by prostaglandins and nitric oxide and modulated by adrenocortical hormones. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289:R1258–1264. 2005.

21. Guionie, O., C. Guillou-Cloarec, D. Courtois, B. S. Bougeard, M. Amelot, and V. Jestin. Experimental infection of Muscovy ducks with highly pathogenic avian influenza virus (H5N1) belonging to clade 2.2. Avian Dis. 54:538–547. 2010.

22. Henning, J., H. Wibawa, J. Morton, T. B. Usman, A. Junaidi, and J. Meers. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. Emerg. Infect. Dis. 16:1244–1250. 2010.

23. Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc. Natl. Acad. Sci. U. S. A. 102:10682–10687. 2005.

24. Jiang, H., H. Yang, and D. R. Kapczynski. Chicken interferon alpha pretreatment reduces virus replication of pandemic H1N1 and H5N9 avian influenza viruses in lung cell cultures from different avian species. Virol. J. 8:447. 2011.

25. Keawcharoen, J., D. van Riel, G. van Amerongen, T. Bestebroer, W. E. Beyer, R. van Lavieren, A. D. Osterhaus, R. A. Fouchier, and T. Kuiken. Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerg. Infect. Dis. 14:600–607. 2008.

26. Kim, J. K., N. J. Negovetich, H. L. Forrest, and R. G. Webster. Ducks: the "Trojan horses" of H5N1 influenza. Influenza Other Respi. Viruses 3:121–128. 2009.

27. Kim, J. K., P. Seiler, H. L. Forrest, A. M. Khalenkov, J. Franks, M. Kumar, W. B. Karesh, M. Gilbert, R. Sodnomdarjaa, B. Douangngeun, E. A. Govorkova, and R. G. Webster. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. J. Virol. 82:11374–11382. 2008.

28. Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430:209–213. 2004.

29. Liang, Q. L., J. Luo, K. Zhou, J. X. Dong, and H. X. He. Immune-related gene expression in response to H5N1 avian influenza virus infection in chicken and duck embryonic fibroblasts. Mol. Immunol. 48:924–930. 2011.

30. Livak, K. J., and T. D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. 2001.

31. Londt, B. Z., A. Nunez, J. Banks, H. Nili, L. K. Johnson, and D. J. Alexander. Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas platyrhynchos) infected experimentally. Avian Pathol. 37:619–627. 2008.

32. Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. Garcia-Sastre, M. G. Katze, and M. Gale Jr. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J. Virol. 82:335–345. 2008.

33. Macari, M., R. L. Furlan, F. P. Gregorut, E. R. Secato, and J. R. Guerreiro. Effects of endotoxin, interleukin-1 beta and prostaglandin injections on fever response in broilers. Br. Poult. Sci. 34:1035–1042. 1993.

34. Magor, K. E. Immunoglobulin genetics and antibody responses to influenza in ducks. Dev. Comp. Immunol. 35:1008–1016. 2011.

35. Marais, M., S. K. Maloney, and D. A. Gray. Brain IL-6- and PG-dependent actions of IL-1β and lipopolysaccharide in avian fever. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301:R791–800. 2011.

36. Marjuki, H., C. Scholtissek, J. Franks, N. J. Negovetich, J. R. Aldridge, R. Salomon, D. Finkelstein, and R. G. Webster. Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks. Arch. Virol. 155:925–934. 2010.

37. Middleton, D., J. Bingham, P. Selleck, S. Lowther, L. Gleeson, P. Lehrbach, S. Robinson, J. Rodenberg, M. Kumar, and M. Andrew. Efficacy

of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology 359:66–71. 2007.

38. Pantin-Jackwood, M. J., D. L. Suarez, E. Spackman, and D. E. Swayne. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. Virus Res. 130:151–161. 2007.

39. Pantin-Jackwood, M. J., and D. E. Swayne. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian Dis. 51:250–259. 2007.

40. Pantin-Jackwood, M. J., and D. E. Swayne. Pathogenesis and pathobiology of avian influenza virus infection in birds. Rev. Sci. Tech. 28:113–136. 2009.

41. Perkins, L. E., and D. E. Swayne. Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. Avian Dis. 46:53–63. 2002.

42. Pfeiffer, J., M. Pantin-Jackwood, T. L. To, T. Nguyen, and D. L. Suarez. Phylogenetic and biological characterization of highly pathogenic H5N1 avian influenza viruses (Vietnam 2005) in chickens and ducks. Virus Res. 142:108–120. 2009.

43. Pfeiffer, J., D. L. Suarez, L. Sarmento, T. L. To, T. Nguyen, and M. J. Pantin-Jackwood. Efficacy of commercial vaccines in protecting chickens and ducks against H5N1 highly pathogenic avian influenza viruses from Vietnam. Avian Dis. 54:262–271. 2010.

44. Phuong, D. Q., N. T. Dung, P. H. Jorgensen, K. J. Handberg, N. T. Vinh, and J. P. Christensen. Susceptibility of Muscovy (Cairina moschata) and mallard ducks (Anas platyrhynchos) to experimental infections by different genotypes of H5N1 avian influenza viruses. Vet. Microbiol. 148:168–174. 2011.

45. Sarmento, L., J. Wasilenko, and M. Pantin-Jackwood. The effects of NS gene exchange on the pathogenicity of H5N1 HPAI viruses in ducks. Avian Dis. 54:532–537. 2010.

46. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252:331–342. 1998.

47. Sims, L. D. Experience in control of avian influenza in Asia. Dev. Biol. (Basel) 130:39–43. 2007.

48. Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerg. Infect. Dis. 12:575–581. 2006.

49. Soubies, S. M., C. Volmer, J. L. Guerin, and R. Volmer. Truncation of the NS1 protein converts a low pathogenic avian influenza virus into a strong interferon inducer in duck cells. Avian Dis. 54:527–531.

50. Spackman, E., D. A. Senne, L. L. Bulaga, S. Trock, and D. L. Suarez. Development of multiplex real-time RT-PCR as a diagnostic tool for avian influenza. Avian Dis. 47:1087–1090. 2003.

51. Stallknecht, D. E., and J. D. Brown. Ecology of avian influenza in wild birds. In: Avian influenza, D. E. Swayne, ed. Blackwell, Ames, Iowa. pp. 43–58. 2008.

52. Steensels, M., M. Bublot, S. Van Borm, J. De Vriese, B. Lambrecht, A. Richard-Mazet, S. Chanavat-Bizzini, M. Duboeuf, F. X. Le Gros, and T. van den Berg. Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI. Vaccine 27:646–654. 2009.

53. Steensels, M., S. Van Borm, B. Lambrecht, J. De Vriese, F. X. Le Gros, M. Bublot, and T. van den Berg. Efficacy of an inactivated and a fowlpox-vectored vaccine in Muscovy ducks against an Asian H5N1 highly pathogenic avian influenza viral challenge. Avian Dis. 51:325–331. 2007.

54. Swayne, D. E. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Ann. N. Y. Acad. Sci. 1081:174–181. 2006.

55. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. J. R. Glisson, Y. M. Saif, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell, Ames, Iowa. 2008. pp 153–184.

56. Swayne, D. E., and M. Pantin-Jackwood. Pathogenicity of avian influenza viruses in poultry. Dev. Biol. (Basel) 124:61–67. 2006.

57. Tang, Y., P. Wu, D. Peng, X. Wang, H. Wan, P. Zhang, J. Long, W. Zhang, Y. Li, W. Wang, X. Zhang, and X. Liu. Characterization of duck H5N1 influenza viruses with differing pathogenicity in mallard (Anas platyrhynchos) ducks. Avian Pathol. 38:457–467. 2009.

58. Tian, G., S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi, K. Yu, and H. Chen. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology 341:153–162. 2005.

59. van der Goot, J. A., M. van Boven, A. Stegeman, S. G. van de Water, M. C. de Jong, and G. Koch. Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. Virology 382:91–97. 2008.

60. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152–179. 1992.

61. Webster, R. G., R. J. Webby, E. Hoffmann, J. Rodenberg, M. Kumar, H. J. Chu, P. Seiler, S. Krauss, and T. Songserm. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology 351:303–311. 2006.

62. Woo, G. H., H. Y. Kim, Y. C. Bae, Y. H. Jean, E. J. Bak, M. J. Kim, E. K. Hwang, and Y. S. Joo. Comparative histopathological characteristics of highly pathogenic avian influenza (HPAI) in chickens and domestic ducks in 2008 Korea. Histol. Histopathol. 26:167–175. 2011.

63. Yao, Q., K. P. Fischer, L. Li, B. Agrawal, Y. Berhane, D. L. Tyrrell, K. S. Gutfreund, and J. Pasick. Immunogenicity and protective efficacy of a DNA vaccine encoding a chimeric protein of avian influenza hemagglutinin subtype H5 fused to CD154 (CD40L) in Pekin ducks. Vaccine 28:8147–8156.

64. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5:730–737. 2004.

ACKNOWLEDGMENTS

The authors appreciate the assistance provided by Scott Lee, Ronald Graham, and Roger Brock in conducting these studies. The authors also thank the Food Safety and Inspection Service's histopathology laboratory at Russell Research Center–USDA for technical assistance. This study was supported by a Specific Cooperative Agreement with the Foreign Agriculture Service of the USDA and Agriculture Research Service CRIS Project 6612-32000-048.

BioOne RESEARCH EVOLVED

Comparison of Pooling 11 or 5 Oropharyngeal Swabbings for Detecting Avian Influenza Virus by Real-Time Reverse Transcription–PCR in Broiler Chickens

Author(s) :Brian S. Ladman, Erica Spackman and Jack Gelb, Jr.
Source: Avian Diseases, 56(1):227-229. 2012.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/9839-062011-ResNote.1
URL: http://www.bioone.org/doi/full/10.1637/9839-062011-ResNote.1

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AVIAN DISEASES 56:227–229, 2012

Research Note—

Comparison of Pooling 11 or 5 Oropharyngeal Swabbings for Detecting Avian Influenza Virus by Real-Time Reverse Transcription–PCR in Broiler Chickens

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Received 21 June 2011; Accepted and published ahead of print 21 August 2011

SUMMARY. The effect of pooling 11 or 5 oropharyngeal (O/P) swabbings on detecting avian influenza virus (AIV) by real-time reverse transcription (RRT)–PCR was evaluated. The model used for the evaluation was designed to minimize viral load and, thus, assess the effect of the pooling on detection. Two-week-old broiler chickens were inoculated via the intranasal route with the low pathogenicity chicken/Maryland/Minh Ma/04 H7N2 strain or remained uninoculated. On days 2, 3, 4, 5, 7, 9, 11, and 14 postinoculation (PI), O/P swabbings were collected from individual infected birds and pooled with either 10 or 4 O/P swabs from uninfected broilers to produce 10 replicate pools of 11 or 5 swabbings, respectively. AIV was readily detected (80%–100%) by RRT-PCR in the pools of 11 and pools of 5 swabbings from days 2 through 5 PI. Detection in pools of both types decreased to similar levels on day 7 (40% for the pools of 11 and 50% for the pools of 5). AIV was not detected on day 9, 11, and 14 PI in pools of either size. On a given sample day PI, mean cycle threshold (Ct) values were consistently higher (lower genome levels) in the pools of 11 compared to the pools of 5. These differences were statistically significant on days 3 and 5 PI, yet Ct values associated with both types of pools were clearly interpretable as AIV positive.

RESUMEN. *Nota de Investigación*—Comparación de muestras agrupadas con 11 ó 5 hisopados orofaríngeos para la detección de virus de la influenza aviar en pollos de engorde mediante transcripción inversa y reacción en cadena de la polimerasa en tiempo real.

Se evaluó el efecto de agrupar 5 o 11 hisopos orofaríngeos (O/P) en la detección del virus de la influenza aviar por transcripción inversa y reacción en cadena en tiempo real (RRT)-PCR. El modelo utilizado para la evaluación fue diseñado para reducir al mínimo la carga viral y por tanto, evaluar el efecto de la agrupación de muestras en la detección. Pollos de engorde de dos semanas de edad, fueron inoculados por vía intranasal con una cepa del virus de la influenza aviar de baja patogenicidad Pollo/Maryland/Minh Ma/04 H7N2, o se mantuvieron como controles no inoculados. En los días 2, 3, 4, 5, 7, 9, 11, y 14 después de la inoculación, se recolectaron hisopos orofaríngeos de las aves infectadas individualmente y se combinaron con cuatro o 10 hisopos de pollos de engorda no infectados para producir 10 replicas de 5 y 11 hisopos, respectivamente. El virus de la influenza aviar fue detectado con facilidad (en un 80% a un 100%) mediante RRT-PCR en las muestra agrupadas con cinco y once hisopos desde los dos a los cinco días después de la inoculación. La detección en las muestras agrupadas de ambos tipos se redujo a niveles similares en el día 7 (40% para muestras agrupadas con 11 y 50% de la muestras agrupadas con 5). El virus de la influenza aviar no se detectó a los 9, 11 o 14 días después de la inoculación. En un día de muestreo determinado los valores promedio de ciclos umbrales (Ct) fueron consistentemente mayores (niveles de genoma mas bajos) en las muestras de 5 hisopos en comparación con las muestras con 11 hisopos. Estas diferencias fueron estadísticamente significativas en los días 3 y 5 después de la inoculación, sin embargo, los valores de Ct asociados con ambos tipos de muestras agrupadas eran claramente interpretables como positivos al virus de la influenza aviar.

Key words: notifiable avian influenza, avian influenza virus, Delmarva Peninsula, chicken, detection, surveillance, real-time reverse transcription–PCR, oropharyngeal swabbing pool, low pathogenicity

Abbreviations: ACIA = antigen capture immunoassay; AI = avian influenza; AIV = avian influenza virus; APHIS = Animal and Plant Health Inspection Service; Ct = cycle threshold; HP = high pathogenicity; ISRCP = Initial State Response and Containment Plan; LP = low pathogenicity; NAI = notifiable avian influenza; O/P = oropharyngeal; PI = postinoculation; RRT-PCR = real-time reverse transcription–polymerase chain reaction; USDA = United States Department of Agriculture; VS = Veterinary Services

Since 2006, all flocks of commercial broiler chickens in the United States have undergone surveillance testing for notifiable avian influenza (NAI) (2). States, in cooperation with the poultry industry, have implemented surveillance programs consisting of active surveillance of healthy flocks prior to slaughter and passive surveillance of sick flocks. Antigen–genome or antibody-based tests (or both) may be used in NAI surveillance programs. For antigen–genome surveillance, 11 swabbings from individual broilers is the statistically determined number of samples needed from a commercial-sized flock (10,000 birds or more) to detect 25% infection with 95% confidence (11). In the states of Delaware and Maryland, the National Poultry Improvement Plan-approved Initial State Response and Containment Plan (ISRCP) and the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Veterinary Services (VS)-approved State Cooperative Agreements for NAI Prevention and Control Program are used in conjunction to address regional NAI programs. These documents specify that active preslaughter surveillance of broilers be performed by testing two pools of oropharyngeal (O/P) swabbings prepared from five and six individual birds from each farm. The pools may be tested by real-time reverse transcription (RRT)–PCR or by antigen

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Table 1. Experimental design for detecting LP H7N2 AIV in pools of 11 or 5 O/P swabbings from broiler chickens using RRT-PCR.

Number of individual broiler swabbings			Swabbings collected on sample day post-inoculation	Number of replicate pools evaluated per sample day
Total in pool	From AIV-infected broilers[A]	From uninfected broilers[B]		
11	1	10	2,3,4,5,7,9,11,14	10
5	1	4	2,3,4,5,7,9,11,14	10

[A]AIV-infected broilers were identified and used to create either the pools of 11 or 5 swabbings for the purpose of minimizing the variability in AIV genome levels between the two types of pools.
[B]Uninfected broilers were randomly selected to create the pools of 11 or 5 swabbings.

capture immunoassay (ACIA). While both ACIA and RRT-PCR testing are used to carry out active (preslaughter) surveillance programs, RRT-PCR is considered to be essential because it is more sensitive than ACIA for detecting avian influenza virus (AIV) infection (3,4,5). The cost of RRT-PCR, which is approximately 2 to 3-fold greater than ACIA, has had the effect of limiting the number of RRT-PCR tests performed in surveillance programs. Moreover, support from USDA/APHIS/VS for NAI surveillance programs has been steadily diminishing and further cuts are predicted in the future. Increasing the number of individual swabbings in pools has been proposed as a way to reduce RRT-PCR costs while still maximizing the level of surveillance. The purpose of this study was to examine the feasibility of using pools of 11 swabbings to perform one RRT-PCR reaction instead of the current program of testing two pools per farm.

MATERIALS AND METHODS

One-hundred and sixty, 1-day-old broiler chickens were obtained from a commercial hatchery. The chicks received standard Marek's disease vaccine (herpesvirus of turkeys and SB1) *in ovo* but did not receive vaccinations for infectious bronchitis virus or Newcastle disease virus. Twenty broilers were randomly assigned to the AIV-infected treatment group. The remaining 140 birds were assigned to the uninfected control group (Table 1). Broilers were maintained under BSL-3 containment in negative-pressure glove-port isolation units with disinfectant dip tanks at the Charles C. Allen Laboratory, University of Delaware, Newark, Delaware. Birds were provided a commercial diet and drinking water *ad libitum*. At 2 wk of age, broilers in the AIV-infected treatment group were inoculated with 10^5 50% embryo infectious dose per bird with the H7N2 low pathogenicity (LP) AIV strain, chicken/Maryland/Minh Ma/04, via the intranasal route. On days 2, 3, 4, 5, 7, 9, 11, and 14 postinoculation (PI), O/P swabbings were collected from individual AIV-infected and uninfected control broilers. A goal of the research was to simulate situations of low viral loads in the pools, thus making detection more difficult. Therefore, only one swab from an AIV-infected broiler from each sample day was pooled with either 10 or 4 individual swabbings from uninfected broilers to create the pools of 11 or 5 swabbings, respectively. The same AIV-infected broilers were used to prepare either the pools of 11 or pools of 5 to minimize variability in AIV genome levels between the two types of pools. Uninfected control broilers were randomly selected for swabbing to create the final pools. A total of 10 replicate pools of 11 or 5 swabbings were prepared on each sample collection day. Swabbings were collected with a plastic shaft Dacron swab (Fisher Scientific, Fairlawn, NJ) and placed into a tube containing 3 ml brain heart infusion broth (Fisher Scientific). To simulate laboratory procedures performed during routine NAI surveillance testing of commercial broilers at National Animal Health Laboratory Network facilities, each swab was agitated 4–5 times and pushed against the side of the tube, expressing the liquid out of the swab as it was drawn out of the tube. Swab pools were frozen at −80 C. After thawing at room temperature, viral RNA was extracted from an aliquot of each O/P swab pool and screened by RRT-PCR for the presence of the AIV matrix gene (8).

Mean AIV RRT-PCR cycle threshold (Ct) values were analyzed between pools of 11 and pools of 5 for a given sample day by *t*-test ($P < 0.05$). The number of AIV-positive pools for a given sample day was analyzed by chi-square analysis ($P < 0.05$). Pools with Ct values >35 were considered negative (10). Pools yielding undetectable Ct values were assigned a Ct value of 46 for the purpose of data analysis. All calculations were made using Excel (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

Our findings demonstrate that H7N2 LP AIV was readily detected by RRT-PCR in pools of 11 O/P swabbings consisting of one swabbing from AIV-infected broiler chickens and the remaining 10 from uninfected broilers (Table 2). On sample days 2, 3, 4, and 5 PI, 80%–100% of both types of pools were found to be avian

Table 2. Comparison of pooling 11 (one positive and 10 negative swabbings) or 5 (one positive and 4 negative swabbings) on detection of LP H7N2 AIV in broiler chickens using RRT-PCR.

	Pool of 11 oropharyngeal swabbings		Pool of 5 oropharyngeal swabbings	
Sample day postinoculation	Mean AIV RRT-PCR Ct values[A]	Number of AIV-positive pools/total replicates[B]	Mean AIV RRT-PCR Ct values[A]	Number of AIV-positive pools/total replicates[B]
2	31.75	9/10	29.51	10/10
3	30.47*	10/10	27.03*	10/10
4	33.36	10/10	30.19	10/10
5	33.34*	8/10	30.04*	10/10
7	35.37	4/10	35.59	5/10
9	44.51	0/10	42.54	0/10
11	45.14	0/10	Undetectable	0/10
14	Undetectable	0/10	Undetectable	0/10

[A]Asterisks (*) within a row indicate that mean AIV RRT-PCR Ct values, analyzed between pools of 11 or 5 for a given sample day, are statistically different (*t*-test, $P < 0.05$).
[B]Number of AIV-positive pools of 11 or 5 swabbings for a given sample day were analyzed (chi-square analysis, $P < 0.05$). Swab pools with Ct values >35 were considered AI negative. Samples yielding undetectable Ct values were assigned a Ct value of 46 for the purpose of data analysis. No statistical differences were found.

influenza (AI) positive. Mean RRT-PCR Ct values of the pools of 11 swabbings were statistically higher ($P < 0.05$) on day 3 and day 5 PI compared to the pools of 5. Although statistically significant (days 3 and 5 PI) and numerical differences (days 2 and 4 PI) were observed when comparing mean Ct values of the pools of 11 or 5, Ct values for both types of pools were still readily interpretable as AIV positive (Ct < 35). By day 7 PI, mean Ct values increased and the number of AIV-positive pools decreased to similar levels in both the pools of 11 or 5, reflecting lower AIV genome levels. The genome levels observed are consistent with previous findings (6,7).

The LP strain used in the study was readily detected in the swab pools of 11 in spite of the reduction in AIV genome levels observed from days 3–7 PI. Compared to high pathogenicity (HP) AIV infections, LP infections may be more difficult to detect. Levels of genome in swabbings are lower than are typically found in HP infections (9). Therefore, it is likely that swab pools of 11 will be sufficient to detect HPAIV as well.

Based on the findings of the current study, the practice of pooling 11 swabbings for active preslaughter surveillance should receive serious consideration. Moreover, in an actual LPAIV field infection, it is likely that more than a single broiler selected for pooled testing in a flock would be positive for AIV. In addition, the ISRCP stipulates that on-farm NAI surveillance consist of sampling mortalities within the prior 24 hr. "Barrel surveillance" swabbing of broilers biases the sampling towards detecting AIV (1).

Cost considerations will continue to drive policy decisions regarding NAI surveillance. There is little doubt that the development of RRT-PCR technology and its application to AIV has been a scientific and, more importantly, a practical success for detecting a disease agent that still threatens a major food animal industry. Further research comparing pooling of 11 or 5 swabbings using other AIV strains and pathotypes is warranted to verify and possibly extend these findings. Policy makers could consider provisional use of pooling 11 swabbings for active preslaughter surveillance testing of healthy flocks until additional data are available.

REFERENCES

1. Akey, B. L. Low-pathogenicity H7N2 avian influenza outbreak in Virginia during 2002. Avian Dis. 47:1099–1103. 2003.

2. Anonymous. Avian influenza info. Washington, D.C., January 12, 2006. Industry to maintain "full confidence" in product safety [Internet]. [cited 2011 June 6]. Available from: http://www.avianinfluenzainfo.com/news/1_12_06.html.

3. Das, A. M., E. Spackman, C. Thomas, D. E. Swayne, and D. L. Suarez. Detection of H5N1 high-pathogenicity avian influenza virus in meat and tracheal samples from experimentally infected chickens. Avian Dis. 52:40–48. 2008.

4. Dormitorio, T. V., J. J. Giambrone, K. Guo, and G. R. Hepp. Evaluation of field and laboratory protocols used to detect avian influenza viruses in wild aquatic birds. Poult. Sci. 88:1825–1831. 2009.

5. Elvinger, F., B. L. Akey, D. A. Senne, F. W. Pierson, B. A. Porter-Spalding, E. Spackman, and D. L. Suarez. Characteristics of diagnostic tests used in the 2002 low-pathogenicity avian influenza H7N2 outbreak in Virginia. J. Vet. Diagn. Invest. 19:341–348. 2007.

6. Ladman, B. S., S. C. Rosenberger, J. K. Rosenberger, C. R. Pope, and J. Gelb Jr. Virulence of low pathogenicity H7N2 avian influenza viruses from the Delmarva Peninsula for broiler and leghorn chickens and turkeys. Avian Dis. 52:623–631. 2008.

7. Spackman, E., J. Gelb, L. A. Preskenis, B. S. Ladman, C. R. Pope, M. J. Pantin-Jackwood, and E. T. Mckinley. The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. Virol. J. 7:331. 2010.

8. Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256–3260. 2002.

9. Swayne, D. E., and J. R. Beck. Experimental study to determine if low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. Avian Dis. 49:81–85. 2005.

10. Takekawa, J. Y., N. J. Hill, A. K. Schultz, S. A. Iverson, C. J. Cardona, W. M. Boyce, and J. P. Dudley. Rapid diagnosis of avian influenza virus in wild birds: use of a portable RRT-PCR and freeze-dried reagents in the field. Immunology and infection. [Internet]. [cited 2011 Nov 7]. Field J. Vis. Exp. 54:e2829. Available from: http://www.JOVE.com/video/2829

11. U.S. Department of Agriculture Animal and Plant Health Inspection Service, June 29, 2007. National Avian Influenza Surveillance Plan [Internet]. [cited 2011 Aug 11]. Available from: http://www.aphis.usda.gov/vs/nahss/poultry/ai/avian_influenza_surveillance_plan_062907.pdf

ACKNOWLEDGMENTS

This research was supported by USDA Multistate Regional Research Project NC-1180. Funding was also provided by the State of Delaware via state lines, "Poultry Disease Research" and "Poultry Diagnostic Laboratory." We thank David Shapiro, Don Ritter, and Heather Hirst for reviewing the manuscript.

AVIAN DISEASES 56:969–975, 2012

Susceptibility of Avian Species to North American H13 Low Pathogenic Avian Influenza Viruses

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Received 10 April 2012; Accepted 14 June 2012; Published ahead of print 14 June 2012

SUMMARY. Gulls are widely recognized reservoirs for low pathogenic avian influenza (LPAI) viruses; however, the subtypes maintained in these populations and/or the transmission mechanisms involved are poorly understood. Although, a wide diversity of influenza viruses have been isolated from gulls, two hemagglutinin subtypes (H13 and H16) are rarely detected in other avian groups, and existing surveillance data suggests they are maintained almost exclusively within gull populations. In order to evaluate the host range of these gull-adapted influenza subtypes and to characterize viral infection in the gull host, we conducted a series of challenge experiments, with multiple North American strains of H13 LPAI virus in ring-billed gulls (*Larus delawarensis*), mallards (*Anas platyrhynchos*), chickens (*Gallus domesticus*), and turkeys (*Meleagris gallopavo*). The susceptibility to H13 LPAI viruses varied between species and viral strain. Gulls were highly susceptible to H13 LPAI virus infection and excreted virus via the oropharynx and cloaca for several days. The quantity and duration of shedding was similar between the two routes. Turkeys and ducks were resistant to infection with most strains of H13 LPAI virus, but low numbers of inoculated birds were infected after challenge with specific viral strains. Chickens were refractory to infection with all strains of H13 LPAI virus they were challenged with. The experimental results presented herein are consistent with existing surveillance data on H13 LPAI viruses in birds, and indicate that influenza viruses of the H13 subtype are strongly host-adapted to gulls, but rare spill-over into aberrant hosts (i.e., turkeys and ducks) can occur.

RESUMEN. Susceptibilidad en diferentes especies de aves a los virus de la influenza aviar de baja patogenicidad subtipo H13 aislados en América del Norte.

Las gaviotas son reservorios ampliamente reconocidos del virus de la influenza aviar de baja patogenicidad, sin embargo, los subtipos mantenidos en estas poblaciones y/o los mecanismos de transmisión involucrados no están bien esclarecidos. Aunque, una amplia diversidad de virus de influenza se han aislado de gaviotas, dos subtipos de hemaglutininas (H13 y H16) rara vez se detectan en otros grupos de aves, y los datos existentes de vigilancia sugieren que se mantienen casi exclusivamente dentro de las poblaciones de gaviotas. Con el fin de evaluar el rango de hospederos de estos subtipos del virus de influenza adaptados en gaviotas y para caracterizar la infección viral en las gaviotas como huéspedes, se llevó a cabo una serie de experimentos con varias cepas del virus de la influenza aviar de baja patogenicidad subtipo H13 aisladas en América del Norte en el desafío de gaviotas Delaware (*Larus delawarensis*), en patos de collar (*Anas platyrhynchos*), en pollos (*Gallus domesticus*) y en pavos (*Meleagris gallopavo*). La susceptibilidad a los virus de influenza aviar de baja patogenicidad H13 variaron entre las especies y las cepas virales. Las gaviotas son muy susceptibles a la infección por el virus de la influenza aviar de baja patogenicidad H13 y el virus se excreta a través de la orofaringe y la cloaca durante varios días. La cantidad y la duración de la eliminación fue similar entre las dos rutas. Los pavos y los patos fueron resistentes a la infección con la mayoría de las cepas de virus de la influenza aviar de baja patogenicidad H13, pero un bajo número de aves inoculadas fueron infectadas después del desafío con las cepas virales específicas. Los pollos fueron refractarios a la infección con las cepas de virus de la influenza de baja patogenicidad H13 con las que fueron inoculados. Los resultados experimentales presentados en este informe son consistentes con los datos de vigilancia existentes con los virus de la influenza aviar de baja patogenicidad H13 en las aves, e indican que los virus de influenza del subtipo H13 están fuertemente adaptados a las gaviotas como hospederos, aunque la diseminación a huéspedes aberrantes (pavos y patos) es rara pero puede ocurrir.

Key words: avian influenza, chickens, gulls, H13, mallards, poultry, turkeys

Abbreviations: AI = avian influenza; bELISA = blocking enzyme-linked immunosorbent assay; BHI = brain-heart-infusion; BSL = biosafety level; EID_{50} = median embryo infectious doses; HA = hemagglutinin; HP = highly pathogenic; LP = low pathogenic; NA = neuraminidase; NP = nucleoprotein; PCD = post-contact day; PID = post-inoculation day; qRT-PCR = quantitative real-time reverse-transcription polymerase chain reaction; SA = sialic acid; SCWDS = Southeastern Cooperative Wildlife Disease Study; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen free; UGA = University of Georgia

The first isolation of avian influenza (AI) virus from a wild bird was during an outbreak of H5N3 highly pathogenic (HP) AI in common terns (*Sterna hirundo*) in South Africa during 1961 (1). In the 50 yr since this event, AI viruses have been commonly isolated from wild birds in the family Laridae (gulls and terns), including HP and low pathogenic (LP) viruses representing numerous hemagglutinin (HA) and neuraminidase (NA) subtypes (9,14,19,23). Two HA subtypes (H13 and H16) have rarely been detected in mammalian or avian hosts outside of the family Laridae, and are thought to be maintained almost exclusively in gull and tern populations (6). Collectively, surveillance data indicate gulls are permissive hosts for a wide diversity of AI viruses and recognized reservoirs for the H13 and H16 subtypes; however, it is currently unclear how these viruses are transmitted or maintained in these avian populations.

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The H13 subtype was first identified and characterized in the early 1980s (12). Although, it is the most common HA subtype detected in gull species around the globe, the epidemiologic patterns of H13 LPAI virus are not well defined. The limited existing experimental and surveillance data indicate the host range of H13 LPAI viruses is unique, but confusing. Other than gulls and terns, natural infections with H13 LPAI virus have sporadically been reported in only a few other species, including domestic turkeys (*Meleagris gallopavo*), shorebirds (family Scolopacidae), and pilot whales (*Globicephala* sp.) (12,13,16,17,24). Experimentally, the infectivity of H13 LPAI viruses for different hosts appears highly variable between species and viral strains. Turkeys and ferrets (*Mustela putorius furo*) were permissive hosts that excreted detectable levels of virus and seroconverted after challenge with H13 LPAI viruses (12,16). Studies in mallards (*Anas platyrhynchos*) and chickens (*Gallus domesticus*) have yielded variable results, ranging from complete resistance to sporadic infections (12,14,16). Interestingly, although gulls are the primary reservoir for H13 LPAI viruses, to date, infection has not been characterized in this host experimentally. This lack of data precludes understanding the mechanisms of viral transmission between gulls and from gulls to aberrant hosts (including domestic animals and other wild avian taxa), and limits any attempts to improve surveillance efficiency for H13 LPAI viruses.

The overall goal of the experimental trials presented herein was to complement existing surveillance data and provide insights into the epidemiology of H13 LPAI virus. The principal objectives were 1) to evaluate the susceptibility of ring-billed gulls (*Larus delawarensis*) to infection with a North American H13 LPAI virus and characterize patterns of viral shedding; and 2) to determine the potential risks for spill-over of North American strains of H13 LPAI virus into important poultry or wild bird species known to be highly susceptible to AI viruses.

MATERIALS AND METHODS

Animals. Four avian species were used in this study: 4-wk-old mallards, 6-wk-old ring-billed gulls, 4-wk-old specific pathogen free (SPF) white-leghorn chickens, and 3-wk-old SPF small Beltsville white turkeys. The mallards, chickens, and turkeys were captive-bred (McMurray Hatchery, Webster City, IA [mallards]) or acquired from in-house flocks at the Southeast Poultry Research Laboratory [SEPRL], Athens, GA [chickens and turkeys]) and cared for under indoor confinement. At 3-wk (turkeys) or 4-wk (mallards and chickens), birds were transferred to a biosafety level (BSL)-2Ag+ facilities at the University of Georgia (UGA; mallards) or the SEPRL (chickens and turkeys), where the experimental challenge trials were conducted.

Ring-billed gulls used in this study were acquired through the Southeastern Cooperative Wildlife Disease Study (SCWDS), UGA, under appropriate federal and state permits. Young nestling ring-billed gulls were hand-caught in St. Lawrence County, New York, and maintained at the UGA under semi-indoor confinement until 6 wk of age, at which time they were transferred to a BSL-2Ag+ facility at the UGA for the experimental challenge trial. Using the methodology described below, all gulls tested negative for antibodies to the nucleoprotein (NP) of AI virus at 48 hr after capture.

The procedures and methods used during the bird capture, husbandry, and experimental trials were performed according to animal care and use proposals approved by the Institutional Animal Care and Use Committee at the UGA and/or SEPRL.

Viruses. The following eight strains of H13 LPAI virus were used in the experimental challenge trials: A/laughing gull/NJ/AI08-1460/2008 (H13N9), A/ring-billed gull/DE/AI10-1708/2010 (H13N9), A/sand (environment)/NJ/1-24/2010 (H13N6), A/turkey/MN/1012/1991 (H13N2), A/ruddy turnstone/NJ/AI09-294/2009 (H13N6), A/laughing gull/NJ/AI08-1388/2008 (H13N9), A/gull/ND/44036/1992 (H13N6), A/laughing gull/NJ/AI08-1460/2008 (H13N9), and A/gull/MD/704/1977 (H13N6). Working stocks for each strain were propagated and titrated in 9-to-11-day-old SPF embryonating chicken eggs (25). The viral inocula used in each trial were prepared by diluting stocks in sterile brain-heart-infusion (BHI) media to the desired titer.

Virus detection. All oropharyngeal and cloacal swabs collected during the trials were stored at −70 C until virus isolations (all trials), titrations (gulls), and/or quantitative real-time reverse transcription PCR (qRRT-PCR [chickens and turkeys]) testing were conducted. Virus isolations and titrations were performed in 9-to-11-day-old SPF embryonating chicken eggs (25). For viral titrations, infectious titers were calculated using the methodology described by Reed and Muench (18) and reported in median embryo infectious doses (EID_{50}) per milliliter.

For qRRT-PCR testing on samples collected during the turkey and chicken trials, RNA was extracted using a previously described combination of Trizol LS reagent (Invitrogen Inc., Carlsbad, CA) and the MagMax AI/ND RNA isolation kit (Ambion, Inc., Austin, TX) (4). The extracted chicken and turkey samples were tested with qRRT-PCR targeting influenza matrix gene (21) using the Applied Biosystems 7500 Fast Real Time PCR instrument (Foster City, CA), and the Applied Biosystems/Ambion AgPath-ID One-Step RT-PCR kit. Samples with a cycle threshold (Ct) less than 38 were considered positive.

Antibody detection. Serum samples were stored at −20 C until testing was performed. All samples collected during the trials were tested for antibodies against the influenza NP using a commercial blocking enzyme-linked immunosorbent assay (bELISA; FlockChek AI MultiS-Screen antibody test kit; IDEXX Laboratories, Westbrook, ME).

Histopathology and immunohistochemistry. Tissues collected during the ring-billed gull trial for histopathologic examination were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. Duplicate sections were cut and immunohistochemically stained using a commercial mouse monoclonal antibody to the NP of influenza A virus at a 1:1000 dilution (Biodesign International, Sako, ME). The methodology used to perform the immunohistochemical staining followed those previously described (5).

Experimental design. *Gulls.* A single viral strain was used in the gull challenge trial. Viral stock for A/laughing gull/NJ/AI08-1460/2008 (H13N9) was diluted in sterile BHI media to a titer of 10^4 EID_{50}/0.2 ml (single-bird inoculum). This lower viral dose (relative to most wild or domestic bird AI virus challenge studies) was chosen to more closely mimic natural levels of exposure.

Eight ring-billed gulls were inoculated with 0.2 ml of the H13N9 LPAI virus inoculum, split evenly between the intranasal and intratracheal routes. Two negative control ring-billed gulls were inoculated with 0.2 ml of sterile BHI media by the same combined routes. After inoculation, all gulls were monitored twice daily for any behavioral changes or overt signs of disease. Oropharyngeal and cloacal swabs were collected from all gulls on post-inoculation day (PID) 0 to 7, 10, 12, and 17, and placed in separate cryogenic vials (Corning Inc., Corning, NY) containing 2.0 ml of BHI media supplemented with antimicrobial drugs (250 μg/ml gentamicin, 500 μg/ml kanamycin, 1000 μg/ml streptomycin, 1000 U/ml penicillin G, and 25 μg/ml amphotericin B). All swab samples were tested with virus isolation and titration. Two LPAI virus–inoculated gulls were humanely euthanatized and necropsied on PID 1, 3, 7, and 17. The two negative control gulls were euthanatized and necropsied on PID 17. At necropsy, samples were collected from all sections of the respiratory and gastrointestinal tracts, liver, and kidneys, and placed in 10% neutral buffered formalin for routine histopathologic and immunohistochemical examination. A sample of blood was collected from the right jugular vein of each gull prior to inoculation and after they were euthanatized. Blood samples were placed into serum separator tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), centrifuged at 1509 × *g* for 15 min, and the serum was harvested.

Mallards. Three viral strains were used in the mallard challenge trials. Viral stocks for A/laughing gull/NJ/AI08-1460/2008 (H13N9), A/ring-billed gull/MN/AI10-1708/2010 (H13N6), and A/sand (envi-

Table 1. Summary of productive infection results obtained from inoculation of mallards, turkeys, and chickens with various H13 LPAI virus strains. No contact birds in any of the challenge groups listed below had detectable evidence of infection based on viral shedding or serologic testing.

Species	Virus	Viral shedding		Serology[A]
		PID 2	PID 4	PID 14
Mallard	A/laughing gull/NJ/AI08-1460/2008 (H13N9)	0/5	0/5	0/5
Mallard	A/ring-billed gull/MN/AI10-1708/2010 (H13N6)	3/5	0/5	3/5
Mallard	A/sand (environment)/NJ/1-24/2010 (H13N6)	0/5	0/5	0/5
Turkey	A/ laughing gull /NJ/AI08-1460/2008 (H13N9)	0/8	0/8	0/8
Turkey	A/ring-billed gull/MN/AI10-1708/2010 (H13N6)	0/8	0/8	0/8
Turkey	A/sand (environment)/NJ/1-24/2010 (H13N6)	0/8	0/8	0/8
Turkey	A/turkey/MN/1012/1991 (H13N2)	1/8	0/8	1/8
Turkey	A/ruddy turnstone/NJ/AI09-294/2009 (H13N6)	0/8	0/8	0/8
Turkey	A/laughing gull/NJ/AI08-1388/2008 (H13N9)	0/8	0/8	0/8
Turkey	A/gull/ND/44036/1992 (H13N6)	1/8	0/8	0/8
Turkey	A/gull/MD/704/1977 (H13N6)	0/8	0/8	0/8
Chicken	A/laughing gull/NJ/AI08-1460/2008 (H13N9)	0/8	0/8	0/8
Chicken	A/ring-billed gull/MN/AI10-1708/2010 (H13N6)	0/8	0/8	0/8
Chicken	A/sand (environment)/NJ/1-24/NJ/2010 (H13N6)	0/8	0/8	0/8
Chicken	A/turkey/MN/1012/1991 (H13N2)	0/8	0/8	0/8
Chicken	A/ruddy turnstone/NJ/AI09-294/2009 (H13N6)	0/8	0/8	0/8
Chicken	A/laughing gull/NJ/AI08-1388/2008 (H13N9)	0/8	0/8	0/8
Chicken	A/gull/ND/44036/1992 (H13N6)	0/8	0/8	0/8
Chicken	A/gull/MD/704/1977 (H13N6)	0/8	0/8	0/8

[A]Serologic testing was performed in all groups using a commercial bELISA.

ronment)/NJ/1-24/2010 (H13N6) were diluted in sterile BHI media to a titer of 10^6 EID_{50}/0.2 ml (single-bird inoculum). Existing field and experimental data suggest that mallards are relatively resistant to H13 LPAI viruses. Consequently, the higher dose used for the mallard trials was chosen to evaluate the potential for this species to become infected with H13 LPAI viruses, even after exposure to high infectious titers.

Twenty mallards were evenly divided into four groups (5 birds/group), and each group was challenged with one of the three LPAI viruses listed above or a sham inoculum. Individual ducks were inoculated with 0.2 ml of H13 LPAI virus or sterile BHI media split evenly between intranasal and intratracheal routes. At PID 1, two additional mallards were placed into each of the isolators containing LPAI virus–inoculated birds. All ducks were monitored twice daily for any behavioral changes or overt signs of disease. Oropharyngeal and cloacal swabs were collected from all ducks on PID 0, 2, and 4 (or post-contact day [PCD] 0, 2, and 4) and placed in separate cryogenic vials containing 2.0 ml of BHI media and antimicrobial drugs, as already described. Swab samples were tested by virus isolation in embryonating chicken eggs. Blood was collected from all ducks prior to inoculation and immediately after euthanasia for serologic testing using the bELISA. The trial was terminated and all ducks humanely euthanatized on PID 14 (PCD 13).

Chickens and turkeys. Eight viral strains were used in the chicken and turkey challenge trials. Viral stocks for A/laughing gull/NJ/AI08-1460/2008 (H13N9), A/ring-billed gull/MN/AI10-1708/2010 (H13N6), A/sand (environment)/NJ/1-24/2010 (H13N6), A/turkey/MN/1012/1991 (H13N2), A/ruddy turnstone/NJ/AI09-294/2009 (H13N6), A/laughing gull/NJ/AI08-1388/2008 (H13N9), A/gull/ND/44036/1992 (H13N6), and A/gull/MD/704/1977 (H13N6) were diluted in sterile BHI media to a titer of 10^7 EID_{50}/0.2 ml (single-bird inoculum). As with mallards, the higher dose used for these trials was selected to provide a sensitive measure of risk for H13 LPAI virus infection in these poultry species.

For both chickens and turkeys, 72 birds were separated into nine groups (8 birds/group), and each group was challenged with one of the viruses listed above or a sham-inoculum consisting of sterile BHI media. At PID 3, two naïve contact birds were placed into each isolator containing LPAI virus–inoculated birds. The methods for inoculation, monitoring, and swab sample collection used in the chicken and turkey trials were identical to those described for the mallard trials.

All swab samples collected during the chicken and turkey trials were initially screened with qRRT-PCR, and all positive samples (Ct value < 38) were further tested with virus isolation in embryonating chicken eggs. Blood was collected from all birds prior to inoculation and after euthanasia for serologic testing using a bELISA. The trial was terminated and all birds humanely euthanized on PID 14 (PCD 11).

Data analysis. In each of these trials, individual birds were considered infected with a H13 LPAI virus based on any one of the following criteria: 1) evidence of seroconversion (i.e. pre-inoculation serum samples tested negative and post-inoculation serum samples tested positive by bELISA); 2) detection of AI viral antigen in immunohistochemically-stained tissues (gull trial only); or 3) virus isolated from cloacal swabs collected on any day or from oropharyngeal swabs collected on or after PID 2. Virus isolated from an oropharyngeal swab at PID 1 was not included as a stand-alone criterion due to the potential for detecting residual inoculum. Samples that were positive by virus isolation but did not have a quantifiable titer were assigned a titer of 10^1 EID_{50}/ml.

RESULTS

No morbidity, mortality, or gross lesions were observed in any of the LPAI virus- or sham-inoculated birds during the four species trials. No virus was isolated from oropharyngeal or cloacal swabs collected from sham-inoculated gulls, mallards, chickens, and turkeys, and all pre- and post-inoculation serum samples from these birds were negative for antibodies to AI virus based on the bELISA.

Based on the infection criteria described above, the susceptibility to H13 LPAI viruses varied among the four species included in this study (Table 1). Ring-billed gulls were highly susceptible to infection, with seven of the eight inoculated birds fulfilling one or more infection criteria. The one uninfected gull was euthanatized on PID 3. This gull was virus isolation positive on the PID 1 oropharyngeal swab but was excreting low concentrations of virus (below the detectable limit of titration), was virus isolation negative on all other collected oropharyngeal and cloacal swabs, did not seroconvert, and had no detectable viral antigen in immunohistochemically stained tissues. Consequently, the PID 1 positive

Table 2. Summary of qRRT-PCR test results on oropharyngeal (OP) and cloacal (CL) swabs collected from turkeys and chickens inoculated with various H13 LPAI virus strains.

		Viral shedding			
		PID 2		PID 4	
Species	Virus	OP	CL	OP	CL
Turkey	A/ laughing gull /NJ/AI08-1460/2008 (H13N9)	1/8 (37.2)	1/8 (37.2)	0/8	0/8
Turkey	A/ring-billed gull/MN/AI10-1708/2010 (H13N6)	0/8	0/8	0/8	0/8
Turkey	A/sand (environment)/NJ/1-24/2010 (H13N6)	0/8	0/8	0/8	0/8
Turkey	A/turkey/MN/1012/1991 (H13N2)	3/8 (33.4–37.7)[A]	1/8 (37.0)	1/8 (26.3)	0/8
Turkey	A/ruddy turnstone/NJ/AI09-294/2009 (H13N6)	0/8	2/8 (37.8–37.9)	0/8	0/8
Turkey	A/laughing gull/NJ/AI08-1388/2008 (H13N9)	2/8 (37.4–37.1)	0/8	0/8	0/8
Turkey	A/gull/ND/44036/1992 (H13N6)	0/8	2/8 (36.5–37.2)[A]	0/8	0/8
Turkey	A/gull/MD/704/1977 (H13N6)	2.8 (35.0–37.3)	0/8	0/8	0/8
Chicken	A/laughing gull/NJ/AI08-1460/2008 (H13N9)	0/8	0/8	0/8	0/8
Chicken	A/ring-billed gull/MN/AI10-1708/2010 (H13N6)	0/8	0/8	0/8	0/8
Chicken	A/sand (environment)/NJ/1-24/NJ/2010 (H13N6)	1/8 (35.9)	0/8	0/8	0/8
Chicken	A/turkey/MN/1012/1991 (H13N2)	2/8 (32.5–35.5)	0/8	0/8	0/8
Chicken	A/ruddy turnstone/NJ/AI09-294/2009 (H13N6)	0/8	0/8	0/8	0/8
Chicken	A/laughing gull/NJ/AI08-1388/2008 (H13N9)	1/8 (37.9)	0/8	0/8	0/8
Chicken	A/gull/ND/44036/1992 (H13N6)	0/8	0/8	0/8	0/8
Chicken	A/gull/MD/704/1977 (H13N6)	0/8	0/8	0/8	0/8

[A]Virus isolated from the qRRT-PCR positive sample.

oropharyngeal swab sample presumably reflects the detection of residual inoculum.

Turkeys and mallards were resistant to infection with most H13 LPAI viral strains, but low numbers of inoculated birds were infected after challenge with specific strains (Table 1). One of eight turkeys inoculated with A/turkey/MN/1012/1991 (H13N2) seroconverted and was virus isolation positive on the PID 2 oropharyngeal swab, and one of the eight turkeys inoculated with A/gull/ND/44036/1992 (H13N6) was virus isolation positive on the PID 2 cloacal swab; post-inoculation antibodies were not detected in the latter turkey. No virus was isolated or antibodies detected in the remaining inoculated or contact turkeys in any group. Three mallards inoculated with A/ring-billed gull/MN/AI10-1708/2010 (H13N6) were virus isolation positive on PID 2 swabs and were also seropositive on post-inoculation serum samples. Two of these mallards were virus isolation positive on both the PID 2 oropharyngeal and cloacal swabs, while the third was positive only on the oropharyngeal swab. All PID 4 swab samples from these three mallards were virus isolation negative. No infections were detected in any of the remaining inoculated or contact mallards in any of the challenge groups. No virus was isolated from PID 2 or 4 swab samples and antibodies were not detected in post-inoculation serum samples collected from any of the inoculated or contact chickens exposed to the eight H13 LPAI viruses (Table 1). Although only two turkeys swab samples were positive by virus isolation, an additional 17 oropharyngeal or cloacal swab samples from chickens and turkeys inoculated with virus tested positive with qRRT-PCR but were negative on virus isolation (Table 2). The following six contact-exposed chicken and turkey samples were qRRT-PCR positive/virus isolation negative on PCD 2: two turkeys (oropharyngeal swabs) in the A/laughing gull/NJ/AI08-1460/2008 (H13N9) group; one turkey (cloacal swab) in the A/laughing gull/NJ/AI08-1388/2008 (H13N9) group; one turkey (oropharyngeal swab) in the A/turkey/MN/1012/1991 (H13N2) group; and two chickens (oropharyngeal) in the A/turkey/MN/1012/1991 (H13N2) group.

Virus was isolated and titrated from all seven ring-billed gulls infected with A/laughing gull/NJ/AI08-1460/2008 (H13N9). In all gulls, viral shedding started between PID 0 and 2 and continued, in surviving birds, up to PID 10, at which time low levels of virus were detected at sporadic time points until the end of the trial at PID 17. All seven infected gulls excreted the H13N9 LPAI virus via both the oropharynx and cloaca. Viral shedding was similar between the two routes, but the concentration and duration were slightly greater via the oropharynx (Fig. 1). Peak titers in oropharyngeal swabs occurred between PID 1 to 3 and ranged from 2.1 to 5.4 $\log_{10}$ EID_{50}/ml. Peak titers in cloacal swabs occurred later, from PID 3 to 6, and ranged from below a quantifiable titer to 4.7 $\log_{10}$ EID_{50}/ml. The two gulls euthanatized on PID 1 had mild tracheal lesions, consisting of patchy deciliation ($n = 2$) and mild lymphocytic tracheitis ($n = 1$). The gull with mild tracheitis had nuclear staining for AI viral antigen in rare tracheal epithelial cells (Fig. 2). No viral antigen or microscopic lesions associated with LPAI viral infection were detected in any of the remaining gull tissues.

DISCUSSION

The experimental results presented herein are consistent with existing field and experimental data and indicate H13 LPAI viruses are strongly adapted to gulls, but rare spill-over into aberrant hosts is possible. The strong adaptation to gulls was evidenced by a high proportion (7/8; 87.5%) of infection in gulls challenged with the reduced titer inoculum (10^4 EID_{50}), and extensive viral shedding via the oropharynx and cloaca without overt clinical signs of disease. This is in contrast to the poor adaptation of all H13 LPAI virus strains used in this study to the remaining three species, which were either refractory or experienced rare infections after challenge with high titer inoculums (10^6–10^7 EID_{50}), had very transient viral shedding, and no intraspecies transmission to contact birds. The mechanisms driving the adaptation of AI viruses to different host species are not completely understood but likely involve multiple interactions associated with viral infection and replication in host tissues, including HA binding to sialic acid (SA) receptors on the host cell, the ability to express viral polymerase genes and utilize the host cell machinery for viral replication, and efficient NA-dependent release of progeny virus from the host cell membrane (15).

In regards to the HA binding, the expression of different avian-type SA receptors in the tissues of laughing gulls, ring-billed gulls,

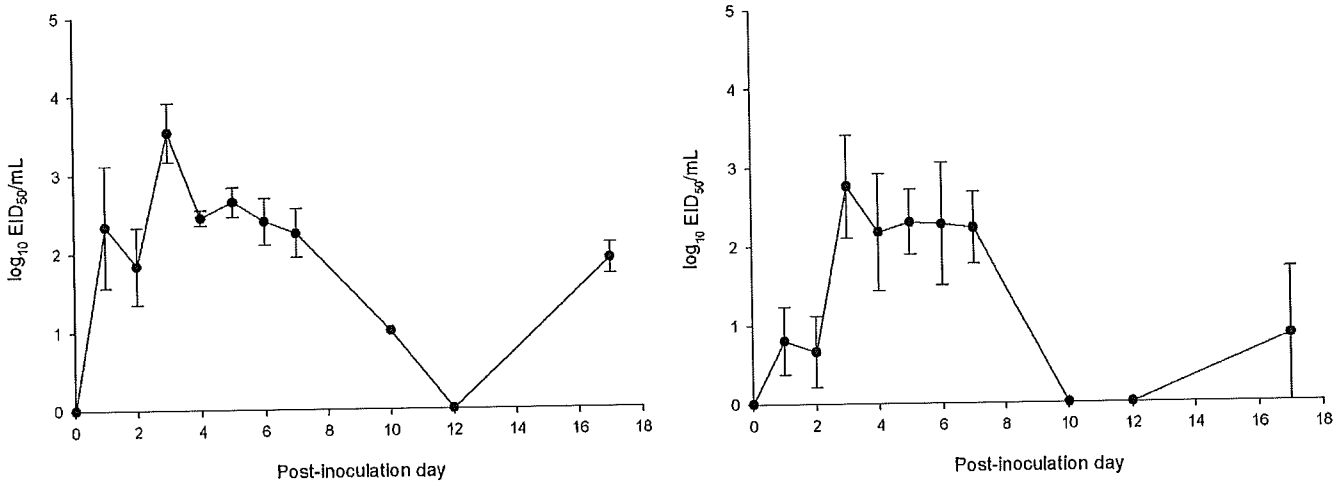

Fig. 1. Mean (± standard error) oropharyngeal and cloacal infectious viral titers excreted by ring-billed gulls (n = 8) experimentally inoculated with A/laughing gull/NJ/AI08-1460/2008 (H13N9). Two gulls were euthanatized and necropsied on PID 1, 3, 7, and 17, resulting in a decreasing sample size over the course of the study: PID 0–1 (n = 8); PID 2–3 (n = 6); PID 4–7 (n = 4); and PID 8–17 (n = 2). Viral titers are reported in $\log_{10}$ EID_{50}/ml.

and mallards generally corresponds with the observed host ranges of wild bird-origin LPAI viruses in these species (7). Fine receptor specificity of HA for SA receptors on the host cell is dependent on the linkage between the SA 2,3Gal-disaccharide and the penultimate N-acetylhexosamine residue of the carbohydrate chain of SA. Based on *in vitro* trials, duck-origin LPAI viruses preferentially bind to SAα2,3Galβ1,3GalNacβ1 (β1,3Gal), whereas gull-origin LPAI viruses prefer SAα2,3Galβ1,4GlcNacβ1 (β1,4Glc) (8). Using lectin histochemistry, Franca *et al.* (7) recently reported laughing gulls and ring-billed gulls strongly express both avian-type SA receptors in the respiratory and intestinal tracts, and consequently, should be susceptible to both duck- and gull-origin AI viruses. In contrast, mallards predominately expressed β1,3Gal in the respiratory and intestinal tracts, suggesting they would be more susceptible to duck-origin viruses. The results of these receptor studies are consistent with field and experimental data on these three species. Although multiple HA subtypes (H1–12) are routinely isolated from mallards, viruses of the H13 and H16 subtypes, to our knowledge, have not. Experimentally, mallards are highly susceptible to duck-origin LPAI viruses, but are comparably much more resistant to gull-adapted strains of the H13 subtype (3,12,26). In contrast, natural infection with numerous HA subtypes have been reported from laughing and ring-billed gulls (9,14,19), including viruses of the H13 and H16 subtypes, and experimentally, gulls are permissive hosts for both duck- and gull-origin LPAI viruses (3).

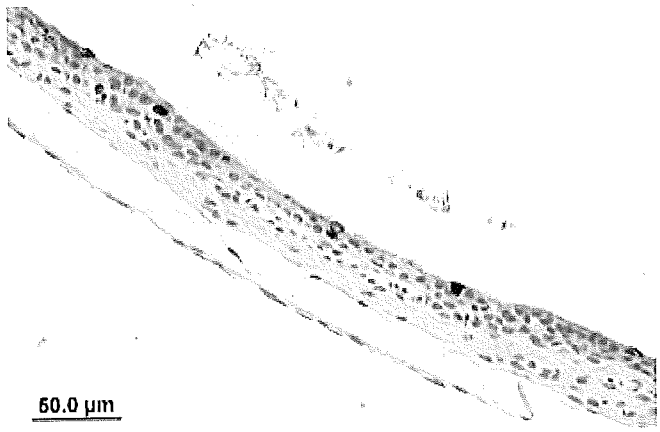

Fig. 2. Trachea from a ring-billed gull infected with A/laughing gull/NJ/AI08-1460/2008 (H13N9) at PID 1. Rare tracheal epithelial cells contain nuclear staining for AI virus NP antigen (brown staining). Immunoperoxidase labeling, hematoxylin counterstain (bar = 50 µm).

Based on virus isolation and serologic testing, none of the chickens became infected after challenge with any of the eight strains of H13 LPAI virus. Two inoculated turkeys had evidence of infection based on virus isolation, and one of these birds seroconverted (Table 1). None of the contact chickens or turkeys in any challenge group had serologic or virologic (isolation) evidence of infection. Twenty-three oropharyngeal or cloacal swab samples collected from inoculated (n = 17) or contact-exposed (n = 6) chickens or turkeys were positive on qRRT-PCR, but attempts to isolate virus from these samples were unsuccessful (Table 2). The majority of these qRRT-PCR positive/virus isolation negative turkey and chicken samples were oropharyngeal swabs (74%; 17/23), samples collected on PID 2 (96%; 22/23), and had a relatively high Ct value of >35 (78%; 18/23). Potential explanations for the discordant test results on these samples include detection of residual inoculum, low levels of replication below the detection threshold of isolation, and/or freeze-thaw artifact. These explanations are not mutually exclusive and all may have contributed to these results. Regardless of the mechanism involved, collectively our data suggest these discordant results are biologically insignificant.

One of the two H13 LPAI virus strains that infected turkeys in this study was originally isolated from a turkey (A/turkey/MN/1012/1991 [H13N2]) (20). Our experimental results with this strain are consistent with a previous study by Laudert *et al.* (16), in which A/turkey/MN/1012/1991 (H13N2) produced infections in turkeys after oculonasal inoculation, but not in chickens. The biologic properties of this H13N2 LPAI virus (i.e., infectivity for turkeys) may reflect some degree of viral adaptation to this species; however, our experimental results suggest this host-range is quite unique because turkeys are generally resistant to infection with most strains of H13 LPAI virus.

Ring-billed gulls excreted the H13N9 LPAI virus via the oropharynx and cloaca for several days; however, the duration and concentration of shedding were slightly greater via the oropharynx. Both gulls at PID 1 had mild microscopic changes in the trachea and

minimal viral antigen was detected in one of these birds. Although the microscopic changes in the trachea may have been associated with viral replication, it is also possible that they were a result of trauma from intratracheal inoculation. The lack of viral antigen detection in the remaining immunohistochemically stained tissues of the infected gulls likely reflects the low level of viral replication and shedding at the time of examination, which ranged from below the detectable limit of titration to 3.1 $\log_{10}$ EID_{50}/ml in oropharyngeal swabs and 0 to 2.9 $\log_{10}$ EID_{50}/ml in cloacal swabs. This is supported by the one gull with positive staining for viral antigen excreting a higher viral titer in the PID 1 oropharyngeal swab (5.4 $\log_{10}$ EID_{50}/ml).

The excretion of virus via the oropharynx is consistent with the results reported for juvenile laughing gulls infected with multiple duck-origin LPAI viruses (3) and is in contrast to LPAI virus infection in mallards, in which fecal shedding is dominant (3,26). Such differences in viral shedding between gulls and mallards may have impacts on routes of transmission within these distinct reservoir groups, as well as the potential risk for virus spillover to aberrant hosts. Within duck populations, LPAI viruses are thought to be transmitted by the fecal-oral route through contaminated aquatic habitats (11). This is an extremely efficient transmission mechanism in ducks due to the high concentrations of virus excreted in the feces (2), the tenacity of LPAI viruses in water (22), and the high concentrations of susceptible ducks that can congregate on aquatic habitats. This is also an efficient means for LPAI virus spillover to domestic animals because both the infected feces and contaminated water can serve as a potential source of virus (10). The routes of LPAI virus transmission in gulls are currently not known; however, considering the extent of viral shedding via the respiratory and intestinal tracts, there may be multiple potential routes, particularly on breeding colonies where the density of naïve chicks is high. The pattern of LPAI virus shedding in gulls also has important implications for sampling these birds. Surveillance efforts focusing on understanding the natural history of AI (i.e., LPAI viruses) generally sample wild bird species by collecting cloacal swabs and/or feces, which is based largely on LPAI virus infection in ducks. However, existing experimental data on gulls suggests that oropharyngeal swabs or pooled oropharyngeal and cloacal swabs may be more efficient samples to collect than cloacal swabs or feces alone.

Collectively, these experimental trials provide valuable data that improve our understanding on the unique biology of H13 LPAI viruses and support ongoing field studies, both for interpreting surveillance results and improving future sampling efforts in gulls. Although not examined in this current study, terns are taxonomically related to gulls and are also frequently cited as reservoirs for AI virus; however, surveillance to date has been limited in this group of birds, relative to gulls. Consequently, although AI viruses have been reported from multiple tern species, there currently is not enough field or experimental data on influenza in terns to understand their role in the ecology of AI virus. Many unanswered questions regarding the biology of H13 LPAI viruses remain and further research is needed to identify mechanisms underlying the unique host-range of these viruses; particularly as such information may have value in understanding the inherent resistance of some poultry species to AI (i.e., chickens).

REFERENCES

1. Becker, W. B. The isolation and classification of tern virus A/Tern/South Africa/61. J. Hyg. 64:309–320. 1966.

2. Brown, J. D., R. D. Berghaus, T. P. Costa, R. Poulson, D. L. Carter, C. Lebarbenchon, and D. E. Stallknecht. Intestinal excretion of a wild bird-origin H3N8 low pathogenic avian influenza virus in mallards (*Anas platyrhynchos*). J. Wildl. Dis. in review.

3. Costa, T. P., J. D. Brown, E. W. Howerth, and D. E. Stallknecht. Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. Avian Pathol. 40:119–124. 2011.

4. Das, A., E. Spackman, M. J. Pantin-Jackwood, and D. L. Suarez. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of avian influenza virus by RT-PCR. J. Vet. Diagn. Invest. 21:771–778. 2009.

5. Driskell, E. A., C. A. Jones, D. E. Stallknecht, E. W. Howerth, and S. M. Tompkins. Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology 399:280–289. 2010.

6. Fouchier, R. A. M., V. J. Munster, J. Keawcharoen, A. D. M. E Osterhaus, and T. Kuiken. Virology of avian influenza in relation to wild birds. J. Wildl. Dis. 43(Suppl.):S7–S14. 2007.

7. Franca, M., D. E. Stallknecht, R. Poulson, J. Brown, and E. W. Howerth. The pathogenesis of low pathogenic avian influenza in mallards. Avian Dis. 56(suppl. 1):676–980. 2012.

8. Gambaryan, A., S. Yamnikova, D. Lvov, A. Tuzikov, A. Chinarev, G. Pazynina, R. Webster, M. Matrosovich, and N. Bovin. Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology 334:276–283. 2005.

9. Graves, I. L. Influenza virnses in birds of the Atlantic flyway. Avian Dis. 36:1–10. 1992.

10. Halvorson, D. A., C. J. Kelleher, and D. A. Senne. Epizootiology of avian influenza: effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota. Appl. Environ. Microbiol. 49:914–919. 1985.

11. Hinshaw, V. S., R. G. Webster, and B. Turner. Water-borne transmission of influenza A viruses? Intervirology 11:66–68. 1979.

12. Hinshaw, V. S., G. M. Air, A. J. Gibbs, L. Graves, B. Prescott, and D. Karunakaran. Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J. Virol. 42:865–872. 1982.

13. Hinshaw, V. S., W. J. Bean, J. Geraci, P. Fiorelli, G. Early, and R. G. Webster. 1986. Characterization of two influenza A viruses from a pilot whale. J. Virol. 58:655–656. 1986.

14. Kawaoka, Y., T. M. Chambers, W. L. Sladen, and R. G. Webster. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163:247–250. 1988.

15. Kuiken, T., E. C. Holmes, J. McCauley, G. F. Rimmelzwaan, C. S. Williams, and B. T. Grenfell. Host species barriers to influenza virus infections. Science 312:394–397. 2006.

16. Laudert, E., V. Sivanandan, D. Halvorson, D. Shaw, and R. G. Webster. Biological and molecular characterization of H13N2 influenza type A viruses isolated from turkeys and surface water. Avian Dis. 37:793–799. 1993.

17. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier. Global patterns of influenza A virus in wild birds. Science 312:384–388. 2006.

18. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497. 1938.

19. Sinnecker, R., H. Sinnecker, E. Zilske, and D. Kohler. Surveillance of pelagic birds for influenza A viruses. Acta Virol. 27:75–79. 1983.

20. Sivanandan, V., D. A. Halvorson, E. Laudert, D. A. Senne, and M. C. Kumar. Isolation of H13N2 influenza A virus from turkeys and surface water. Avian Dis. 35:974–977. 1991.

21. Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256–3260. 2002.

22. Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. Persistence of avian influenza virus in water. Avian Dis. 34:406–411. 1990.

23. Stallknecht, D. E., and J. D. Brown. Ecology of avian influenza in wild birds. In: Avian influenza, 1st ed. Blackwell Publishing, Ames, IA. pp. 43–58. 2008.

24. Stallknecht, D. E., M. P. Luttrell, R. Poulson, V. Goekjian, L. Niles, A. Dey, S. Krauss, and R. G. Webster. Surveillance and testing approaches to detect and recover avian influenza viruses from shorebird populations. J. Wildl. Dis 48:382–393. 2012.

25. Swayne, D. E., D. A. Senne, and D. L. Suarez. Influenza. In: A laboratory manual for the isolation and identification of avian pathogens, 5th ed. American Association of Avian Pathologists, Kennett Square, PA. pp. 128–134. 2008.

26. Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84:268–278. 1978.

ACKNOWLEDGMENT

We thank Whitney Kistler, Shamus Keeler, Lee Harper, and the personnel of the New York Department of Environmental Conservation for assistance in acquiring the ring-billed gulls used in this study. Additionally, we thank the faculty and staff of the Southeastern Cooperative Wildlife Disease Study (UGA), Poultry Diagnostic and Research Center (UGA), and SEPRL for support with animal husbandry and laboratory testing. We also acknowledge the histotechnicians in the Department of Pathology at the UGA, particularly Abbie Butler and Patricia Rowe for performing the hematoxylin and eosin and immunohistochemical staining. Funding for this work was provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health and Human Services, under Contract No. HHSN266200700007C and the National Science Foundation under grant No. DEB-0917853. The opinions expressed herein are those of the authors and do not necessarily reflect the views of any of the funding agencies.

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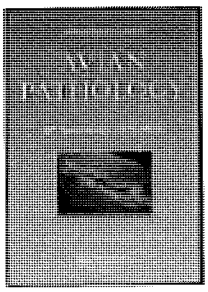

Avian Pathology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cavp20

Viral diagnostics: will new technology save the day?

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Accepted author version posted online: 21 Mar 2012. Version of record first published: 18 Jun 2012

To cite this article: Erica Spackman (2012): Viral diagnostics: will new technology save the day?, Avian Pathology, 41:3, 251-258

To link to this article: http://dx.doi.org/10.1080/03079457.2012.675051

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Avian Pathology (June 2012) **41(3)**, 251–258

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REVIEW

Viral diagnostics: will new technology save the day?

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Technology for infectious agent detection continues to evolve, particularly molecular methods that first emerged in the mid-1970s. The goals of new technology in diagnostics, whether in humans or in animals, including poultry, are to achieve the highest sensitivity and specificity possible to accurately identify the infection status of an individual or flock in the shortest time possible. Ease of use, low cost and increased information from a single test (e.g. multiplexing) are also critical areas frequently targeted for improvement. New tests and modifications of current tests are reported often, and diagnostic tests are now commonly developed by commercial companies. As one would expect, most advances in diagnostic technology are applied first to human health, and then may be adapted to animal health if practical. In the present review the trends and novel innovative technologies in primarily viral diagnostics are reviewed and the practicality of these methods and application for poultry health are discussed briefly. Also, influenza will seem to be over-represented in viral diagnostics since it is frequently used as a proof-of-concept target for novel technology due to its importance for animal and public health. Finally, the review is intended to be a brief survey of some of the innovative diagnostic technologies reported in recent years. It is not entirely comprehensive of all technology and the author makes no claims or endorsements of any of the technology or products mentioned.

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A Brief History of Diagnostics

The classical age. It is noteworthy how rapidly technology for viral diagnostics has evolved over the past century. However, some of the oldest classical or molecular methods are still in use, although with some modifications.

Viruses were first identified as filterable agents in 1898, when foot and mouth disease was discovered by Loeffler & Frosch (1898). The ability to propagate viruses outside an animal host was an enormous advance for virology and was first reported in embryonated chicken eggs with Rous sarcoma virus in 1911 (Rous & Murphy, 1911); however, it was not until the 1930s, after it was reported that fowlpoxvirus could be propagated in embryonated chicken eggs (Woodruff & Goodpasture, 1931), that the method began to be used more widely. By the mid-1930s methods to propagate Variola virus, mumps and herpesviruses were reported (Goodpasture *et al.*, 1931; Lazarus *et al.*, 1937; Nelson, 1939). It was not until 1948 that cell culture was first reported when Weller and Enders developed cell culture for mumps and influenza (Weller & Enders, 1948).

Antibody-based assays constituted the first era in viral diagnostics. Complement fixation was described in 1929 for several viruses (Bedson & Bland, 1929) and a fluorescent antibody test for influenza was reported in 1956 (Liu, 1956). Electron microscopy using negative staining was first described for virus detection in 1959 (Brenner & Horne, 1959), and then a vast improvement in electron microscopy came about in 1967 when Best *et al.* described the use of specific antibody, in what is now known as immune electron microscopy, for rubella virus detection (Best *et al.*, 1967). Monoclonal antibodies, first reported in 1975 (Kohler & Milstein, 1975), were the next great technological leap and led the way for improved antibody-based tests for virus detection.

Protein separation by electrophoresis was developed in the mid-1960s (Davis, 1964; Ornstein, 1964) and allowed for virus proteins to be identified by weight and, more specifically, by antibody once the western blot was established (Towbin *et al.*, 1979). The original protein assays were primarily used in research settings, but western blotting has been used to diagnose virus infection by antibody detection for viruses that establish chronic infections such as human immunodeficiency virus (Burke *et al.*, 1987) and even feline immunodeficiency virus (Egberink *et al.*, 1991). Western blot and enzyme-linked immunosorbent assay technology evolved into lateral-flow antigen-detection immunoassays in the mid-1980s by utilizing the antibody in the solid phase and the antigen in the liquid phase (Gary *et al.*, 1985; Rivera *et al.*, 1986). Antigen-detection immunoassays were soon optimized to be substantially cheaper and easier to run. Faster, point-of-care commercial kits were marketed and validation studies began to be reported (Dascal *et al.*, 1989) and are now among the most common commercial diagnostic test formats in human and veterinary medicine. For example, in North America at least five companies manufacture kits for seasonal influenza and six companies manufacture kits for respiratory syncytial virus.

This review was presented as the Houghton Lecture at the 17th Congress of the World Veterinary Poultry Association in Cancun, Mexico, 2011
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Received 14 November 2011
ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/12/030251-08 © 2012 Houghton Trust Ltd
http://dx.doi.org/10.1080/03079457.2012.675051

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The molecular era. The development of molecular biological methods represented a substantial change in microbiological research and diagnostics by making the genetic material of an organism accessible and identifiable. The initial advantage of molecular diagnostic tests was that they could achieve a level of specificity well beyond that of classical methods. As the technology has been refined, molecular tests are now able to exceed classical tests in sensitivity and most can provide additional specific relevant information about the target, such as type, subtype, lineage, drug resistance, serotype, and so forth.

During the mid-1970s, molecular methods began to emerge when Southern blotting was described. Material could now be probed with radio-labelled nucleic acids for specific DNA sequences (Southern, 1975). Related methods such as northern blotting (Alwine *et al.*, 1977), restriction fragment-length polymorphism analysis (Mears *et al.*, 1978) and refinements of the original methods soon followed. Although there were some reports of the application of this technology for the detection of infectious agents, Southern and northern blotting were never widely adopted by clinical diagnostic laboratories because they are slow, expensive and at that time were further complicated by necessity of using radionuclides to visualize the results. The potential advantages of molecular methods over classical methods thus did not offset the disadvantages.

The method of polymerase chain reaction (PCR) was first reported in 1985 (Saiki *et al.*, 1985) for primer-mediated enzymatic amplification of specific target sequences in genomic DNA. The technique was revolutionary in that it enabled the rapid and accurate detection of specific DNA sequences in pathogenic organisms. At that time, PCR reactions had to be carried out by hand; tubes were manually moved between water baths at different temperatures to complete each cycle and fresh polymerase enzyme needed to be added after each cycle because thermostable polymerases had not yet been utilized. The method was greatly enhanced when thermostable polymerases were introduced in 1988 (Saiki *et al.*, 1988), which also improved the specificity of PCR because higher cycling temperatures were possible. Another problem was resolved when reverse transcription (RT)-PCR was developed in 1988, making it possible to produce DNA copies of target RNA viruses (Rappolee *et al.*, 1988). Soon after its development, PCR was applied to the detection of human papillomavirus (Shibata *et al.*, 1988) and RT-PCR was applied to human immunodeficiency virus type 1 detection (Hart *et al.*, 1988). A multiplex test for influenza A and influenza B followed a few years later (Fan *et al.*, 1998). Real-time PCR, which visualizes the results as the reaction is running, was introduced in 1996 (Heid *et al.*, 1996) and was first applied to viral diagnostics in 1999 when tests for SV40 and hepatitis B were first reported (Abe *et al.*, 1999; Shi *et al.*, 1999). However, as with earlier molecular tests, PCR-based methods were not picked up quickly by clinical diagnostic laboratories.

Not surprisingly, as molecular technologies have expanded, commercial companies producing tests have multiplied; in 2011 there were approximately 350 companies that manufacture *in vitro* molecular medical tests (Carlson, 2011). However, commercial molecular tests are primarily found only in the developed world, with 79% of the market being in the US and Europe (Carlson, 2011).

Molecular diagnostics for poultry diseases. The early 1990s was also the time when PCR methods were first introduced for the detection of poultry viruses with tests for chicken infectious anaemia virus, infectious bronchitis virus, infectious bursal disease virus, Marek's disease virus, infectious laryngotracheitis virus and avian leucosis viruses (Tham & Stanislawek, 1992; Todd *et al.*, 1992; van Woensel *et al.*, 1992; Williams *et al.*, 1992; Kwon *et al.*, 1993; Stauber *et al.*, 1995; Jackwood *et al.*, 1996). The first RT-PCR for avian influenza virus (AIV) was reported in 1995 (Horimoto & Kawaoka, 1995), but as a method to amplify the haemagglutinin for sequencing to evaluate pathotype rather than for initial virus detection. In addition to identifying infected flocks, some of the earlier applications of PCR methods in poultry health were aimed at detecting vaccine contaminants (Bruckner *et al.*, 1996; Reimann & Werner, 1996). As with human medicine, research laboratories adopted the technology much earlier than diagnostic laboratories.

Current Capabilities

Current achievable detection limits are a single molecule: virion, protein, RNA or DNA. However, few tests with that level of sensitivity and specificity are available clinically. In general the adoption of new technology lags behind its development, so the state of the art does not necessarily correlate with routine practice. One reason is that there are numerous barriers to adoption that must be overcome for any new technology to be adopted by diagnosticians. These include: cost of new equipment; fit into workflow; new skills/training needed for personnel; and validation and regulatory approval.

Finally, because of wide variation in resources and needs among veterinary (and public health) laboratories worldwide, it is difficult to identify a "standard" method or technology. However, it is apparent that in both veterinary medicine and human health two of the most widely used methods for virus detection are PCR-based methods and virus culture, depending on the target.

It is also interesting to point out a difference between veterinary and human health laboratories, where the veterinary laboratories have the advantage in adopting new technology. Although veterinary diagnostic programmes, particularly for food animals, generally do not have the same resources as public health, less strict regulations can promote more rapid adoption of new technologies (and frequently there are grey areas as regulations often lag behind the emergent technology). In public health, *in vitro* diagnostic tests are typically commercial kits, which frequently require some form of licensing (e.g. approval by the Food and Drug Administration in the US and the CE-IVD mark in Europe). In veterinary medicine, in-house tests are used much more commonly as there are fewer commercial kits available, due to the smaller market (but note that commercial veterinary kits often do require licensing as well). However, this extra freedom has also meant that harmonization has been difficult to achieve. Some harmonization has been established since the World Organisation for Animal Health (OIE) has been able to recommend specific methods for agents that are listed diseases (OIE, 2011). An additional difference between human and animal diagnostic tests is that tests for animal diseases can be tested and validated using

experimentally-generated specimens from the target host. This allows for better information on test performance, an easier paradigm for refining tests and better validation data.

In poultry health laboratories, in-house tests (i.e. any test that is not available as a commercial kit, including peer-reviewed published assays and unpublished assays) are not uncommon. Commercially produced test kits have become increasingly available for important animal diseases, including tests for poultry (Table 1). By far the most targeted agent by commercial kits is AIV due to its zoonotic potential and because it is an OIE-listed disease. In some cases the same kit for AIV (type A influenza) marketed for poultry has been validated and is available for humans, swine, horses, and so forth, and *vice versa*. An interesting illustration of this is shown in Figure 1, which compares graphically the number of publications in the PubMed database for AIV PCR tests, Newcastle disease virus (NDV) PCR tests and Marek's disease virus PCR tests by year. Although not all papers are necessarily for diagnostic tests, this demonstrates the focus on AIV versus NDV and Marek's disease virus, both of which are historically more important for poultry production but are not zoonotic concerns; market potential has thus driven discovery to some degree. Commercialization of new kits for poultry diseases will probably be slow due to limited markets compared with public health. However, bacterial agents that are important for food safety (e.g. *Salmonella enteriditis, Campylobacter jejuni*) have also been targeted widely for commercial kits.

Among the in-house tests, real-time PCR-based methods are probably the assay of choice. In veterinary diagnostics, real-time PCR is the preferred test for numerous OIE-listed diseases (Hoffmann *et al.*, 2009). Currently real-time RT-PCR is probably the most common test for AIV and NDV, where it is used as a screening test with virus isolation in embryonated chicken eggs being used as a confirmatory test. PCR-based tests (real-time or conventional) have been reported for detecting almost all poultry viral and bacterial diseases of poultry, and a few have been developed to further characterize isolates such as (not a comprehensive list): tests that differentiate NDV lineages (Wise *et al.*, 2004; Farkas *et al.*, 2009), AIV subtypes (Spackman *et al.*, 2002; Das & Suarez, 2007) and infectious bursal disease virus pathotypes (Ghorashi *et al.*, 2011; Hernandez *et al.*, 2011) and that identify infectious bronchitis virus serotypes/genotypes (Jackwood *et al.*, 1997; Keeler *et al.*, 1998; Moscoso *et al.*, 2005; Wang & Khan, 1999; Jones *et al.*, 2011). Although PCR-based methods, including conventional and real time, are used widely, kits for few poultry agents have been commercialized.

Novel Technologies

In diagnostics there are limited numbers of targets to choose for agent detection: proteins from the target agent, the nucleic acid of the target agent, or the host's specific immune response to the target agent. Detection assays can either amplify the target or the signal to improve sensitivity, and refinements to these can substantially improve sensitivity and specificity of the established tests. We now have the technology to detect a single molecule of RNA, DNA or protein, including a single virion. Thus we have hit what is probably the limit for sensitivity and specificity, and now the trend in technology is to improve convenience (time, cost, ease of use, multiplexing, reusable or disposable), or to specialize for a specific situation ("personalized" medicine) in order to provide in-depth information.

PCR-based assays are the most common tests that target agent nucleic acids, and most protein detection assays utilize antibody for specific target detection. Of course improvements and modifications continue to be developed for each target type, which increase sensitivity, specificity, and information, and which can reduce cost and time to result. Overall trends in diagnostic assay

Table 1. *Commercial test kits available for poultry diagnostics (availability varies regionally).*

Format	Target	Agents	
Enzyme-linked immunosorbent assay	Antibody	Avian encephalomyelitis virus	*Bordetella avium*
		Avian influenza virus	*Mycoplasma gallisepticum*
		Avian leucosis virus	*Mycoplasma synoviae*
		Avian pneumovirus	*Mycoplasma meleagridis*
		Avian reovirus	*Ornithobacterium rhinotracheale*
		Chicken anaemia virus	*Pasteurella multocida*
		Infectious bronchitis virus	*Salmonella enteriditis*
		Infectious bursal disease	
		Infectious laryngotracheitis virus	
		Haemorrhagic enteritis virus	
		Newcastle disease virus	
		Reticuloendotheliosis virus	
Serum plate agglutination	Antibody	*Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasma meleagridis, Salmonella pullorum*	
Agar gel immunodiffusion assay	Antibody or antigen	Avian influenza virus	
Lateral flow immunoassay	Antigen	Avian influenza virus	
PCR (including real-time PCR)	Nucleic acid	Avian influenza virus, Newcastle disease virus, *Salmonella* spp.	
Nucleic acid sequence-based amplification	Nucleic acid	Avian influenza virus, Newcastle disease virus	

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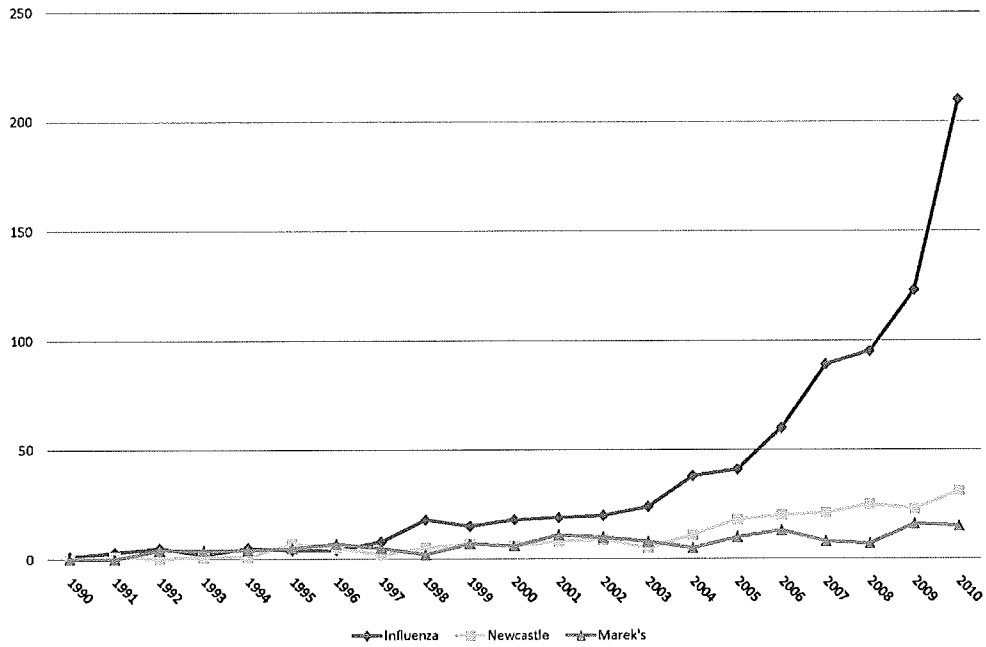

Figure 1. *Number of publications in the PubMed database by year with "PCR" and "avian influenza", "Newcastle disease" or "Marek's disease".*

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improvements are presented in Table 2. To be viable, and also successful, technologies must involve improvements in numerous areas compared with any current tests. Instrumentation is a major area of development, and new assays frequently rely on test-kit-specific instruments (which is also due to commercialization).

Nucleic acid detection. There are numerous nucleic acid detection technologies available: PCR based, isothermic amplification, and microarray—but PCR-based assays are probably the most widely used. Assays to further characterize nucleic acids include sequencing, microarray and restriction fragment-length polymorphism, and are usually only utilized by reference laboratories. A full examination of nucleic acid detection technology is beyond the scope of this review, so the focus is on some of those that have had the most impact.

Isothermic amplification methods such as loop-mediated isothermal amplification and nucleic acid sequence-based amplification have been used for years, and have the advantage that thermo-cyclers are not necessary and the reactions can be incubated in a simple water bath. They have similar sensitivity and specificity to PCR, but can be more expensive to run due to the cost of the reagents.

Microarrays have been proposed as a diagnostic tool that can provide a high level of information, so not only can one identify the target agent but also crucial information such as strain, lineage, pathotype, and so forth, can be gained. Microarrays are similar to Southern blots as they both rely on DNA hybridization, but microarrays use a glass slide that has hundreds of DNA probes attached to it instead of a single probe in the liquid phase. The microarray slide is incubated with nucleic acid from a specimen, and if the nucleic acid in the sample matches (a complementary match) a probe sequence on the microarray slide it will bind, which produces a fluorescent signal. The signal can be detected and differentiated from that of all the other sequences on the slide, so the specific sequence that is bound can be identified from among the hundreds on the slide, thus providing specific sequence information on the target. If multiple sequences from the specimens bind they can be read separately, which can provide additional information by multiplexing. Unfortunately, however, microarrays have relatively poor sensitivity and require prior enrichment of the target; they also take more time to run than PCR tests, and cost substantially more per sample. If some of these problems can be resolved, microarrays would be a useful tool for reference laboratories to investigate new disease outbreaks (such as an index case), or even new variants of infectious agents. However, gene sequencing has become so fast and cheap that in-depth analysis of PCR products is common and can provide much of the same information that a microarray can, and more efficiently. Currently, microarrays have the most diagnostic utility for genetic conditions such as cancer, rather than for infectious disease.

Numerous modifications have been reported to improve PCR-based methods and many involve improving the sensitivity and specificity of the reaction. Real-time PCR probes can be constructed in many ways (hairpins, two probe systems, different dyes), all of which have different advantages and disadvantages for cost, sensitivity and specificity that can be optimized for a given test or instrument. The simplest modifications are those that optimize the temperature cycling parameters to exploit the characteristics of the enzyme and template. Some examples are linear-after-the-exponential PCR and touch-down PCR. Other modifications involve using molecularly altered primers (and sometimes probes with real-time PCR). An example is minor groove binding protein (Kutyavin *et al.*, 2000), which enhances binding allowing for shorter probes by raising the melting temperature. Probes with minor groove binding protein have been applied to the detection of several viral

Table 2. *Trends in improving diagnostic tests and some common approaches.*

Description	Goal	Approach
Specificity	Improved accuracy	Modifications to proteins and nucleic acids Cycling parameters
Sensitivity	Improved accuracy	Detection of analyte Cycling parameters Probe design
Stability	Lower cost Increased utility	Isothermic reactions Stable enzymes Stable dyes
Smaller volumes	Lower cost per test Higher capacity Faster results	Instrumentation
Automation	Reduce operator error and cost Reduce errors with interpretation of results Remote analysis possible	Instrumentation
Multiplexing	Cheaper More information per test	Instrumentation Reporter molecules (dyes, nanoparticles, quantum dots, etc.)
Point of care	Convenience Provides immediately actionable information	Instrumentation Stable reagents

diseases of poultry including AIV, infectious laryngotracheitis virus and NDV (Di Trani *et al.*, 2006; Lu *et al.*, 2008; Farkas, *et al.*, 2009; McMenamy *et al.*, 2011). This approach works well for highly variable targets where conserved regions are too short for a conventional probe.

Ultra-small particles such as nanoparticles, quantum dots and microspheres have also been utilized to modify PCR-based assays and have also been employed widely for antibody-based assays. Each of these has different features and needs to be used with specific instrumentation, but can be uniquely labelled and used to specifically identify a PCR amplification product. One of the most practical features of nanoparticles, microspheres and quantum dots is that there are many unique labels that can be used so, in theory, reactions can be highly multiplexed, even into hundreds. Of course there are other technical issues with interference and competition when PCR is highly multiplexed and the instrumentation is expensive, but as the technology improves it will become more practical.

Owing to advances in microfluidics in recent years, improved PCR instrumentation and reaction automation has been developed. One innovation is all-in-one/self-contained cartridges that extract the DNA or RNA and run the reaction. The cartridges are run in vendor-specific, dedicated instruments that analyse the results; for example, Gene Xpert (Cephied Inc., Sunnyvale, CA, USA) and Film Array (Idaho Technologies Inc., Salt Lake City, UT, USA). Microfluidics have also allowed sample volumes to decrease, which increases the sample capacity, decreases the cost per reaction and reduces the time needed to run the reaction; one example is the Integrated Cycler (3M, Focus and Simplexa, Focus Diagnostics, Inc. Cypress, CA, USA), which runs 96 samples in a small single-use carousel and heats with infrared radiation. Digital PCR (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) uses microfluidics to partition a PCR reaction into volumes so low that only a single molecule of analyte should be present, thus allowing absolute quantitation.

Biosensors. Biosensors are instruments that use everything from chemical reactions to electrical resistance to detect a wide range of analytes as diverse as chemicals, viruses and bacteria. Currently most commercially available biosensors are for chemicals, such as drugs and explosives, but perhaps the most common biosensors are blood glucose monitors. Biosensors have been reported for several viruses using DNA and viral proteins as targets (Wang *et al.*, 1996; Inoue *et al.*, 1999; Baeumner *et al.*, 2002; Zhou *et al.*, 2002; Xu *et al.*, 2007), but none have been adopted clinically. If the technology can be validated for viruses, portable biosensors would provide an advantage over other technologies, as samples frequently require minimal processing, making them appropriate for point-of-care testing. Most are reusable or have minimal consumables; the costs of individual tests should therefore be low.

Reporter cell lines. Reporter cell lines are cell lines that have been modified to produce a measurable signal when they are infected by a target virus. For example, the enzyme-linked virus inducible system cell line (Proffitt & Schindler, 1995), which is now commercially available, is engineered to produce β-galactosidase when infected with herpes simplex virus. The β-galactosidase gene was inserted into the cell line and linked to a promoter that is controlled by viral proteins, so β-galactosidase is only produced when replicating herpes simplex virus is present. In some cases, cell lines that would not be naturally susceptible to infection with a virus can be engineered to be susceptible by inserting the appropriate receptor.

Although reporter cell lines are specific and sensitive, they take a relatively long time to yield results and require a high degree of technical skill and resources. Also, there is some limitation for the agents these cell lines can target because a high degree of detail of the virus replication cycle has to be known, and the disease in question needs to be important enough to merit the skill and resources required to make the tests practical and cost-effective.

Detection of a specific immune response. One novel approach is to detect the host-specific anamnestic immune response to a pathogen, instead of the pathogen itself. A commercially available kit using this technology is the "Quantiferon" kit (Cellestis Ltd., Melbourne, Australia), a tuberculosis test. A cytomegalovirus test is also available and is marketed for pre-organ transplant testing. Some of the details of the test are proprietary, but essentially blood from the patient is exposed to antigen from the target pathogen, and the test then evaluates the level of interferon induced from immune cells in the blood. The advantage of this format is that both active and latent infection can be detected in individuals while tests for the agent itself (including culture) or the agent's nucleic acid would be negative in a latent infection. In some ways Quantiferon is similar to the tube or plate agglutination tests used for avian

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mycoplasma, except that it detects active or latent infection instead of antibody. This format would probably not work well for acute infections and so far has not been applied to poultry infections.

Sample collection, transport and processing. One critical area, which has been largely ignored by diagnostic test developers, is sample collection and processing. Sampling methods for viruses (i.e. media, swab types, sample transport and storage) were originally based on bacterial methods and there are relatively few reports in veterinary or human medicine of validation studies of sample collection and transport. Similarly there are few reports of improvements in sample collection technology. Two relatively recent notable technologies are the use of flocked swabs and FTA cards (Whatman Inc. Whatman Plc, Maidstone, Kent UK). Flocked swabs are composed of a nylon material that is claimed to have superior capture and release characteristics compared with conventional swabs, thus improving sensitivity by increasing the amount of analyte in the sample (Daley *et al.*, 2006; Dalmaso *et al.*, 2008; Scansen *et al.*, 2010). FTA cards have probably had the greatest impact on sample collection and transport of any technology in past decade. They can be used to collect any fluid that can be spotted on the card. Chemicals in the cards inactivate most pathogens and stabilize the nucleic acid, which can then be safely shipped at ambient temperatures and without International Air Transport Association classification as a live pathogen, which saves money. Nucleic acids are extracted from discs punched from the card at the destination laboratory and can be processed for any nucleic acid detection assay. The use of FTA cards has been reported for specimen collection, shipping and subsequent detection of AIV, infectious bronchitis virus, infectious bursal disease virus and NDV (Moscoso *et al.*, 2005, 2006; Perozo *et al.*, 2006; Purvis *et al.*, 2006; Abdelwhab *et al.*, 2011; Kraus *et al.*, 2011).

Some sample processing and collection innovations are too new to have been widely adopted or are still in commercial development. A novel method for nucleic acid purification is syncronous coefficient of drag alternatives (Marziali *et al.*, 2005), which uses the unique electro-chemical properties of DNA and RNA to purify them from cells and contaminants that could be inhibitory for PCR and other assays. This method has been described by its inventor, Andre Marziali, as "focusing DNA in a spot in a gel while all other molecules are excluded" due to their unique behaviour in an electric field (Roberts, 2011). Another approach to sample collection that is still in development is a mask to collect respiratory agents from coughs, which concentrates them in a buffered solution (Deton Corp., Pasadena, CA, USA) (Roberts, 2011). The latter will probably never be practical for poultry, but is a good example of emergent sample collection technology.

What Does the New Technology Mean for Poultry Health?

Regardless of technological capabilities the market will always determine what tests are implemented. Many of the above technologies were developed with human health in mind, where an individual is the target. In contrast, the approach to poultry and food animal health differs somewhat when the target is a population. Also, just because we can do something does not mean it is necessary or practical; for example, sensitivity to the point of single-molecule detection. Would detection of a single AIV virion or *Mycoplasma gallisepticum* cell really improve control or would it complicate control? Is detection of a single molecule clinically relevant? Is such detection necessary and can the cost be justified? The answers to these questions vary by agent and situation, and the utility and success of a test is heavily reliant on whether it is fit-for-purpose: the best test is the one that fulfils the needs of the situation. The needs vary among different compartments and regions, whether testing is for flock health versus food safety and trade, the importance of the test target, and so forth. For example, a test that works well for the broiler industry in Mexico may not be as practical for layers in the US. This leads to perhaps one of the most important questions when implementing a test, and one that should be considered during development: *how will the results be used?*

Looking at how new technology has been implemented in the past shows an interesting trend; molecular methods have not completely supplanted classical methods, such as culture. AIV and NDV are good examples where molecular tests have improved detection in the field, so that immediate action can be taken in the case of a positive flock, but culture is still required to obtain a live agent for regulatory action, to characterize the virus present, and for development of vaccines. This paradigm, where molecular tests and point-of-care tests are used as screening tests and then additional confirmatory tests (often culture) must be conducted, is utilized for numerous veterinary and human diseases. The new technology has added to our arsenal, but in many cases has not truly replaced the original test. This is likely to be true with the newest innovations; the molecular methods could be modified or replaced, but the classical methods, specifically culture, will not be abandoned.

In general, many novel technologies will not be easily adopted due to cost or an impractical format. But if one was to select one of the technologies outlined above as having the most promise for poultry diagnostics, the biosensor would probably be the best because it could make pen-side testing possible. However, biosensors are among those that are furthest from adoption (development is still nascent for virus and bacteria detection with biosensors). Microfluidics could also be useful for the diagnostic laboratory to increase capacity and decrease costs if the equipment is affordable. As some of the new technologies become cheaper the improvements may be able to justify the cost of adoption. At the very least there will definitely be improvements for poultry diagnostics from some of the simple modifications becoming available for nucleic acid detection assays even if substantial changes in format are unlikely in the near future.

Acknowledgements

This review was presented as the Houghton Lecture at the 17th Congress of the World Veterinary Poultry Association in Cancun, Mexico, 2011.

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References

Abdelwhab, E. M., Lüschow, D., Harder, T.C. & Hafez, H.M. (2011). The use of FTA(R) filter papers for diagnosis of avian influenza virus. *Journal of Virololgical Methods*, 174, 120–122.

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R. & Kohara, M. (1999). Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *Journal of Clinical Microbiology*, 37, 2899–2903.

Alwine, J.C., Kemp, D.J. & Stark, G.R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proceedings of the National Academy of Sciences USA*, 74, 5350–5354.

Baeumner, A J., Schlesinger, N.A., Slutzki, N.S., Romano, J., Lee, E.M. & Montagna, R.A. (2002). Biosensor for dengue virus detection: sensitive, rapid, and serotype specific. *Analytical Chemistry*, 74, 1442–1448.

Bedson, S. & Bland, J. (1929). Complement-fixation with filterable viruses and their antisera. *British Journal of Experimental Pathology*, 10, 393–404.

Best, J., Banatvala, J.E., Almeida, J. & Waterson, A.P. (1967). Morphological characteristics of rubella virus. *The Lancet*, 290, 237–239.

Brenner, S. & Horne, R.W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochimica et Biophysica Acta*, 34, 103–110.

Bruckner, L., Stauber, N., Brechtbuhl, K. & Hofmann, M. A. (1996). Detection of extraneous agents in vaccines using the polymerase chain reaction of Newcastle disease virus in poultry biologicals. *Developments in Biololgical Standards*, 86, 175–182.

Burke, D.S., Brandt, B.L., Redfield, R.R., Lee, T.H., Thorn, R.M., Beltz, G.A. & Hung, C.H. (1987). Diagnosis of human immunodeficiency virus infection by immunoassay using a molecularly cloned and expressed virus envelope polypeptide. Comparison to Western blot on 2707 consecutive serum samples. *Annals of Internal Medicine*, 106, 671–676.

Carlson, B. (2011). Molecular diagnostics: potential and reality. *Genetic Engineering and Biotechnology News*, 31, 18–19.

Daley, P., Castriciano, S., Chernesky, M. & Smieja, M. (2006). Comparison of flocked and rayon swabs for collection of respiratory epithelial cells from uninfected volunteers and symptomatic patients. *Journal of Clinical Microbiology*, 44, 2265–2267.

Dalmaso, G., Bini, M., Paroni, R. & Ferrari, M. (2008). Qualification of high-recovery, flocked swabs as compared to traditional rayon swabs for microbiological environmental monitoring of surfaces. *PDA Journal of Pharmaceutical Science and Technology*, 62, 191–199.

Das, A. & Suarez, D.L. (2007). Development and bench validation of real-time reverse transcription polymerase chain reaction protocols for rapid detection of the subtypes H6, H9, and H11 of avian influenza viruses in experimental samples. *Journal of Veterinary Diagnostic Investigation*, 19, 625–634.

Dascal, A., Chan-Thim, J., Morahan, M., Portnoy, J. & Mendelson, J. (1989). Diagnosis of herpes simplex virus infection in a clinical setting by a direct antigen detection enzyme immunoassay kit. *Journal of Clinical Microbiology*, 27, 700–704.

Davis, B.J. (1964). Disc electrophoresis-II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 121, 404–427.

Di Trani, L., Bedini, B., Donatelli, I., Campitelli, L., Chiappini, B., De Marco, M. A., Delogu, M., Buonavoglia, C. & Vaccari, G. (2006). A sensitive one-step real-time PCR for detection of avian influenza viruses using a MGB probe and an internal positive control. *BMC Infectious Diseases*, 6, 87.

Egberink, H.F., Lutz, H. & Horzinek, M.C. (1991). Use of western blot and radioimmunoprecipitation for diagnosis of feline leukemia and feline immunodeficiency virus infections. *Journal of the American Veterinary Medical Association*, 199, 1339–1342.

Fan, J., Henrickson, K.J. & Savatski, L.L. (1998). Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). *Clinical Infectious Diseases*, 26, 1397–1402.

Farkas, T., Szekely, E., Belak, S. & Kiss, I. (2009). Real-time PCR-based pathotyping of Newcastle disease virus by use of TaqMan minor groove binder probes. *Journal of Clinical Microbiology*, 47, 2114–2123.

Gary, G.W., Jr., Kaplan, J.E., Stine, S.E. & Anderson, L.J. (1985). Detection of Norwalk virus antibodies and antigen with a biotin-avidin immunoassay. *Journal of Clinical Microbiology*, 22, 274–278.

Ghorashi, S.A., O'Rourke, D., Ignjatovic, J. & Noormohammadi, A.H. (2011). Differentiation of infectious bursal disease virus strains using real-time RT-PCR and high resolution melt curve analysis. *Journal of Virololgical Methods*, 171, 264–271.

Goodpasture, E.W., Woodruff, A.M. & Buddingh, G.J. (1931). The cultivation of vaccine and other viruses in the chorioallantoic membrane of chick embryos. *Science*, 74, 371–372.

Hart, C., Schochetman, G., Spira, T., Lifson, A., Moore, J., Galphin, J., Sninsky, J. & Ou, C. Y. (1988). Direct detection of HIV RNA expression in seropositive subjects. *Lancet*, 332, 596–599.

Heid, C.A., Stevens, J., Livak, K.J. & Williams, P.M. (1996). Real time quantitative PCR. *Genome Research*, 6, 986–994.

Hernandez, M., Tomas, G., Hernandez, D., Villegas, P., Banda, A., Maya, L., Panzera, Y. & Perez, R. (2011). Novel multiplex RT-PCR/RFLP diagnostic test to differentiate low- from high-pathogenic strains and to detect reassortant infectious bursal disease virus. *Avian Diseases*, 55, 368–374.

Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A., van Rijn, P.A., Slomka, M J., Banks, J., Brown, I.H., Alexander, D.J. & King, D.P. (2009). A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Veterinary Microbiology*, 139, 1–23.

Horimoto, T. & Kawaoka, Y. (1995). Direct reverse transcriptase PCR to determine virulence potential of influenza A viruses in birds. *Journal of Clinical Microbiology*, 33, 748–751.

Inoue, K., Arai, T. & Aoyagi, M. (1999). Sensitivity of real time viral detection by an optical biosensor system using a crude home-made antiserum against measles virus as a ligand. *Biological and Pharmaceutical Bulletin*, 22, 210–213.

Jackwood, D.J., Hanes, G. & Miller, S. H. (1996). Infectious bursal disease viral RNA amplification using RT/PCR from bursa tissue following phenol:chloroform inactivation of the virus. *Avian Diseases*, 40, 457–460.

Jackwood, M.W., Yousef, N.M. & Hilt, D.A. (1997). Further development and use of a molecular serotype identification test for infectious bronchitis virus. *Avian Diseases*, 41, 105–110.

Jones, R.M., Ellis, R.J., Cox, W.J., Errington, J., Fuller, C., Irvine, R.M. & Wakeley, P.R. (2011). Development and validation of RT-PCR tests for the detection and S1 genotyping of infectious bronchitis virus and other closely related gammacoronaviruses within clinical samples. *Transboundary and Emerging Diseases*, 58, 411–420.

Keeler, C.L., Jr., Reed, K.L., Nix, W.A. & Gelb, J. Jr. (1998). Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. *Avian Diseases*, 42, 275–284.

Kohler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256, 495–497.

Kraus, R.H., van Hooft, P., Waldenstrom, J., Latorre-Margalef, N., Ydenberg, R.C. & Prins, H.H. (2011). Avian influenza surveillance with FTA cards: field methods, biosafety, and transportation issues solved. *Journal of Visualized Experiments*, 54, e2832.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B. & Hedgpeth, J. (2000). 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research*, 28, 655–661.

Kwon, H.M., Jackwood, M.W. & Gelb, J. Jr. (1993). Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. *Avian Diseases*, 37, 194–202.

Lazarus, A S., Eddie, B. & Meyer, K.F. (1937). Propagation of variola virus in the developing egg. *Proceedings of the Society of Experimental Biology and Medicine*, 36, 7–8.

Liu, C. (1956). Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labeled antibody. *Proceedings of the Society of Experimental Biology and Medicine*, 92, 883–887.

Loeffler, F. & Frosch, P. (1898). Berichte der Kommission zur Erforschung der Maul und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. *Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten*, 23, 371–391.

Lu, Y.Y., Yan, J.Y., Feng, Y., Xu, C.P., Shi, W. & Mao, H.Y. (2008). Rapid detection of H5 avian influenza virus by TaqMan-MGB real-time RT-PCR. *Letters in Applied Microbiology*, 46, 20–25.

Marziali, A., Pel, J., Bizzotto, D. & Whitehead, L.A. (2005). Novel electrophoresis mechanism based on synchronous alternating drag perturbation. *Electrophoresis*, 26, 82–90.

McMenamy, M.J., McKillen, J., Hjertner, B., Kiss, I., Yacoub, A., Leijon, M., Duffy, C., Belak, S., Welsh, M. & Allan, G. (2011). Development and comparison of a Primer-Probe Energy Transfer based assay and a 5' conjugated Minor Groove Binder assay for sensitive real-time PCR detection of infectious laryngotracheitis virus. *Journal of Virological Methods*, 175, 149–155.

Mears, J.G., Ramirez, F., Leibowitz, D., Nakamura, F., Bloom, A., Konotey-Ahulu, F. & Bank, A. (1978). Changes in restricted human cellular DNA fragments containing globin gene sequences in thalassemias and related disorders. *Proceedings of the National Academy of Sciences USA*, 75, 1222–1226.

Moscoso, H., Alvarado, I. & Hofacre, C.L. (2006). Molecular analysis of infectious bursal disease virus from bursal tissues collected on FTA filter paper. *Avian Diseases*, 50, 391–396.

Moscoso, H., Raybon, E.O., Thayer, S.G. & Hofacre, C.L. (2005). Molecular detection and serotyping of infectious bronchitis virus from FTA filter paper. *Avian Diseases*, 49, 24–29.

Nelson, J. B. (1939). The behavior of pox viruses in the respiratory tract. II. The response of mice to the nasal instillation of variola virus. *Journal of Experimental Medicine*, 70, 107–116.

OIE. (2011). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011 Retrieved November 14, 2011, from http://www.oie.int/index.php?id=170

Ornstein, L. (1964). Disc Electrophoresis. I. Background and Theory. *Annals of the New York Academy of Sciences*, 121, 321–349.

Perozo, F., Villegas, P., Estevez, C., Alvarado, I. & Purvis, L. B. (2006). Use of FTA filter paper for the molecular detection of Newcastle disease virus. *Avian Pathology*, 35, 93–98.

Proffitt, M.R. & Schindler, S. A. (1995). Rapid detection of HSV with an enzyme-linked virus inducible system (ELVIS) employing a genetically modified cell line. *Clinical and Diagnostic Virology*, 4, 175–182.

Purvis, L.B., Villegas, P. & Perozo, F. (2006). Evaluation of FTA paper and phenol for storage, extraction and molecular characterization of infectious bursal disease virus. *Journal of Virololgical Methods*, 138, 66–69.

Rappolee, D.A., Mark, D., Banda, M.J. & Werb, Z. (1988). Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. *Science*, 241, 708–712.

Reimann, I. & Werner, O. (1996). Use of the polymerase chain reaction for the detection of reticuloendotheliosis virus in Marek's disease vaccines and chicken tissues. *Zentralblatt für Veterinarmedizin B*, 43, 75–84.

Rivera, E., Sjoland, L. & Karlsson, K.A. (1986). A solid phase fluorescent immunoassay for the rapid detection of virus antigen or antibodies in fetuses infected with porcine parvovirus. *Archives of Virology*, 88, 19–26.

Roberts, J. (2011). Streamlining nucleic acid sample prep: advances in long-read technology, target enrichment, and collection reshape operations. *Genetic Engineering and Biotechnology News*, 31, 26–28.

Rous, P. & Murphy, J. (1911). Tumor implantations in the developing embryo. *Journal of the American Medical Association*, 56, 741–742.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239, 487–491.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. & Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230, 1350–1354.

Scansen, K.A., Bonsu, B.K., Stoner, E., Mack, K., Salamon, D., Leber, A. & Marcon, M.J. (2010). Comparison of polyurethane foam to nylon flocked swabs for collection of secretions from the anterior nares in performance of a rapid influenza virus antigen test in a pediatric emergency department. *Journal of Clinical Microbiology*, 48, 852–856.

Shi, L., Ho, J., Norling, L. A., Roy, M. & Xu, Y. (1999). A real time quantitative PCR-based method for the detection and quantification of simian virus 40. *Biologicals*, 27, 241–252.

Shibata, D.K., Arnheim, N. & Martin, W. J. (1988). Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *Journal of Experimental Medicine*, 167, 225–230.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, 98, 503–517.

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T. & Suarez, D.L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology*, 40, 3256–3260.

Stauber, N., Brechtbuhl, K., Bruckner, L. & Hofmann, M.A. (1995). Detection of Newcastle disease virus in poultry vaccines using the polymerase chain reaction and direct sequencing of amplified cDNA. *Vaccine*, 13, 360–364.

Tham, K.M. & Stanislawek, W.L. (1992). Detection of chicken anaemia agent DNA sequences by the polymerase chain reaction. *Archives of Virology*, 127, 245–255.

Todd, D., Mawhinney, K.A. & McNulty, M.S. (1992). Detection and differentiation of chicken anemia virus isolates by using the polymerase chain reaction. *Journal of Clinical Microbiology*, 30, 1661–1666.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences USA*, 76, 4350–4354.

van Woensel, P.A., van Blaaderen, A., Moorman, R.J. & de Boer, G.F. (1992). Detection of proviral DNA and viral RNA in various tissues early after avian leukosis virus infection. *Leukemia*, 6(Suppl 3), 135S–137S.

Wang, J., Cai, X., Rivas, G., Shiraishi, H., Farias, P.A. & Dontha, N. (1996). DNA electrochemical biosensor for the detection of short DNA sequences related to the human immunodeficiency virus. *Analytical Chemistry*, 68, 2629–2634.

Wang, X. & Khan, M.I. (1999). A multiplex PCR for Massachusetts and Arkansas serotypes of infectious bronchitis virus. *Molecular and Cellular Probes*, 13, 1–7.

Weller, T.H. & Enders, J.F. (1948). Production of hemagglutinin by mumps and influenza A viruses in suspended cell tissue cultures. *Proceedings of the Society of Experimental Biology and Medicine*, 69, 124–128.

Williams, R.A., Bennett, M., Bradbury, J.M., Gaskell, R.M., Jones, R.C. & Jordan, F.T.W. (1992). Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. *Journal of General Virology*, 73, 2415–2420.

Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R. & Spackman, E. (2004). Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *Journal of Clinical Microbiology*, 42, 329–338.

Woodruff, A.M. & Goodpasture, E.W. (1931). The susceptibility of the chorio-allantoic membrane of chick embryos to infection with the fowl-pox virus. *American Journal of Pathology*, 7, 209–222.

Xu, J., Suarez, D. & Gottfried, D.S. (2007). Detection of avian influenza virus using an interferometric biosensor. *Analytical and Bioanalytical Chemistry*, 389, 1193–1199.

Zhou, X., Liu, L., Hu, M., Wang, L. & Hu, J. (2002). Detection of hepatitis B virus by piezoelectric biosensor. *Journal of Pharmaceutical and Biomedical Analysis*, 27, 341–345.

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AVIAN DISEASES 56:1010–1016, 2012

Research Note—

Avian Influenza Virus Wild Bird Surveillance in the Azov and Black Sea Regions of Ukraine (2010–2011)

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Received 11 April 2012; Accepted 16 July 2012; Published ahead of print 17 July 2012

SUMMARY. The Azov and Black Sea basins are part of the transcontinental wild bird migration routes from Northern Asia and Europe to the Mediterranean, Africa, and Southwest Asia. These regions constitute an area of transit, stops during migration, and nesting for many different bird species. From September 2010 to September 2011, a wild bird surveillance study was conducted in these regions to identify avian influenza viruses. Biological samples consisting of cloacal and tracheal swabs and fecal samples were collected from wild birds of different ecological groups, including waterfowl and sea- and land-based birds, in places of mass bird accumulations in Sivash Bay and the Utlyuksky and Molochniy estuaries. The sampling covered the following wild bird biological cycles: autumn migration, wintering, spring migration, nesting, and postnesting seasons. A total of 3634 samples were collected from 66 different species of birds. During the autumn migration, 19 hemagglutinating viruses were isolated, 14 of which were identified as low pathogenicity avian influenza (LPAI) virus subtypes H1N?, H3N8, H5N2, H7N?, H8N4, H10N7, and H11N8. From the wintering samples, 45 hemagglutinating viruses were isolated, 36 of which were identified as LPAI virus subtypes H1N1, H1N? H1N2, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H12N2, and H15N7. Only three viruses were isolated during the spring migration, nesting, and postnesting seasons (serotypes H6, H13, and H16). The HA and NA genes were sequenced from the isolated H5 and N1 viruses, and the phylogenetic analysis revealed possible ecological connections between the Azov and Black Sea regions and Europe. The LPAI viruses were isolated mostly from mallard ducks, but also from shellducks, shovelers, teals, and white-fronted geese. The rest of the 14 hemagglutinating viruses isolated were identified as different serotypes of avian paramyxoviruses (APMV-1, APMV-4, APMV-6, and APMV-7). This information furthers our understanding of the ecology of avian influenza viruses in wild bird species.

RESUMEN. *Nota de Investigación*—Vigilancia para el virus de la influenza aviar en aves silvestres en las regiones del Mar de Azov y del Mar Negro en Ucrania (años 2010–2011).

Las cuencas del Mar de Azov y del Mar Negro son parte de las rutas transcontinentales de aves silvestres migratorias procedentes del norte de Asia y Europa hacia el Mediterráneo, África y el suroeste de Asia. Estas regiones constituyen un área de tránsito, de descanso durante la migración y de anidación para varias especies de aves diferentes. Desde septiembre 2010 hasta septiembre 2011, un estudio de vigilancia de aves silvestres se llevó a cabo en estas regiones con el fin de identificar los virus de la influenza aviar. Las muestras biológicas que consistieron de hisopos cloacales y traqueales y de muestras fecales de aves silvestres de diferentes grupos ecológicos, incluyendo aves acuáticas además de aves marinas y terrestres, en los lugares de acumulación masiva en la Bahía de Sivash y en los estuarios de Utlyuksky y Molochniy. El muestreo abarcó los siguientes ciclos biológicos de aves silvestres: la migración de otoño, el descanso invernal, la migración de primavera, temporada de anidación, y las estaciones posteriores a la anidación. Se recolectaron un total de 3634 muestras de 66 especies diferentes de aves. Durante la migración otoñal, se aislaron 19 virus hemaglutinantes de los cuales 14 fueron identificados como virus de influenza aviar de baja patogenicidad, de los subtipos H1N?, H3N8, H5N2, H7N?, H8N4, H10N7 y H11N8. De las muestras de invierno, se aislaron 45 virus hemaglutinantes, 36 de los cuales fueron identificados como virus de baja patogenicidad subtipos H1N1, H1N? H1N2, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H12N2 y H15N7. Sólo tres virus fueron aislados durante la migración de primavera, de anidación, y de las estaciones posteriores a la anidación (serotipos H6, H13, y H16). Los genes HA y NA se secuenciaron de los virus aislados con subtipos H5 y N1, y el análisis filogenético reveló posibles conexiones ecológicas entre los mares de Azov y las regiones del Mar Negro y Europa. Los virus de baja patogenicidad fueron aislados en su mayoría de patos de collar, pero también en patos tarros de blancos, patos pico de cuchara, cercetas y gansos de frente blanca. El resto de los 14 virus hemaglutinantes aislados fueron identificados como diferentes serotipos de paramixovirus aviar (APMV-1, APMV-4, APMV-6, y APMV-7). Esta información amplía el conocimiento de la ecología de los virus de influenza aviar en las especies de aves silvestres.

Key words: avian influenza, avian influenza virus, surveillance, wild birds, Crimea

Abbreviations: AI = avian influenza; AIV = avian influenza virus; APMV = avian paramyxovirus; HA = hemagglutinating; HP = highly pathogenic; LP = low pathogenicity; NA = neuraminidase; nt = nucleotide

Avian influenza viruses (AIVs) are type A influenza viruses belonging to the family *Orthomyxoviridae* (13). AIVs are divided into subtypes based on the two surface glycoproteins, the HA (subtypes H1–H16) and the NA (subtypes N1–N9). Because of their segmented genome, AIVs can reassort and consequently lead to the emergence of new virus strains. Wild aquatic birds are the natural reservoirs of AIVs and play an important role in the epizootology of avian influenza (AI) (13). Low pathogenicity (LP) AIVs are maintained in wild bird reservoirs, predominately the aquatic birds in the orders Anseriformes and Charadriiformes (9,15). These viruses are passed within and between species of birds that share the same ecosystems, and such infections are typically not associated with disease (14). In rare cases isolates of the Asian H5N1 highly

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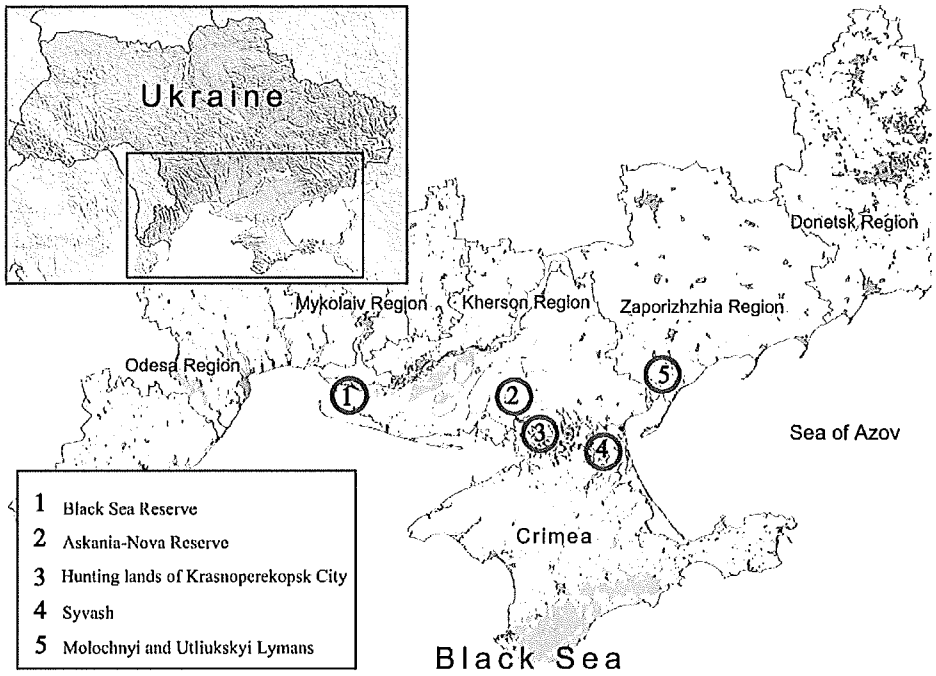

Fig. 1. Map of Ukraine indicating the regions included in the AIV wild bird surveillance study.

pathogenic (HP) AIV lineage have caused disease and mortality in some wild bird species (2). Poultry species (chickens, turkeys, quail, guinea fowl, etc.) reared in proximity to wild bird ecosystems can become infected by viruses carried by the wild birds.

Commercial poultry is an important industry in Ukraine; in addition backyard poultry is common in the southern regions. It is very important for Ukraine to remain free of AI, and one of the components for AI control is to monitor AIV infections in wild bird populations. The importance of conducting AIV surveillance in wild birds became evident during the outbreaks of H5N1 HPAI in Ukraine in 2005, 2006. and 2008, when the virus was introduced into the country by wild birds during the autumn migration (16). The role of wild birds was considerable in the further dissemination of H5N1 HPAIV into territories of Autonomous Republic of Crimea. Due to a very cold winter in 2005–2006, and other unfavorable weather factors, wild birds constantly moved in many directions, dispersing the virus and originating new outbreaks in the different geographic areas of Crimea (7,11).

Ukraine occupies an unique geographical location in Central and Eastern Europe, as the transcontinental migrations of numerous wild bird species pass from North Asia and Europe to the Mediterranean, Africa, and Southwest Asia and cross from the Baltic and Caspian seas to the Black and Mediterranean seas, and from Western Siberia and Kazakhstan to Western Europe and North Africa.

The Azov and Black Sea regions were selected for monitoring wild birds for AIV and avian paramyxovirus (APMV) infections because of the abundance of migratory birds. The Azov and Black Sea basins and the peninsula of Crimea are historically areas of nesting, flight, migratory stops, and wintering for many bird species. The Azov–Black Sea coast is one of the densest territories of Eastern Europe from an ornithological point of view. In Ukraine there are 19 wetlands of international importance with an area of more than 590,000 ha. More than 320 types of birds inhabit the wetlands, representing almost 84% of bird species in Ukraine. In this study we present the results of wild bird AIV and APMV surveillance in the Azov and Black Sea regions of Ukraine between September 2010 and September 2011.

MATERIALS AND METHODS

Wild bird surveillance. Sampling of wild birds was conducted from September 2010 to September 2011 in the following regions of the Azov and Black Sea regions: Sivash Bay, Utliukskyi and Molochnyi estuaries, Askania-Nova Reserve, Black Sea Biosphere Reserve, and hunting lands of Krasnoperekopskyi, Nyznygorsky, and Djankoysky districts of Crimea (Fig. 1). The collection of biological material from wild birds covered the following annual bird life cycles: autumn migration (September to November 2010), wintering (December 2010 to February 2011), and spring migration, nesting, and postnesting movements (March 2011 to August 2011).

Sample collection. Sampling from wild birds was carried out in cooperation with ornithologists, who helped determine the bird species identity. Cloacal and tracheal swabs were collected from captured birds and from birds shot by hunters. Fresh feces were collected from certain species of birds in places of mass bird accumulations. Feces were collected only if the origin and type of bird had been established. Samples of feces were taken in a checkerboard pattern at a distance of at least 1.5–2 m from each other, to avoid selecting feces from the same bird.

The samples (cloacal swabs, tracheal swabs, and feces) were taken from adult birds, regardless of gender. The sample size depended on the size of the flock and was at least 25 samples if the flock was up to 500 birds and at least 50 samples per 1000 birds if the number of birds in the flock was more than 1000. Estimates of the number of birds were conducted by ornithologists.

Samples were collected in cryotubes containing 1.0 ml of transport media (phosphate-buffered saline:glycerin, 1:1) with antibiotics (penicillin 2000 U/ml, streptomycin 2 mg/ml, gentamicin 50 μg/ml, and nystatin 1000 U/ml), and a fivefold concentration of antibiotics was used for the fecal samples and cloacal swabs (5). Samples were stored at −196 C in liquid nitrogen, where they were kept until processing.

Virus isolation. Virus isolation was conducted in accordance with the OIE procedures (5). Cloacal swabs, tracheal swabs, and fecal samples were inoculated into the allantoic cavity of 9–10-day-old SPF chicken embryonated eggs. Every sample was passaged three times. The presence of hemagglutinating (HA) viruses in allantoic fluid was determined by the hemagglutination test with a 1% suspension of chicken red blood cells (5).

Virus identification. The HA virus subtype was determined by hemagglutination inhibition tests as previously described (1,5). Determi-

Table 1. Number of samples of biological material and the results of virus isolation data (total/number positive) of wild birds of different ecological groups in the central part of the Azov and Black Sea regions from September 2010 to September 2011.

Bird species	Sampling periods			Total samples (%)
	Autumn migration	Wintering	Spring migration, nesting, postnesting movements	
Pelecaniformes				
Cormorant (*Phalacrocorax carbo*)	0/15	—	—	0/15
Ciconiiformes				
Gray heron (*Ardea cinerea*)	—	0/4	0/1	0/5
Purple heron (*Ardea purpurea*)			0/1	0/1
Anseriformes				
Mute swan (*Cygnus olor*)	0/1	—	—	0/1
White-fronted goose (*Anser albifrons*)	0/216	9/649 H9N2(3), H6N1(6)[A]	—	9/865 (1.04)[B]
Graylag goose (*Anser anser*)	—	0/20	—	0/20
Branta ruficollis (*Rufibrenta ruficollis*)	—	0/215	—	0/215
Shelduck (*Tadorna tadorna*)	1/8 H8N4	7/238 H1N2, H1N? (4), H11N2 (2)	—	8/246 (3.25)
Ruddy shelduck (*Tadorna ferruginea*)	0/90	4/190 H4N?, H11N2 (2), H11N6	—	4/280 (1.42)
Wild duck[C]	0/4	2/45 H8N2, H10N4	—	2/49 (4.08)
Mallard duck (*Anas platyrhynchos*)	11/366 H1N?, H3N8 (5), mixH3/H4N8, H7N?, H8N4, H10N7, H11N8	13/392 H1N1(2), H7N3 (3), H7N6 (2), H7N7, H10N7 (2), mixH10/APMV-7, H12N2/N8, H15N7	—	24/758 (3.16)
Red-crested pochard (*Netta rufina*)	0/1	—	—	0/1
Wigeon (*Anas penelope*)	0/130	—	—	0/130
Pochard (*Aythya ferina*)	0/3	—	—	0/3
Pintail (*Anas acuta*)	0/3	—	—	0/3
Garganey (*Anas querquedula*)	0/3	—	—	0/3
Teal (*Anas crecca*)	1/18 H5N2	—	0/1	1/19 (5.26)
Shoveler (*Anas clypeata*)	1/4 H8N4	—	—	1/4 (25.0)
Gadwall (*Anas strepera*)	0/3	—	—	0/3
Galliformes				
Gray partridge (*Perdix perdix*)	0/3	0/1	—	0/4
Gruiformes				
Crane (*Grus grus*)	0/7	—	—	0/7
Coot (*Fulica atra*)	0/34	—	0/1	0/34
Water rail (*Rallus aquaticus*)	—	0/1		0/1
Moorhen (*Gallinula chloropus*)	—	—	0/2	0/2
Little crake (*Porzana parva*)	—	—	0/2	0/2
Charadriiformes				
Yellow-legged gull (*Larus cachinnans*)	0/9	1/39 H1/H5	1/42 (H6N?)	2/90 (2.22)
Slender-billed gull (*Larus genei*)	0/12	—	0/32	0/44
Mediterranean gull (*Larus melanocephalus*)	—	0/2	0/141	0/143
Common gull (*Larus canus*)	—	0/9		0/9
Black-headed gull (*Larus ridibundus*)	—	—	1/131 (H16N?)	1/131 (0.76)
Sandwich tern (*Thalasseus sandvicensis*)	0/2	—	0/15	0/17
Gull-billed tern (*Gelochelidon nilotica*)	—	—	0/85	0/85
Gray plover (*Pluvialis squatarola*)	0/4	—	0/5	0/9
Kentish plover (*Charadrius alexandrinus*)	0/1	—	—	0/1
Sanderling (*Calidris alba*)	0/3	—	—	0/3
Dunlin (*Calidris alpina*)	0/23	—	0/2	0/25
Little stint (*Calidris minuta*)	—	—	0/2	0/2
Temminck's stint (*Calidris temminckii*)	—	—	0/3	0/3

Table 1. Continued.

Bird species	Sampling periods			Total samples (%)
	Autumn migration	Wintering	Spring migration, nesting, postnesting movements	
Common sandpiper (*Actitis hypoleucos*)	—	—	0/5	0/5
Wood sandpiper (*Tringa glareola*)	—	—	1/37 (H13N?)	1/37 (2.70)
Green sandpiper (*Tringa ochropus*)	—	—	0/5	0/5
Marsh sandpiper (*Tringa stagnatilis*)	—	—	0/1	0/1
Greenshank (*Tringa nebularia*)	—	—	0/6	0/6
Black-winged stilt (*Himantopus himantopus*)	—	—	0/4	0/4
Oystercatcher (*Haematopus ostralegus*)	—	—	0/4	0/4
Collared pratincole (*Glareola pratincola*)	—	—	0/1	0/1
Snipe (*Gallinago gallinago*)	—	—	0/1	0/1
Ruff (*Phylomachus pugnax*)	—	—	0/91	0/91
Coraciiformes				
Kingfisher (*Alcedo atthis*)	—	—	0/3	0/3
Passeriformes				
Sand martin (*Riparia riparia*)	—	—	0/3	0/3
Swallow (*Hirundo rustica*)	—	—	0/14	0/14
Jackdaw (*Corvus monedula*)	—	0/10	—	0/10
Calandra lark (*Melanocorypha calandra*)	—	0/60	—	0/60
Pied wagtail (*Motacilla alba*)	—	—	0/1	0/1
Yellow wagtail (*Motacilla flava*)	—	—	0/2	0/2
Magpie (*Pica pica*)	—	0/35	—	0/35
Rook (*Corvus frugilegus*)	—	—	0/30	0/30
Chaffinch (*Fringilla coelebs*)	—	0/2	—	0/2
Reed bunting (*Emberiza schoeniclus*)	—	0/20	0/1	0/21
Starling (*Sturnus vulgaris*)	—	0/35	0/2	0/37
Reed warbler (*Acrocephalus scirpaceus*)	—	—	0/5	0/5
Great reed warbler (*Acrocephalus arundinaceus*)	—	—	0/11	0/11
Sedge warbler (*Acrocephalus schoenobaenus*)	—	—	0/1	0/1
Savi's warbler (*Locustella luscinioides*)	—	—	0/1	0/1
Bearded tit (*Panurus biarmicus*)	—	—	0/8	0/8
Icterine warbler (*Hippolais icterina*)	—	—	0/1	0/1
Olivaceous warbler (*Hippolais pallida*)	—	—	0/1	0/1
Total, no. (%)	14/962 (1.45)	36/1966 (1.83)	3/706 (0.42)	53/3634 (1.46)

[A]Total number/number of isolated viruses, HA:NA (number of viruses of this subtype).
[B]Percentage of positive samples.
[C]The species could not be identified because of bad weather.

nation of neuraminidase (NA) subtype was conducted by the neuraminidase activity inhibition test (8). For these studies, the following antisera were used: H1N1, H2N3, H3N8, H4N6, H5N1, H6N8, H7N1, H8N4, H9N2, H10N7, H10N9, H11N6, H12N5, H13N6, H14N6, H15N9, H16N3, APMV-1, APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by Veterinary Laboratories Agency (Weybridge, UK) and the antisera H1N1, H2N3, H3N8, H4N8, H5N3, H6N2, H7N3, H8N4, H9N7, H10N1, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3, APMV-1, APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by the Istituto Zooprofilattico Sperimentale delle Venezie (Padova, Italy).

Sequencing. Gene sequence analysis was conducted on the H5 and N1 genes of the AIV isolates. Sequencing templates for individual influenza genes were produced by amplifying partial HA or NA genes with degenerate and end-specific primers by RT-PCR as previously described (12). Templates were then purified by agarose gel extraction with the QIAquick gel extraction kit (Qiagen, Valencia, CA). The BigDye terminator kit (Applied Biosystems, Foster City, CA) was used for direct cycle sequencing and subsequently run on an AB 3730 (Applied Biosystems).

Phylogenetic analysis. Phylogenetic analysis included the portion HA1 of the H5 gene from nucleotide (nt) 12 of the coding region to approximately nt 570 (GenBank accession numbers JX262650–JX262652). The portion of the N1 NA gene analyzed corresponded to nt 60 to nt 580 of the coding region (GenBank accession numbers JX262653–JX262657). Isolates representing other known major lineages were included in the phylogenetic reconstruction, and the Basic Local Alignment Search Tool search was used to identify the most closely related isolates. Sequences were aligned with Clustal V (Lasergene, v. 8.0.2; DNAStar, Madison, WI). Trees were constructed with BEAST v. 1.4.8 (4) using Hasegawa-Kishino-Yano 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.

RESULTS

From September 2010 to December 2011, 3857 samples were collected from 66 different species of wild birds in the Azov and Black Sea regions of Ukraine for virological examination. The wild bird species belonged to eight orders: Pelecaniformes, Ciconiiformes, Anseriformes, Galliformes, Gruiformes, Charadriiformes, Coraciiformes, and Passeriformes (Table 1). Of the 66 species, 46 represent the local avifauna breeding in the territory of the Azov and Black Sea

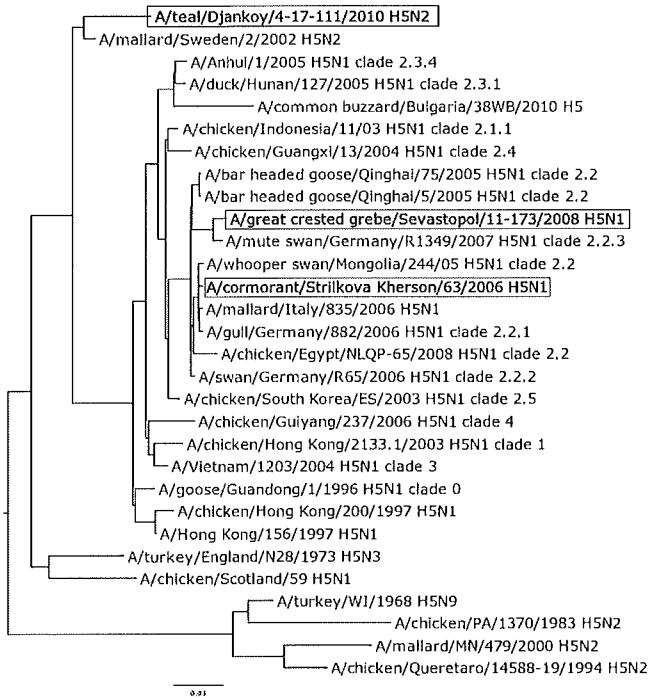

Fig. 2. Phylogenetic tree of the HA1 region of the H5 genes based on nucleotide sequence. Sequences from isolates collected in this study are shown in boldface.

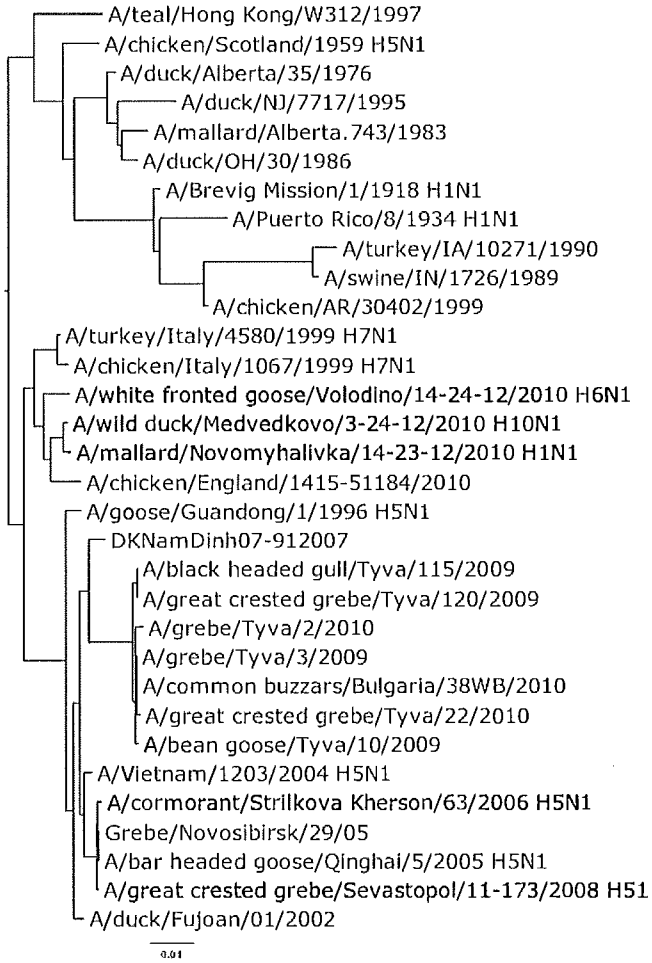

Fig. 3. Phylogenetic tree of the N1 genes based on nucleotide sequence. Sequences from isolates collected in this study are shown in boldface.

regions. Twenty species do not breed in the territory of study, stopping only during migration, including 11 short-range migrants from the northern territories of Ukraine, Belarus, Russia, and other neighboring areas and nine long-distance migrants from the Russian tundra, Southern Europe (probably the Volga Delta), and Kazakhstan. Most of the species represented in the samples in one way or another are connected with wetlands: 17 species of waterfowl and another 28 species belonging to wetland birds. All other birds are terrestrial species. Before beginning the study, a census of the number of birds in the projected areas of surveillance was conducted. By ornithologists' estimations, during October 2010 there were 421,711 birds belonging to 90 species in the Eastern Sivash (17). The most widespread bird species had a wetland habitat, particularly Anseriformes.

From September to November 2010, during the autumn migration, 962 samples were collected from 25 species of birds in the wetlands of Sivash (Kherson and Zaporizhia regions of Ukraine and Crimea), as well as from hunter-killed birds in Nizhnegorsky, Dzhankojsky, and Krasnoperekopskyi districts of Crimea (Fig. 1). During this period, 14 influenza viruses of different subtypes were isolated: H1N?, H3N8, H5N2, H7N?, H8N4, H10N7, and H11N8. For the H1N? and H7N? viruses we could not determine the subtype of the neuraminidase by serological tests because they did not react with any of the antisera. All of these viruses were isolated from waterfowl: shelduck (1), mallard (11), teal (1), and shoveler (1).

During the winter (December 2010–February 2011) the number of birds in the Azov and Black Sea regions of Ukraine changed. According to the ornithologists, in the wetlands of Sivash the total number of waterfowl (Anseriformes) was approximately 62,000 birds in December 2010, with approximately 28,000 additional birds belonging to other genera and other habitat groups. Seventy-six bird species were identified. When reevaluated in February 2011, the total number of wild birds in the Sivash decreased to 48,000, and the number of species was 73. During this period, 1966 samples were collected from 19 species of birds wintering in this region. These samples were collected in the Askania-Nova Reserve and in the Sivash wetlands (Fig. 1). Forty-five HA viruses were isolated from these samples. Thirty-six of them were AIVs of subtypes H1N1, H1N2, H1N?, H4N?, H6N1, H7N3, H7N6, H7N7, H8N2, H9N2, H10N7, H10N4, H11N2, H11N6, H12N2, and H15N7. We were not able determine the neuraminidase subtype for the H1N? and H4N? viruses with the available antisera. The AIVs were isolated from wild waterfowl samples: white-fronted goose (9), shelduck (7), ruddy shelduck (4), mallard (13) and yellow-legged gull (1). Two other AIVs were isolated from wild ducks whose species could not be identified because of bad weather.

The last set of samples were collected during the spring migration, nesting, and postnesting seasons (March to August 2011). This period is characterized by a lower density of wild birds in the areas surveyed. Spring migration was short, and wild bird density was low. During nesting, most birds did not form large clusters or colonies, especially waterfowl (geese and ducks). As during the postnesting, the concentration of birds depended on the species, which was reflected in the surveillance results. In total, during this period 706 samples were collected from 44 species of birds. Most of the

biological material collected during the spring migration and breeding were from the Askania-Nova Reserve and the Black Sea Biosphere Reserve. Samples from wild birds in the postbreeding movements were collected in wetlands in the Utlyuksky and Molochniy estuaries of the Velykyi Utluk River (Zaporizhia region). Only three HA viruses were isolated from the samples collected and were identified as AIV subtypes H6, H13, and H16 (NA not determined). These viruses were collected from two gull species and a wood sandpiper (*Tringa glareola*).

The remaining 14 HA viruses that were isolated between September 2010 and August 2011 were identified as different serotypes of avian paramyxoviruses: APMV-1, APMV-4, APMV-6, and APMV-7. Details of these isolates will be presented elsewhere.

DISCUSSION

In this study, 14 of the 16 (no H2 or H14) known HA subtypes of AIV were isolated from samples collected from wild birds from the Azov and Black Sea regions, indicating that AIVs circulate among different wild bird species in this area. Similarly all NA subtypes except N5 and N9 were isolated. The most common HA subtypes isolated were H1 (eight viruses; H1N1, H1N2), H6 (seven viruses; H6N1), H7 (seven viruses; H7N3, H7N6, H7N7), H11 (six viruses; H11N8, H11N2, H11N6), H3 (five viruses; H3N8), H8 (four viruses; H8N4, H8N2), H10 (four viruses; H10N7, H10N4), and H9 (three viruses; H9N2). Only one virus was isolated from each of the other HA subtypes (H5, H12, H13, H15, and H16).

Most of the viruses (49 isolates) were isolated from Anseriformes and only four viruses from Charadriiformes. No AIV was isolated from any of the other six orders sampled. For the different seasons, the virus prevalence, based on virus isolation, varied from 0.42% to 1.83%. The average prevalence for the whole year was 1.46%. The greatest number of AIVs were isolated during the winter (36 isolates; 1.83%), and during the autumn migration, when 14 AIVs were detected (1.45%). In contrast, only three AIVs (0.42%) were isolated from wild birds during spring migration, nesting, and postnesting movements.

These results on virus isolations may be related to geographic and climatic features of the Azov and Black Sea regions of Ukraine, which determine the abundance and diversity of bird species. Thus, according to ornithologists, the largest concentration of wild birds in a short period of time in a limited area, especially Anseriformes and Charadriiformes from different geographical regions of Siberia, Asia, and the northern part of Eurasia, happens during the autumn migration and wintering. This underlines the importance of this season and region in the ecology of viruses such as AIV and APMV. Studies like this can further our understanding of the circulation of viruses in wild birds and predict possible introductions of viruses into other geographic regions. As for the spring migration, nesting, and postnesting season, the number of birds is much lower. Therefore, in our opinion, this season is not as significant as the autumn and winter seasons for the spread of viruses.

The HA and NA genes of the H5 or N1 viruses from this study were sequenced as well as some earlier H5N1 viruses from the Institute's repository. The H5 HA genes clustered within two lineages (Fig. 2). One lineage included A/teal/Djankoy/40-17-11/2010, the most recent H5 isolate (H5N2), and was most closely related to wild bird LPAIVs found in wild birds in Europe. The other two H5 isolates collected earlier, A/great created grebe/Sevastopol/11-173/2008 (H5N1) and A/cormorant/Strilkova/Kherson/63/2006 (H5N1), were most closely related to the clade 2.2 Asian H5N1 HPAIV lineage that spread from the Qinghai Lake outbreak into Europe and Northern Asia (3). The N1 genes followed a similar pattern where the earlier H5N1 isolates, A/great created grebe/Sevastopol/11-173/2008 and A/cormorant/Strilkova/Kherson/63/2006, were closely related to the Qinghai Lake lineage. The N1 NAs from the more recent isolates, A/white fronted goose/Volodino/14-24-12/2010 H6N1, A/wild duck/Mevedkovo/3-24-12/2010 H10N1, and A/mallard/Novomyhalivka/14/-23-12/2010 H10N1, appear to be most closely related to LPAIVs from Europe (Fig. 3). This demonstrates that these regions of Ukraine are ecologically connected with Europe and underlines the importance of this region as an area of convergence of different flyways, also noted in a previous study (6).

Wild bird surveillance studies were previously conducted in Ukraine from 2006 to 2008 in the Crimea region (6). Compared with these studies, we identified a larger number of influenza virus subtypes H6, H7, H8, and H11 and a smaller number of H3 and H4 viruses. This confirms that even between years, different results might be obtained in the same region. It is therefore very important to continue the monitoring of wild birds at the intersection of migration routes. Similar to other wild bird surveillance studies, most of our viruses were isolated from Anseriformes and Charadriiformes, with the highest isolation rates from mallards. Isolation rates vary widely among wild bird surveillance studies, but the highest rates, which were observed in the autumn, were consistent with previous studies of waterfowl from North America (10). On the other hand, the lowest rates of virus isolation are typically seen in wintering birds in both Europe and North America, unlike what was observed in this study.

REFERENCES

1. Capua, I., and D. J. Alexander. Avian influenza and Newcastle Disease: a field and laboratory manual. Springer, New York. 2009.

2. Chen, H., G. D. J. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. M. Peiris, and Y. Guan. H5N1 virus outbreak in migratory waterfowl: a worrying development could help to spread this dangerous virus beyond its stronghold in southeast Asia. Nature 436:191–192. 2005.

3. Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J. Virol. 80:5976–5983. 2006.

4. Drummond, A. J., and A. Rambaut. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7:214. 2007.

5. [O.I.E.] World Organisation for Animal Health. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals 2011. World Organisation for Animal Health, Paris. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf. 2012.

6. Kulak, M. V., F. A. Ilinykh, A. V. Zaykovskaya, A. V. Epanchinzeva, I. L. Evstaphiev, N. N. Tovtunec, K. A. Sharshov, A. G. Durimanov, N. A. Penkovskaya, A. M. Shestopalov, A. I. Lerman, I. G. Drozdov, and D. E. Swayne. Surveillance and identification of influenza A viruses in wild aquatic birds in the Crimea, Ukraine (2006–2008). Avian Dis. 54:1086–1090.

7. Muzyka, D. V., B. T. Stegniy, V. O. Busol, A. N. Rula, K. F. Mayorova, and S. V. Homenko. Epizootic situation concerning highly-pathogenic avian influenza (H5N1) among wild birds in Ukraine. Vet. Med. 87:128–138. 2006.

8. Pedersen, J. C. Neuraminidase-inhibition assay for the identification of influenza A virus neuraminidase subtype of neuraminidase antibody specificity. In: Avian influenza viruses: methods in molecular biology. E. Spackman, ed. Humana Press, Totowa, NJ. pp. 67–75. 2008.

9. Stallknecht, D. E. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. Fouth International Symposium on Avian Influenza. 61–69. 1998.

10. Stallknecht, D. E., and J. D. Brown. Ecology of avian influenza in wild birds. In: Avian influenza. D. E. Swayne, ed. Blackwell, Ames, IA. pp. 43–58. 2008.

11. Stegniy, B. T., A. I. Buzun, D. V. Muzyka, M. V. Miroshnichenco, E. N. Belyavtseva, N. M. Pasyuga, and S. M. Kuzemko. Influenza of natural water reservoirs factors on the rise of highly pathogenic avian influenza in AR Crimea. Vet. Med. 87:186–192. 2006.

12. Suarez, D. L., M. Garcia, J. Latimer, D. Senne, and M. Perdue. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. J. Virol. 73:3567–3573. 1999.

13. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. J. R. Glisson, Y. M. Saif, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell, Ames, Iowa. pp. 153–184. 2008.

14. Swayne, D. E., and M. Pantin-Jackwood. Pathobiology of avian influenza virus infections in birds and mammals. In: Avian influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA. pp. 87–122. 2008.

15. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152–179. 1992.

16. Stegniy, B. T., I. Yu. Bisuk, A. P. Gerilovych, A. I. Buzun, and D. V. Muzyka. Analysis of epizootic situation concerning highly pathogenic avian influenza areas on the territory of the AR Crimea, isolation and identification of the agent. Vet. Med. Ukraine 1:14–17. 2007.

17. Chernichko, I. I., and V. A. Kostyushin, eds. Results of the regional ornithological monitoring (Azov–Black Sea coast of Ukraine). Bulletin ROM 6 (October). 24 p. 2010.

ACKNOWLEDGMENTS

The authors express their sincere thanks to ornithologists R. M. Chernichko, I. I. Chernichko, Y. A. Andryushchenko, and V. M. Popenko for help in catching birds and identifying bird species as well as providing relevant information. This research was funded by USDA Project P444 through the Science and Technology Center in Ukraine (STCU).

Virus Genes
DOI 10.1007/s11262-011-0696-3

Comparative full genome analysis of four infectious laryngotracheitis virus (*Gallid herpesvirus-1*) virulent isolates from the United States

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Received: 1 October 2011 / Accepted: 28 November 2011
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Abstract *Gallid herpesvirus-1* (GaHV-1), commonly named infectious laryngotracheitis (ILT) virus, causes the respiratory disease in chickens known as ILT. The molecular determinants associated with differences in pathogenicity of GaHV-1 strains are not completely understood, and a comparison of genomic sequences of isolates that belong to different genotypes could help identify genes involved in virulence. Dideoxy sequencing, 454 pyrosequencing and Illumina sequencing-by-synthesis were used to determine the nucleotide sequences of four genotypes of virulent strains from GaHV-1 groups I–VI. Three hundred and twenty-five open reading frames (ORFs) were compared with those of the recently sequenced genome of the Serva vaccine strain. Only four ORFs, ORF C, U_L37, ICP4 and U_S2 differed in amino acid (aa) lengths among the newly sequenced genomes. Genome sequence alignments were used to identify two regions (5′ terminus and the unique short/repeat short junction) that contained deletions. Seventy-eight synonymous and 118 non-synonymous amino acid substitutions were identified with the examined ORFs. Exclusive to the genome of the Serva vaccine strain, seven non-synonymous mutations were identified in the predicted translation products of the genes encoding glycoproteins gB, gE, gL and gM and three non-structural proteins U_L28 (DNA packaging protein), U_L5 (helicase-primase) and the immediate early protein ICP4. Furthermore, our comparative sequence analysis of published and newly sequenced GaHV-1 isolates has provided evidence placing the cleavage/packaging site (*a*-like sequence) within the inverted repeats instead of its placement at the 3′ end of the U_L region as annotated in the GenBank's entries NC006623 and HQ630064.

Keywords Infectious laryngotracheitis · *Gallid herpesvirus-1* · DNA sequence · Virulence · Attenuation

Electronic supplementary material The online version of this article (doi:10.1007/s11262-011-0696-3) contains supplementary material, which is available to authorized users.

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Introduction

Infectious laryngotracheitis (ILT) is a respiratory disease of chickens occurring worldwide and is caused by *Gallid herpesvirus-1* (GaHV-1) [18]. This virus of the genus *Iltovirus* in the *Alphaherpesvirinae* subfamily of *Herpesviridae* [5] is responsible for the highly contagious disease characterized by acute respiratory symptoms that include gasping, coughing,

Published online: 16 December 2011

sneezing, depression, nasal discharge and conjunctivitis [17]. The disease is largely controlled through mass vaccination via aerosol or drinking water, with live attenuated strains that have been attenuated by multiple passages in either embryonated eggs [chicken embryo origin (CEO)] or tissue culture [tissue culture origin (TCO)] [11, 46]. To date, differentiating vaccine strains from virulent field isolates has involved (i) restriction fragment length polymorphisms of PCR products (multilocus PCR–RFLP analyses) [4, 31, 40, 43] and (ii) limited nucleotide sequence analysis [19, 40]. Overall, U.S. strains can be grouped into nine genotypes (I–IX) according to Oldoni et al. [43]. In a recent study in the U.S., 63% of field isolates from commercial farms were shown to be closely related to the CEO vaccine strains (groups IV and V) [18]. Because of this, it is believed that vaccine strains in North America have reverted to virulence and that these revertants have become established in the poultry population, capable of re-emerging as non-attenuated CEO-related viral isolates [42]. Moreover, in Europe, a study of strains collected over 35 years showed that 94% (98/104) of EU strains were also related to ILT vaccines [40].

The molecular biology of GaHV-1 has recently been reviewed [9, 27]. The GaHV-1 genome was initially characterized using restriction endonuclease analysis [22, 29, 38] and was shown to contain a type D arrangement with two unique regions (U_L and U_S) with the U_S region flanked by inverted repeat sequences. In the late 1980s, Sanger-based DNA sequencing data of GaHV-1 became publicly available [13–16]. However, it was not until 2006 that the first complete genomic sequence of GaHV-1 was assembled by Thureen and Keeler [48] from overlapping genomic contigs of different strains analyzed in six different laboratories [6–8, 10, 13–16, 23–26, 30, 33–36, 44, 53–55]. Recently, Lee et al. [37] have determined the complete *non-composite* nucleotide sequence of the European CEO Serva vaccine strain of GaHV-1 and reported significant differences in the lengths of 12 ORFs in comparison with those published for the composite sequence. To expand on this and investigate the potential genetic basis for GaHV-1 pathogenicity, full genome sequences of four virulent U.S. isolates, including the USDA standard challenge strain (group I genotype), broiler breeder isolate 81658 (group III genotype), broiler isolate 63140 (group V genotype) and broiler isolate 1874C5 (group VI genotype), were obtained, and a comparative sequence analysis of these with the genome of the Serva vaccine strain was performed.

Materials and methods

Virus and cells

The GaHV-1 strain used in this study (USDA reference strain, obtained from the National Veterinary Services Laboratory, Ames, IA, USA) was propagated on confluent monolayers of primary chicken embryo fibroblast cells (CEF). Fibroblasts were isolated from 11-day-old chicken embryos by the warm trypsinization methods [48] and plated in Dulbecco's modified essential medium supplemented with penicillin (50 μg/ml), streptomycin (50 μg/ml) and 8% fetal bovine serum. Group III strain 81658 previously genotyped as closely related to tissue culture origin (TCO) vaccine was obtained from a 60-week-old non-vaccinated broiler breeder flock with severe signs of the disease, where males vaccinated with the TCO vaccine (Schering-Plough Animal Health, Omaha, NE, USA) were introduced 51 weeks prior to the outbreak. GaHV-1 isolate 63140 (group V), previously genotypes as closely related to the CEO vaccine, was obtained in 2006 from an unvaccinated broiler flock in Georgia with signs of severe upper respiratory disease and high mortality. GaHV-1 group VI strain 1874C5 was obtained in 2005 from an unvaccinated broiler flock in Delaware—as with previous isolates the flock showed severe respiratory disease and high mortality. All isolates were propagated in chicken kidney (CK) cells as described previously by Tripathy and Garcia [49]. Virus DNA from cells infected with strains USDA reference and 63140 was purified using the modified Hirt's supernatant protocol [20]. The micrococcal nuclease/polyethylene glycol procedure [50] was used to isolate nucleocapsid DNA from strains 81658, 63140 and 1874C5.

Determination of the TR_S/U_L junction in GaHV-1 genomic concatamers

Standard PCR was used to amplify the TR_S/U_L junction sequence using the forward primer GATTCTGTGATC GCGCTTCGAGG (isolate 63140 nucleotide coordinates 153217–153239) and reverse primer TGCTTGCCTGCA TATACCGGATGC (isolate 63140 nucleotide coordinates 273–296) and Hirt's supernatant DNA of strain 63140 as the template. High-fidelity platinum Taq polymerase (Invitrogen, Carlsbad CA) was used in standard amplification reactions containing 2 mM Magnesium, 1× PCR buffer and 62°C annealing temperatures. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The nucleotide sequence of the PCR product was determined using standard Sanger-based sequencing.

High-throughput DNA sequencing

Sequencing of the viral genomes was accomplished using three DNA sequencing technologies: Sanger dideoxy, 454 pyrosequencing and Illumina sequence-by-synthesis. The USDA reference strain was sequenced using dideoxy chain terminator sequencing chemistry from pUC19 libraries of

cloned random DNA fragments (>1.0 Kb) generated by incomplete enzymatic digestions with either *Taq*I or *Aci*I restriction endonucleases (New England BioLabs, Beverly, MA). Plasmid DNA was purified by alkaline lysis according to the manufacturer's instructions (Eppendorf 5 Prime, Boulder CO) and sequenced from both ends with M13 forward and reverse primers using dideoxy chain termination and the ABI PRISM 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA).

Strain 63140 was initially sequenced commercially on a pyrosequencing platform, the Genome Sequencer 20 (GS20) system (454 Life Sciences). This involved the construction of random libraries from 5.0 micrograms of nucleocapsid-purified DNA using the methodology described by Margulies et al. [39]. Subsequently, strain 1874C5 and strain 81658 were sequenced using the Illumina HISeq 2000 sequencing platform (Illumina Inc., San Diego, CA) at two different facilities. Isolate 1874C5 was sequenced at the University of Delaware Sequencing and Genotyping Center in Newark, DE, and isolate 81658 was sequenced at the Genome Services Lab at Hudson Alpha Institute for Biotechnology (Huntsville, Alabama). Sequencing of both isolates was carried out on a HiSeq2000, running HiSeq Control Software (HCS) v1.4.8. Single-read genomic (1874C5) DNA libraries were prepared from 5 μg of partially purified genomic DNA that was sheared by nebulization (1874C5) or sonication (81658) to an average length of 200 bp. The samples were processed according to Illumina Library Creation kit's instructions and sequenced on one lane of a HiSeq Flowcell v1.5. Libraries were clustered on a cBot v1.4.36.0 using Illumina's Truseq PE Cluster Kit v2.0 and sequenced using a 200 cycle TruSeq SBS HS v2 kit. The clustered flow-cell was sequenced for 206 cycles and broken down into 3 separate reads. The first read was 100 cycles in length, followed by a six-cycle index read. Following the index read, paired end re-synthesis was performed also using Truseq PE Cluster Kit v2.0, which was then followed by another 100 cycles. Image analysis and base calling were performed using the standard Illumina Pipeline consisting of Real time Analysis (RTA) version v1.12.4.2 and Casava v1.8 with default settings.

GaHV-1 DNA sequence analysis

The genome of the USDA reference strain was assembled with the Phrap and CAP3 software programs (http://www.phrap.org) [21], and sequence gap closure was achieved by primer walking of gap-spanning clones and sequencing of PCR products. PCR products were generated using a battery of oligonucleotides and Platinum Taq polymerase (Life Technologies, Carlsbad, CA).

The 63140 genome was assembled from 28,100 reads using GS De Novo Assembler (Roche, Indianapolis, IN), Sequencher (Gene Codes, Ann Arbor, MI) and the Mira Assembler [2]. Ambiguities in the sequencing data and problematic regions containing mononucleotide reiterations which mapped to the existing 5,454 scaffolds using Mira were resolved through resequencing using Illumina technology. The complete genomic sequence of 63140 therefore represents hybrid assembly from two sequencing technologies. The sequence of strain 81658 was assembled from the Illumina dataset using Mira in de novo assembly mode, and the sequence of 1874C5 was determined by read alignment to the published ILTV reference sequence using Genome Studio (Illumina) and CLC Genomics Workbench (CLC bio, Cambridge, MA).

All genomic sequences were maintained and analyzed using Lasergene (DNASTAR, Madison, WI), NCBI Entrez and other web-based tools. Homology searches were conducted using the NCBI program blastP with default settings. Multiple alignments of proteins and nucleotide sequences were generated using MAFFT [28], Multalin [3] and SCAN2 (Softberry.com).

Results and discussion

Sequencing, coverage and assembly

Genomic sequencing using classical Sanger dideoxy chemistry of individual clones enabled the generation of a consensus sequence of 137,997 bp after de novo assembly of the GaHV-1 USDA reference strain. This included the genomic subregions U_L, TR_L U_S and 800 bp of the U_S/TR_S junction. The TR_S subregion was generated by reverse complementing the TR_L sequencing data. Final DNA consensus sequence represented, on average, 10-fold redundancy at each base position, with a Consed estimated error rate of less than 0.01 error per 10 kbp [12]. Using 454 pyrosequencing, 58,924 reads were generated from strain 63140 with a mean length of 249 bp, of which 44,398 reads aligned to the ILTV genome. The assembled sequence represented approximately 77-fold coverage at each base pair, but sequence coverage was uneven with a number of gaps. Illumina sequencing was used to fill these gaps as well as resolve homopolymer stretches ambiguous in the 454 data. 7,817,890 100 mer Illumina reads aligned to the 63140 genome for an average fold coverage of 5,363. A similar average fold coverage of 5,759 was obtained from the assembly of 8,370,909 reads for the 81658 genome. With an average read length of 41.5 nucleotides (147,530 reads), the 6,122,687 nucleotides of 1874C5 sequence generated by Illumina technology represented a 41-fold coverage of the genome. The comparatively low 1874C5 coverage corresponded to the concentration of the viral DNA in the initial preparation. Eighty-five percent of the

Illumina reads represented chicken chromosomal DNA sequence. The GenBank accession numbers for the GaHV-1 genomes are as follows: 1874C5 (JN542533), USDA reference strain (JN542534), 81658 (JN542535) and 63140 (JN542536).

Genomic organization

The genomes of the USDA reference strain (group I), 81658 (group III), 63140 (group V) and 1874C5 (group VI) were determined to be 151769, 150335, 153633 and 150691 nucleotides in length. These sizes were comparable to the lengths of the composite genome (148,687 bp) and the genome of the Serva vaccine strain (152,630 bp). To further characterize these differences in genomic lengths, the sizes of the genomic subregions unique long [U_L], internal repeat short [IR_S], unique short [U_S] and terminal repeat short [TR_S]) were defined (Table 1). The unique long (U_L) sequences were 109,580 (USDA reference strain), 109,575 (81658), 112,915 (63140) and 113,030 (1874C5) bp in length and contained two 102-bp inverted repeats separated by a 726-bp sequence (Fig. 1) encoding microRNA miR-l1-5p and miR-l1-3P [45]. Flanking the unique long were two inverted repeat sequences (ATTCGAAACCCCTCGCG and CGCGAGGGGTTTCGAAT) of 17 nucleotides in length. Although this would qualify the GaHV-1 genome as a class E genome, only two isomers of the genome have been reported [22, 38].

The unique short (U_S) and flanking inverted regions (IR), respectively, were 13,095 and 14,547 (USDA reference strain), 13,094 and 13,833 (81658), 13,094 and 13,812 (63140) and 13,094 and 12,283/12,284 (1874C5) nucleotides in length. The Serva genome is annotated to contain the largest unique long region (113,930 bp) with relatively short inverted repeats (12,803 bp) and a standard length U_S region (13,094 bp) (Table 1). The average length of the GaHV-1 genomes sequenced in this report is 151,607 bp in length with the lengths of the U_S genomic subregions to be the most conserved among all GaHV-1 genomes.

Interestingly, the genome of the Serva strain, sequenced using the SOLiD^{TM} system and assembled based on the composite sequence, contains a truncated terminal repeat short (TR_S) region. A 1,015-bp sequence annotated to be at the 3′ end of the unique long of the Serva genome, when reverse-complemented, mapped to the end (TR_S) of the linear genomes of all four virulent viruses. Alignment of the reads using 454 pyrosequencing clearly defines the ends of the linear GaHV-1 genome since all the data are flush with the 3′ end. Furthermore, the sequencing data of the ICP4 genes of the GaHV-1 strains SA-2 and CSW-1 (accession numbers U27243 and U27244) are in full agreement with our definition of the GaHV-1 genomic termini [24]. Therefore, these data suggest that the genomic subregion (U_L) of the Serva may not be 113,930 bp but rather 112,915 nucleotides in length, and the inverted repeats may not be 12,803 bp but rather 13,818 bp for a genomic unit length of 153,645 bp rather than 152,630 bp as previously indicated [37] (Table 1).

To experimentally define the 3′ terminus of the GaHV-1 genome, forward and reverse oligonucleotides were used in an amplification reaction with DNA isolated from infected CK cells using the Hirt's supernatant protocol. The forward primer was specific for sequences in the disputed 1,015-bp sequence, while the reverse primer was specific for the 5′ terminus of the unique long. Since a PCR product of approximately 700 bp (Fig. 2c) was amplified, this clearly demonstrated that the 1,015-bp sequence is present at the 3′ end of the TR_S region and not at the 3′ end of the U_L region as annotated in the Serva genome. Sequencing of the PCR product (Fig. 2d) revealed that an extra thymidine residue is present between the sequences at the 3′ terminus (TR_S) and the 5′ terminus of the U_L in concatameric GaHV-1 (63140) genomes. A comparison of the termini sequences of GaHV-1, including cleavage and packaging sites, relative to sequences found at the ends of other herpesvirus genomes is presented in Fig. 3.

Open reading frames within the genomes of virulent GaHV-1 strains

The genomes of the newly sequenced virulent strains of GaHV-1 contain over 300 ORFs that are at least 60 amino acids in length. Each predicted translation products of these 325 ORFs were analyzed using NCBI BLAST program to determine the percentage similarity to other proteins found in the GenBank data base. Of these, only 80 ORFs showed

Table 1 Comparison of the lengths of the genomes and genomic subregions of the *Gallid herpesvirus-1* strains USDA (group I), 80658 (group III), 63140 (group V), 1874C5 (group VI) and the Serva vaccine strain

Genome	UL	IRS	US	TRS	Total
USDA	109,580	14,547	13,095	14,547	151,769
81658	109,575	13,833	13,094	13,833	150,335
63140	112,915	13,812	13,094	13,812	153,633
1874C5	113,030	12,283	13,094	12,284	150,691
Averages	111,275	13,619	13,094	13,619	151,607
Serva	113,930	12,803	13,094	12,803	152,630

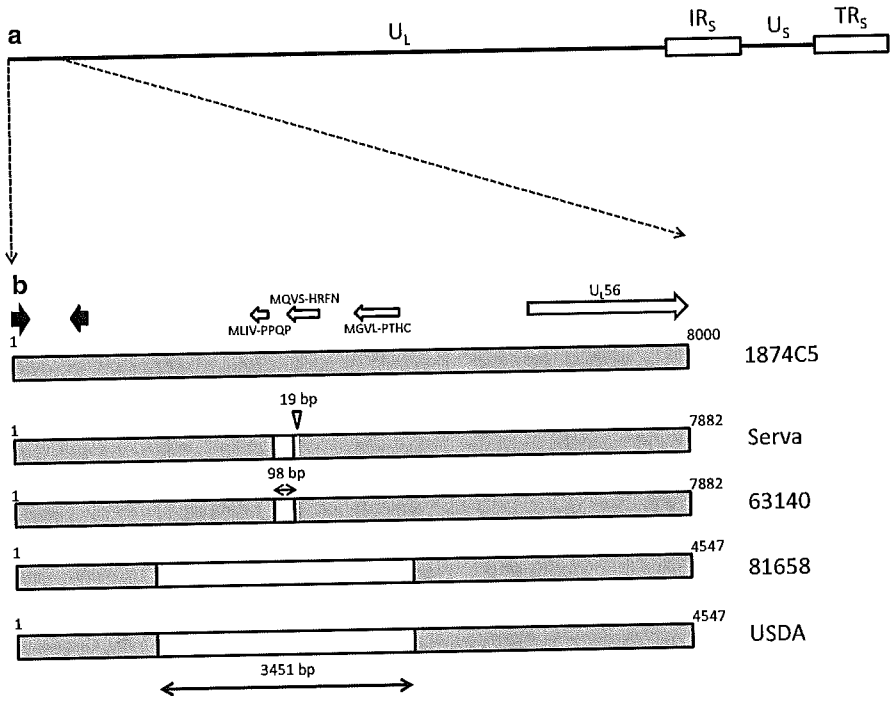

Fig. 1 Schematic alignments of the 5′ terminus of five Gallid herpesvirus 1 genomes. **a** The D-type organization of the GaHV-1 genome as described previously. **b** The locations of the two 102-bp inverted repeats (*black arrows*) and the U_L56 ORF (*open arrow*). Three short ORFs MLIV-PPQP, MQVS-HRFN and MGVL-PTHC are shown in *open gray arrows*. The ORFs are named according to their first and last four amino acids. The positions of the 98-bp deletions in the Serva (after nucleotide 3,265) and 63140 (after nucleotide 3,264) genomes and the larger 3,451-bp deletions within the genomes of 81658 and USDA after nucleotide positions 2,153 and 2,155, respectively, are shown as *open white boxes*. The smaller 19-bp downstream deletions (CCCTTTTTAACAAAAACAG) after nucleotide positions 3,459 and 3,458 of the Serva and 63,140 genomes, respectively, are shown as *white lines*

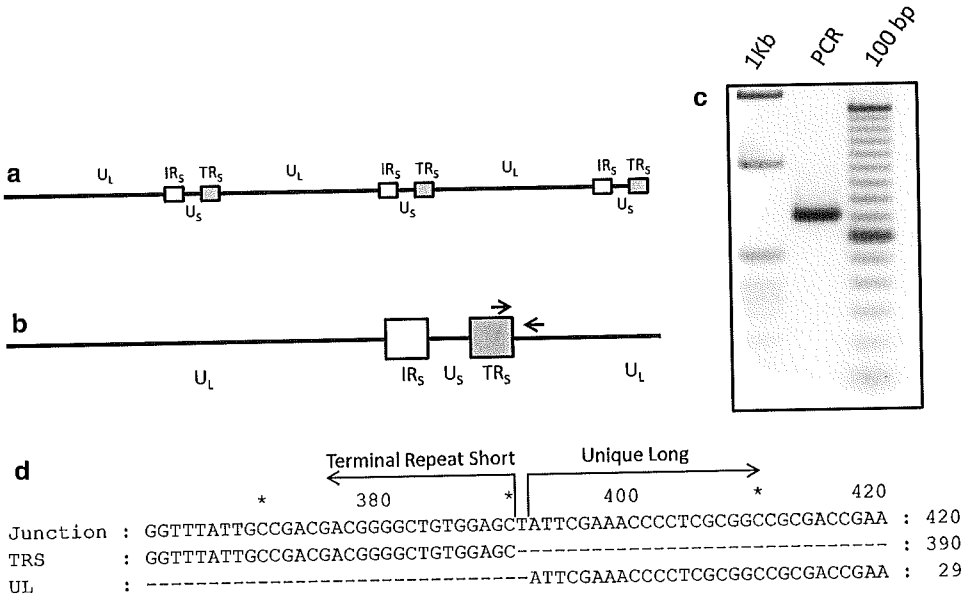

Fig. 2 Amplification scheme used to define the 3′ terminus of the GaHV-1 genome. **a** Cartoon of the concatameric GaHV-1 genomes. **b** The location of the forward and reverse oligonucleotides used in the amplification reaction (PCR) with templates prepared from Hirt's supernatants. **c** Visualization of the PCR product in an ethidium bromide–stained 1.5% agarose gel. **d** The nucleotide sequence at the junction between the terminal repeat short (TR_S) and the unique long (U_L)

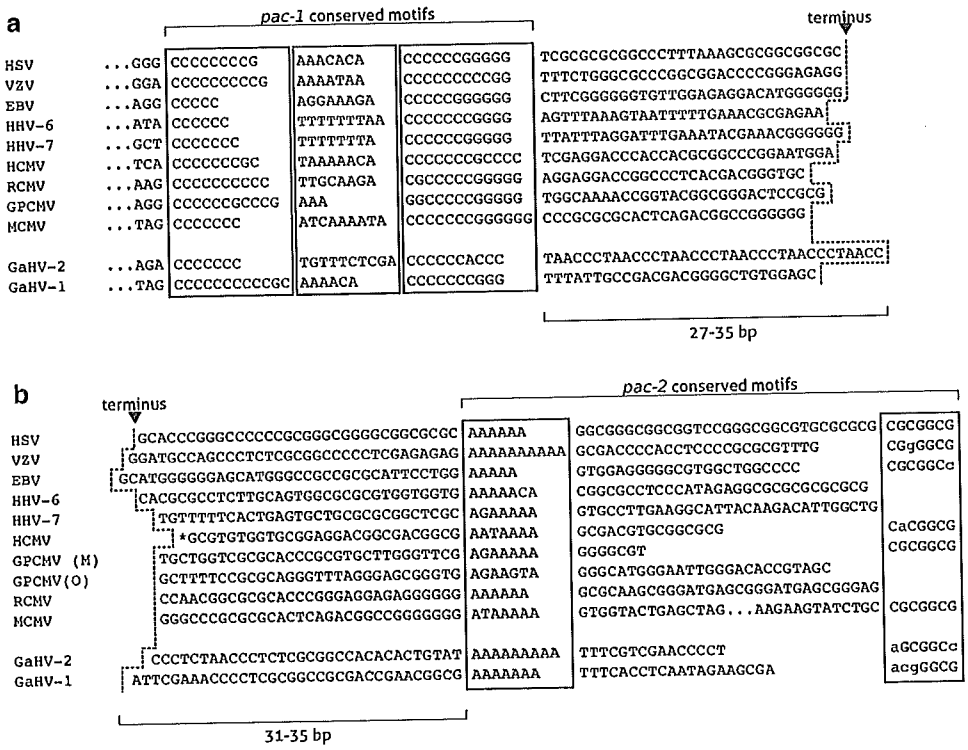

Fig. 3 The proposed cleavage/packaging sequence of GaHV-1 aligned with *a*-like sequences of other alpha-, gamma- and beta-herpesviruses. Domains conserved among members of the family *Herpesviridae* are *boxed*. **a** Alignments of *pac-1* sites at the 3′ terminus of *Herpesviridae* genomes. **b** Alignments of the *pac-2* sites at the 5′ terminus of *Herpesviridae* genomes. HSV (Herpes simplex virus-1 or *Human herpesvirus-1*), VZV (Varicella-zoster virus or *Human herpesvirus-3*), EBV (Epstein-Barr virus or *Human herpesvirus-4*), HHV-6 (*Human herpesvirus-6*), HHV-7 (*Human herpesvirus-7*), HCMV (Human cytomegalovirus or *Human herpesvirus-5*), RCMV (Rhesus cytomegalovirus or *Macacine herpesvirus-3*) GPCMV (Guinea pig cytomegalovirus or *Caviid herpesvirus-2*), MCMV (Mouse cytomegalovirus or *Murid herpesvirus-1*), GaHV-2 (*Gallid herpesvirus-2* or Marek's disease virus-1) and GaHV-1 (*Gallid herpesvirus-1 or* Infectious laryngotracheitis virus)

significant sequence similarity to protein sequence found in GenBank. No novel ORFs with significant similarity to protein sequences within GenBank were discovered other than those already described by Thureen and Keeler [48]. Although the number of genes within the GaHV-1 genome has been established since 2006, discrepancies in the lengths of some ORF have been reported by Spatz et al. in 2010 [47] and confirmed by Lee [37]. Differences in the size of predicted translation products of 18 genes were identified between the composite genome and the genomes of the four virulent strains (Table 2). The lengths of groups I, II, V and VI ORFs are all in agreement with their orthologues found within the Serva genome, except for the reported sequence length heterogeneity of the ICP4 ORF [1, 24, 52].

In the analysis of the GaHV-1 ORFs within the four sequenced genomes, only four ORFs (ORF C, U_L37, U_S2 protein kinase and ICP4) differed among the U.S. virulent isolates and Serva vaccine strain (Table 2). A truncated variant of ORF C (303 aa) is encoded within the genome of group III (81658). This variant is missing the last 31 amino acids due to a cytosine to thymidine mutation at nucleotide position 913 resulting in the introduction of an ocher stop codon. Also, within the group III genome, three nucleotides CTA are missing for the UL37 gene after nucleotide position 1,383. This in-frame deletion of the codon for leucine occurs in a region containing four CTA codons. Within the U_S2 gene of the USDA reference strain, an extra thymidine base after nucleotide position 638 is predicted to cause a reading frame shift resulting in a truncated U_S2 protein of 217 amino acids, while all other U_S2 genes of GaHV-1 are predicted to encode a 229-amino-acid protein. The lengths of ORFs 66 and 80 encoding ICP4 vary among virulent U.S. isolates and the Serva vaccine strain: 1,482 bp (Serva and 93140), 1,486 bp (USDA and 81658) and 1,492 bp (1874C5) (Table 2, Fig. 4).

Insertions and deletions (INDELS)

To investigate the presence of INDELS within the genomes of virulent GaHV-1 strains, a multiple sequence alignment was generated using the web-based alignment program MAFFT [28]. Sequence heterogeneity was localized within two genomic subregions (U_L and IR): specifically the

Table 2 Summary of ORFs that differ in lengths between the published composite sequence (GenBank # NC006623) and the sequences of genomes of the *Gallid herpesvirus-1* strains Serva, USDA, 81658, 63140 and 1874C5

Gene	Region	Homolog	Composite	USDA	81658	1874C5	63140	Serva
ORF1	U_L	U_L56	**284**	274	274	274	274	274
ORF2	U_L	ORF F	**752**	746	746	746	746	746
ORF3	U_L	U_L54	**420**	544	544	544	544	544
ORF4	U_L	U_L53	**337**	339	339	339	339	339
ORF15	U_L	ORFC	334	334	**303**	334	334	334
ORF21	U_L	U_L25	**572**	592	592	592	592	592
ORF26	U_L	U_L29	**999**	1,176	1,176	1,176	1,176	1,176
ORF27	U_L	U_L30	**1,008**	1,092	1,092	1,092	1,092	1,092
ORF28	U_L	U_L31	**339**	314	314	314	314	314
ORF33	U_L	U_L36	**2,556**	2,784	2,784	2,784	2,784	2,784
ORF34	U_L	U_L37	**890**	1,022	**1,021**	1,022	1,022	1,022
ORF35	U_L	U_L38	**412**	482	482	482	482	482
ORF36	U_L	U_L39	**785**	786	786	786	786	786
ORF38	U_L	U_L41	**398**	399	399	399	399	399
ORF39	U_L	U_L42	**432**	436	436	436	436	436
ORF40	U_L	U_L43	**395**	394	394	394	394	394
ORF 66	IRs	RS1	**1,463**	**1,486**	**1,486**	**1,492**	**1,482**	**1,482**
ORF69	Us	US2	229	**217**	229	229	229	229
ORF80	TRs	RS1	**1,463**	**1,486**	**1,486**	**1,492**	**1,482**	**1,482**

Bold denotes amino acid lengths that differ among the majority of the open reading frames

diploid ICP4 loci near the IR/U_S junctions and the 5′ end of the unique long region. As shown in Fig. 4, INDELS map throughout the inverted repeats. Heterogeneity within the ORFs encoding ICP4 of the four GaHV-1 strains has been previously described [1, 52]. The ICP4 ORF of the composite genome, which was based on the sequencing data of Johnson et al. [24], is 1,463 aa in length. The size of the ICP4 ORF within the Serva vaccine genomes is 1,482 aa [37]. We now report two additional lengths of 1,486 (USDA and 81658) and 1,492 (1874C5) aa. These size differences can be attributed to three in-frame indels. The sequence AAGAACCGCAACAACCGC is only present at the 3′ end of the ICP4 gene within the 1874C5 genome and is deleted after nucleotide positions 149,368, 146,076, 149,371 and 147,501 within the genomes of 63140, 81658, Serva and USDA, respectively, while an additional sequence (GCGGCCCAAGAC) is missing from the genomes of 63140 and Serva. The deletion of the second sequence occurs after nucleotide position 147,069 within the Serva genome and after nucleotide position 147,066 in the 63140 isolate's genome. The sequence AGATT is present after nucleotide position 4,380 in the ICP4 gene of the Australian vaccine strain SA-2 as sequenced by Johnson et al. [24] and results in a premature termination yielding a truncated form of ICP4. In the analysis of 28 full-length GenBank sequences of ICP4 genes from various GaHV-1 strains, the most common length was 1,482 aa, occurring in 42.8% of the sequences examined, while 1,492, 1,486 and 1,463 length variants comprised 28.6, 25 and 3.6% of the total sequences examined, respectively. The variant encoding the 1,463 ICP4 appears to be unique to the SA-2 strain and was used in the composite genome sequence [48]. Since neither the SA-2 genome nor its ICP4 gene has been resequenced, it is not known whether this is true variation or a sequencing artifact.

In addition to the small in-frame deletions within the ICP4 genes, two large deletions were identified between the promoters of the ICP4 and U_S10 genes (Fig. 4, white boxes). The largest deletion of 1,582 bp was identified upstream of the U_S10 promoter in the 1874C5 genome starting after nucleotide position 141,322. Within the genome of the USDA reference strain, a small deletion of 143 bp (after nucleotide position 141,578) was identified which overlaps the 3′ region of the 1,582-bp deletion mapped within the 1874C5 genome. Surprisingly, these deletions are within the 280-bp hairpin structure comprising the origin of DNA replication (OriS) as first identified by Walter Fuchs (GenBank number AM238250). Although PCR products of approximately 556 bp could be amplified and cloned using flanking primers (GGTTGCTCATGTGAGAAGACAATG ACC and AATACGCTCTTGCGACAGACCGTGG) with USDA and 1874C5 DNA (data not shown), numerous attempts to sequence these clones using Sanger-based sequencing failed to resolve the 280-bp palindrome. This region was also absent in the composite sequence as reported by Thureen and Keeler [48]. However, since PCR products could be generated and cloned, this region is likely present in the USDA and 1874C5 genomes, but difficult to sequence due to its thermostability (dG values of −190.87, −190.03, −190.67 and −185.45 kcal/mol for AM238250, Serva,

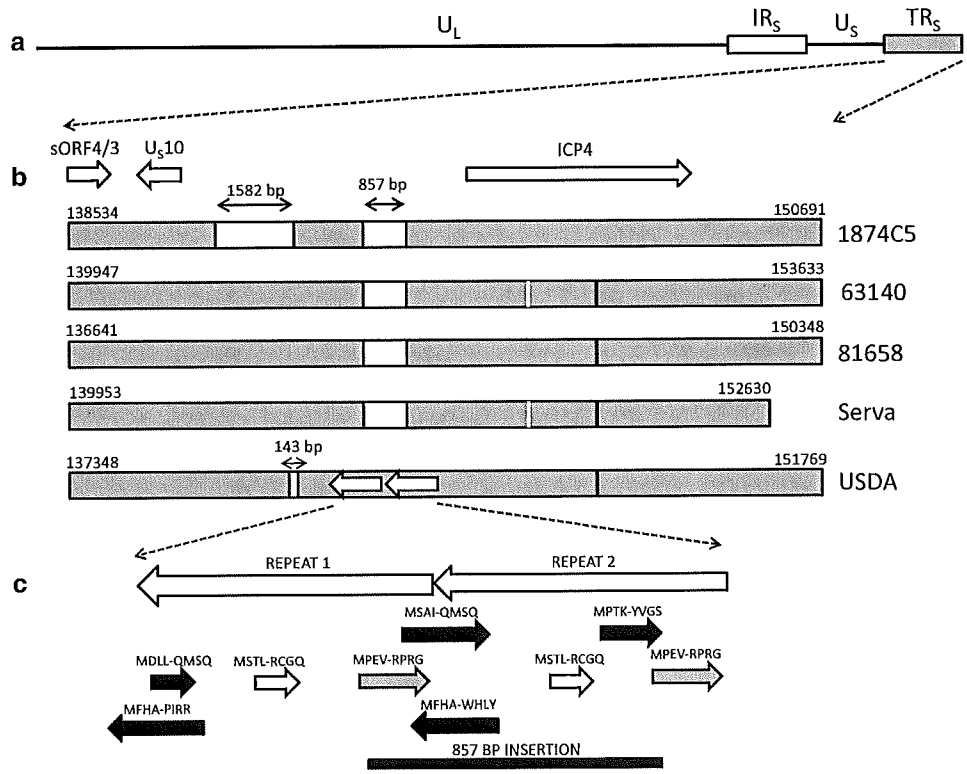

Fig. 4 Schematic alignments of the 3′ terminus of five Gallid herpesvirus 1 genomes. **a** The D-type organization of the GaHV-1 genome showing the unique long (U_L) region and the internal repeat short, (IR_S) and terminal repeat short (TR_S) regions bracketing the unique short (U_S) region. **b** The location of three genes encoding sORF4/3, U_S10 and ICP4 (*open gray arrows*). The positions of the 1,582-, 857- and 143-bp deletions are denoted by *open white boxes*. Two near-perfect direct repeats of 855 and 857 bp within the USDA genome are denoted by *open white arrows*. The location of small in-frame deletions within the ICP4 genes of 63140, 81658, Serva and USDA is marked by *vertical black* (AAGAACCGCAACAACCGC) or *white* (GCGGCCCAAGAC) *lines*. **c** ORFs within the repeat regions. The ORFs are named according to their first and last four amino acids. ORFs that are duplicated are *shaded* identically

81658 and 63140). A multiple sequence alignment of the OriS within the inverted repeat regions is shown in Fig. 5. Within the stem regions of the hairpin structures, base pair substitutions occur only when matched with complementary nucleotides on the reverse strand. Only the loop region contains mismatched nucleotides.

A smaller 857-bp deletion upstream of the ICP4 promoter region was identified in the genomes of 1874C5, 63140, 81658 and Serva after nucleotide positions 143,133, 146,109, 142,801 and 146,112, respectively (Fig. 4b). This deletion mapped to a region in the USDA genome containing two near-perfect direct repeats of 855 and 857 bp. Four single nucleotide polymorphisms and a 2-bp insertion in the larger 857-bp repeat differentiate the two repeats. Several small novel ORFs map within these repeats. The MPTK-YVGS novel ORF is solely present in the 857-bp repeat due to a 2-bp deletion in the 855-bp repeat immediately downstream of the start codon (Fig. 4c). These repeats have been found in varying copy numbers in other sequenced GaHV-1 strains [9, 53] and represent an expansion region similar to the 132-bp repeat region found in the GaHV-2 genome [41].

The largest deletions identified in this study mapped to the 5′ terminus of two GaHV-1 genomes in a region devoid of large open reading frames (ORFs) and microRNAs (Fig. 1). These 3,451-bp deletions within the genomes of 81658 and USDA initiate after nucleotide positions 2,153 and 2,155, respectively. Similarly, a large deletion of 3,575 bp relative to the Australian vaccine strain SA-2 has been reported in the genome of the virulent GaHV-1 strain A489 by Johnson and others [25]. Three short ORFs (MLIV-PPQP, MQVS-HRFN and MGVL-PTHC of 83, 143 and 199 aa, respectively), which are absent in the 81658 and USDA genomes, map to this region within the 1874C5 genome. BLAST analysis of these ORFs however failed to reveal any meaningful similarity to known proteins. Lastly, two smaller deletions of 98 and 19 bp also mapped to this general region within the genomes of the Serva and 63140 strains of GaHV-1.

Single nucleotide polymorphisms

In order to identify amino acid changes associated with attenuation and/or virulence, the nucleotide sequence of the

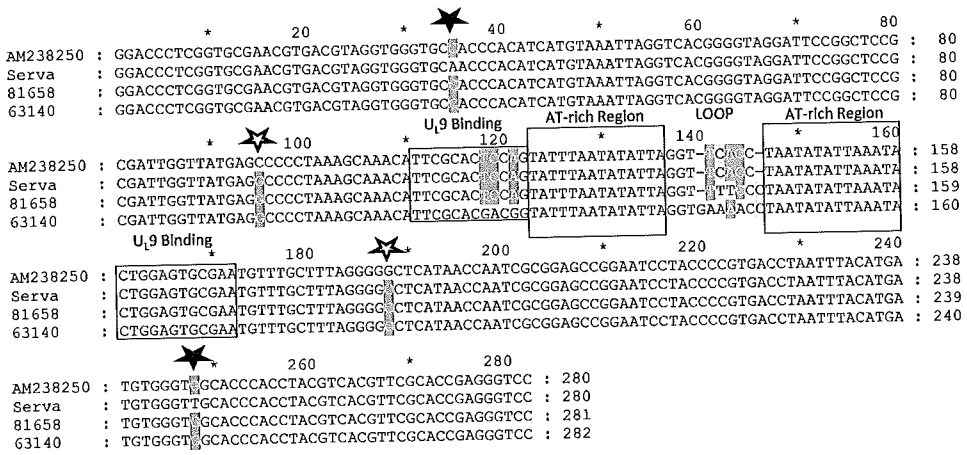

Fig. 5 Alignment of the sequences comprising the near-perfect hairpin structure at the origin of DNA replication (OriS) within the inverted repeat regions of AM238250, Serva, 81658 and 63140. The AT-rich regions are boxed as well as domains likely to be involved in the binding of the U_L9 protein. Base pair substitutions with matched complementary nucleotides are indicated by *open* and *closed stars*

vaccine strain Serva was aligned to nucleotide sequences of the four sequenced virulent strains (USDA, 81658, 1874C5 and 63140) using the web-based programs, Multalin and MAFFT. Over 190 single nucleotide polymorphisms (SNPs) were identified in the predicted translation products. Of these, 76 occurred within coding regions without amino acid substitutions (synonymous). Relative to codon used in the Serva genes, 39, 49, 13, and 27 synonymous substitutions were identified in the corresponding genes within the USDA, 1874C5, 63140 and 81685 genomes, respectively (Sup. 1). A list of the 118 non-synonymous mutations is presented in Table 3. Non-synonymous substitutions exclusively found in the Serva genome were identified in the genes U_L27-, U_L1-, U_L10-, U_S8-encoded glycoproteins B, L, M and E, respectively, and the non-structural proteins were involved in DNA packaging (U_L28), DNA replication (U_L5 helicase-primase) and the immediate early protein ICP4. The most significant substitutions that are likely to affect structure and/or function are $Thr^{42} \rightarrow Ala^{42}$ (U_L10 encoding gM), $Lys^{343} \rightarrow Glu^{343}$ (U_L5 encoding the helicase-primase protein) and $Gln^{141} \rightarrow Pro^{141}$ (U_L1 encoding gL).

Conclusions

Sequence analysis of the genomes of four virulent GaHV-1 isolates and their comparison within the composite and Serva vaccine strain genomes generated an ORF consensus for 80 genes (out of 325 examined) that showed homology to well-characterized alphaherpesvirus genes and unique GaHV-1 genes previously described [9]. Sixty ORFs had amino acid lengths identical to those found in the composite sequence, while 18 differed. Excluding the composite sequence, only four ORFs were predicted to encode proteins that differed in amino acid lengths among the newly sequenced genomes. This study has identified differences in the lengths of the ORFs encoding the transcriptional activator ICP4 (1,482, 1,486 and 1,492 aa), ORF C (303 and 334 aa), U_L37 (1021 and 1022 aa) and U_S2 protein kinase (217 and 229 aa). Relative to the group VI genome (1874C5), 3,451-bp deletions at the 5′ terminus were identified in genotype groups I USDA and III 81658 genomes, while smaller deletions of 98 bp were identified in the same region of the Serva vaccine and virulent genotype group V 63140. Relative to the group I genome (USDA), 857 bp were missing from the genomes of Serva and groups III, V and VI isolates. Within the genomes of the groups I and VI, deletions within the inverted repeats mapped to the origin of replication. These deletions are likely due to the inability to resolve highly structured regions containing inverted repeats using Sanger sequencing. The lack of the 5′ proximal deletions in the Serva and the virulent groups V 63140 genomes, and the 857-bp deletions of both the vaccine strain and virulent isolates suggest that neither changes in these areas of the genome are related to virulence.

In a comparison with the predicted translation products of >80 ORF within the genomes of groups I, III, V, VI and the Serva vaccine strain, 78 synonymous and 118 non-synonymous amino acid substitutions were identified. A total of seven non-synonymous amino acid substitutions in U_L27, U_L1, U_L10 and U_S8 genes encoding glycoproteins gB, gL, gM and gE, respectively, and three non-structural proteins (U_L28, U_L5 and ICP4) genes were identified that could differentiate the Serva vaccine strain from all four GaHV-1 virulent isolates collectively. In particular, the surface glycoprotein changes may reflect the different geographical origin of the Serva strain (Europe) and the

Table 3 Single nucleotide polymorphisms resulting in non-synonymous amino acid substitutions in ORFs within the genomes of the GaHV-1 strains Serva, USDA, 81658, 63140 and 1874C5

	Non-synonymous				
	Serva	USDA	1874C5	63140	81685
ORF U_L56					
Trp190 Pro190	tgg	*cca*	tgg	tgg	*cca*
ORF F					
Ser78 Thr78	tca	tca	tca	*aca*	tca
Cys271 Tyr271	tgt	*tat*	tgt	tgt	*tat*
ORF U_L54					
Tyr50 Cys50	tat	*tgt*	tat	tat	*tgt*
ORF U_L52					
Ala167 Glu167	gcg	*gag*	gcg	gcg	*gag*
Thr263 Ala263	aca	*gca*	aca	aca	*gca*
Tyr527 Ser527	tat	*tct*	tat	tat	*tct*
ORF U_L50					
Phe320 Leu320	ttc	ttc	*ctc*	ttc	ttc
ORF U_L46					
Tyr327 Asp327	tac	tac	tac	tac	*gac*
ORF A					
Pro126 Ser126	ccc	ccc	*tcc*	ccc	ccc
ORF B					
Tyr2 His2	tat	*cat*	tat	tat	*cat*
Cys83 Arg83	tgc	tgc	*cgc*	tgc	tgc
Glu129 Asp129	gag	gag	*gat*	gag	gag
ORF E					
Gly133 Ala133	ggc	ggc	ggc	*gcc*	ggc
Gln245 Arg245	caa	caa	*cga*	caa	caa
Leu405 Arg405	ctg	*cgg*	ctg	ctg	*cgg*
ORF U_L22					
Leu568 Ser568	tta	tta	*tca*	tta	tta
ORF U_L23					
Arg97 Trp97	cgg	cgg	cgg	*tgg*	cgg
Asp212 Asn212	gac	gac	*aac*	gac	gac
ORF U_L26					
Gln179 Arg179	cag	cag	cag	*cgg*	cag
Ile210 Met210	att	att	*atg*	att	att
ORF U_L27					
Val116 Ala116	**gtc**	*gcc*	*gcc*	*gcc*	*gcc*
Met348 Thr348	atg	atg	atg	atg	*acg*
Arg496 His496	cgc	cgc	cgc	*cac*	cgc
Ile644 Thr644	ata	*aca*	*aca*	*aca*	ata
Pro799 Ser799	ccc	ccc	*tcc*	ccc	ccc
Lys805 Arg805	aag	aag	*aga*	aag	aag
Glu809	gag	gag	gag	*N/A*	gag
ORF U_L28					
Glu363 Gly363	gaa	*gga*	gaa	gaa	gaa
Gln374 Arg374	caa	caa	caa	caa	*cga*
Ile509 Thr509	att	*act*	*act*	att	att

Table 3 continued

	Non-synonymous				
	Serva	USDA	1874C5	63140	81685
Val638 Ala638	**gtc**	*gcc*	*gcc*	*gcc*	*gcc*
Asp735 Gly735	gac	gac	gac	*ggc*	gac
ORF U_L29					
Gly905 Asp905	ggc	ggc	ggc	ggc	*gac*
ORF U_L30					
Arg91 Gly91	aga	*gga*	*gga*	aga	*gga*
Ala312 Val312	gcc	*gtc*	*gtc*	gcc	*gtc*
Ala578 Val578	gcg	gcg	*gtg*	gcg	gcg
Glu1010 Lys1010	gaa	gaa	*aaa*	gaa	gaa
Thr1011 Leu1011	act	act	*ctt*	act	act
Ser1012 Leu1012	tct	tct	*ctt*	tct	tct
Leu1013 Met1013	ttg	ttg	*atg*	ttg	ttg
ORF U_L36					
Asp84 Ala84	gac	gac	*gcc*	gac	gac
Ser359 Gly359	agc	agc	agc	*ggc*	agc
ORF U_L10					
Thr42 Ala42	**acc**	*gcc*	*gcc*	*gcc*	*gcc*
Pro248 Ser248	cct	cct	*tct*	cct	cct
ORF U_L9					
Val10 Ala10	gtc	gtc	*gcc*	gtc	gtc
ORF U_L6					
Ala258 Thr258	gcc	gcc	*acc*	gcc	gcc
Glu693 Lys693	gaa	gaa	*aaa*	gaa	gaa
ORF U_L5					
Lys343 Glu343	**aaa**	*gaa*	*gaa*	*gaa*	*gaa*
Ala391 Thr391	gca	gca	gca	gca	*aca*
ORF U_L1					
Pro8 Gln8	ccg	*cag*	ccg	ccg	*cag*
Gln141 Pro141	**cag**	*ccg*	*ccg*	*ccg*	ccg
ORF U_L0					
Thr18 Ala18	acg	*gcg*	acg	acg	*gcg*
Val117 Ala117	gtt	*gct*	gtt	gtt	*gct*
Pro153 Ser153	cca	*tca*	cca	cca	cca
Ser273 Phe273	tcc	tcc	*ttc*	tcc	tcc
Leu341 Pro341	cta	cta	*cca*	cta	cta
Thr378 Ala378	aca	*gca*	aca	aca	aca
ORF ilt240					
Gly2 Asp2	ggt	*gat*	*gat*	ggt	*gat*
Asn11 Ile11	aac	*atc*	*atc*	aac	*atc*
Arg18 Cys18	cgc	*tgc*	*tgc*	cgc	*tgc*
Leu24 Pro24	ctt	*cct*	*cct*	ctt	*cct*
Asn40 Lys40	aat	*aaa*	*aaa*	aat	*aaa*
Lys41 Gln41	aag	*cag*	*cag*	aag	*cag*
ORF ilt239					
Phe6 Ile6	ttt	*att*	*att*	ttt	*att*
Stop10 Cys10	tga	*tgc*	*tgc*	tga	*tgc*

Table 3 continued

	Non-synonymous				
	Serva	USDA	1874C5	63140	81685
ORF ilt244					
His21 Tyr21	cac	*tac*	cac	cac	cac
Asp40 Val40	gat	*gtt*	gat	gat	gtt
ORF ilt246					
Ala37 Val37	gcg	gcg	*gtg*	gcg	gcg
ORF ilt247					
Gly3 Trp3	ggg	*tgg*	ggg	ggg	*tgg*
ORF ilt248					
His6 Pro6	cat	cat	*cct*	cat	cat
Gln16 Arg16	cag	cag	*cgg*	cag	cag
ORF ilt249					
Leu26 Trp26	ttg	ttg	*N/A*	ttg	*tgg*
ORF U_S10					
Met136 Ile136	atg	atg	atg	atg	*ata*
ORF U_S3					
Ala99 Thr99	gct	*act*	gct	gct	*act*
Lys291 Thr291	aag	aag	*acg*	aag	aag
ORF sORF1					
Gly279 Asp279	ggc	ggc	ggc	ggc	*gac*
ORF U_S4					
His98 Asn98	cac	cac	*aac*	cac	cac
Val115 Gly115	gtg	gtg	*ggg*	gtg	gtg
Gly220 Leu220	ggt	ggt	*ctt*	ggt	ggt
ORF U_S5					
Arg162 Trp162	cgg	*tgg*	cgg	cgg	*tgg*
Val293 Ala293	gtg	*gcg*	gtg	gtg	*gcg*
Ala340 Thr340	gcc	gcc	gcc	*acc*	gcc
Ala684 Thr684	gcc	gcc	*acc*	gcc	gcc
ORF U_S7					
Ala178 Asp178	gct	gct	*gat*	gct	gct
ORF U_S8					
Lys210 Arg210	**aaa**	*aga*	*aga*	*aga*	*aga*
Thr386 Ala386	acc	*gcc*	acc	acc	*gcc*
ORF U_S9 (8A?)					
Thr91 Ile91	aca	aca	*ata*	aca	aca
Gly115 Asp115	ggc	ggc	*gac*	ggc	ggc
ORF ICP4					
Ala267 Thr267	gcc	gcc	*acc*	gcc	gcc
Gln414 His414	cag	cag	*cat*	cag	cag
Arg618 Pro618	cgc	cgc	*ccg*	cgc	cgc
Ser716 Thr716	agc	agc	*acg*	agc	agc
Asp743 Asn743	aac	*gac*	*gac*	aac	aac
Arg781 His781	**cac**	*cgc*	*cgc*	*cgc*	*cgc*
Gln860 Arg860	caa	caa	*caa/cga*	caa	caa
Ser876 Tyr876	tct	tct	*tct/tat*	tct	tct
Ala1131 Val1131	gtg	*gcg*	*gcg*	gtg	*gcg*
Pro1292 Leu1292	ctg	*ccg*	*ccg*	ctg	*ccg*

Table 3 continued

	Non-synonymous				
	Serva	USDA	1874C5	63140	81685
Gly1328 Arg1328	agg	*ggg*	agg	agg	*ggg*
Gly1329 Asp1329	gac	*ggc*	gac	gac	gac
Pro1423 Leu1423	ccc	ccc	*ctc*	ccc	ccc
Thr1437 Ser1437	tcc	*acc*	tcc	tcc	*acc*

All Serva ORFs encoding amino acid of lengths >60 were examined for non-synonymous amino acids substitutions in comparison with homologous ORFs found within the genomes of the four virulent strains. The list includes 80 ORFs as defined by Lee et al. within the Serva genome as well as seven additional short ORFs (ilt239, ilt240, ilt244, iltv246, ilt247, ilt248 and ilt249, located between the ICP4 and U_S10 genes) that contained non-synonymous substitutions. Codons in bold are exclusive for Serva genes and not used in the corresponding genes in the genomes of the virulent isolates. Codons that differ relative to the Serva codons are italicized

U.S. virulent isolates, while difference in the U_L28, U_L5 and ICP4 genes may be related to the attenuation of the Serva strain or virulence of the genotypes I, III, V and VI U.S. isolates. Interestingly, no mutations were found in regions that encode microRNAs [32, 45, 51]. The identification of both intragenic and intergenic deletions and single nucleotide polymorphism mutations that differentiate the Serva vaccine genome from the genomes of four virulent isolates will further advance our understanding of genes involved in virulence.

Acknowledgments We thank Robert Mullis for his contributions in the bioinformatic analysis of the sequencing data. This research was funded by the United States Department of Agriculture CRIS program (project number 6612-32000-052) and the US Poultry and Egg Association (Project F032).

References

1. P.C. Chang, Y.L. Lee, J.H. Shien, H.K. Shieh, Rapid differentiation of vaccine strains and field isolates of infectious laryngotracheitis virus by restriction fragment length polymorphism of PCR products. J. Virol. Methods **66**, 179–186 (1997)
2. B. Chevreux, T. Wetter, S. Suhai, Genome sequence assembly using trace signal and additional sequence information. Comput. Sci. Biol. GCB **1**, 45–56 (1999)
3. F. Corpet, Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**, 10881–10890 (1988)
4. J.L. Creelan, V.M. Calvert, D.A. Graham, S.J. McCullough, Rapid detection and characterization from field cases of infectious laryngotracheitis virus by real-time polymerase chain reaction and restriction fragment length polymorphism. Avian Pathol. **35**, 173–179 (2006)
5. A.J. Davison, R. Eberle, G.S. Hayward, D.J. McGeoch, A.C. Minson, P.E. Pellet, B. Roizman, M.J. Studdert, E. Thiry, Family Herpesviridae, in *Virus Taxonomy: Eight Report of the International Committee on Taxonomy of Viruses*, ed. by C.M. Fauquet,

M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Academic Press, San Diego, 2005), pp. 193–212
6. W. Fuchs, T.C. Mettenleiter, DNA sequence and transcriptional analysis of the UL1 to UL5 gene cluster of infectious laryngotracheitis virus. J. Gen. Virol. **77**(Pt 9), 2221–2229 (1996)
7. W. Fuchs, T.C. Mettenleiter, DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL10 gene product as a nonglycosylated and nonessential virion protein. J. Gen. Virol. **80**(Pt 8), 2173–2182 (1999)
8. W. Fuchs, T.C. Mettenleiter, The nonessential UL49.5 gene of infectious laryngotracheitis virus encodes an *O*-glycosylated protein which forms a complex with the non-glycosylated UL10 gene product. Virus Res. **112**, 108–114 (2005)
9. W. Fuchs, J. Veits, D. Helferich, H. Granzow, J.P. Teifke, T.C. Mettenleiter, Molecular biology of avian infectious laryngotracheitis virus. Vet. Res. **38**, 261–279 (2007)
10. W. Fuchs, K. Ziemann, J.P. Teifke, O. Werner, T.C. Mettenleiter, The non-essential UL50 gene of avian infectious laryngotracheitis virus encodes a functional dUTPase which is not a virulence factor. J. Gen. Virol. **81**, 627–638 (2000)
11. E. Gelenczei, E. Marty, Strain stability and immunologic characteristics of tissue-culture modified infectious laryngotracheitis virus. Avian Dis. **9**, 44–56 (1962)
12. D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing. Genome Res. **8**, 195–202 (1998)
13. A.M. Griffin, The complete sequence of the capsid p40 gene from infectious laryngotracheitis virus. Nucleic Acids Res. **18**, 3664 (1990)
14. A.M. Griffin, Identification of 21 genes of infectious laryngotracheitis virus using random sequencing of genomic DNA. J. Gen. Virol. **70**(Pt 11), 3085–3089 (1989)
15. A.M. Griffin, The nucleotide sequence of the glycoprotein gB gene of infectious laryngotracheitis virus: analysis and evolutionary relationship to the homologous gene from other herpesviruses. J. Gen. Virol. **72**(Pt 2), 393–398 (1991)
16. A.M. Griffin, M.E. Boursnell, Analysis of the nucleotide sequence of DNA from the region of the thymidine kinase gene of infectious laryngotracheitis virus; potential evolutionary relationships between the herpesvirus subfamilies. J. Gen. Virol. **71**(Pt 4), 841–850 (1990)
17. J.S. Guy, T.J. Bagust, Laryngotracheitis, in *Disease of Poultry*, 11th edn., ed. by Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, D.E. Swayne (Iowa State Press, Ames, 2003), pp. 121–134
18. J.S. Guy, M. Garcia, *Infectious Laryngotracheitis Virus*, 12th edn. (Wiley-Blackwell, Hoboken, 2008)
19. M.G. Han, S.J. Kim, Analysis of Korean strains of infectious laryngotracheitis virus by nucleotide sequences and restriction fragment length polymorphism. Vet. Microbiol. **83**, 321–331 (2001)
20. B. Hirt, Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**, 365–369 (1967)
21. X. Huang, A. Madan, CAP3: a DNA sequence assembly program. Genome Res. **9**, 868–877 (1999)
22. M.A. Johnson, C.T. Prideaux, K. Kongsuwan, M. Sheppard, K.J. Fahey, Gallid herpesvirus 1 (infectious laryngotracheitis virus): cloning and physical maps of the SA-2 strain. Arch. Virol. **119**, 181–198 (1991)
23. M.A. Johnson, C.T. Prideaux, K. Kongsuwan, S.G. Tyack, M. Sheppard, ICP27 immediate early gene, glycoprotein K (gK) and DNA helicase homologues of infectious laryngotracheitis virus (gallid herpesvirus 1) SA-2 strain. Arch. Virol. **140**, 623–634 (1995)
24. M.A. Johnson, S.G. Tyack, C. Prideaux, K. Kongsuwan, M. Sheppard, Nucleotide sequence of infectious laryngotracheitis virus (gallid herpesvirus 1) ICP4 gene. Virus Res. **35**, 193–204 (1995)
25. M.A. Johnson, S.G. Tyack, C.T. Prideaux, K. Kongsuwan, M. Sheppard, Nucleotide sequence of the left-terminus of infectious laryngotracheitis virus (Gallid herpesvirus 1) SA-2 strain. Arch. Virol. **142**, 1903–1910 (1997)
26. M.A. Johnson, S.G. Tyack, C.T. Prideaux, K. Kongsuwan, M. Sheppard, Sequence characteristics of a gene in infectious laryngotracheitis virus homologous to glycoprotein D of herpes simplex virus. DNA Seq. **5**, 191–194 (1995)
27. R.C. Jone, Viral respiratory diseases (ILT, aMPV infections, IB): are they ever under control? Br. Poult. Sci. **51**, 1–11 (2010)
28. K. Katoh, K. Kuma, H. Toh, T. Miyata, MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. **33**, 511–518 (2005)
29. C.L. Keeler Jr., J.W. Hazel, J.E. Hastings, J.K. Rosenberger, Restriction endonuclease analysis of Delmarva field isolates of infectious laryngotracheitis virus. Avian Dis. **37**, 418–426 (1993)
30. D.H. Kingsley, J.W. Hazel, C.L. Keeler Jr, Identification and characterization of the infectious laryngotracheitis virus glycoprotein C gene. Virology **203**, 336–343 (1994)
31. N.C. Kirkpatrick, A. Mahmoudian, D. O'Rourke, A.H. Noormohammadia, Differentiation of infectious laryngotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. Avian Dis. **50**, 28–34 (2006)
32. B.W. Kong, Identification of virus encoding microRNAs using 454 FLX sequencing platform. Methods Mol. Biol. **733**, 81–91 (2011)
33. K. Kongsuwan, M.A. Johnson, C.T. Prideaux, M. Sheppard, Identification of an infectious laryngotracheitis virus gene encoding an immunogenic protein with a predicted M(r) of 32 kilodaltons. Virus Res. **29**, 125–140 (1993)
34. K. Kongsuwan, M.A. Johnson, C.T. Prideaux, M. Sheppard, Use of lambda gt11 and monoclonal antibodies to map the gene for the 60, 000 dalton glycoprotein of infectious laryngotracheitis virus. Virus Genes **7**, 297–303 (1993)
35. K. Kongsuwan, C.T. Prideaux, M.A. Johnson, M. Sheppard, K.J. Fahey, Nucleotide sequence of the gene encoding infectious laryngotracheitis virus glycoprotein B. Virology **184**, 404–410 (1991)
36. K. Kongsuwan, C.T. Prideaux, M.A. Johnson, M. Sheppard, S. Rhodes, Nucleotide sequence analysis of an infectious laryngotracheitis virus gene corresponding to the US3 of HSV-1 and a unique gene encoding a 67 kDa protein. Arch. Virol. **140**, 27–39 (1995)
37. S.W. Lee, P.F. Markham, J.F. Markham, I. Petermann, A.H. Noormohammadi, G.F. Browning, N.P. Ficorilli, C.A. Hartley, J.M. Devlin, First complete genome sequence of infectious laryngotracheitis virus. BMC Genomics **12**, 197 (2011)
38. D.A. Leib, J.M. Bradbury, C.A. Hart, K. McCarthy, Genome isomerism in two alphaherpesviruses: Herpesvirus saimiri-1 (Herpesvirus tamarinus) and avian infectious laryngotracheitis virus. Brief Report. Arch. Virol. **93**, 287–294 (1987)
39. M. Margulies, M. Egholm, W.E. Altman, S. Attiya, J.S. Bader, L.A. Bemben, J. Berka, M.S. Braverman, Y.J. Chen, Z. Chen, S.B. Dewell, L. Du, J.M. Fierro, X.V. Gomes, B.C. Godwin, W. He, S. Helgesen, C.H. Ho, G.P. Irzyk, S.C. Jando, M.L. Alenquer, T.P. Jarvie, K.B. Jirage, J.B. Kim, J.R. Knight, J.R. Lanza, J.H. Leamon, S.M. Lefkowitz, M. Lei, J. Li, K.L. Lohman, H. Lu, V.B. Makhijani, K.E. McDade, M.P. McKenna, E.W. Myers, E. Nickerson, J.R. Nobile, R. Plant, B.P. Puc, M.T. Ronan, G.T. Roth, G.J. Sarkis, J.F. Simons, J.W. Simpson, M. Srinivasan, K.R. Tartaro, A. Tomasz, K.A. Vogt, G.A. Volkmer, S.H. Wang, Y. Wang, M.P. Weiner, P. Yu, R.F. Begley, J.M. Rothberg, Genome sequencing in microfabricated high-density picolitre reactors. Nature **437**, 376–380 (2005)
40. C.J. Neff, C. Sudler, R.K. Hoop, Characterisation of western European field isolates of avian infectious laryngotracheitis virus by restriction fragment length polymorphisms and sequencing. Avian Dis. **52**, 278–283 (2008)

41. M. Niikura, J.B. Dodgson, H.H. Cheng, Stability of Marek's disease virus 132-bp repeats during serial in vitro passages. Arch. Virol. **151**, 1431–1438 (2006)
42. D. Ojkic, J. Swinton, M. Vallieres, E. Martin, J. Shapiro, B. Sanei, B. Binnington, Characterization of field isolates of infectious laryngotracheitis virus from Ontario. Avian Pathol. **35**, 286–292 (2006)
43. I. Oldoni, A. Rodriguez-Avila, S. Riblet, M. Garcia, Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP). Avian Dis. **52**, 59–63 (2008)
44. D.J. Poulsen, C.R. Burton, J.J. O'Brian, S.J. Rabin, C.L. Keeler Jr, Identification of the infectious laryngotracheitis virus glycoprotein gB gene by the polymerase chain reaction. Virus Genes **5**, 335–347 (1991)
45. R. Rachamadugu, J.Y. Lee, A. Wooming, B.W. Kong, Identification and expression analysis of infectious laryngotracheitis virus encoding microRNAs. Virus Genes **39**, 301–308 (2009)
46. Y. Samberg, I. Aronovici, The development of a vaccine against avian infectious laryngotracheitis I. Modification of a laryngotracheitis virus. Refug. Vet. **26**, 54–59 (1969)
47. S.J. Spatz, M. Garcia, C.L. Keeler Jr., C.L. Afonso, E. Mundt, S. Riblet, L. Zsak, D.L. Rock, in Presented at the 5th international workshop on the molecular pathogenesis of Marek's disease virus and 1st symposium on avian herpesviruses, Athens, GA, USA.
48. D.R. Thureen, C.L. Keeler Jr, Psittacid herpesvirus 1 and infectious laryngotracheitis virus: comparative genome sequence analysis of two avian alphaherpesviruses. J. Virol. **80**, 7863–7872 (2006)
49. D.N. Tripathy, M. García, Laryngotracheitis, in *Isolation and Identification of Avian Pathogens*, 5th edn., ed. by D.E. Swayne, J.R. Glisson, M.W. Jackwood, W.M. Reed (American Association of Avian Pathologist, University of Pennsylvania, New Bolton Center, 2008), pp. 94–98
50. J.D. Volkening, S.J. Spatz, Purification of DNA from the cell-associated herpesvirus Marek's disease virus for 454 pyrosequencing using micrococcal nuclease digestion and polyethylene glycol precipitation. J. Virol. Methods **157**, 55–61 (2009)
51. L.A. Waidner, J. Burnside, A.S. Anderson, E.L. Bernberg, M.A. German, B.C. Meyers, P.J. Green, R.W. Morgan, A microRNA of infectious laryngotracheitis virus can downregulate and direct cleavage of ICP4 mRNA. Virology **411**, 25–31 (2011)
52. W. Wanasawaeng, N. Chansiripornchai, Molecular classification of infectious laryngotracheitis virus from chick embryo origin vaccine, tissue culture origin vaccine and field isolates. Thai J. Vet. Med. **40**, 393–398 (2010)
53. M.A. Wild, S. Cook, M. Cochran, A genomic map of infectious laryngotracheitis virus and the sequence and organization of genes present in the unique short and flanking regions. Virus Genes **12**, 107–116 (1996)
54. K. Ziemann, T.C. Mettenleiter, W. Fuchs, Gene arrangement within the unique long genome region of infectious laryngotracheitis virus is distinct from that of other alphaherpesviruses. J. Virol. **72**, 847–852 (1998)
55. K. Ziemann, T.C. Mettenleiter, W. Fuchs, Infectious laryngotracheitis herpesvirus expresses a related pair of unique nuclear proteins which are encoded by split genes located at the right end of the UL genome region. J. Virol. **72**, 6867–6874 (1998)

Chapter 9
Avian Astroviruses

Mary Pantin-Jackwood, Daniel Todd, and Matthew D. Koci

Abstract Avian astroviruses comprise a diverse group of viruses affecting many avian species and causing enteritis, hepatitis, and nephritis. To date, six different astroviruses have been identified in avian species based on the species of origin and viral genome characteristics: two turkey-origin astroviruses [Turkey Astrovirus type 1 (TAstV-1) and type 2 (TAstV-2)]; two chicken-origin astroviruses [Avian Nephritis Virus (ANV) and Chicken Astrovirus (CAstV)]; and two duck-origin astrovirus [Duck Astrovirus type 1 (DAstV-1) and type 2 (DAstV-2)]. ANV has also been detected in turkeys, ducklings, pigeons, and guinea fowl; and TAstrovirus-2-like viruses have also been found in guinea fowl. Astroviruses are commonly associated with enteric disease syndromes in poultry including runting-stunting syndrome of broilers (RSS), poult enteritis complex or syndrome (PEC or PES), poult enteritis mortality syndrome (PEMS), and enteritis in guinea fowl. The molecular characterization of the different avian astroviruses shows great genetic variability among each type, and this variability influences the ability to detect these viruses by molecular and serological techniques. In this chapter, we review the different aspects related to avian astroviruses, including molecular biology, pathogenesis, diagnosis, and control.

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S. Schultz-Cherry (ed.), *Astrovirus Research: Essential Ideas, Everyday Impacts, Future Directions*, DOI 10.1007/978-1-4614-4735-1_9,
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Introduction

Avian astroviruses comprise a diverse group of viruses affecting many avian species and causing enteritis, hepatitis, and nephritis. Reports of astroviruses as cause of illness in birds predate that of mammals. In 1965, a fatal hepatitis was described in ducklings [2] and was eventually shown to be caused by an astrovirus [22, 23]. Another astrovirus, avian nephritis virus (ANV), was first isolated in 1976 and was associated with growth depression and kidney lesions in young chickens. Initially classified as an *Enterovirus*, it was later reclassified as an astrovirus following the complete sequencing of the viral genome [30]. To date, six different astroviruses have been identified in avian species based on the species of origin and viral genome characteristics: two turkey-origin astroviruses [Turkey Astrovirus type 1 (TAstV-1) and type 2 (TAstV-2)]; two chicken-origin astroviruses [ANV and Chicken Astrovirus (CAstV)]; and two duck-origin astrovirus [Duck Astrovirus type1 (DAstV-1) and type 2 (DAstV-2)]. ANV has also been detected in turkeys [18, 70], ducklings [6], pigeons [45, 116], and guinea fowl [8]; and TAstV-2-like viruses also have been found in guinea fowl [9, 10].

As in mammals, astroviruses are commonly associated with enteritis in avian species. Enteric diseases cause substantial economic losses in commercial poultry, and many enteric disease syndromes have been described including runting-stunting syndrome of broilers (RSS), poult enteritis complex or syndrome (PEC or PES), and poult enteritis mortality syndrome (PEMS). Astroviruses are among the most common viruses found in cases of PEC, PES, and PEMS in turkeys [2, 3, 34–37, 42, 68, 69, 75, 76, 79, 81, 99]. Astroviruses have also been isolated from cases of RSS in broilers and have been previously associated with poor weight gain, enteric disease, and kidney disease in chickens [4, 15, 27, 83, 85, 86, 105], as well as in guinea fowl suffering from enteritis [10].

Astroviruses were found in turkeys in the UK in 1980 by McNulty et al. [56], and later in the 1980s, in the USA (TAstV-1) [75, 79, 80]. A second TAstV, antigenically and genetically distinct from the previously described isolates, was later characterized [44] and this and similar astroviruses have been designated TAstV-2 [70, 71, 98]. Beyond this classification, data from genetic analyses of field viruses detected in turkeys indicate the circulation of multiple lineages of TAstV-2 [9, 18, 33, 70, 94].

Avian nephritis virus has also been shown to be widely distributed in chicken flocks [30, 53, 70, 103]. Under field conditions, clinical signs associated with infection with this virus broiler chickens have varied from subclinical to outbreaks of RSS and baby chick nephropathy [26]. A second type of astrovirus affecting chickens was shown to be antigenically and genetically distinct from ANV and was named chicken astrovirus (CastV, 4). CAstVs that share high levels of nucleotide sequence identity with this first characterized CAstV have been detected in the USA in broiler chickens affected with RSS [67]. Recently, molecular and antigenic characterization of enterovirus-like viruses (ELVs) demonstrated that they were CAstV, some of them closely related to the first characterized CAstV, and some of them more distantly related [102].

The molecular characterization of the different avian astroviruses shows great genetic variability among each type, and this variability influences the ability to

detect these viruses by molecular and serological techniques. It is known that ANV and TAstV-2 each comprise at least two serotypes but many more are suspected. Some studies have shown that TAstV-2, ANV, and CAstV display substantial levels of capsid protein sequence variability, possibly indicating that there are numerous serotypes of each virus [33, 71, 72, 88, 94, 104]. Concomitant infections with different strains have been reported for avian astroviruses and this can result in recombination between strains [71, 72, 94, 104]. Recombination is likely to provide an important mechanism by which the sequence diversity of avian astroviruses is increased. In this chapter, we will review the different aspects related to avian astroviruses, including molecular biology, pathogenesis, diagnosis, and control.

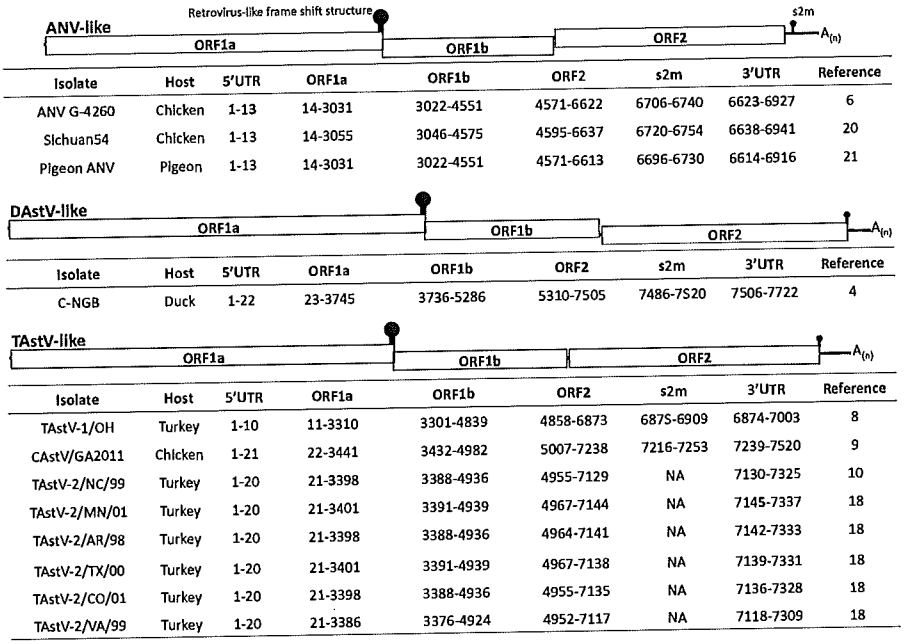
Virus Aspects

Genomic Organization of Avian Astroviruses

The complete genomic sequence of 22 avian astrovirus isolates have been reported, including the genomes of TAstV-1,TAstV-2, ANV, DAstV, and CAstV [20, 30, 38, 39, 44, 94, 113, 115]. Like all other members of the *Astroviridae* family, avian astroviruses or avastroviruses are composed of a positive sense RNA ranging from 6,623 to 7,722 nt in length, excluding the poly-A tail (Fig. 9.1). While the specific lengths of individual regions vary among isolates, in general each comprises a short 5′ untranslated region (UTR), a large open reading frame (ORF) predicted to encode several nonstructural proteins (ORF1a), a second reading frame predicted to encode the RNA dependent RNA polymerase (ORF1b), a third reading frame which encodes the capsid protein (ORF2), and a 3′UTR, followed by a poly-A tail (Fig. 9.1).

Interestingly, while these basic features are well conserved across all of *Astroviridae*, examination of each of the reported genomes demonstrates essentially three basic genome configurations within the avian astroviruses. The ANV-like genome in which ORF1a and ORF2 are in the same frame and ORF1b is in the +1 reading frame; the TAstV-like genome in which ORF1b and ORF2 are both in the +1 reading frame; and the DAstV-like genome in which each ORF is in a distinct reading frame (Fig. 9.1). In addition to these organizational differences, the TAstV-2-like viruses are the only members of Avastrovirus missing the conserved s2m RNA stem-loop motif [44, 94]. While not unprecedented, as the bat, rat, and MBL1 astroviruses are missing this motif [11, 12], its absence suggests this feature is not essential. The prevalence of the s2m motif among *Astroviridae* as well as other viruses led Monceyron et al. to propose that it was introduced into an astrovirus progenitor via a recombination event between non-related viruses [62]. The biological significance of the loss of s2m or the differences in the organization of the ORFs is unclear, but may reflect differences in evolutionary pressures.

Phylogenetic comparison of the Avastrovirus genomes demonstrates that individual isolates cluster based primarily on the host they have been described to infect. One exception is that of TAstV-1, which is found more closely associated with



Isolate	Host	5'UTR	ORF1a	ORF1b	ORF2	s2m	3'UTR	Reference
ANV G-4260	Chicken	1-13	14-3031	3022-4551	4571-6622	6706-6740	6623-6927	6
Sichuan54	Chicken	1-13	14-3055	3046-4575	4595-6637	6720-6754	6638-6941	20
Pigeon ANV	Pigeon	1-13	14-3031	3022-4551	4571-6613	6696-6730	6614-6916	21

Isolate	Host	5'UTR	ORF1a	ORF1b	ORF2	s2m	3'UTR	Reference
C-NGB	Duck	1-22	23-3745	3736-5286	5310-7505	7486-7520	7506-7722	4

Isolate	Host	5'UTR	ORF1a	ORF1b	ORF2	s2m	3'UTR	Reference
TAstV-1/OH	Turkey	1-10	11-3310	3301-4839	4858-6873	6875-6909	6874-7003	8
CAstV/GA2011	Chicken	1-21	22-3441	3432-4982	5007-7238	7216-7253	7239-7520	9
TAstV-2/NC/99	Turkey	1-20	21-3398	3388-4936	4955-7129	NA	7130-7325	10
TAstV-2/MN/01	Turkey	1-20	21-3401	3391-4939	4967-7144	NA	7145-7337	18
TAstV-2/AR/98	Turkey	1-20	21-3398	3388-4936	4964-7141	NA	7142-7333	18
TAstV-2/TX/00	Turkey	1-20	21-3401	3391-4939	4967-7138	NA	7139-7331	18
TAstV-2/CO/01	Turkey	1-20	21-3398	3388-4936	4955-7135	NA	7136-7328	18
TAstV-2/VA/99	Turkey	1-20	21-3386	3376-4924	4952-7117	NA	7118-7309	18

Fig. 9.1 Genomic organization and lengths of regions for each fully characterized avastrovirus genome. Diagrams represent the three basic organizations of the avian astrovirus genomes, and list the specific length of each feature of the fully characterized avian astrovirus isolates. The numbers presented for ORF1a and ORF2 are the nucleotide positions of the start and stop codons. The numbers presented for ORF1a are the nucleotides from the beginning of the "slippery sequence" (AAAAAAC) to the ORF1a stop codon

chicken astroviruses than the other turkey astroviruses (Fig. 9.2). This discrepancy, however, is not unexpected as TAstV-1 has been classified as a member genogroup I (see Taxonomy) with the ANV-like viruses based on phylogenetic comparisons of the capsid genes [6]. The one non-ANV-like CAstV isolate included in this alignment, however, was found to group between genogroups I and II. At present, it is unclear if this represents a distinct genogroup or an evolutionary transition as it has some elements more consistent with genogroup I member TAstV-1 (genomic organization, Fig. 9.1) and others more consistent with genogroup II members TAstV-2 and DAstV (overall length and 5′ UTR, Figs. 9.1 and 9.3).

RNA Functional Elements

Comparison of the 5′ UTR of the different avastrovirus isolates demonstrates a conserved motif in spite of the differences in the lengths between the different viruses (Figs. 9.1 and 9.3). The conserved motif is even more prominent when viewed in

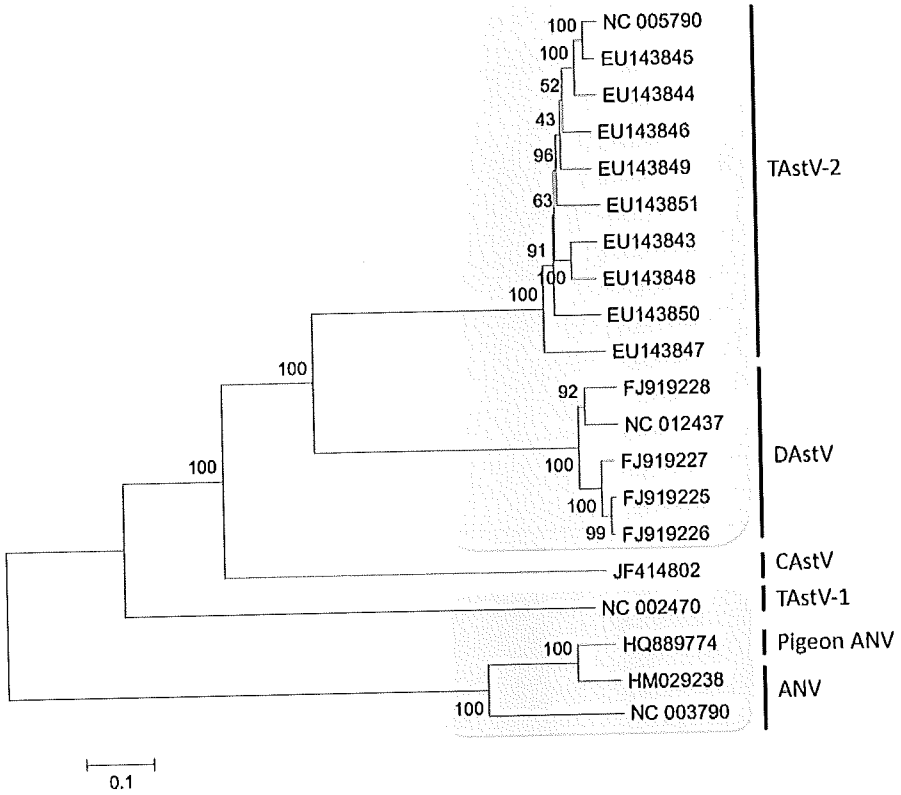


Fig. 9.2 Phylogenetic relationship of avastroviruses. The full-length genomes of each of the 22 avastroviruses were aligned using ClustalW2, and the phylogeny estimated neighbor using the neighbor joining method and 1,000 bootstrap replicates were calculated. The viruses that have been assigned to one of the two genogroups have been highlighted (genogroup I=*brown*, genogroup II=*blue*)

the context of its predicted secondary structure (Fig. 9.3b). The conserved stem-loop structure has been proposed to function as a promoter element for RNA transcription [38, 107]. This idea is supported by the fact that a similar highly conserved sequence can be found immediately upstream of ORF2 in the area where RNA transcription of the subgenomic message would begin (Fig. 9.3c). The function of these elements has not been demonstrated experimentally in any astrovirus isolate; however, given their conservation within the genome as well as across *Astroviridae* this suggests a functional role.

The formation of another RNA structural element, the retrovirus-like frame shift stem-loop, has been a defining characteristic of *Astroviridae* [32]. The presence or absence of this structure, along with the other basic genomic organization, has been one of the items used in the classification of novel viruses to the family [20, 30, 39, 44]. The stem-loop formed has been described to play a key role in the translation of the ORF1b as part of the ORF1a polyprotein in human astrovirus infected cells

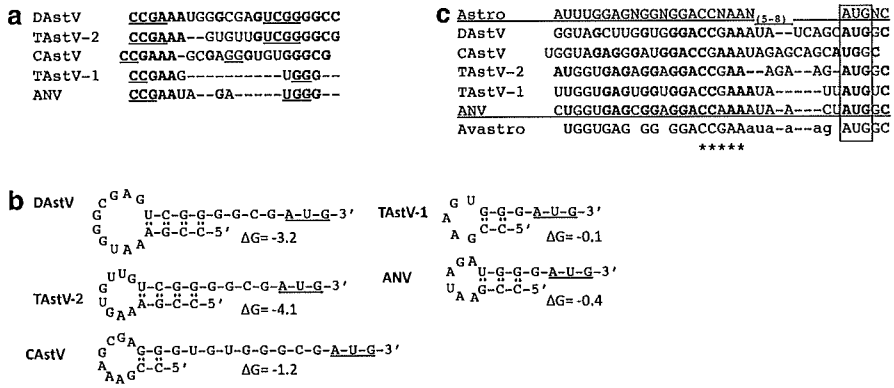


Fig. 9.3 Comparison of the avastrovirus 5′ UTRs and putative "promoter element." (**a**) The 5′ UTR sequence from representative ANV (NC003790), CAstV (JF414802), TAstV-1 (NC002470), DAstV (NC012437), and TAstV-2 (NC005790) genomes were aligned using ClustalW. *Bolded* nucleotides represent positions conserved across a majority of isolates. *Underlined* positions are predicted to complex to form hairpin. (**b**) The 5′ UTR sequences were analyzed for secondary structure using mFold. The start codon for ORF1a is *underlined*. (**c**) The putative promoter sequence upstream of ORF2 was aligned based on the previously reported astrovirus consensus sequence (REF) which is reported above the alignment (Astro). Positions which match the astrovirus consensus sequence are shown in bold. The *boxed* positions highlight the ORF2 start codon. The consensus sequence of the avastrovirus isolates (Avastro) is shown at the bottom of the alignment. Lower case letters denote consensus in the variable region. The CCGAA motif found at the very beginning of the 5′ UTR and in the putative promoter region are denoted by *asterisk*

[48–50, 55]. Experimental evidence demonstrated that the stem-loop formed allowed for a frame shift to occur upstream of the ORF1a stop-codon, and that brought ORF1b into frame with ORF1a resulting in the translation of one polyprotein [21, 49]. Based on this observation, it has been assumed that the presence of this structure in Avastrovirus genomes meant a similar mechanism allowed for the translation of the avian astrovirus RNA-dependent RNA polymerase (RdRp). Recently, studies by Kang et al. have suggested that the avian astroviruses may not use this strategy [39]. Comparison of the stem-loop predicted for human astrovirus to that of the avian viruses demonstrates differences in their ability to induce the needed frame shift (Fig. 9.4). In human astrovirus, the loop structure is between the "slippery sequence" (AAAAAAC) and the ORF1a stop codon (Fig. 9.4). Conversely, in the avian astroviruses, the ORF1a stop codon is immediately downstream of the "slippery sequence," and upstream of the stem loop structure (Fig. 9.4). Therefore, any frame shift induced may not allow for translation of ORF1a to read through to ORF1b. Kang et al. proposed that translation of ORF1b in the CAstV could initiate via a start codon just upstream of the "slippery sequence" [39]; however, this is the only avian virus to have a start site immediately upstream of the "slippery sequence" and would require the stem-loop structure not to be formed. Examination of these viruses for other alternative start sites revealed the first ATG in ORF1b was between 68 and 257 nt downstream from the ORF1a stop codon (Fig. 9.4). It is possible that a more complex stem-loop and frame shift structure is produced in these viruses that

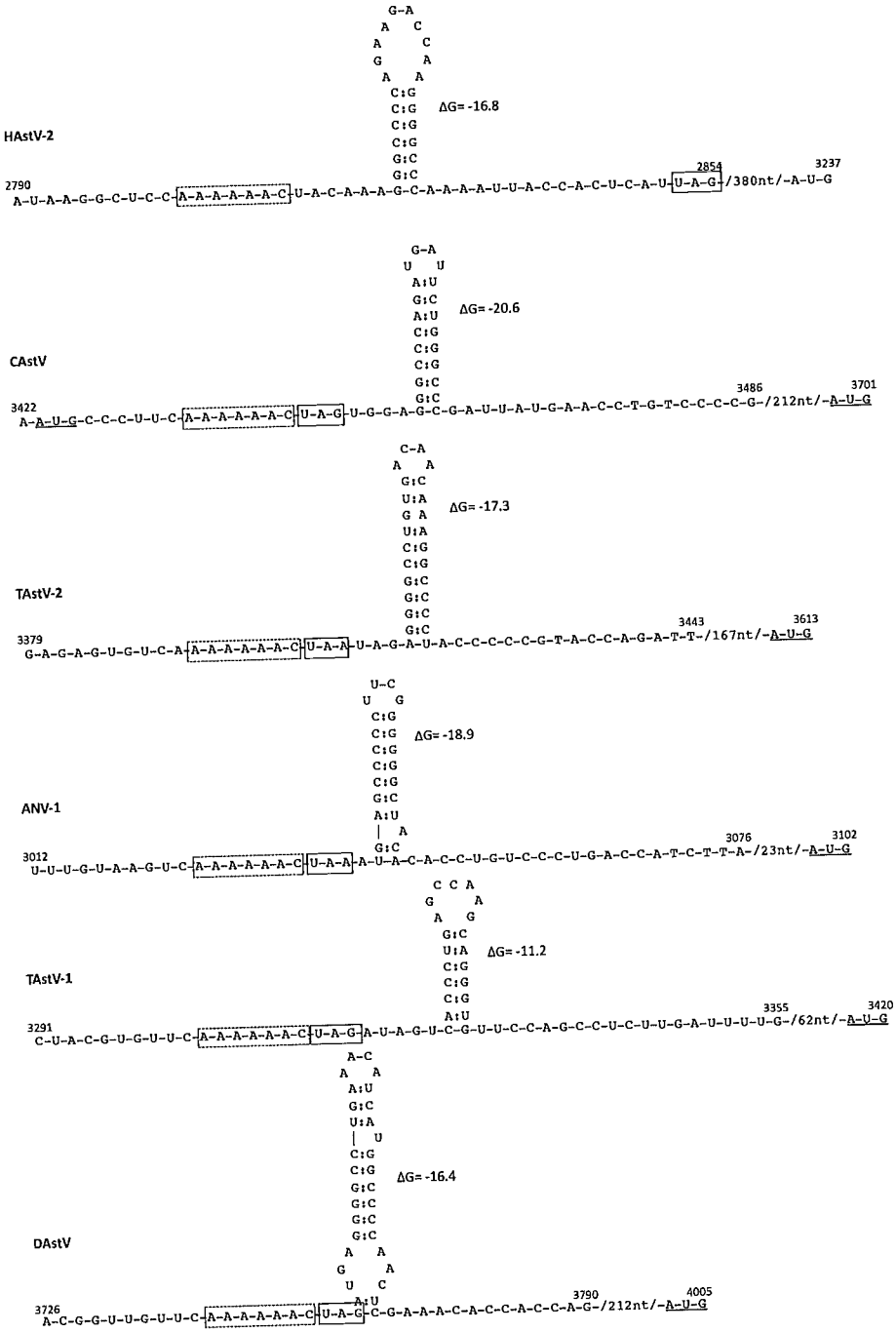


Fig. 9.4 Avastroviruses have conserved frame shift structure, but may have alternative translational strategy. The ORF1a-ORF1b region reported to form a frame shift structure for the translation of ORF1b was analyzed by mFold for ANV (NC003790), ChAstV (JF414802), TAstV-1 (NC002470), DAstV (NC012437), and TAstV-2 (NC005790) genomes, and compared with HAstV-1 (AY720892). For each genome, the predicted structure formed from a 65 nt region starting 10 nt upstream of the slippery sequence (AAAAAAC, highlighted by *dashed box*). The ORF1a stop codon is highlighted by a *solid box*, and potential start codons in the ORF1b frame are *underlined*

allows for the translation of ORF1a–1b or, as proposed by Kang et al., perhaps the avian astroviruses use a different strategy for the translation of their RdRp [39].

Viral Proteins

ORF1a and ORF1b are thought to encode six or more mature non-structural proteins; however, much of what is known about these proteins is based on sequence comparisons with human astroviruses. Analyses of the Avastrovirus genomes have found evidence of transmembrane proteins, a nuclear localization signal, and the serine protease in ORF1a and the RdRp in ORF1b [20, 30, 38, 44]. While additional studies are needed to further characterize the nonstructural proteins encoded by astroviruses in general, and avastroviruses specifically, recent studies by Nighot et al. and Meyerhoff et al. describe the use of an antibody directed against one the non-structural proteins encoded by TAstV-2 [59, 66]. The development of these reagents and others will facilitate our ability to identify these proteins and understand their role in the virus life cycle.

The best characterized astrovirus protein is the capsid protein; however, even here much of our understanding of the avian astroviruses is based on studies of the human astroviruses. Across the different avian astroviruses, ORF2 encodes a protein ranging in size from 670 to 744 amino acids in length resulting in a precursor protein of 73–81 kDa. It is unclear how this capsid precursor protein is processed to result in mature capsid subunit proteins, or how the subunits then assemble into intact virions, but it is largely assumed that similar to the human astroviruses, the precursor is cleaved by trypsin-like proteases to produce 2–3 capsid subunits ranging in size from 25 to 34 kDa [7]. This assumption is supported by the reports of Tang et al. who identified structural proteins of 31 and 34.5 kDa [98].

While detailed structural and functional studies of the avian astrovirus capsids are lacking, this region of the virus has been the most intensely examined at the genetic level and much of what we know about these viruses and their relationship to each other has come from these bioinformatic analyses. Comparisons among the capsid sequences of avastroviruses and mamastroviruses, as well as within avastroviruses, indicate that all of the astrovirus capsid sequences have essentially three main domains (Fig. 9.5). The N-terminal end of the capsid protein is rich in basic amino acids; 23–35 of the first 100 residues are basic, primarily lysine or arginine. The numbers of basic residues is higher among TAstV-2, DAstV, and CAstV isolates, which have 32–35 positive residues, and contain a conserved SRSRSRSRSRSR motif. The TAstV-1 and ANV isolates have 23–25 positive residues but do not have the SR repeat region. Highly basic N-terminal regions have been demonstrated to play a key role in the encapsidation of viral RNAs for several viruses, and therefore it is assumed that these residues play a similar role in astroviruses [46].

Analysis of the rest of the avian astrovirus capsid protein demonstrates, as previously described for human astroviruses, the N-terminal half of the protein is far

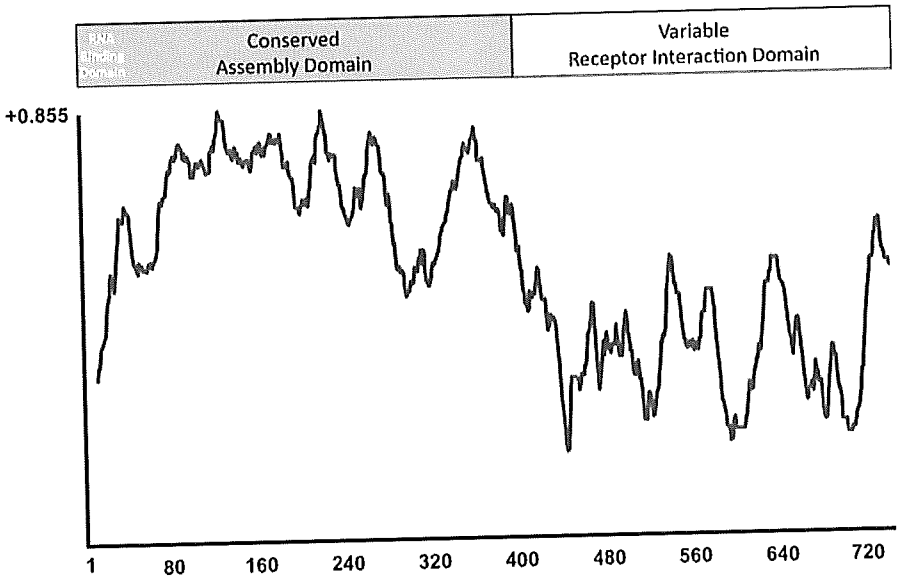


Fig. 9.5 Avastrovirus capsid domains. The relative position of the three functional domains of the astrovirus capsid protein, as described by Krishna (2005), are represented by the *blue bar* and is superimposed on percent similarity plot demonstrating the level of amino acid homology across 34 avastrovirus capsid sequences (see Fig. 9.5). The similarity plot was constructed using Vector NTI 10.0.1 and using a 25 amino acid window

more conserved than the C-terminal half (Fig. 9.5). Analysis of the human astroviruses indicate that the more conserved region of the capsid is important in virion assembly while the more variable region is found on the outer surface of the virion and is involved in interacting with cellular receptors and the immune system [46]. This suggests that these different regions of the capsid protein are under different evolutionary pressures. The first approximately 400 residues are important for encapsidation of the viral RNA and formation of the virion. As such, there are strong structural constraints to the sequence and therefore potential selection against amino acid substitutions. Conversely, the last approximately 300 residues would be exposed to antibodies produced by the host as well as binding to potential new host cells. Especially in the context of interspecies transmission, which appears to occur with some regularity among the avian astroviruses (see "Epidemiology" and "Pathogenesis" sections), one could imagine considerable evolutionary pressure driving amino acid changes in this region. Studies by Strain et al. support this idea [94]. Strain et al. identified 12 codons within the TAstV-2 capsid with evidence of positive selection. Among these 12 codons, 10 were located in the C-terminal half. Additional studies are required to better understand the roles each of these regions play in the life cycle of avian astroviruses and specifically how these different roles affect their evolution.

Taxonomy

Currently, avastrovirus species are based on the hosts from which they were isolated [7]; however, at present the International Committee on Taxonomy of Viruses (ICTV) only recognizes three species within the genus: Chicken astroviruses (ANV-1, ANV-2, ANV-3), Duck astrovirus (DAstV-C-NGB), and Turkey astrovirus (TAstV-1). As such, the species do not correspond to genetic phylogenies (Figs. 9.2 and 9.6), and all the other related viruses are considered members of the genus but have not been approved as species. To address these issues, the classification of avastrovirus species is currently being redefined based on the complete amino acid sequence of the capsid protein. Based on this analysis, the avian astroviruses can be divided into two main genogroups: genogroup I and genogroup II (Fig. 9.6).

Comparison of the predicted phylogeny of avian astroviruses based on the full-length capsid amino acid sequence (Fig. 9.6) with that based on the available full-length genomic sequence (Fig. 9.1) shows two similar evolutionary relationships. In general, all of the isolates are from a particular type of cluster in distinct clades, and these clades largely correspond with the species the viruses were isolated from. The one major exception is the TAstV-1 isolate, which is found more similar to the ANV viruses (Figs. 9.2 and 9.6). However, more careful inspection of the branch order of the TAstV-2 isolates between Figs. 9.2 and 9.6 demonstrates potentially important differences. Most notably, in Fig. 9.6 the CO/2001 and MI/2001 isolates are on the same branch; however, in Fig. 9.2 they were found to be more divergent. This discrepancy is due to a potential recombination event which occurred between these two viruses at the beginning of ORF2 [94]. This results in two isolates which appear to be identical based on the capsid analysis, but, in fact, are more divergent, and suggests analysis of the capsid region in most cases may be sufficient to assign an individual isolate to a particular group; understanding how that isolate may be related to others within that group requires more extensive comparisons.

As we identify new types of avian astroviruses, such as the CAstVs, it may be necessary to adopt new criteria for determining which genogroup it should be assigned to. Based on the full-genome phylogenetic analysis (Fig. 9.2) CAstVs appear to be closer to the genogroup II (DAstV and TAstV-2) viruses than the genogroup I viruses (ANV and TAstV-1). This observation is supported by the alignment of the capsid sequences (Fig. 9.6); however, there appears to be considerably more capsid diversity among the CAstV isolates than other avian astroviruses. In fact, based on the capsid there appears to be two distinct groups (Fig. 9.6). Comparisons of these groups based on limited regions of ORF1b demonstrate far more homology among these viruses [90], providing more evidence that understanding how individual isolates are related to other astroviruses may require comparisons at the whole genome level.

Epidemiology

Information about the range of avian species infected with astroviruses and the prevalence of avian astrovirus infections has been accumulated from a relatively small number of serological studies and an increasing number of recent studies

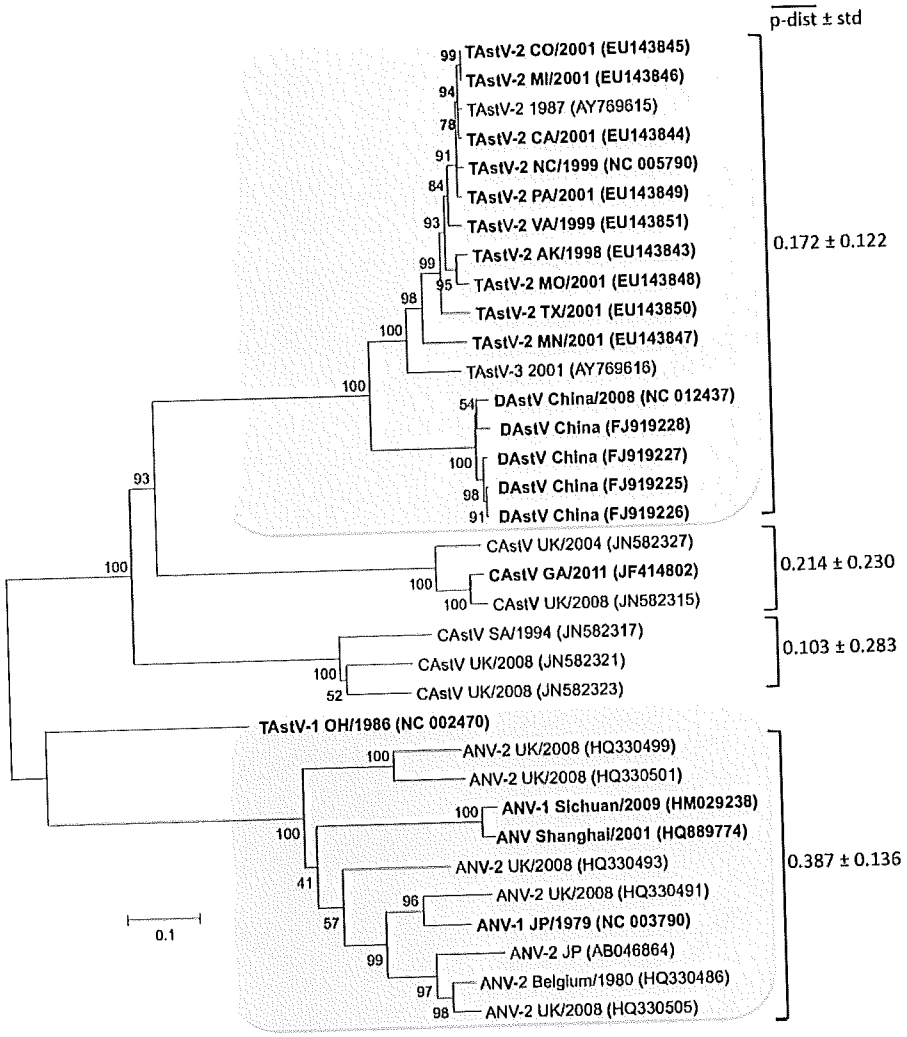


Fig. 9.6 Phylogenetic relationship of avastroviruses. The full-length amino acid sequences of the capsid proteins from 34 avastroviruses were aligned using ClustalW2, and the phylogeny estimated using the neighbor joining method and 1,000 bootstrap replicates were calculated using MEGA5. The viruses that have been assigned to one of the two genogroups have been highlighted (genogroup I=*brown*, genogroup II=*blue*). Isolates in *bold* denote those with full genomic sequence represented in Fig. 9.2. The mean amino acid genetic distance (p-dist)±standard deviation for each of the major clades was calculated using MEGA5

based on the use of virus-specific RT-PCR tests. Using indirect immunofluorescence (IF) performed with the reference ANV-1 virus (G-4260), investigations in the 1980s and 1990s have shown that ANV infections are highly prevalent in commercial chickens in Japan and in several European countries [13, 31]. Antibody to ANV was also detected in turkey flocks and in SPF flocks [13, 58]. More recently, application

of two indirect IF tests developed to separately detect antibodies to two antigenically different CAstVs demonstrated that infections with both CAstV antigenic groups were very common in UK broilers and in broiler parent flocks. Antibodies to both were also detected in parent flocks from many European countries, and were also detected in some UK turkeys [105]. Using a recently developed serological test for DAstV-1, it was found that 63.2% and 51% sera samples from two commercial flocks of ducks that survived DAstV-1 were positive for DAstV [108]. Interestingly, DAstV-specific antibodies were also detected in 28.6% of 42 sera samples from a healthy flock, indicating a wide distribution of subclinical infections caused by DAstV [108]. To our knowledge, seroprevalence studies have not been reported for the TAstVs.

The majority of RT-PCR tests developed for detecting TAstV, ANV, CAstV, and DAstV have used primers that are located in the comparatively well-conserved RNA polymerase (ORF 1b) gene. Based on ORF 1b RT-PCR testing, ANVs have not only been detected in commercial chickens in the USA, UK, and Hungary but have also been detected in turkeys [14], pigeons [116], ducks [6], and guinea fowl [8]. Although the ANVs detected in these species appear to have closely related ORF 1b sequences, it remains to be determined whether the ANVs detected in other bird types are the same as those detected in chickens in terms of their capsid protein sequences. Irrespective of the capsid sequence findings, ANV appears to have a relatively wide host range including non-poultry species [45]. Their ability to infect more than one avian species is likely to add to the diversity exhibited by this virus, and through RNA recombination, allow new variants to emerge.

Other avian astroviruses also appear to be capable of infecting more than one avian species. The detection of TAstV-2 ORF1b fragments by RT-PCR in samples from guinea fowl supports the view that TAstV-2 may have a wider host range [9]. However, knowledge of capsid protein sequences may be required to determine whether the astrovirus from guinea fowl can be classified as a TAstV-2. Full-genome sequence analysis indicated that the DA93 DAstV possessed capsid and RNA polymerase protein sequences that shared ~70% amino acid identities with that of TAstV-2. It remains to be determined whether ducks and turkeys are infected with the same variants. Although CAstV-like RNA has not yet been detected in other avian species, the detection by indirect IF of CAstV antibodies in turkeys suggests these birds could be infected with CAstV or antigenically related viruses [105]. The host range of TAstV-2 and CAstV appears to extend beyond turkeys and chickens, respectively, and, like ANV, may further extend to non-poultry species.

Although infections with ANV and CAstV are highly prevalent in commercially reared chickens, and infections with TAstVs are also highly prevalent in commercially reared turkeys, there is little or no information about the prevalence of these viruses in other avian species or about the prevalence of DAstVs in ducks. RT-PCR detection rates of 96% and 100% were obtained for CAstV and ANV when sample sets (n=~50) from UK broiler flocks affected by enteritis and growth retardation were investigated [88, 103]. Although large numbers of samples from "normal" chickens have not yet been tested, RT-PCR findings to date suggest ANV and CAstV infections are endemic in chickens. TAstVs have a wide geographic distribution and

are considered one of the most prevalent virus infections in 1–5-week-old poults suffering from diarrhea [34, 67, 70, 76, 77, 79, 94]. The prevalence of TAstV varies from 40% to 80% in symptomatic poults [9, 18, 34, 76] to less than 50% in healthy flocks [34, 76]. Field observations indicate that TAstV-2 is currently more prevalent in turkeys than TAstV-1 [9, 70] and its occurrence is often associated with other enteric viruses [34, 67, 69, 76].

The application of RT-PCR tests to longitudinal survey samples has shown that ANV and CAstV can be detected in the intestinal contents from chickens at all ages from 4 days of age to slaughter [89, 90, 103]. Given that as high as 10^8 virus RNA copies/extracted sampled are detected at some time points, it is likely that high levels of virus will be excreted, and, since astroviruses are relatively resistant to chemical and physical inactivation, environmental contamination will be high [89]. Another longitudinal survey conducted to detect enteric viruses in commercial turkey operations demonstrated the presence of TAstV-2, ANV, and TAstV-1 from 2 to 12 weeks of age [69].

With astroviruses being primarily considered as causing enteric infections, spread via the fecal–oral route is likely to be the major means of horizontal transmission. However, horizontal transmission by respiratory or reproductive (male to female via semen) routes cannot be ruled out. Young broiler or turkey poult flocks can become horizontally infected if the housing and equipment within is contaminated with virus due to inadequate cleaning and disinfection between flocks or if the virus is introduced into the house due to poor on-farm biosecurity. Young flocks can also become infected with virus that is acquired vertically. The detection of ANV infections in chickens maintained under quarantine conditions provided indirect evidence of vertical transmission [13], but recently, the detections of low levels of ANV in late-dead duck embryos [6] and chick embryos (Todd et al., unpublished) have provided direct evidence. CAstV has also been detected by RT-PCR in similar dead-in-shell embryo samples (Todd et al., unpublished). Astrovirus excreted from vertically infected chicks will spread horizontally within the flocks.

Pathogenesis

Diseases Caused by Avian Astroviruses

Avian astroviruses, as in mammalian species, have been not only associated with enteritis, but can also cause extra-intestinal diseases including hepatitis and nephritis. Direct correlation of astroviruses identified in feces with enteric disease in birds is complicated by the presence of other enteric pathogens. When separated from other viruses, experimental infection with avian astroviruses can cause illness but usually attenuated when compared to what is seen clinically. The difficulty in obtaining pure astrovirus isolates has hindered pathogenesis studies on some of these viruses.

Duck Astrovirus

Duck astrovirus (DAstV) is an important duck pathogen and causes a highly contagious and fatal hepatitis in ducklings [109]. To date, two different astroviruses causing hepatitis have been recognized in ducks, both of which were initially classified as picornaviruses and named duck hepatitis viruses 2 and 3 (DHV-2 and DHV-3). Duck hepatitis virus 2 (DHV-2), isolated originally by Asplin (1965) [1], was morphologically characterized as an astrovirus in 1984 and renamed duck astrovirus 1 (DAstV-1) [22, 23]. The virus was later definitively identified as an astrovirus [102] and completely sequenced [20]. Duck hepatitis virus 3 (DHV-3), first reported by Toth in 1969 [106] and characterized by Haider and Calnek in 1979 [24], was recently identified by sequencing as a second astrovirus in ducks (DAstV-2) [102].

DAstV-1 (DHV-2) can cause a severe acute disease in young ducks which can result in up to 50% mortality [109]. Infection occurs through oral and cloacal routes. Deaths occur within 1–4 days, usually within 1–2 h after the appearance of clinical signs, which include polydypsia with loose droppings, excessive urate excretion, and sometimes convulsions and acute opisthotonos. Affected ducks usually die in good condition and both the mean death time and the mortality rate (10–50%) depend on the age of the ducks [22]. Survivors excrete virus for at least 1 week after infection and rear normally, with little evidence of retarded growth. The target organs for DAstV-1 appear to be liver and kidneys. The liver is usually pale pink with hemorrhages, and the kidneys are often swollen. Microscopic changes are characterized by extensive necrosis of hepatocytes and bile duct hyperplasia [22]. DAstV-2 has a low pathogenicity for ducklings experimentally infected, and only ducklings appear to be infected by the virus. Mortality rarely exceeds 30% [109] and mature ducks are refractory to the disease [22].

In a recent study, very low mortality rates (4.67% and 0.63%) were observed in two flocks affected by DAstV-1 [108], in sharp contrast with the high mortality rate (up to 50%) in a recent Chinese outbreak and in the 1983 UK outbreak [20, 22]. This may suggest differences in pathogenicity among DAstVs. Co-infections of DAstV and other viruses may also play a role in high mortality observed in some outbreaks.

Turkey Astrovirus

Turkey astroviruses are commonly detected in commercial turkeys experiencing viral enteritis. TAstV-2 has been associated with many enteric disease syndromes in turkeys including poult enteritis complex or syndrome (PEC or PES), and poult enteritis mortality syndrome (PEMS) [8, 18, 34, 44, 70, 75, 112]. Clinical signs of the disease usually develop between 1 and 3 weeks of age and generally last up to 2 weeks [78]. Severity is usually mild to moderate and is characterized by diarrhea, listlessness, litter eating, and nervousness. Although mortality rate is low, morbidity occurring as decreased growth is of great economic concern [2, 78]. The exact role of TAstV-2 as the etiological agent of enteritis in turkeys is difficult to estimate since it can also be

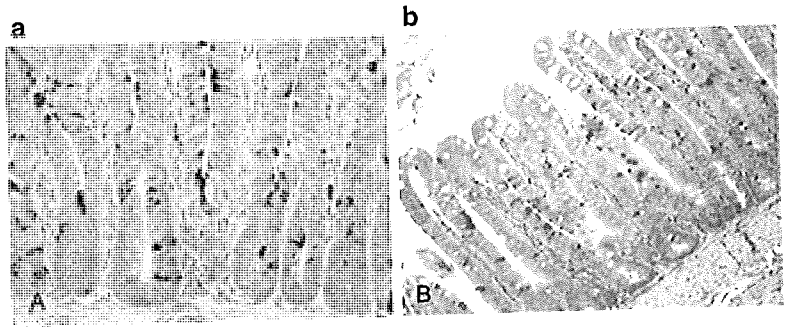


Fig. 9.7 Photomicrographs of jejunum sections from a turkey infected with TAstV-2, 3 days after inoculation; immunohistochemistry (IHC) and in situ hybridization (ISH) staining for detection of the virus. (**a**) Viral antigen staining present in the cytoplasm of the enterocytes at the middle section of the villi (*dark brown*); immunoperoxidase labeling, hematoxylin counterstain. (**b**) Viral RNA staining present in the cytoplasm of the enterocytes (*red*); digoxygenin labeling, BCIP-NBT staining. Magnification 200×. Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poults. M. Pantin-Jackwood, E. Spackman and J.M. Day. Avian Pathology 37, 193-201. Reprinted with permission of the publisher (Taylor & Francis Ltd, http://tandf.co.uk/journals)

detected in apparently healthy turkeys [69, 76]. TAstV-1 has also been detected in healthy turkeys and turkeys with enteric disease, but is less prevalent than TAstV-2.

Experimentally, young turkeys infected with TAstV-2 develop a diarrhea by 2 days post infection which continues for several days [41, 63, 68, 92]. TAstV-2 infection results in overall growth depression of infected birds. Common lesions observed at necropsy are dehydration, distended intestines filled with watery contents and undigested feed, and dilated ceca with foamy contents. Microscopic lesions present in the intestines consist of mild crypt hyperplasia, mild villous atrophy, and mild lymphocytic infiltration, and were most common in the jejunum [5, 41, 68]. Presence of the viruses has been demonstrated by immunohistochemistry (IHC) and by in situ hybridization (ISH) in both villi and crypt enterocytes in the duodenum, jejunum, ileum, and ceca (Fig. 9.7) [5, 41, 59, 66, 68]. Mild lymphocytic infiltration was also observed in other organs including pancreas, liver, spleen, and kidneys. Viremia occurred after infection with TAstV-2 [41, 68]; however, only intestinal cells appear to support viral replication. The possibility exists that low levels of viral replication may occur in other tissues and may not be detected with the tests used which would explain the extra-intestinal lesions observed.

Mild-to-moderate atrophy of the bursa of Fabricius and thymus was found in TAstV-2-infected poults [5, 41, 63, 68, 74, 81, 92, 99], and was associated with the depletion of lymphoid areas. The cause of these changes in the primary immune organs is unclear and could result in the immune suppression that some have reported with TAstV-2 infections (see section "Immunity"); however, the lymphoid depletion could also be a consequence of maldigestion and malabsorption leading to nutritional deficiencies in these rapidly growing poults.

Early mortality has been reported in poults experimentally inoculated with TAstV-2 viruses [68, 111]. The mortality in these cases most likely occurred because of the high

viral challenge the poults received at a very young age, which induced a moderately severe lethargy and feed and water withdrawal that resulted in death. In an earlier study, the pathogenicity of two turkey astrovirus isolates was compared in older turkeys (14–28 days old). Both viruses caused enteritis and growth depression but no mortality [99].

Based on RT-PCR, TAstV-2-like viruses have also been detected in guinea fowl presenting enteric disease but these viruses require further genetic characterization to confirm their identity as TAstV-2 [10].

Avian Nephritis Virus

Avian nephritis virus (ANV) was first isolated from rectal contents of apparently healthy broiler chicks [110] and experimental infections revealed that it mainly results in a sub-clinical disease, although mild growth depression and mortality have been reported for infections with ANV strains including the serotype 1G-4260 strain (ANV-1) [29, 52, 110]. ANV typically causes histological changes in the kidneys although the viral antigen can be detected in other organs including liver, spleen, pancreas, jejunum, and rectum [27, 29, 84, 86]. Young chicks are the most susceptible and become resistant to disease after the first month of life [28]. ANV infections in chicks can vary from subclinical to outbreaks of runting stunting syndrome (RSS) and nephropathy [19, 31, 86, 95, 96, 110]. However, the role of ANV infection on RSS is not clear. Although ANV affected body gain in SPF chicks [85], an experimental study on the effect of ANV and several unclassified enterovirus-like viruses (ELVs) in commercial chicks demonstrated that only one ELV, later also characterized as an ANV, analyzed among the broad panel produced clinical signs in inoculated chicks [57]. Similarly, infection of 1-day-of-age chicks with ELVs, which were later identified as the FP3 and 612 isolates of CAstV and an ANV, showed that the ELVs are able to induce more severe lesions in the intestine, the kidneys, and the pancreas than the G-4260 strain of ANV [89]. Passive and active surveys indicate that ANV is widely distributed worldwide and that serological evidence can be found in turkeys as well [13, 16, 58, 65, 95]. Although an ANV genetically related to ANV-1 was detected in the feces of turkeys [70, 72], to date the infection has never been associated with the disease in turkeys. An attempt to infect poults with the chicken-derived ANV-1 strain was unsuccessful [61].

In chickens experimentally infected with ANV, the virus was first detected in feces 2 days after inoculation, with maximum virus shedding at 4–5 days after inoculation. The virus was widely distributed in tissues, with high titers in the kidney and jejunum and lower titers in the bursa of Fabricius, spleen, and liver [26]. Transient diarrhea and weight gain depression are common signs among 1-day-old infected chickens. At necropsy, mild-to-severe discoloration and swelling in the kidneys are observed, and in dead chicks visceral urate deposits are found [26]. The microscopic lesions described in infected chickens include necrosis and degeneration of epithelial cells of the proximal convoluted tubules of the kidney, interstitial lymphocyte infiltration, and moderate fibrosis. ANV virus particles and antigen

were demonstrated in the degenerating kidney epithelium by electron microscopy and IF respectively [26].

Recently, novel ANVs have been isolated from chickens and turkeys suffering from runting and stunting-like and/or locomotory disease [15]. One of these isolates, isolate 19, induced diarrhea and runting and stunting in 3-week-old SPF layer chickens; however, the clinical tenosynovitis, which was seen regularly in the broilers and poults from the field, was not seen in the experimentally infected layers [15]. The isolation of these new avian astrovirus strains from chickens and turkeys with acute tenosynovitis and arthritis without the isolation of any other detectable pathogen suggests that they also might play a role in leg disorders. The pathogenicity of this virus was further demonstrated by the mortality and histopathological findings and seems to be broader than is reported for ANV-1 [15]. Many studies have shown that the pathogenicity of ANV-1 is limited to the kidney of chickens, in contrast to the damage observed in the kidney, pancreas, duodenum, thymus, bursa, and liver seen after infection with this new ANV [15]. Other ANVs have been shown to vary in pathogenicity and tropism. A Belgian PV2 isolate, originally recognized as an ELV, was shown to cause substantial growth retardation, and severe and long-lasting intestinal lesions following experimental infection [17]. Lesions were detected in the pancreas and proventriculus and were absent in the kidneys from chickens experimentally infected with PV2, suggesting that some ANV isolates such as PV2 may have different tissue tropisms [17].

The presence of ANV has also been demonstrated by RT-PCR in organ samples collected from 30-day-old ducklings and from dead-in-shell ducklings [6]. Histological examination of many tissues indicated that lesions were confined to the kidney and intestine. The finding of ANV in ducklings dead in shell indicates naturally occurring vertical infection and it is possible that ANV may play a role in the death of duckling embryos. ANVs were also detected by RT-PCR in 89% of fecal samples from young pigeons suffering from diarrhea, whereas samples from healthy pigeons were negative [115]. In another study, a very high prevalence of ANV was found among feral pigeons and wood pigeons [45]. Astrovirus was detected in almost all juvenile birds, while the detection rate among the adults was 62%. In this case, most birds appeared healthy, and it was not possible to correlate the presence of a virus to any illness in the birds. ANV-like viruses have also been detected in guinea fowl but the role they play in disease is not clear [8].

Chicken Astrovirus

The common detection of CAstV by RT-PCR in cases of broiler chickens affected by growth retardation suggests that infections with this avian astrovirus are contributing to poor growth performance [89]. High levels of CAstV RNA and CAstV antigen have also been detected in kidney tissue from chickens affected by nephritis and visceral gout (Todd, unpublished results).

Experimental infections of 1-day-of-age and 5-day-of-age SPF layer chickens with CAstV caused mild diarrhea and, except for some areas of limited damage at the

base of the villi of the small intestine in some birds, no histological visible damage in the liver, kidney, spleen, thymus, or bursa was observed [4]. Used ELV-4 isolate 612, later typed as a CAstV [102], to infect commercial 1-day-old broilers with maternal antibodies against the virus and found only a growth depression of 11.3%, 20.4%, and 9.6% in the three trials that were performed [56]. Experimental infections of 1-day-old SPF layer chickens with CAstV strain 612 caused only very mild histological changes in the intestine and liver, and mild-to-moderate lesions in the kidney [87]. An experimental infection with the FP3 CAstV strain, which was originally known as ELV-3 and which is genetically and antigenically distinct from the 612 strain isolate, caused no clinical signs in 1-day-old broilers [57]. However, experimental infections of 1-day-old SPF layer chickens with the FP3 isolate caused mild-to-moderate histological changes in the kidney, pancreas, and intestine, with virus-specific antigen being detected in these tissues [87].

Mechanisms of Viral Pathogenesis

Our understanding of how astroviruses cause disease trails behind our knowledge of its molecular and epidemiologic properties and is confined mainly to work done with TAstV. Studies by Thouvenelle et al. and Reynolds et al. suggested that astrovirus infection induces changes in the expression of constituent enzymes of the brush border, specifically maltase, with a significant decrease in intestinal D-xylose absorption, resulting in disaccharide maldigestion, malabsorption, and subsequently osmotic diarrhea, even though it causes only mild microscopic lesions [75, 101]. The cellular mechanisms responsible for these changes are still unknown.

Most of the studies done with TAstV have demonstrated that astrovirus infection induces diarrhea in the absence of significant changes in the intestinal morphology. Furthermore, there is little evidence of inflammation and little or no increase in cell death following astrovirus infection [5, 41, 101], further suggesting astrovirus clinical signs result from submicroscopic changes in the intestine. Initial studies to identify physiological changes in infected poults demonstrated changes to ultrastructure of the epithelial cells including rearrangement of F-actin. These changes were associated with decreased apical expression of the sodium/hydrogen exchanger 3 (NHE3) with a concomitant decrease in sodium absorption [66]. Collectively, these data suggest that astrovirus infection induces a malabsorption, osmotic diarrhea, possibly through redistribution of specific brush border enzymes and transporters resulting from dysregulation of the actin cytoskeleton [64, 66, 75, 101].

Immunity

The immune response to astroviruses has not been well characterized in most species. In humans, it has been postulated that anti-astrovirus antibodies developed following natural infections are key mediators in protecting normal healthy adults

[47, 60]. In birds; however, this is far less clear and may differ among host species and/or types of avian astroviruses.

Studies with ANV, CAstV, and DAstV suggest that recovery from natural infection and/or vaccination leads to an immune response that protects the host from subsequent infection [1, 22, 28, 83, 109]. Multiple serotypes have been reported for each of these astrovirus types [4, 86, 104]. There are reports of at least two serotypes of TAstV-2; however, curiously there is no experimental evidence of an anamnestic response [41, 100]. This lack of an adaptive immune response has led several laboratories to investigate its effects on the immune system. These studies suggest TAstV-2 may induce immune dysregulation and/or localized inhibition [40, 41, 73, 74].

Studies by Qureshi et al. examined the response of lymphocytes from TAstV-2 or mock-infected poults to the mitogen concanavalin A [74]. These studies demonstrated a decrease in the *ex vivo* proliferative response to ConA by peripheral blood lymphocytes from TAstV-2 infected poults as compared with controls. Additionally, thymocytes and splenocytes that were exposed to the virus *in vitro* also demonstrated a reduced proliferative response [74]. In subsequent studies, Qureshi et al. reported TAstV-2 infections also resulted in impaired migration, phagocytic activity, and bacterial killing activity of innate immune cells, as compared with controls [73]. Functional examination of the innate immune cells (macrophages and heterophils) from TAstV-2 infected poults demonstrated reduced expression of the pro-inflammatory cytokines IL-1 and IL-6 [73]. Interestingly, studies by Koci et al. demonstrated significant increases in the levels of active TGF-β in the serum of TAstV-2 infected poults [41]. Collectively, these findings suggest that TAstV-2 infection leads to some sort of immune dysfunction, the specific mechanism of which is still not understood.

In spite of the immune inhibitory effects reported with TAstV-2 infection, the clinical disease is largely resolved within 12–14 days [41], suggesting that some anti-viral host factors are produced. Studies by Qureshi et al. and Koci et al. have both demonstrated that macrophages and adherent splenocytes from TAstV-2-infected poults produce more nitric oxide (NO) following *ex vivo* stimulation with lipopolysccharide than do cells from uninfected controls [40, 73]. NO has long been recognized as a potent anti-viral effector molecule; however, histological examination of TAstV-2 infected poults does not demonstrate large influxes of macrophages or other innate immune cells. To determine if NO could play a role in the regulation of TAstV-2 replication, the virus was exposed to NO donor compounds *in vitro* prior to inoculating embryos, and found to inactivate the virus [40]. Conversely, when embryos were pretreated with an inhibitor of the inducible nitric oxide synthase (iNOS) enzyme virus replication increased significantly [40, 51]. Finally, to determine if TAstV-2-infected intestines demonstrated an increase in the expression of iNOS, tissues from TAstV-2-infected turkey poults were stained for the presence of TAstV-2 antigen and turkey iNOS. These experiments demonstrated an increase in iNOS expression following TAstV-2 infection, with the majority of the staining localized to the intestinal epithelium suggesting that in the absence of a response from either innate or adaptive immune cells the intestine is capable of mounting its own innate defense [59].

Diagnosis

Astrovirus infections of commercially reared chickens and turkeys are endemic and this may also be the case with commercially reared ducks. Although infections are common, many are likely to be subclinical, with the incidence of clinical disease that can be directly attributed to a single avian astrovirus being relatively small. Therefore, with regard to diagnosis, it is important to differentiate whether astrovirus-related diseases are being diagnosed or whether astrovirus infections are being diagnosed.

The detection of clinical signs, gross pathological findings, and histological lesions is required before disease can be diagnosed. However, in the case of astroviruses, these in themselves may not be sufficient to identify the causal virus. For example, hepatitis in ducklings may be caused by one of the two different astroviruses: DAstV-1 and DAstV-2, originally known as duck hepatitis virus types 2 and 3, or the picornavirus, originally known as duck hepatitis virus type 1 [109]. Similarly, histological lesions consistent with nephritis in chickens can be caused by ANV, infectious bronchitis virus (IBV), and CAstV (Todd et al., unpublished results). In cases of hepatitis and nephritis, the presence of a specific astrovirus needs to be demonstrated in the affected tissue, preferably at the site of the histological lesion. This can be achieved using immunohistochemistry (IHC), undertaken with paraffin-embedded formalin-fixed tissue, or immunofluorescence (IF) performed with frozen tissue or tissue impression smears. However, due to the limited availability of avian astrovirus-specific antibodies, to date these methods have received limited application. IF is used to diagnose hepatitis in ducklings caused by DAstV-2 [24]. Very recently, a prototype immunoperoxidase-based IHC method has been applied to demonstrate the presence of CAstV antigen in nephritis-associated histological lesions (Todd et al., unpublished results; Fig. 9.8). This work suggested that CAstV alone was capable of causing nephritis in chickens and highlighted the need to identify the virus responsible in cases of nephritis and visceral gout. Imada et al. has reported that IF is a useful diagnostic procedure for ANV, detecting the group antigens and staining infected kidneys during the early acute phase of the disease [26]. *In situ* hybridization (ISH) can also be used to detect the presence of viral nucleic acid at the site of the histological lesion. Because probes generated from cloned virus-specific cDNA fragments may be more easily produced than virus-specific antibodies, ISH may prove to be an easier approach to develop. ISH has been used to demonstrate the presence of TAstV-2 RNA within the enterocytes present on the sides of the villi from virus-infected turkeys, but its use to detect other avian astroviruses has not yet been reported [5, 41, 68].

Diagnosing astrovirus infections is considerably easier than diagnosing astrovirus-associated disease. The method first used was negative contrast electron microscopy (EM), which depended on the visualization of the characteristic five- and six-pointed star morphology that characterizes astroviruses. EM performed with enteric samples has been used for the diagnosis of TAstVs and for the diagnosis of DAstV-1 in liver samples from ducks with hepatitis [23, 56]. However, astrovirus

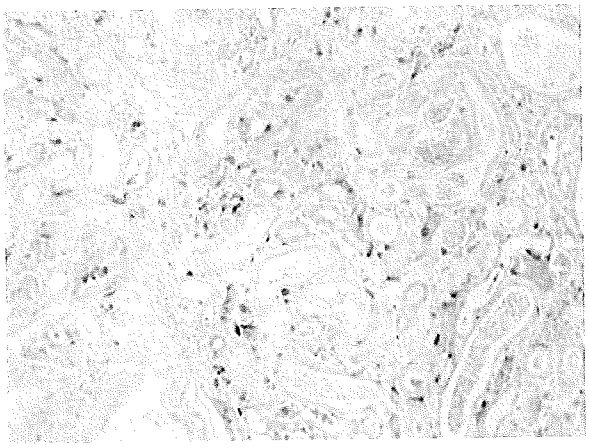


Fig. 9.8 Photomicrograph of a kidney section from a broiler chicken naturally infected with CAstV; immunohistochemistry (IHC) staining for detection of the virus. Viral antigen staining present in kidney epithelium (*dark brown*); immunoperoxidase labeling, hematoxylin counterstain. Magnification 200×

diagnosis by EM has a number of drawbacks, the most important being that some avian astroviruses fail to display the characteristic star morphology. This appears to be the case for ANV, DAstV-2, and CAstV, which are detected as small spherical viruses without obvious surface structure, and, on this basis, these were originally considered to be picornaviruses (ANV) or enterovirus-like viruses (CAstV, DAstV-2) [57]. Additional drawbacks of EM include its relative lack of sensitivity, and its unsuitability for the testing of large samples due to the time, labor, and equipment required.

Virus isolation in embryonated eggs or cell culture has been successful for most of the avian astroviruses including ANV, CAstV, TAstV, and DAstV. TAstV and DAstV can be propagated in turkey and duck embryos, respectively, and sometimes in chick embryos as well [98, 109]. Although the propagation of DAstV-1 (formerly DHV-2) in cell culture has been reported [4], the propagation of TAstV in cell cultures has not. The growth of high yields of TAstV isolates in turkey embryos has facilitated the production of purified virus, with which virus-specific antibodies have been prepared [97]. The astroviruses from chickens can be propagated in chicken embryos and in cell culture. CAstVs can be propagated in SPF chick embryos following yolk sac inoculation to produce variable effects including early embryo death, dwarfing, hemorrhages, edema, and feather changes [91]. ANVs can be propagated in SPF embryos following egg inoculation using a variety of routes. Isolation of ANV in cell cultures has been reported in a number of studies [19, 25, 110], and induces a cell rounding CPE in primary chicken kidney cells typically within 72 h post infection [26] . Experience from some laboratories suggests that some ANVs may be non-cultivable and the inconsistent growth of some ANVs in primary cell culture and embryos has prompted speculation that embryos containing ANV antibody may inhibit in vitro propagation (Todd et al. unpublished results). Baxendale and Mebatsion have reported the isolation and propagation of CAstVs in primary chick embryo liver cells and LMH cells, in which plaques similar to those produced by DAstV (DHV-2) were obtained [4]. The CAstV isolates have been

used in virus neutralization tests, with which antibody to CAstV was detected [4]. There is also evidence that CAstVs can non-productively infect when inoculated into primary chick embryo liver cells [105]. In these cases, the inoculated CAstV undergoes partial replication, producing virus proteins that can be detected by indirect IF without producing infectious virus and CPE [105]. The effects produced by particular avian astroviruses in cell culture or in experimentally inoculated embryos are relatively nonspecific and cannot easily be distinguished from the effects caused by other avian astroviruses or other viruses. As such, the cultured virus needs to be identified; this can be accomplished through the detection of virus-specific antigens or nucleic acid. Although cultivable virus isolates are essential for biological characterization and pathogenesis studies, virus isolation is not routinely used to detect avian astroviruses, the principal reason being that astrovirus-containing samples are frequently contaminated with viruses such as adenoviruses or reoviruses, which replicate faster than the astroviruses in cell culture and/or in embryos. In addition, virus isolation and identification is time-consuming and labor-intensive, making it unsuitable for testing large samples.

A microtiter plate-based ELISA has been developed for the detection of TAstV-2 antigen in feces or intestinal content samples [97]. This test depended on the use of rabbit polyclonal antibodies that were raised by immunization with two different TAstV-2, which had been purified following replication in turkey embryos. Results compared favorably with those obtained by EM in terms of the numbers of positive samples recorded. Despite their convenience and low cost, the absence of virus-specific antibodies for other avian astroviruses has restricted the development of antigen-detecting ELISAs.

Attention has focused increasingly on the use of molecular diagnostic tests for detecting avian astroviruses. Since the first report describing an RT-PCR test for detecting TAstV [43], conventional and/or real-time tests have been described for ANV [14, 54, 103], CAstV [70]; [89], and DAstV [6] as well as TAstV-1 and TAstV-2 [14, 41, 93, 97]. The primers for these tests are usually located in the genomic regions encoding nonstructural proteins such as the RNA polymerase (ORF 1b), which are more conserved than the capsid protein encoding region (ORF 2). However, the high levels of sequence diversity displayed by avian astroviruses species make it difficult to select primers that will detect all variants within one type. Therefore, primer design should be based on comparing sequences from as large a number of virus variants as possible and the need to incorporate degenerate primers recognized. The importance of primer selection was highlighted when it was shown that the RT-PCR test using primers located in the 3′ untranslated region (3′ UTR) was more successful in detecting ANV-positive samples than tests based on primers located in the nonstructural protease or RNA polymerase [89, 103]. Similarly, CAstV from all genetic groupings was detected by the conventional and real-time RT-PCR tests described by Smyth et al. [88, 89], whereas one CAstV grouping was not detected by another published test [14]. Applications of these molecular tests have demonstrated that TAstV, ANV, and CAstV are highly prevalent in samples from their respective hosts, and there is a strong likelihood that the

vast majority of commercial chicken and turkey flocks will become infected at some point in their lifetimes [18, 69, 70, 89]. Therefore, since all enteric samples are likely to be positive using these highly sensitive RT-PCR methods, knowing that an enteric sample is astrovirus positive is of questionable importance. However, with regard to the impact that the infection may be having on the flock or bird, there may be considerably more value in knowing the amounts of virus present, and in knowing whether internal tissues such as the kidney or liver are virus positive.

In comparison with other avian viruses, the serological diagnoses of avian astroviruses have received relatively little attention. A major reason for this is the limited number of avian astrovirus strains that have been adapted to cell culture. Although a number of ANV isolates can be propagated in cell culture, with very few exceptions cell culture isolations of CAstVs, TAstVs, and DAstVs have not been described. The lack of cell culture methods limits the development of indirect IF assays and virus neutralization tests to analyze chicken, turkey, and duck sera for antibodies. Additionally, the development of other serological assays such as ELISAs requires the ability to produce ample quantities of virus and virus antigen. With limited cell culture propagation methods, only a few laboratories have developed these assays for understanding astrovirus serology. An ELISA for detecting ANV antibody was described by Decaesstecker and Meulemans [16]. Sellers et al. have reported the use of an ELISA for detecting antibodies to CAstV using recombinantly expressed capsid protein [83]. Wang et al. [108] and Zhao et al. [114] also developed similar ELISA tests using recombinant capsid proteins for detecting DAstV and ANV-specific antibodies in duck and chicken serum samples.

The antigenic diversities displayed by avian astrovirus species need to be taken into consideration when using serological tests. For example, Shirai et al. [85] have demonstrated that ANV strains representing serotype 1 (ANV-1) and serotype 2 (ANV-2) exhibit very low levels of cross-reactive antibodies using indirect IF. Todd et al. [104] have recently reported the common occurrence of ANV variants, which displayed very different capsid protein sequences from those of ANV-1 and ANV-2, and which, on this basis, are likely to be antigenically different. This view is supported by a recent work by de Wit et al. [16], who showed that serum samples containing antibodies to a novel ANV isolate (isolate 19) were not detected by an ANV-1 specific indirect IF assay. It is recognized that the CAstVs can be subdivided into two major antigenic groups, designated A and B, which display relatively low levels of cross-reactivity by indirect IF [90, 105]. For this reason, a recent CAstV seroprevalence study involved the application of two separate indirect IF tests, one based on isolate 612 for detecting antibodies to the CAstVs from Group A, and the second based on isolate 11672 for detecting antibodies to the Group B CAstVs [105]. Therefore, the antigenic diversities exhibited by ANV and CAstV need to be considered in relation to their serological diagnosis. Serological tests based on the use of single ANV or CAstV strains will not detect antibodies to all ANV or CAstV infections. This is of particular importance when testing SPF flocks to demonstrate freedom from infection.

Control

Although it has been possible to produce and maintain specific pathogen free (SPF) flocks that are free from ANV infection as determined by serological testing, the common and widespread occurrence of avian astroviruses such as ANV, CAstV, and TAstVs in commercial poultry, and, in the case of ANV, their occurrence in wild birds [45], when combined with their potential for vertical transmission, strongly suggests that their eradication from commercial poultry would not be feasible. In addition, astroviruses are very stable in the environment and resistant to inactivation by most routinely used disinfectants, which complicates its elimination from infected premises [82]. Strict biosecurity, increased down time between flocks, and effective disinfection of the premises would help reduce the likelihood of contracting avian astrovirus infections.

Convalescent antisera have been used to control outbreaks of DAstV-1 and DAstV-2 [109]. Although experimental live attenuated vaccines were also developed for these astroviruses, these were not commercially produced [109]. Similarly, commercial vaccines have not been developed for ANV, CAstV, and TAstVs. In the case of ANV and CAstV, which are known to be vertically transmitted, vaccine protocols focused on the parent flocks during rear should be considered as a method to prevent or limit transmission to progeny in ovo. In addition, breeder vaccination should ensure uniformly higher levels of maternal antibody, which would better equip the young birds to withstand early viral challenges. The development of live attenuated or inactivated avian astrovirus vaccines will both depend on the availability of cell or embryo culture methods. Alternatively, recombinant expression systems, such as recombinant baculovirus system, might be used to produce astrovirus capsid subunit vaccines. Sellers et al. [83] recently described the production of a prototype recombinant baculovirus-derived CAstV vaccine, which, following multiple administrations to breeders, offered some protection to progeny chicks against experimentally induced RSS.

Conclusions

In comparison with other avian viruses, such as fowl adenoviruses, avian paramyxoviruses, and avian coronaviruses, relatively little is known about avian astroviruses and their impact on birds. A major reason for this has been the absence in most cases of cell culture growth systems, with which viruses can be isolated and biologically characterized; however, the level of genetic diversity, species affected, and permutations of clinical diseases caused by the various members of *Astroviridae* complicate our ability to develop a cohesive narrative as other avian viruses. In spite of these challenges, within the last decade, considerable advances have been made. The different avian astroviruses are second only to the human astroviruses in terms of their level of characterization, and in many cases, such a pathogenic mechanism and immunity are the best characterized. Regardless, considerably more research is needed.

Most notably, a standardized process for naming and classifying both existing as well as new members of the genera needs to be developed. As avian virology laboratories continue to develop new detection methods, and identify and characterize novel avian astroviruses in commercial poultry as well as wild bird species, a more consistent method of comparing (molecularly or antigenically) would aid in our ability to understand the relationship of viruses within the genera as well as identifying potential virulence determinants. As part of this effort to better understand avian astroviruses, the avian virology community should be encouraged to more fully characterize representative members of each genotype, serotype, and subgroup. This would include not only sequencing, but also the development of antibodies and other reagents. Together these efforts would all be in support of increasing our understanding of the cellular, physiological, and immunological changes astroviruses induce in their various host species so that we can develop more effective prevention, control, and therapeutic strategies.

References

1. Asplin FD. Duck hepatitis: vaccination against two serological types. Vet Rec. 1965;77: 1529–30.
2. Barnes GL, Guy JS, Vaillancourt JP. Poult enteritis complex. Rev Sci Tech. 2000;19: 565–88.
3. Barnes GL, Guy JS. Poult enteritis-mortality syndrome. In: Saif YM, Barnes HJ, Glisson JR, Faddly AM, MacDougald LR, Swayne DE, editors. Diseases of poultry. 11th ed. Ames IA: Blackwell Publishing; 2003.
4. Baxendale W, Mebatsion T. The isolation and characterisation of astroviruses from chickens. Avian Pathol. 2004;33:364–70.
5. Behling-Kelly E, Schultz-Cherry S, Koci M, Kelley L, Larsen D, Brown C. Localization of astrovirus in experimentally infected turkeys as determined by in situ hybridization. Vet Pathol. 2002;39:595–8.
6. Bidin M, Lojkic I, Bidin Z, Tisljar M, Majnaric D, Mikec M. Detection and characterization of avian nephritis virus in ducklings. Avian Pathol. 2011;40:173–7.
7. Bosch A, Guix S, Krishna NK, Méndez E, Monroe SS, Pantin-Jackwood M, et al. Family Astroviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy: ninth report of the international committee on taxonomy of viruses. London: Elsevier Inc; 2012.
8. Canelli E, Cordioli P, Barbieri I, Catella A, Pennelli D, Ceruti R, et al. Astroviruses as causative agents of poultry enteritis: genetic characterization and longitudinal studies on field conditions. Avian Dis. 2012;56(1):173–82.
9. Cattoli G, De Battisti C, Toffan A, Salviato A, Lavazza A, Cerioli M, et al. Co-circulation of distinct genetic lineages of astroviruses in turkeys and guinea fowl. Arch Virol. 2007;152:595–602.
10. Cattoli G, Toffan A, De Battisti C, Salviato A, Terregino C, Capua I. Astroviruses found in the intestinal contents of guinea fowl suffering from enteritis. Vet Rec. 2005;156:220.
11. Chu DK, Chin AW, Smith GJ, Chan KH, Guan Y, Peiris JS, et al. Detection of novel astroviruses in urban brown rats and previously known astroviruses in humans. J Gen Virol. 2010;91:2457–62.
12. Chu DK, Poon LL, Guan Y, Peiris JS. Novel astroviruses in insectivorous bats. J Virol. 2008;82:9107–14.

13. Connor TJ, McNeilly F, McFerran JB, McNulty MS. A survey of avian sera from Northern Ireland for antibody to avian nephritis virus. Avian Pathol. 1987;16:15–20.
14. Day JM, Spackman E, Pantin-Jackwood M. A multiplex RT-PCR test for the differential identification of turkey astrovirus type 1, turkey astrovirus type 2, chicken astrovirus, avian nephritis virus, and avian rotavirus. Avian Dis. 2007;51:681–4.
15. de Wit JJ, Dam GB, de Laar JM, Biermann Y, Verstegen I, Edens F, et al. Detection and characterization of a new astrovirus in chicken and turkeys with enteric and locomotion disorders. Avian Pathol. 2011;40:453–61.
16. Decaesstecker M, Meulemans. An ELISA for the detection of antibodies to avian nephritis virus and related entero-like viruses. Avian Pathol. 1991;20:523–30.
17. Decaesstecker M, Charlier G, Peeters J, Meulemans G. Pathogenicity of fowl enteroviruses. Avian Pathol. 1989;18:697–713.
18. Domanska-Blicharz K, Seroka A, Minta Z. One-year molecular survey of astrovirus infection in turkeys in Poland. Arch Virol. 2011;156:1065–72.
19. Frazier JA, Howes K, Reece RL, Kidd AW, Cavanagh D. Isolation of noncytopathic viruses implicated in the aetiology of nephritis and baby chick nephropathy and serologically related to avian nephritis virus. Avian Pathol. 1990;19:139–60.
20. Fu Y, Pan M, Wang X, Xu Y, Xie X, Knowles NJ, et al. Complete sequence of a duck astrovirus associated with fatal hepatitis in ducklings. J Gen Virol. 2009;90:1104–8.
21. Gibson CA, Chen J, Monroe SA, Denison MR. Expression and processing of nonstructural proteins of the human astroviruses. Adv Exp Med Biol. 1998;440:387–91.
22. Gough RE, Borland ED, Keymer IF, Stuart JC. An outbreak of duck hepatitis type II in commercial ducks. Avian Pathol. 1985;14:227–36.
23. Gough RE, Collins MS, Borland E, Keymer LF. Astrovirus-like particles associated with hepatitis in ducklings. Vet Rec. 1984;114:279.
24. Haider SA, Calnek BW. In-vitro isolation, propagation and characterisation of duck hepatitis virus type III. Avian Dis. 1979;23:715–29.
25. Hewson KA, O'Rourke D, Noormohammadi AH. Detection of avian nephritis virus in Australian chicken flocks. Avian Dis. 2010;54:990–3.
26. Imada T. Avian nephritis. In: Saif YM, Faddly AM, Glisson JR, MacDougald LR, Nolan LK, Swayne DE, editors. Diseases of poultry. Ames, IA: Blackwell Publishing; 2008.
27. Imada T, Maeda M, Furuta K, Yamaguchi S, Kawamura H. Pathogenicity and distribution of avian nephritis virus (G-4260 strain) in inoculated laying hens. Natl Inst Anim Health Q (Tokyo). 1983;23:43–8.
28. Imada T, Taniguchi T, Yamaguchi S, Minetoma T, Maeda M, Kawamura H. Susceptibility of chickens to avian nephritis virus at various inoculation routes and ages. Avian Dis. 1981;25:294–302.
29. Imada T, Yamaguchi S, Kawamura H. Pathogenicity for baby chicks of the G-4260 strain of the picornavirus "avian nephritis virus". Avian Dis. 1979;23:582–8.
30. Imada T, Yamaguchi S, Mase M, Tsukamoto K, Kubo M, Morooka A. Avian nephritis virus (ANV) as a new member of the family Astroviridae and construction of infectious ANV cDNA. J Virol. 2000;74:8487–93.
31. Imada T, Yamaguchi S, Miura N, Kawamura H. Antibody survey against avian nephritis virus among chickens in Japan. Natl Inst Anim Health Q (Tokyo). 1980;20:79–80.
32. Jiang B, Monroe SS, Koonin EV, Stine SE, Glass RI. RNA sequence of astrovirus: distinctive genomic organization and a putative retrovirus-like ribosomal frameshifting signal that directs the viral replicase synthesis. Proc Natl Acad Sci USA. 1993;90:10539–43.
33. Jindal N, Patnayak DP, Chander Y, Ziegler AF, Goyal SM. Comparison of capsid gene sequences of turkey astrovirus-2 from poult-enteritis-syndrome-affected and apparently healthy turkeys. Arch Virol. 2011;156:969–77.
34. Jindal N, Patnayak DP, Chander Y, Ziegler AF, Goyal SM. Detection and molecular characterization of enteric viruses from poult enteritis syndrome in turkeys. Poult Sci. 2010;89: 217–26.

35. Jindal N, Patnayak DP, Ziegler AF, Lago A, Goyal SM. Duration of growth depression and pathogen shedding in experimentally reproduced poult enteritis syndrome. Avian Dis. 2009;53:517–22.
36. Jindal N, Patnayak DP, Ziegler AF, Lago A, Goyal SM. Experimental reproduction of poult enteritis syndrome: clinical findings, growth response, and microbiology. Poult Sci. 2009;88:949–58.
37. Jindal N, Patnayak DP, Ziegler AF, Lago A, Goyal SM. A retrospective study on poult enteritis syndrome in Minnesota. Avian Dis. 2009;53:268–75.
38. Jonassen CM, Jonassen TT, Sveen TM, Grinde B. Complete genomic sequences of astroviruses from sheep and turkey: comparison with related viruses. Virus Res. 2003;91:195–201.
39. Kang KI, Icard AH, Linnemann E, Sellers HS, Mundt E. Determination of the full length sequence of a chicken astrovirus suggests a different replication mechanism. Virus Genes. 2012;44(1):45–50.
40. Koci MD, Kelley LA, Larsen D, Schultz-Cherry S. Astrovirus-induced synthesis of nitric oxide contributes to virus control during infection. J Virol. 2004;78:1564–74.
41. Koci MD, Moser LA, Kelley LA, Larsen D, Brown CC, Schultz-Cherry S. Astrovirus induces diarrhea in the absence of inflammation and cell death. J Virol. 2003;77:11798–808.
42. Koci MD, Schultz-Cherry S. Avian astroviruses. Avian Pathol. 2002;31:213–27.
43. Koci MD, Seal BS, Schultz-Cherry S. Development of an RT-PCR diagnostic test for an avian astrovirus. J Virol Methods. 2000;90:79–83.
44. Koci MD, Seal BS, Schultz-Cherry S. Molecular characterization of an avian astrovirus. J Virol. 2000;74:6173–7.
45. Kofstad T, Jonassen CM. Screening of feral and wood pigeons for viruses harbouring a conserved mobile viral element: characterization of novel astroviruses and picornaviruses. PLoS One. 2011;6:e25964.
46. Krishna NK. Identification of structural domains involved in astrovirus capsid biology. Viral Immunol. 2005;18:17–26.
47. Kurtz J, Lee T. Astrovirus gastroenteritis age distribution of antibody. Med Microbiol Immunol. 1978;166:227–30.
48. Lewis TL, Matsui SM. An astrovirus frameshift signal induces ribosomal frameshifting in vitro. Arch Virol. 1995;140:1127–35.
49. Lewis TL, Matsui SM. Astrovirus ribosomal frameshifting in an infection-transfection transient expression system. J Virol. 1996;70:2869–75.
50. Lewis TL, Matsui SM. Studies of the astrovirus signal that induces (-1) ribosomal frameshifting. Adv Exp Med Biol. 1997;412:323–30.
51. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15:323–50.
52. Maeda M, Imada T, Taniguchi T, Horiuchi T. Pathological changes in chicks inoculated with the picornavirus "avian nephritis virus". Avian Dis. 1979;23:589–96.
53. Mandoki M, Bakonyi T, Ivanics E, Nemes C, Dobos-Kovacs M, Rusvai M. Phylogenetic diversity of avian nephritis virus in Hungarian chicken flocks. Avian Pathol. 2006;35:224–9.
54. Mandoki M, Dobos-Kovacs M, Bakonyi T, Rusvai M. Molecular diagnosis of avian nephritis: preliminary report. Acta Vet Hung. 2006;54:51–60.
55. Marczinke B, Bloys AJ, Brown TD, Willcocks MM, Carter MJ, Brierley I. The human astrovirus RNA-dependent RNA polymerase coding region is expressed by ribosomal frameshifting. J Virol. 1994;68:5588–95.
56. McNeilly F, Connor TJ, Calvert VM, Smyth JA, Curran WL, Morley AJ, Thompson D, Singh S, McFerran JB, Adair BM, McNulty MS. Avian Pathol. 1994 Jun;23(2):313-27.
57. McNulty MS, Curran WL, McFerran JB. Detection of astroviruses in turkey faeces by direct electron microscopy. Vet Rec. 1980;106:561.
58. McNulty MS, Connor TJ, McNeilly F, McFerran JB. Biological characterization of avian enteroviruses and enterovirus-like viruses. Avian Pathol. 1990;19:75–87.

59. McNulty MS, Connor TJ, McNeilly F. A survey of specific pathogen-free chicken flocks for antibodies to chicken anemia agent, avian nephritis virus and group A rotavirus. Avian Pathol. 1989;18:215–20.
60. Meyerhoff RR, Nighot PK, Ali RA, Blikslager AT, Koci MD. Characterization of turkey inducible nitric oxide synthase and identification of its expression in the intestinal epithelium following astrovirus infection. Comp Immunol Microbiol Infect Dis. 2012;35(1):63–9.
61. Mitchell DK. Astrovirus gastroenteritis. Pediatr Infect Dis J. 2002;21:1067–9.
62. Mockett AP, Huggins MB, Woods M, Orbell S. A compariosn of three serological methods to detect chicken and turkey antibodies to avian nephritis virus and the use of virus-specific monoclonal antibodies. Avian Pathol. 1993;22(4):751–70.
63. Monceyron C, Grinde B, Jonassen TO. Molecular characterisation of the 3′-end of the astrovirus genome. Arch Virol. 1997;142:699–706.
64. Mor SK, Abin M, Costa G, Durrani A, Jindal N, Goyal SM, et al. The role of type-2 turkey astrovirus in poult enteritis syndrome. Poult Sci. 2011;90:2747–52.
65. Moser LA, Carter M, Schultz-Cherry S. Astrovirus increases epithelial barrier permeability independently of viral replication. J Virol. 2007;81:11937–45.
66. Nicholas RAJ, Goddard RD, Luff PR. Prevalence of avian nephritis virus in England. Vet Rec. 1988;123:398.
67. Nighot PK, Moeser A, Ali RA, Blikslager AT, Koci MD. Astrovirus infection induces sodium malabsorption and redistributes sodium hydrogen exchanger expression. Virology. 2010;401:146–54.
68. Pantin-Jackwood MJ, Day JM, Jackwood MW, Spackman E. Enteric viruses detected by molecular methods in commercial chicken and turkey flocks in the United States between 2005 and 2006. Avian Dis. 2008;52:235–44.
69. Pantin-Jackwood MJ, Spackman E, Day JM. Pathogenesis of type 2 turkey astroviruses with variant capsid genes in 2-day-old specific pathogen free poults. Avian Pathol. 2008;37:193–201.
70. Pantin-Jackwood MJ, Spackman E, Day JM, Rives D. Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of astrovirus and rotavirus on the farms. Avian Dis. 2007;51:674–80.
71. Pantin-Jackwood MJ, Spackman E, Woolcock PR. Molecular characterization and typing of chicken and turkey astroviruses circulating in the United States: implications for diagnostics. Avian Dis. 2006;50:397–404.
72. Pantin-Jackwood MJ, Spackman E, Woolcock PR. Phylogenetic analysis of turkey astroviruses reveals evidence of recombination. Virus Genes. 2006;32:187–92.
73. Pantin-Jackwood MJ, Strother KO, Mundt E, Zsak L, Day JM, Spackman E. Molecular characterization of avian astroviruses. Arch Virol. 2011;156:235–44.
74. Qureshi MA, Saif YM, Heggen-Peay CL, Edens FW, Havenstein GB. Induction of functional defects in macrophages by a poult enteritis and mortality syndrome-associated turkey astrovirus. Avian Dis. 2001;45:853–61.
75. Qureshi MA, Yu M, Saif YM. A novel "small round virus" inducing poult enteritis and mortality syndrome and associated immune alterations. Avian Dis. 2000;44:275–83.
76. Reynolds DL, Saif YM. Astrovirus: a cause of an enteric disease in turkey poults. Avian Dis. 1986;30:728–35.
77. Reynolds DL, Saif YM, Theil KW. Enteric viral infections of turkey poults: incidence of infection. Avian Dis. 1987;31:272–6.
78. Reynolds DL, Saif YM, Theil KW. A survey of enteric viruses of turkey poults. Avian Dis. 1986;31:89–98.
79. Reynolds DL, Schultz-Cherry SL. Astrovirus infections. In: Saif YM, Faddly AM, Glisson JR, MacDougald LR, Nolan LK, Swayne DE, editors. Diseases of poultry. Ames, IA: Blackwell Publishing; 2008.
80. Saif LJ, Saif YM, Theil KW. Enteric viruses in diarrheic turkey poults. Avian Dis. 1985;29:798–811.
81. Saif YM, Saif LJ, Hofacre CL, Hayhow C, Swayne DE, Dearth RN. A small round virus associated with enteritis in turkey poults. Avian Dis. 1990;34:762–4.

82. Schultz-Cherry S, Kapczynski DR, Simmons VM, Koci MD, Brown C, Barnes HJ. Identifying agent(s) associated with poult enteritis and mortality syndrome: importance of the thymus. Avian Dis. 2000;44:256–65.
83. Schultz-Cherry S, King DJ, Koci MD. Inactivation of an astrovirus associated with poult enteritis mortality syndrome. Avian Dis. 2001;45:76–82.
84. Sellers H, Linneman E, Icard AH, Mundt E. A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. Vaccine. 2010;28:1253–63.
85. Shirai J, Nakamura K, Narita M, Furuta K, Hihara H, Kawamura H. Visceral urate deposits in chicks inoculated with avian nephritis virus. Vet Rec. 1989;124:658–61.
86. Shirai J, Nakamura K, Shinohara K, Kawamura H. Pathogenicity and antigenicity of avian nephritis isolates. Avian Dis. 1991;35:49–54.
87. Shirai J, Tanimura N, Uramoto K, Narita M, Nakamura K, Kawamura H. Pathologically and serologically different avian nephritis virus isolates implicated in etiology of baby chick nephropathy. Avian Dis. 1992;36:369–77.
88. Smyth JA, Connor TJ, McNeilly F, Moffet DA, Calvert VM, McNulty MS. Studies on the pathogenicity of enterovirus-like viruses in chickens. Avian Pathol. 2007;36:119–26.
89. Smyth VJ, Jewhurst HL, Adair BM, Todd D. Detection of chicken astrovirus by reverse transcriptase-polymerase chain reaction. Avian Pathol. 2009;38:293–9.
90. Smyth VJ, Jewhurst HL, Wilkinson DS, Adair BM, Gordon AW, Todd D. Development and evaluation of real-time TaqMan(R) RT-PCR assays for the detection of avian nephritis virus and chicken astrovirus in chickens. Avian Pathol. 2010;39:467–74.
91. Smyth VJ, Todd D, Trudgett J, Lee A, Welsh MD. Capsid protein sequence diversity of chicken astrovirus. Avian Pathol. 2012;41(2):151–9.
92. Spackman D, Gough RE, Collins MS, Lanning D. Isolation of an enterovirus-like agent from the meconium of dead-in-shell chicken embryos. Vet Rec. 1984;114:216–8.
93. Spackman E, Day JM, Pantin-Jackwood MJ. Astrovirus, reovirus, and rotavirus concomitant infection causes decreased weight gain in broad-breasted white poults. Avian Dis. 2010; 54:16–21.
94. Spackman E, Kapczynski D, Sellers H. Multiplex real-time reverse transcription-polymerase chain reaction for the detection of three viruses associated with poult enteritis complex: turkey astrovirus, turkey coronavirus, and turkey reovirus. Avian Dis. 2005;49:86–91.
95. Strain E, Kelley LA, Schultz-Cherry S, Muse SV, Koci MD. Genomic analysis of closely related astroviruses. J Virol. 2008;82:5099–103.
96. Takase K, Shinohara K, Tsuneyoshi M, Yamamoto M, Yamada S. Isolation and characterization of cytopathic avian enteroviruses from broiler chickens. Avian Pathol. 1989;18:631–42.
97. Takase K, Uchimura T, Yamamoto M, Yamada S. Susceptibility of embryos and chicks, derived form immunized breeding hens, to avian nephritis virus. Avian Pathol. 1994;23: 117–25.
98. Tang Y, Ismail MM, Saif YM. Development of antigen-capture enzyme-linked immunosorbent assay and RT-PCR for detection of turkey astroviruses. Avian Dis. 2005;49:182–8.
99. Tang Y, Murgia AM, Saif YM. Molecular characterization of the capsid gene of two serotypes of turkey astroviruses. Avian Dis. 2005;49:514–9.
100. Tang Y, Murgia MV, Ward L, Saif YM. Pathogenicity of turkey astroviruses in turkey embryos and poults. Avian Dis. 2006;50:526–31.
101. Tang Y, Saif YM. Antigenicity of two turkey astrovirus isolates. Avian Dis. 2004;48: 896–901.
102. Thouvenelle ML, Haynes JS, Reynolds DL. Astrovirus infection in hatchling turkeys: histologic, morphometric, and ultrastructural findings. Avian Dis. 1995;39:328–36.
103. Todd D, Smyth VJ, Ball NW, Donnelly BM, Wylie M, Knowles NJ, et al. Identification of chicken enterovirus-like viruses, duck hepatitis virus type 2 and duck hepatitis virus type 3 as astroviruses. Avian Pathol. 2009;38:21–30.

104. Todd D, Trudgett J, McNeilly F, McBride N, Donnelly B, Smyth VJ, et al. Development and application of an RT-PCR test for detecting avian nephritis virus. Avian Pathol. 2010;39: 207–13.
105. Todd D, Trudgett J, Smyth VJ, Donnelly B, McBride N, Welsh MD. Capsid protein sequence diversity of avian nephritis virus. Avian Pathol. 2011;40:249–59.
106. Todd D, Wilkinson DS, Jewhurst HL, Wylie M, Gordon AW, Adair BM. A seroprevalence investigation of chicken astrovirus infections. Avian Pathol. 2009;38:301–9.
107. Toth TE. Studies of an agent causing mortality among ducklings immune to duck virus hepatitis. Avian Dis. 1969;13:834–46.
108. Walter JE, Briggs J, Guerrero ML, Matson DO, Pickering LK, Ruiz-Palacios G, et al. Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children. Arch Virol. 2001;146:2357–67.
109. Wang X, Wang Y, Xie X, Zhang B, Zhang D. Expression of the C-terminal ORF2 protein of duck astrovirus for application in a serological test. J Virol Methods. 2011;171:8–12.
110. Woolcock PR. Duck hepatitis. In: Saif YM, Faddly AM, Glisson JR, MacDougald LR, Nolan LK, Swayne DE, editors. Diseases of poultry. Ames, IA: Blackwell Publishing; 2008.
111. Yamaguchi S, Imada T, Kawamura H. Characterisation of a picornavirus isolated from broiler chicks. Avian Dis. 1979;23:571–81.
112. Yu M, Ismail MM, Qureshi MA, Dearth RN, Barnes HJ, Saif YM. Viral agents associated with poult enteritis and mortality syndrome: the role of a small round virus and a turkey coronavirus. Avian Dis. 2000;44:297–304.
113. Yu M, Tang Y, Guo M, Zhang Q, Saif YM. Characterization of a small round virus associated with the poult enteritis and mortality syndrome. Avian Dis. 2000;44:600–10.
114. Zhao W, Hua XG, Yuan L, Cui L, Shan TL, Dai XQ, et al. Sequence analyses of the representative Chinese-prevalent strain of avian nephritis virus in healthy chicken flocks. Avian Dis. 2011;55:65–9.
115. Zhao W, Zhu AL, Yu Y, Yuan CL, Zhu CX, Lan DL, et al. Segmentation expression of capsid protein as an antigen for the detection of avian nephritis virus infection in chicken flocks. J Virol Methods. 2012;179(1):57–61.
116. Zhao W, Zhu AL, Yu Y, Yuan CL, Zhu CX, Yang ZB, et al. Complete sequence and genetic characterization of pigeon avian nephritis virus, a member of the family Astroviridae. Arch Virol. 2011;156:1559–65.
117. Zhao W, Zhu AL, Yuan CL, Yu Y, Zhu CX, Lan DL, et al. Detection of astrovirus infection in pigeons (Columbia livia) during an outbreak of diarrhoea. Avian Pathol. 2011;40:361–5.

AVIAN DISEASES 56:276–281, 2012

Low Pathogenicity Avian Influenza Viruses Infect Chicken Layers by Different Routes of Inoculation

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Received 27 September 2011; Accepted 28 November 2011; Published ahead of print 29 November 2011

SUMMARY. In order to develop better control measures against avian influenza, it is necessary to understand how the virus transmits in poultry. In a previous study in which the infectivity and transmissibility of the pandemic H1N1 influenza virus was examined in different poultry species, we found that no or minimal infection occurred in chicken and turkeys intranasally (IN) inoculated with the virus. However, we demonstrated that the virus can infect laying turkey hens by the intracloacal (IC) and intraoviduct (IO) routes, possibly explaining the drops in egg production observed in turkey breeder farms affected by the virus. Such novel routes of exposure have not been previously examined in chickens and could also explain outbreaks of low pathogenicity avian influenza (LPAI) that cause a decrease in egg production in chicken layers and breeders. In the present study, 46-wk-old specific-pathogen-free chicken layers were infected by the IN, IC, or IO routes with one of two LPAI viruses: a poultry origin virus, A/chicken/CA/1255/02 (H6N2), and a live bird market isolate, A/chicken/NJ/12220/97 (H9N2). Only hens IN inoculated with the H6N2 virus presented mild clinical signs consisting of depression and anorexia. However, a decrease in number of eggs laid was observed in all virus-inoculated groups when compared to control hens. Evidence of infection was found in all chickens inoculated with the H6N2 virus by any of the three routes and the virus transmitted to contact hens. On the other hand, only one or two hens from each of the groups inoculated with the H9N2 virus shed detectable levels of virus, or seroconverted and did not transmit the virus to contacts, regardless of the route of inoculation. In conclusion, LPAI viruses can also infect chickens through other routes besides the IN route, which is considered the natural route of exposure. However, as seen with the H9N2 virus, the infectivity of the virus did not increase when given by these alternate routes.

RESUMEN. Los virus de la influenza aviar de baja patogenicidad infectan a gallinas de postura por diferentes vías de inoculación.

Con el fin de desarrollar mejores medidas de control contra la influenza aviar, es necesario entender cómo se transmite el virus dentro de la avicultura comercial. En un estudio previo en el que se examinó la infectividad y la transmisibilidad del virus de la influenza pandémico H1N1 entre diferentes especies de aves, se encontró que la infección es nula o mínima en pollos y pavos inoculados con este virus por vía intranasal. Sin embargo, se ha demostrado que el virus puede infectar pavas reproductoras en producción por las rutas intracloacal e intraoviductal, lo que puede explicar las bajas en la producción de huevos observadas en las granjas de pavos reproductoras afectadas por el virus. Estas nuevas rutas de exposición no habían sido previamente examinadas en los pollos y también podrían explicar los brotes de influenza aviar de baja patogenicidad, que causan una disminución en la producción de huevos en las gallinas de postura y en las aves reproductoras. En el presente estudio, se infectaron aves de postura de 46 semanas de edad libres de patógenos específicos por las rutas intranasal, intracloacal, o intraoviductal con uno de dos virus de la influenza aviar de baja patogenicidad: un virus de origen aviar, el aislamiento denominado A/pollo/CA/1255/02 (H6N2), y un aislamiento obtenido de un mercado de aves vivas, A/pollo/NJ/12220/97 (H9N2). Solamente las gallinas inoculadas por vía intranasal con el virus H6N2 presentaron leves signos clínicos consistentes en depresión y anorexia. Sin embargo, se observó disminución en el número de huevos producidos en todos los grupos inoculados con los virus cuando se compararon con las gallinas del grupo control. Se encontró evidencia de infección en todos los pollos inoculados con el virus H6N2 por cualquiera de las tres rutas y el virus se transmitió a gallinas expuestas por contacto. Por otro lado, sólo una ó dos gallinas procedentes de cada uno de los grupos inoculados con el virus H9N2 eliminaron virus con niveles detectables o seroconvirtieron y no transmitieron el virus a las aves expuestas por contacto, independientemente de la vía de inoculación. En conclusión, los virus de influenza aviar baja patogenicidad pueden infectar a los pollos a través de otras vías, además de la vía intranasal, que se considera la vía natural de exposición. Sin embargo, como se observó con el virus H9N2, la infectividad del virus no aumentó cuando se administró por estas rutas alternas.

Key words: low pathogenic avian influenza, H6N2 type, H9N2 type, routes of inoculation, chickens

Abbreviations: AI = avian influenza; BHI = brain heart infusion; C = cloacal; dpi = days postinoculation; EID_{50} = 50% egg infectious dose; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; IC = intracloacal; IHC = immunohistochemistry; IN = intranasal; IO = intraoviduct; LPAI = low pathogenicity avian influenza; OP = oropharyngeal; pH1N1 = pandemic H1N1; qRRT-PCR = quantitative real-time reverse transcriptase-polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free

Avian influenza (AI) continues to be a threat to commercial poultry. AI viruses occasionally transmit from their natural reservoirs, wild aquatic birds, to domestic birds producing subclinical infections and sometimes respiratory disease and drops in egg production. These viruses are typically termed low pathogenicity AI (LPAI) viruses and can be a combination of most 16 hemagglutinin and nine neuraminidase subtypes. Some H5 and H7 LPAI viruses, after circulating in domestic poultry, mutate into highly pathogenic AI (HPAI) viruses and cause high mortality (34). Most LPAI viruses produce subclinical infections in experimental

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studies but, under commercial rearing conditions and probably complicated by secondary pathogens, environmental stress, and immunosuppression, these viruses can also produce mild to moderate disease. Secondary bacterial, fungal, or viral infections are usually necessary to produce sufficiently severe respiratory damage to result in illness or death; however, some LPAI virus strains, such as certain Asian H9N2 lineages adapted to efficient replication in poultry, can cause more-prominent signs and also significant mortality (1,15,24).

Typically, LPAI viruses have limited local replication in the respiratory and alimentary tracts (33). Rarely, LPAI viruses have spread outside the respiratory or alimentary tract causing infection and damage in epithelial-containing tissues of visceral organs such as kidney, pancreas, and oviduct. Clinical signs will differ with the virus strain, host species, and age of the host and pathophysiologic changes can be found in the respiratory, digestive, urinary, and reproductive systems (18,33).

The intranasal (IN) route of exposure is routinely used to emulate the natural route of infection for AI viruses in experimental settings (31). However, in studies done to evaluate the pathogenicity of the 2009 pandemic H1N1 (pH1N1) virus in breeder turkeys, infection was not produced easily by IN inoculation. Therefore, intracloacal (IC) and intraoviduct (IO) inoculation were tested in an attempt to explain infection of breeder turkeys with the virus and were successful in infecting the turkeys (19,35). In another study, more than 50% of 8-to-10-wk-old turkeys inoculated by the cloacal (C) route with the pH1N1 virus became infected and virus replication was demonstrated in the cecal tonsil and bursa (2). Cloacal exposure has been shown to be important to the transmission of other pathogens in poultry. For example, cloacal contact with feces that have been contaminated with *Histomonas meleagridis* is thought to be one way in which blackhead is transmitted from bird to bird (14), and IC inoculation or vaccination has been used experimentally with Newcastle disease virus and infectious laryngotracheitis virus (4,5,26). Infection following alimentary tract exposure has also been demonstrated in chickens and ducks infected with an H5N1 HPAI virus (11). The initiation of H5N1 HPAI virus infection in birds is still favored by a respiratory route of exposure, but alimentary initiated infection is possible if birds consume high doses of virus, such as by cannibalizing infected carcasses (11). Little is known about the ability of LPAI viruses to infect chickens by alternate routes of exposure.

LPAI viruses are known to affect lay of eggs and replicate in the reproductive tract of turkeys and, as with turkeys infected with the pH1N1 virus, drops in egg production are commonly reported with LPAI virus outbreaks in turkey breeders (8,13,15,22,29,32,37). Decreases in egg production have also been observed in chicken breeders and layers infected with LPAI viruses (3,12,15,24,40). In 2000, an outbreak of H6N2 LPAI occurred in California and 12 separate incidences were reported primarily involving layer-type birds, but the virus was also isolated from backyard chickens and a ptimary broiler breeder (7,39). Drops in egg production and increased mortality were among the clinical signs reported in the layer flocks. Yolk peritonitis was a feature also described (39).

The IO route of exposure provided an explanation of how turkey breeders became infected with the pH1N1 virus through the practice of artificial insemination and highlighted the importance of exploring alternate routes of infection for AI viruses in poultry (19). In 1970, Samadieh and Bankowski showed that egg production was severely affected in laying turkey hens inseminated with semen contaminated with a turkey influenza virus (25), but no other studies have been published exploring the transmission of AI virus by semen. The presence of virus antigen has been found in testicles of birds infected with HPAI viruses (6; Pantin-Jackwood, pers. obs.) but has not been reported for LPAI viruses.

To evaluate whether LPAI viruses could infect chickens through the cloacal route and reproductive tract, we inoculated chicken layers with two different LPAI viruses given by three different routes, IN, IC, and IO, and examined them for the presence of clinical signs, gross and microscopic lesions, virus in tissues, virus shedding, seroconversion, and transmission to contacts.

MATERIALS AND METHODS

Viruses. Two LPAI viruses from the Southeast Poultry Research Laboratory (SEPRL) repository were used in this study: A/chicken/CA/1255/02 (H6N2) and A/chicken/NJ/12220/97 (H9N2). The H6N2 virus was isolated from 118-week-old layers (39) and the H9N2 virus was a chicken isolate from a live bird market. The H6N2 virus was chosen because of its history of producing drops in egg production in layer flocks and its known ability to infect chickens experimentally (10,39). The H9N2 isolate had not been previously examined in chickens and was chosen to see if the route of inoculation would have an effect on infectivity in hens. The viruses were propagated in 9-day-old specific-pathogen-free (SPF) embryonating chicken eggs and titrated as described (27,38). Allantoic fluid was harvested from the eggs 48 hr postinoculation and diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to obtain 10^6 50% egg infectious dose (EID_{50}) per 0.1 ml/bird. A sham inoculum was made using allantoic fluid from noninfected eggs diluted 1:300 in BHI. All experiments were performed in biosecurity level-2 enhanced facilities at SEPRL.

Experimental design. Seventy 46-wk-old, SPF white leghorn chickens (*Gallus domesticus*) in lay, from SEPRL's in-house flock, were used in this study. The hens were housed in self-contained isolation units that were ventilated under negative pressure with inlet and exhaust HEPA-filtered air and maintained under continuous lighting. Feed and water were provided with *ad libitum* access. Hens were cared for in accordance to SEPRL's Institutional Animal Care and Use Committee-approved animal use protocol. Hens were acclimated to the isolators for 3 days before the beginning of the study to ensure that they were all laying eggs.

Hens were divided into 7 groups of 10 birds each. Hens from groups 1–3 were inoculated with 0.1 ml of inoculum containing 10^6 EID_{50} of the H6N2 virus IN via the choanal slit ($n = 10$), IC by applying the inoculum to the cloacal lips ($n = 10$), and IO by depositing the virus inside the vagina ($n = 10$). Hens from groups 4–6 were similarly inoculated: IN ($n = 10$), IC ($n = 10$), and IO ($n = 10$) with 0.1 ml of inoculum containing 10^6 EID_{50} of the H9N2 virus. Hens from group 7 were IN inoculated with sham inoculum ($n = 10$; Table 1).

Hens were observed daily for clinical signs of disease and laid eggs were counted. Eggs were collected at 4 days postinoculation (dpi) for virus detection. Oropharyngeal (OP) and C swabs were collected at 2, 4, 7, and 14 dpi from all birds to determine viral shedding by quantitative real-time RT-PCR (qRRT-PCR). Swab samples were suspended in 2 ml sterile BHI broth containing an antibiotic-antimycotic mixture and frozen at −70 C until processing. Two hens from each group were euthanatized at 3 dpi with 0.2 ml sodium pentobarbital (5 g/ml) per bird, gross lesions recorded, and the following tissues: lung, spleen, heart, kidney, and oviduct, were collected separately in BHI for virus isolation and stored frozen at −70 C. Trachea, lungs, bursa, kidneys, adrenal gland, thymus, brain, liver, heart, ventriculus, pancreas, intestine, spleen, ovary, oviduct, beak, and thigh tissue were also collected at necropsy. Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry (IHC) was used to stain duplicate sections to determine influenza viral antigen distribution in individual tissues as described previously (17). Two new hens were added to each group at 3 dpi, serving as contacts to study virus transmission. At 14 dpi, all the hens were bled, euthanatized, and necropsied to examine the oviduct.

Table 1. Number of eggs laid by hens inoculated through different routes with 10^6 EID_{50}/0.1 ml of H6N2 or H9N2 viruses or by sham-inoculated controls.

Groups[A]	No. eggs laid 1 to 7 dpi	No. eggs laid 8 to 14 dpi	Total no. of eggs	No. of hens with active oviducts at 14 dpi
1. H6N2 IN	21	6	27	3/10
2. H6N2 IC	26	15	41	4/10
3. H6N2 IO	19	10	29	4/10
4. H9N2 IN	19	11	30	6/10
5. H9N2 IC	21	15	36	6/10
6. H9N2 IO	25	10	35	7/10
7. Controls	33	23	56	8/10

[A]IN = intranasal; IC = intracloacal, IO = intraoviduct.

Serology. Hemagglutination inhibition (HI) assays were performed with serum collected from chickens 14 days after virus challenge to quantify the antibody response. The assay antigens were prepared by inactivating the allantoic fluid from virus-infected chicken eggs with 0.1% beta-propriolactone and adjusting the pH to 7.0 with sodium-bicarbonate. The HI assays were performed in accordance with standard procedures (20). Titers were calculated as the reciprocal of the last HI-positive serum dilution, and samples with HI titers of 4 (2^2) or below were considered negative. A one-way ANOVA with a Tukey's post-test was used to analyze HI titers, after confirming normality, using Prism v.5.01 software (GraphPad Software Inc., La Jolla, CA) and values expressed as the mean ± SD ($P < 0.05$).

Virus detection. RNA was extracted from OP and C swabs using a previously described combination of Trizol LS reagent (Invitrogen Inc., Carlsbad, CA) and the MagMax AI/ND RNA isolation kit (Ambion, Inc., Austin, TX) (9). A qRRT-PCR targeting the influenza M gene (30) was performed using the SmartCycler® 2 (Cepheid Inc., Sunnyvale, CA) and the OneStep RT-PCR kit (Qiagen®, Valencia, CA). A standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the viruses in duplicate and results reported as EID_{50}/ml equivalents (21). The calculated qRRT-PCR detection limit was 10^2 EID_{50}/ml per reaction.

Tissues (lung, spleen, heart, kidney, and sections of the oviduct) were normalized by weight and homogenized in BHI medium, and titers of infectious virus were subsequently determined in embryonated chicken eggs as previously described (36). Similarly, albumin collected from eggs laid on 4 dpi was also examined for the presence of virus. The minimal detectable titer was $10^{0.9}$ EID_{50}/ml from swabs and $10^{1.97}$ EID_{50}/g from tissues.

RESULTS

Clinical signs and gross lesions. Only hens IN-inoculated with the H6N2 virus presented clinical signs consisting of mild depression and a decrease in feed consumption which lasted until 7 dpi. The hens from all groups were laying at the beginning of the experiment and eggs were collected from all groups until the end of the study. However, a decrease in the number of eggs laid was observed in most groups during the second week of the study (Table 1). A decrease in total eggs laid was observed in all virus-inoculated groups when compared to sham-inoculated controls. Hens inoculated with the H6N2 virus produced, in total, 27%–52% fewer eggs and hens inoculated with the H9N2 virus produced 36%–46% less eggs. Hens inoculated with the H6N2 virus by the IN and IO route had the largest decrease in eggs laid. When oviducts were examined at the end of the experiment, 6–7 out of 10 hens per group inoculated with the H6N2 virus had stopped laying eggs *vs.* 3–4 hens per group from the H9N2-inoculated hens and two from the sham-inoculated hens.

Gross and microscopic lesions and viral antigen staining in tissues. Two hens per group were euthanatized at 3 dpi and necropsied. No gross lesions were observed in sham-inoculated control hens. As for the virus-inoculated hens, the only changes observed consisted of congestion of the oviduct. All hens examined had active oviducts. Tissues collected from the hens were also examined for microscopic lesions and viral antigen staining. No, or minimal, lesions were observed in most hens with the exception of hens IN-inoculated with the H6N2 virus in which the trachea presented mild to moderate degenerative changes of the epithelium and the lungs had mild congestion, mild interstitial inflammation with mixed mononuclear cells, and mild catarrhal bronchitis. Mild to moderate hyperplasia of the intestinal epithelium and mild proliferation of gut-associated lymphoid tissues was also present in many hens. Remaining organs, including the oviduct, lacked significant histopathologic lesions. No viral antigen staining, detected by IHC, was present in tissues of any of the hens examined.

Virus detection and serology. No virus was detected in swabs or tissues from the sham-inoculated control birds. The virus shedding and seroconversion data for the virus-inoculated groups is presented in Table 2. All hens became infected with the H6N2 virus when exposed by any of the three routes; IN, IC, or IO. This was demonstrated by seroconversion, with hens from all three groups showing antibodies to the virus at 14 days after inoculation. No statistical difference in HI titers was found between the groups (data not shown). However, differences in numbers of birds shedding virus were observed between hens inoculated with the H6N2 virus by the three different routes. Six to 8 out of 8–10 hens inoculated shed virus through the oropharyngeal route at 2, 4, and 7 dpi, but only 3 of 8 hens shed virus through the cloaca and only at 4 dpi. In hens IC-inoculated, most viral shedding occurred at 4 dpi, and by

Table 2. Virus detection in OP and C swabs and seroconversion in hens inoculated through different routes with 10^6 EID_{50}/0.1 ml of H6N2 or H9N2 viruses.

	Virus shedding at 2 dpi; no. of positive hens/total hens (log_{10} titer)[B]		Virus shedding at 4 dpi; no. of positive hens/total hens (log_{10} titer)		Virus shedding at 7 dpi; no. of positive hens/total hens (log_{10} titer)		Seroconversion at 14 dpi
Groups[A]	OP	C	OP	C	OP	C	HI positive hens/ total hens (titer[C])
H6N2 IN	8/10 (4.8±.5[D])	0/10	8/8 (5.3 ± 0.5)	3/8 (3.0 ± 0.5)	6/8 (1.2 ± 0.3)	0/8	8/8 (6.6 ± 1.9)
H6N2 IC	1/10 (4.2)	3/10 (4.3 ± 0.8)	5/8 (4.3 ± 1.7)	4/8 (4.4 ± 0.1)	1/8 (2.1)	1/8 (1.8)	8/8 (5.5 ± 1.5)
H6N2 IO	1/10 (4.0)	2/10 (4.7 ± 0.4)	4/8 (4.1 ± 1.6)	6/8 (4.3 ± 1.1)	5/8 (2.9 ± 0.6)	4/8 (1.5 ± 0.6)	8/8 (6.8 ± 1.8)
H9N2 IN	–	–	1/8 (3.2)	0/8	0/8	0/8	1/8 (7)
H9N2 IC	–	–	0/8	0/8	0/8	0/8	2/8 (2)
H9N2 IO	–	–	0/8	0/8	0/8	0/8	2/8 (3.0±.7)

[A]IN = intranasal; IC = intracloacal; IO = intraoviduct.
[B]OP = oropharyngeal; C = cloacal.
[C]Mean log_2 HI titer of the sera ± standard deviation.
[D]Mean virus titer ± standard deviation for positive samples as determined by qRRT-PCR and reported as log_{10} EID_{50}/ml.

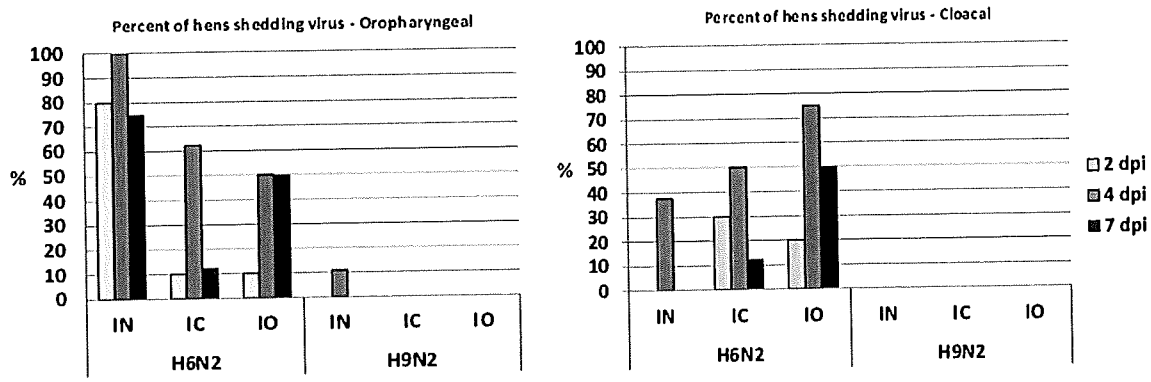

Fig. 1. Percentage of hens shedding virus at 2, 4, and 7 days after being inoculated through different routes with H6N2 or H9N2 viruses.

both the OP and the C route, but at 7 dpi only one hen was shedding virus. In hens IO-inoculated, virus was shed by half or more of the hens at both 4 and 7 dpi and by both routes. The highest virus titers were shed by the OP route from hens IN-inoculated. The percentage of hens shedding viruses at each time point can be better appreciated in Fig. 1. More than 75% of the hens IN-inoculated shed virus through the OP route at all time points, with 37% of hens shedding virus though the cloaca at only 4 dpi. Only 30% of the hens IC-inoculated shed virus initially though the cloaca, but virus was also detected in the oropharynx at 2, 4, and 7 dpi in 10%, 62.5%, and 12.5% of the hens, respectively. In addition, only 10%–20% of hens IO-inoculated shed virus at 2 dpi but more than 50% shed virus through both routes at 4 and 7 dpi. As for hens inoculated with the H9N2 virus, only 1 or 2 hens from each group shed detectable levels of the virus or seroconverted.

The two contact hens from the H6N2 IN- and IC-inoculated groups were positive for virus shedding at 4 days after being introduced into the isolators and seroconverted (Table 3). Only one of the contacts from the IO-inoculated group got infected. None of the contacts from the H9N2 groups showed evidence of infection.

Virus replication was also examined at 2 dpi in tissues collected from hens inoculated with the H6N2 LPAI virus. Virus was only detected in the lung of the two hens IN-inoculated (titers of 1.97 and 2.7 EID_{50}/g), in one hen IC-inoculated (titer of 1.97 EID_{50}/g), in the oviduct of one hen IN-inoculated (titer of 1.97 EID_{50}/g), and in one hen IO-inoculated (titer of 2.7 EID_{50}/g). No virus was detected in albumin samples from all eggs collected at 4 dpi. Tissues and eggs from hens inoculated with the H9N2 virus were not examined because most did not show evidence of infection by virus shedding and seroconversion.

Table 3. Virus detection in OP and C swabs of contact hens, examined at 4 days after introduction into the different groups, and HI titers measured at 12 days.

Groups[A]	Virus shedding; no. of positive hens/total hens (titer)[B]		Seroconversion; no. of positive hens/total hens (titer)
	OP	C	
H6N2 IN	2/2 (3.7, 3.1[C])	0/2	2/2 (7.0, 7.0[D])
H6N2 IC	2/2 (4.5, 3.2)	0/2	2/2 (6.0, 5.0)
H6N2 IO	1/2 (3.7)	0/2	1/2 (10.0)
H9N2 IN	–	–	0/2
H9N2 IC	–	–	0/2
H9N2 IO	–	–	0/2

[A]IN = intranasal; IC = intracloacal; IO = intraoviduct.
[B]OP = oropharyngeal; C = cloacal.
[C]Mean virus titers as determined by qRRT-PCR and reported as $\log_{10}$ EID_{50}/ml.
[D]Mean $\log_2$ HI titer of the sera ± standard deviation.

DISCUSSION

As demonstrated in this study, LPAI viruses can also infect chickens through other routes besides the IN route, which has been traditionally considered the natural route of exposure. The H6N2 virus infected all hens regardless of the route of inoculation and, although most hens didn't show signs of infection when given the H9N2 virus, one or two hens got infected when inoculated by any of the three routes. Hens inoculated IN with the H6N2 virus presented mild clinical signs and shed higher titers of the virus through the OP route at 2 and 4 dpi than did hens inoculated by the IC or IO routes. Interestingly, in hens inoculated with the virus by the IC or IO routes, the respiratory tract eventually got infected, reflected by virus shed by the OP route at 4 dpi. Likewise, hens IN-inoculated shed virus by the cloaca at 4 dpi. This most likely indicates infection by re-exposure to the virus by the different routes, probably by virus present in the isolators after being shed by the hens. The virus infected the hens when given through the cloaca or the oviduct; however, it is not clear in what tissues or cells the virus replicated in, as no virus staining was observed in tissues collected at 3 dpi. Nevertheless, the virus did not initially replicate as well when given by the IC or IO route when compared to the IN route, as more hens inoculated by this route were shedding virus at the earlier time point.

The H6N2 virus was originally isolated from 118-wk-old layers presenting with drops in egg production, increased mortality, and decrease of feed consumption (39). In our study, we saw a decrease in egg production in hens inoculated with this virus but only found evidence of virus replication in the oviduct of two of the hens examined at 3 dpi. However, at the end of the study, half of the hens inoculated with the H6N2 virus had stopped laying eggs compared to eight out of 10 of the sham-inoculated controls. Hens inoculated with the H9N2 virus also laid fewer eggs than did the controls, in spite of the lack of evidence of infection in most of the hens inoculated with this virus. A study examining the pathogenesis of an H4N8 LPAI virus in SPF laying hens indicated that this virus had a tissue tropism for the respiratory and urogenital systems, but lesions were only observed in the oviduct of some of the virus-inoculated hens at 5 and 8 dpi and none at 1.5 and 3 dpi (28). Similar to our study, no virus was isolated from the internal contents of eggs; however, virus was recovered from ovarian and oviduct tissues (28). Those authors concluded that AI virus isolated from the kidney,

magnum, and ovary of hens resulted from a localized infection of the respiratory tract and was not the result of systemic influenza infection but was probably the result of viral infection of the air-sac epithelium associated with the serosal surface of the internal organs. Our study indicates a second alternative: the direct or retrograde infection of the intestine and the oviduct by exposure to the virus by the IC or IO routes. Environmental exposure to the virus by the cloacal route is feasible, and IO exposure is a possibility in chicken breeders if the reproductive organs of the males are infected with the virus or via transmission by semen as reported previously for turkeys (25).

When conducting controlled experiments, it is difficult to reproduce the conditions present in the farms and many factors, including the number of birds used and the presence of secondary infectious agents, could explain the differences observed in the presentation of the disease. For instance, in breeder turkeys, LPAI infections commonly result in decreased egg production, and co-infection with other agents can worsen the clinical presentation and, in some cases, induce complete cessation of egg production (8,13,16,22,29,32,37). Environmental factors such as animal density and housing can also contribute to the differences in infection and transmission observed between the field and experimental situations (2). On the other hand, Pillai *et al.* showed that a triple reassortant H3N2 virus associated with drops in egg production in turkey breeders can, by itself, cause drastic reduction or complete cessation of egg production and pathology of the reproductive tract in IN-inoculated 26-wk-old breeder turkeys (22) and that this virus could be detected from internal egg contents following experimental infection (23). In our study, we did not observe virus staining in the oviduct as seen in studies in turkeys (19,22), and virus isolation from the oviduct or egg albumin was low or under the levels of detection. Therefore, it is not clear why the hens infected with the LPAI viruses produced fewer eggs than did the controls. One explanation could be that the mild disease produced by infection caused distress or affected feed and water consumption enough to affect lay. More studies are needed to better understand the effect of LPAI on the reproductive tract of hens.

In conclusion, LPAI viruses can also infect chickens when given by the IC or IO routes, can locally replicate, and can re-infect by the respiratory route, which is considered the natural route of exposure. This has implications in the transmission and pathogenesis of AI viruses in poultry and can inform future management decisions for control of the virus, among them better cleaning and disinfection of the premises and examination of alternate routes of transmission, including through AI virus-infected males.

REFERENCES

1. Bano, S., K. Naeem, and S. A. Malik. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. Avian Dis. 47:817–822. 2003.

2. Berhane, Y., D. Ojkic, J. Neufeld, M. Leith, T. Hisanaga, H. Kehler, A. Ferencz, H. Wojcinski, C. Cottam-Birt, M. Suderman, K. Handel, S. Alexandersen, and J. Pasick. Molecular characterization of pandemic H1N1 influenza viruses isolated from turkeys and pathogenicity of a human pH1N1 isolate in turkeys. Avian Dis. 54:1275–1285. 2010.

3. Bowes, V. A., S. J. Ritchie, S. Byrne, K. Sojonky, J. J. Bidulka, and J. H. Robinson. Virus characterization, clinical presentation, and pathology associated with H7N3 avian influenza in British Columbia broiler breeder chickens in 2004. Avian Dis. 48:928–934. 2004.

4. Brandly, C. A., and L. D. Bushell. A report of some investigations of infectious laryngotracheitis. Poult. Sci. 13:212–217. 1934.

5. Bwala, D. G., S. Clift, N. M. Duncan, S. P. Bisschop, and F. F. Oludayo. Determination of the distribution of lentogenic vaccine and virulent Newcastle disease virus antigen in the oviduct of SPF and commercial hen using immunohistochemistry. Res. Vet. Sci. 2011. (E-pub ahead of print).

6. Capua, I., and F. Mutinelli. A color atlas and text on avian influenza. Papi Editore, Edizioni Tecnico Scientifiche, Bologna, Italy. p. 47. 2001.

7. Cardona, C. J., B. R. Charlton, and P. R. Woolcock. Persistence of immunity in commercial egg-laying hens following vaccination with a killed H6N2 avian influenza vaccine. Avian Dis. 50:374–379. 2006.

8. Choi, Y. K., J. H. Lee, G. Erickson, S. M. Goyal, H. S. Joo, R. G. Webster, and R. J. Webby. H3N2 influenza virus transmission from swine to turkeys, United States. Emerg. Infect. Dis. 10:2156–2160. 2004.

9. Das, A., E. Spackman, M. J. Pantin-Jackwood, and D. L. Suarez. Removal of real-time reverse transcription-polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of avian influenza virus by RT-PCR. J. Vet. Diagn. Invest. 21:771–778. 2009.

10. Jackwood, M. W., D. L. Suarez, D. Hilt, M. J. Pantin-Jackwood, E. Spackman, P. Woolcock, and C. Cardona. Biologic characterization of chicken-derived H6N2 low pathogenic avian influenza viruses in chickens and ducks. Avian Dis. 54:120–125. 2010.

11. Kwon, Y. K., and D. E. Swayne. Different routes of inoculation impact infectivity and pathogenesis of H5N1 high pathogenicity avian influenza virus infection in chickens and domestic ducks. Avian Dis. 54:1260–1269. 2010.

12. Lu, H., P. A. Dunn, E. A. Wallner-Pendleton, D. J. Henzler, D. C. Kradel, J. Liu, D. P. Shaw, and P. Miller. Investigation of H7N2 avian influenza outbreaks in two broiler breeder flocks in Pennsylvania, 2001–02. Avian Dis. 48:26–33. 2004.

13. Ludwig, S., A. Haustein, E. F. Kaleta, and C. Scholtissek. Recent influenza A (H1N1) infections of pigs and turkeys in northern Europe. Virology 202:281–286. 1994.

14. McDougald, L. R. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis. 49:462–476. 2005.

15. Mutinelli, F., I. Capua, C. Terregino, and G. Cattoli. Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenicity avian influenza epidemics in Italy during 1999 and 2000. Avian Dis. 47:844–848. 2003.

16. Olsen, C. W., A. I. Karasin, S. Carman, Y. Li, N. Bastien, D. Ojkic, D. Alves, G. Charbonneau, B. M. Henning, D. E. Low, L. Burton, and G. Broukhanski. Triple reassortant H3N2 influenza A viruses, Canada, 2005. Emerg. Infect. Dis. 12:1132–1135. 2006.

17. Pantin-Jackwood, M. J., and D. E. Swayne. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian Dis. 51:250–259. 2007.

18. Pantin-Jackwood, M. J., and D. E. Swayne. Pathogenesis and pathobiology of avian influenza virus infection in birds. Rev. Sci. Tech. 28:113–136. 2009.

19. Pantin-Jackwood, M., J. L. Wasilenko, E. Spackman, D. L. Suarez, and D. E. Swayne. Susceptibility of turkeys to pandemic-H1N1 virus by reproductive tract insemination. Virol. J. 7:27. 2010.

20. Pedersen, J. C. Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantitation of serum antibodies to the avian influenza virus. Methods Mol. Biol. 436:53–66. 2008.

21. Pfeiffer, J., D. L. Suarez, L. Sarmento, T. L. To, T. Nguyen, and M. J. Pantin-Jackwood. Efficacy of commercial vaccines in protecting chickens and ducks against H5N1 highly pathogenic avian influenza viruses from Vietnam. Avian Dis. 54:262–271. 2010.

22. Pillai, S. P., M. Pantin-Jackwood, S. J. Jadhao, D. L. Suarez, L. Wang, H. M. Yassine, Y. M. Saif, and C. W. Lee. Pathobiology of triple reassortant H3N2 influenza viruses in breeder turkeys and its potential implication for vaccine studies in turkeys. Vaccine 27:819–824. 2009.

23. Pillai, S. P., Y. M. Saif, and C. W. Lee. Detection of influenza A in eggs laid by infected turkeys. Avian Dis. 54:830–833. 2010.

24. Roussan, D. A., G. Y. Khawaldeh, R. H. Al Rifai, W. S. Totanji, and I. A. Shaheen. Avian influenza virus H9 subtype in poultry flocks in Jordan. Prev. Vet. Med. 88:77–81. 2009.

25. Samadieh, B., and R. A. Bankowski. Effect of avian influenza-A viruses upon egg production and fertility of turkeys. Avian Dis. 14:715–722. 1970.

26. Seal, B. S., D. J. King, D. P. Locke, D. A. Senne, and M. W. Jackwood. Phylogenetic relationships among highly virulent Newcastle disease virus isolates obtained from exotic birds and poultry from 1989 to 1996. J. Clin. Microbiol. 36:1141–1151. 1998.

27. Senne, D. Virus propagation in embryonating eggs. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 5th ed. D. E. Swayne, L. Dofour-Zavala, J. R. Gilsson, J. E. Pearson, M. W. Jackwood, W. M. Reed, and P. R. Woolcock, eds. American Association of Avian Pathologists, Jacksonville, FL. pp. 204–208. 2008.

28. Shalaby, A. A., R. D. Slemons, and D. E. Swayne. Pathological studies of A/chicken/Alabama/7395/75 (H4N8) influenza virus in specific-pathogen-free laying hens. Avian Dis. 38:22–32. 1994.

29. Skeeles, J., J. N. Beasley, P. Blore, and S. Klopp. Severe egg-production drops in turkey breeders in Southcentral Missouri. Avian Dis. 25:764–767. 1981.

30. Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256–3260. 2002.

31. Spickler, A. R., D. W. Trampel, and J. A. Roth. The onset of virus shedding and clinical signs in chickens infected with high-pathogenicity and low-pathogenicity avian influenza viruses. Avian Pathol. 37:555–577. 2008.

32. Suarez, D. L., P. R. Woolcock, A. J. Bermudez, and D. A. Senne. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis. 46:111–121. 2002.

33. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. J. R. Glisson, Y. M. Saif, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell Publishing, Ames, Iowa. 2008.

34. Swayne, D. E., and M. Pantin-Jackwood. Pathobiology of avian influenza virus infections in birds and mammals. In: Avian influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, Iowa. pp. 87–122. 2008.

35. Swayne, D. E., M. Pantin-Jackwood, D. Kapczynski, E. Spackman, and D. L. Suarez. Susceptibility of poultry to pandemic (H1N1) 2009 virus [letter]. Emerg. Infect. Dis. 15:2061–2063. 2009.

36. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian influenza. In: A labortatory manual for the isolation and identification of avian pathogens, 4th ed. J. R. Glisson, D. E. Swayne, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 150–155. 1998.

37. Tang, Y., C. W. Lee, Y. Zhang, D. A. Senne, R. Dearth, B. Byrum, D. R. Perez, D. L. Suarez, and Y. M. Saif. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis. 49:207–213. 2005.

38. Villegas, P. Titration of virological suspensions. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 5th ed. D. E. Swayne, L. Doufour-Zavala, J. R. Gilsson, J. E. Pearson, M. W. Jackwood, W. M. Reed, and P. R. Woolcock, eds. American Association of Avian Pathologists Jacksonville, FL. pp. 217–221. 2008.

39. Woolcock, P. R., D. L. Suarez, and D. Kuney. Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000–02. Avian Dis. 47:872–881. 2003.

40. Ziegler, A. F., S. Davison, H. Acland, and R. J. Eckroade. Characteristics of H7N2 (nonpathogenic) avian influenza virus infections in commercial layers, in Pennsylvania, 1997–98. Avian Dis. 43:142–149. 1999.

ACKNOWLEDGMENTS

We appreciate the assistance provided by Ronald Graham and Scott Lee in conducting these studies. We also thank the FSIS histopathology laboratory at Russell Research Center–United States Department of Agriculture for technical assistance.

Journal of Virology

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J. Virol. 2012, 86(13):7118. DOI: 10.1128/JVI.00674-12.
Published Ahead of Print 2 May 2012.

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Seasonal Trivalent Inactivated Influenza Vaccine Protects against 1918 Spanish Influenza Virus Infection in Ferrets

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The influenza virus H1N1 pandemic of 1918 was one of the worst medical catastrophes in human history. Recent studies have demonstrated that the hemagglutinin (HA) protein of the 1918 virus and 2009 H1N1 pandemic virus [A(H1N1)pdm09], the latter now a component of the seasonal trivalent inactivated influenza vaccine (TIV), share cross-reactive antigenic determinants. In this study, we demonstrate that immunization with the 2010-2011 seasonal TIV induces neutralizing antibodies that cross-react with the reconstructed 1918 pandemic virus in ferrets. TIV-immunized ferrets subsequently challenged with the 1918 virus displayed significant reductions in fever, weight loss, and virus shedding compared to these parameters in nonimmune control ferrets. Seasonal TIV was also effective in protecting against the lung infection and severe lung pathology associated with 1918 virus infection. Our data demonstrate that prior immunization with contemporary TIV provides cross-protection against the 1918 virus in ferrets. These findings suggest that exposure to A(H1N1)pdm09 through immunization may provide protection against the reconstructed 1918 virus which, as a select agent, is considered to pose both biosafety and biosecurity threats.

The Spanish influenza pandemic of 1918 resulted in an estimated 20 to 50 million deaths worldwide and a 10-year reduction in the average life expectancy (13). During that year in the United States, outbreaks of respiratory disease occurred simultaneously in humans and swine; whether the H1N1 virus jumped species from humans to pigs or vice versa remains unclear. Recently, it has been suggested that the 1918 pandemic virus was derived from an influenza virus of swine origin and that the precursor of this virus was a descendant of a distinct avian H1N1 virus (1). The H1N1 virus became established in domestic pigs after 1918, forming the classical swine H1N1 lineage, and continued to circulate as the dominant influenza virus in North American swine populations until 1998 (7, 29). Although the human 1918 and swine H1N1 viruses evolved separately in different hosts and thus diverged genetically (32, 33), the hemagglutinin (HA) of the H1N1 viruses maintained significant antigenic similarities (40, 42).

In April 2009, a novel influenza A H1N1 virus [A(H1N1)pdm09] was first detected in humans and spread throughout the world to cause the first influenza pandemic of the 21st century. This pandemic virus of apparent swine origin was derived from a reassortment in nature of avian, human, and swine influenza A viruses and contains an HA gene belonging to the classical swine H1N1 lineage (12). During the subsequent 2009-2010 and 2010-2011 winter seasons, the 2009 H1N1 subtype virus continued to circulate throughout the world and appears to have replaced the seasonal H1N1 virus (30). In September 2009, the Food and Drug Administration (FDA) licensed the first A(H1N1)pdm09 vaccine, available as a monovalent vaccine used during the 2009-2010 season. Subsequently, the WHO recommended that vaccine strains for the 2010-2011 trivalent influenza vaccine contain a pandemic 2009 H1N1 (A/California/7/2009-like) component (8). For the inactivated trivalent influenza vaccine (hereinafter referred to as TIV), each 0.5-ml dose of TIV contains 15 μg of influenza HA of each strain (45 μg total) from three viral strains: two influenza A virus subtypes (H1N1 and H3N2) and one influenza B virus strain. The seasonal inactivated TIV is the most commonly administered influenza vaccine, and each year hundreds of millions of individuals are vaccinated worldwide (20, 21).

Several serological studies of the A(H1N1)pdm09 virus have provided evidence for the presence of preexisting cross-reactive antibodies to a 1918-like H1N1 virus from previous vaccinations or infections (15, 18, 26). Using stored serum samples from trials of seasonal TIV predating the 2009 pandemic, Hancock et al. showed that vaccination with contemporary seasonal influenza vaccines containing former seasonal H1N1 viruses provided little cross-reactive immunity to the A(H1N1)pdm09 virus; however, subjects who were born before 1930 (and therefore were probably exposed to a 1918-like H1N1 virus) possessed cross-reactive antibodies to the A(H1N1)pdm09 virus (15). Thus, despite more than 90 years of separation between the pandemic viruses, the 1918 and A(H1N1)pdm09 viruses induced antibodies that demonstrated cross-neutralization. Moreover, immunization of mice with a 1918 virus vaccine conferred protection against the A(H1N1)pdm09 virus, documenting cross-protection *in vivo* (25, 42). Conversely, using the mouse model, a recent study addressed the impact of A(H1N1)pdm09 influenza monovalent vaccination on 1918 virus protection (26). However, this study did not test the efficacy of contemporary seasonal TIV against 1918 virus challenge in ferrets.

Ferrets are considered to be the most suitable animal model for influenza vaccine efficacy studies (5, 17, 36), and this animal model has been used to demonstrate the inherent virulence of the

Received 16 March 2012 Accepted 12 April 2012
Published ahead of print 2 May 2012
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doi:10.1128/JVI.00674-12

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TABLE 1 Serum antibody neutralizing responses following seasonal TIV administration in ferrets

Group	Neutralizing antibody titer (GMT)[a]			
	A/Perth/16/2009	A/Mexico/4482/2009	B/Brisbane/60/2008	1918
Experiment 1				
Prevaccination	<10	<10	<10	<10
Pre-1st booster vaccination	<10	<10	20–80 (40)	<10
Pre-2nd booster vaccination	10–20 (11)	10–40 (17)	160–320 (211)	<10–10 (8)
Prechallenge	20–80 (35)	40–320 (121)	160–640 (485)	10–40 (23)
Experiment 2				
Prevaccination	<10	<10	<10	<10
Pre-1st booster vaccination	<10	<10–20 (5.9)	20–80 (38)	<10
Pre-2nd booster vaccination	ND	ND	ND	ND
Prechallenge	20–160 (47)	40–320 (136)	40–320 (143)	20–80 (28)

[a] Serum neutralizing antibody responses following primary and booster immunizations of 2010-2011 seasonal TIV are shown. ND, not done.

1918 influenza viruses in comparison to that of other influenza viruses (41). Here, we demonstrate that vaccination with the seasonal 2010-2011 TIV induced neutralizing antibodies to the 1918 virus and that immune ferrets displayed a significant reduction in viral shedding and fever following 1918 virus challenge. Influenza vaccination with TIV also prevented significant virus replication in the lung and trachea tissues of ferrets on subsequent challenge, further demonstrating that the current influenza vaccine would provide protection against the 1918 pandemic virus. These findings have implications for biosafety and biosecurity precautions that are designed to protect laboratorians and the public from possible exposure to this virus.

MATERIALS AND METHODS

Viruses. Stocks for the H1N1 influenza viruses used in the study, A/Mexico/4482/2009 (Mex/4482) and the rescued 1918 virus, were propagated in Madin-Darby canine kidney (MDCK) cells as previously described (39, 43). Mex/4482 has been found to be antigenically and genetically similar to the prototype A(H1N1)pdm09 vaccine virus, A/California/7/2009 (12). The seasonal human H3N2 virus, A/Perth/16/2009 (Perth/16), and the influenza B virus, B/Brisbane/60/2008 (B/Brisbane/60), were grown in the allantoic cavities of 10-day-old embryonating hens' eggs at 34°C for 48 to 72 h. Pooled cell supernatant or allantoic fluid was clarified by centrifugation and stored at −70°C. All stocks were titrated in a standard plaque assay and expressed as PFU (43). All experiments were performed in biosafety level 3 laboratories with enhancements (BSL3+) as outlined in *Biosafety in Microbiological and Biomedical Laboratories* (9). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Vaccination and challenge. Adult male Fitch ferrets, 5 to 12 months of age (Triple F Farms, Sayre, PA), serologically negative by hemagglutination inhibition (HI) assay for currently circulating influenza viruses, were used in this study. For vaccinations, ferrets were each injected 3 times (4 to 5 weeks between injections) intramuscularly (i.m.) with an adult-human dose (0.5 ml) of the 2010-2011 seasonal inactivated split-product TIV or phosphate-buffered saline (PBS) (controls). To determine viral replication in nasal washes, as well as clinical signs of infection, ferrets were randomly assigned to be challenged with 1918 virus (5 TIV inoculated and 5 PBS controls), Perth/16 virus (3 TIV inoculated and 4 PBS controls), or Mex/4482 (3 TIV inoculated and 4 PBS controls). In a second experiment (experiment 2) to study the effect of vaccination on virus replication in respiratory tissues and lung histopathology, ferrets were TIV inoculated ($n = 6$) or given PBS ($n = 6$) on the schedule detailed above.

Prior to initial vaccination, vaccine boosts, and viral challenge, all ferrets were bled for collection of serum. Following anesthesia with an i.m. injection of a ketamine-xylazine-atropine cocktail, ferrets were challenged intranasally with 10^6 PFU of virus in a total volume of 1 ml (500 μl per nostril) diluted in PBS (31). Challenge with 1918, Mex/4482, or Perth/16 virus occurred 5 to 8 weeks after final vaccine boost. Following challenge, ferrets were monitored daily for changes in body weight and temperature, as well as clinical signs of illness (24). The statistical significance of differences in weight loss and virus titers (nasal washes and tissues) between vaccinated and control animals was determined by Student's *t* test.

Histopathology. Necropsies were performed on three ferrets from each group at three and 5 days postinoculation (d.p.i.). Three 5-mm lung sections of each left lung lobe were collected for histopathology. Lung sections were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Duplicate 5-μm sections were immunohistochemically (IHC) stained to demonstrate influenza A nucleoprotein (NP) by first microwaving the sections in Citra antigen retrieval solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for type A influenza virus NP was used as the primary antibody for a streptavidin-biotin-alkaline phosphatase complex-based IHC method as previously described (35, 38). Only cells with red-staining nuclei were considered positive with the IHC assay.

Antibody assays. All sera were initially diluted 1:10 in receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo, Japan) and tested for neutralizing antibody titers against 1918, Mex/4482, Perth/16, or B/Brisbane/60 virus as described previously (27). Titers were calculated as the reciprocal of the highest dilution of serum that neutralized 100 PFU of virus in MDCK cell cultures. The neutralization titers are also presented as the geometric mean titers (GMT) from vaccinated or control ferrets.

RESULTS

Immunogenicity of 2010-2011 seasonal inactivated TIV in ferrets. We first assessed the level of cross-reactive antibody to 1918 (H1N1) influenza virus following vaccination with the 2010-2011 seasonal TIV. Prior to vaccine boost and viral challenge, ferret sera were collected to assess neutralization antibody responses against the 1918 virus and against the three homologous viruses in the 2010-2011 TIV. As shown in Table 1 (experiment 1), a single dose of vaccine did not induce detectable neutralizing antibody titers to 1918, seasonal H3N2 (Perth/16), or H1N1 (Mex/4482) virus. Neutralizing antibody titers to influenza B (B/Brisbane/60) virus were detected that ranged from 20 to 80 (geometric mean titer

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TABLE 2 Clinical symptoms observed in TIV-immunized ferrets challenged with homologous virus or heterologous 1918 virus

Group	No. of animals with indicated result following challenge/total no. of animals[a]								
	A/Perth/16/2009 (homologous)			A/Mexico/4482/2009 (homologous)			1918 (heterologous)		
	Wt loss (%)	Fever (°C)	Virus detected (peak titer)	Wt loss (%)	Fever (°C)	Virus detected (peak titer)	Wt loss (%)	Fever (°C)	Virus detected (peak titer)
TIV immunized	3/3 (4.6)	3/3 (1.3)	3/3 (5.2)	3/3 (6.5)	3/3 (1.2)	3/3 (5.2)	5/5 (2.8)	5/5 (1.1)	5/5 (3.7)
Mock vaccinated	4/4 (5.7)	4/4 (1.5)	4/4 (5.3)	4/4 (16.8)	4/4 (1.1)	4/4 (4.9)	5/5 (13.2)	5/5 (1.4)	5/5 (5.6)

[a] Ferrets were vaccinated i.m. three times with the 2010-2011 seasonal trivalent influenza vaccine (TIV) or with PBS. Data show either the number of animals in which weight loss was observed, with mean maximum weight loss percentage in parentheses, the number of animals in which fever was observed, with mean maximum increase over baseline in parentheses, or the number of animals in which virus was detected in nasal wash samples, with mean peak titer ($\log_{10}$ PFU/ml) in parentheses.

[GMT] = 40). Following vaccine boost, TIV elicited low neutralizing antibody (<10 to 40) titers to 1918, Perth/16, and Mex/4482 virus, whereas a single vaccine boost led to substantial increases in antibody titers (160 to 320) to B/Brisbane/60 virus (GMT = 211). A second vaccine boost was required to obtain detectable homologous antibody levels in all ferrets, measured 3 days before challenge. The prechallenge neutralizing titers were highest against B/Brisbane/60 virus (GMT = 485), followed by Mex/4482 virus (GMT = 121) and then Perth/16 (GMT = 35) virus. Neutralizing activity to 1918 virus was also detected, at titers that ranged from 10 to 40 (GMT = 23). As expected, all sera from PBS control or prevaccinated ferrets failed to neutralize any of the viruses in the test.

In a second vaccine experiment aiming to evaluate TIV-induced protection against viral replication in respiratory tract tissues, preboost and prechallenge ferret neutralizing titers against 1918 virus were determined (Table 1, experiment 2). Similar to the antibody responses in the first experiment, a single immunization with 2010-2011 seasonal TIV failed to elicit a significant antibody response to 1918, H3N2 (Perth/16), or Mex/4482 (H1N1) virus. Again, a second vaccine boost elicited measurable homologous antibody levels in all ferrets. The prechallenge neutralizing antibody titers ranged from 20 to 160 (GMT = 47) against Perth/16 virus and from 40 to 320 (GMT = 136) to Mex/4482 virus. The prechallenge virus neutralizing antibody titers to B/Brisbane/60 ranged from 40 to 320 (GMT = 143). Importantly, neutralizing activity to 1918 virus that ranged from 20 to 80 (GMT = 28) was detected in all ferrets (Table 1, experiment 2). Overall, these data indicate that the licensed vaccine for seasonal 2010-2011 influenza elicits neutralizing antibodies to the 1918 pandemic virus.

Protective efficacy of seasonal TIV to homologous virus challenge. We first determined the level of protection against homologous virus challenge induced by seasonal 2010-2011 TIV. Vaccine protection was measured by reductions in fever, weight loss, and viral shedding in ferrets after challenge with Perth/16 or Mex/4482 virus. Following either virus challenge, no significant differences in body temperatures were detected between TIV-immunized and unimmunized control ferrets observed for 14 days postchallenge (p.c.). All TIV-immunized ferrets displayed a peak in body temperature on days 1 and 2 p.c., with mean maximum fever of 1.2 or 1.3°C above the baseline (Table 2). Similarly, Perth/16 or Mex/4482 virus challenge induced an increase in temperature among all corresponding control ferrets, with a mean maximum fever of 1.5 or 1.1°C above the baseline, respectively.

Viral challenge with the seasonal Perth/16 virus resulted in minimal morbidity for vaccinated and control ferrets, causing 4.6% and 5.7% maximum weight loss, respectively (Fig. 1A). In contrast, the more pathogenic Mex/4482 virus caused greater morbidity among unvaccinated control ferrets (16.8% mean maximum weight loss) and TIV provided partial protection against weight loss; none of the vaccinated animals lost more than 9.7% of their initial body weight (6.5% mean maximum weight loss) during any point in the study (Fig. 1B and Table 2).

The extent of virus replication in the upper respiratory tract was determined by titrating nasal wash samples collected from immune and control ferrets following challenge with Perth/16 or Mex/4482 virus. In comparison to unimmunized control animals, TIV-immunized ferrets displayed a significant reduction

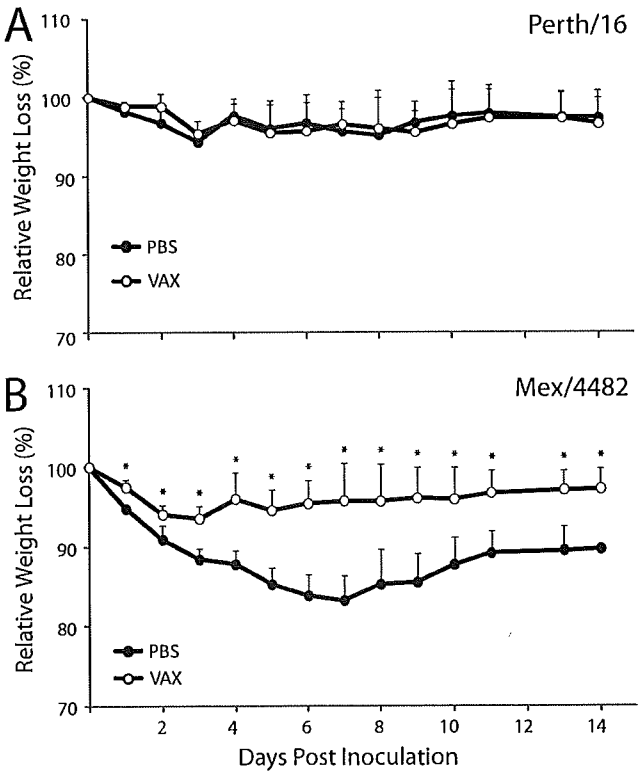

FIG 1 Protective efficacy of seasonal TIV (VAX) in ferrets following homologous virus challenge. The morbidity of ferrets was determined for 14 days following challenge with seasonal human H3N2 virus A/Perth/16/2009 (Perth/16) (A) or H1N1 virus A/Mexico/4482/2009 (Mex/4482) (B). For each virus challenge, three TIV-inoculated ferrets (open circle) and four PBS control ferrets (filled circle) were challenged with 10^6 PFU of virus. Differences in weight loss between groups were analyzed by Student's t test (*, $P \leq 0.005$). Error bars show standard deviations.

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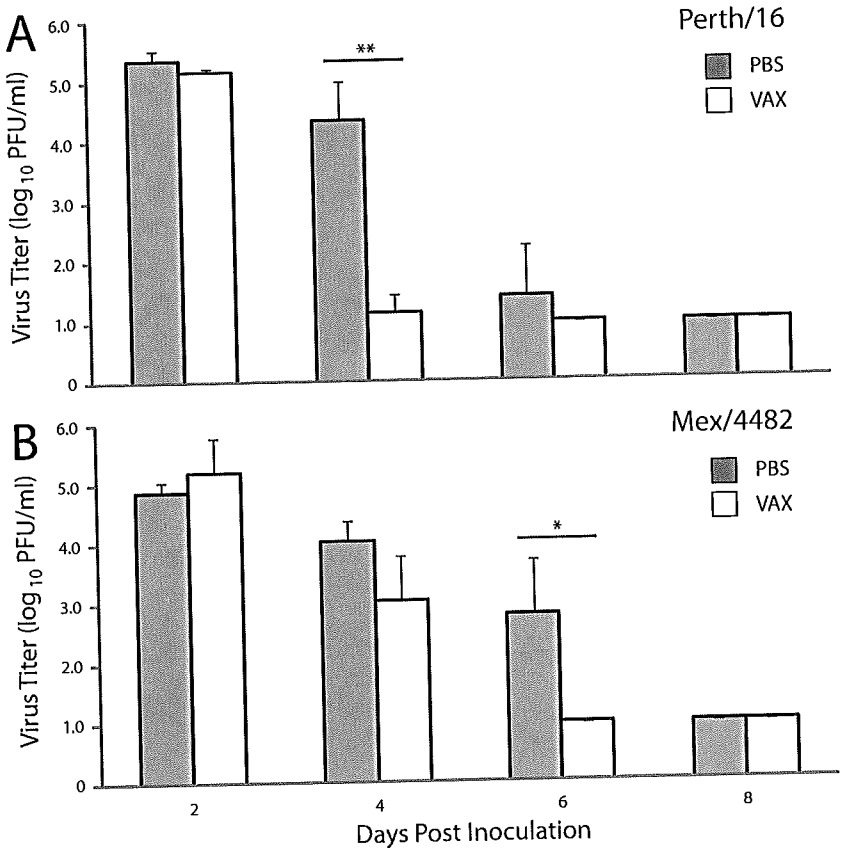

FIG 2 Seasonal TIV efficacy against virus replication of A/Perth/16/2009 (Perth/16) or A/Mexico/4482/2009 (Mex/4482) virus. The nasal cavities of ferrets were washed with 1 ml of PBS every other day starting 2 days p.c., and virus titers were determined in a standard plaque assay. Gray-shaded bars represent the results for unimmunized (PBS) control ferrets, and open bars represent the results for TIV-immunized ferrets. The data shown are mean values plus standard deviations (error bars) for 3 or 4 ferrets per group (A and B). Differences in viral titers of nasal washes between groups were analyzed by Student's *t* test (*, $P \leq 0.020$; **, $P \leq 0.0005$).

in viral shedding among Perth/16 virus-challenged ferrets on day 4 p.c. ($P \leq 0.0005$) and Mex/4482 virus-challenged ferrets on day 6 p.c. ($P \leq 0.020$) (Fig. 2). Moreover, TIV-immunized ferrets cleared Perth/16 and Mex/4482 virus 2 days earlier than control ferrets. These data indicate that although TIV does not provide sterilizing immunity against homologous virus challenge, the seasonal influenza vaccine offers some level of protection against viral shedding and against A(H1N1)pdm09-induced severe morbidity. Such data can be compared to the level of cross-protection against heterologous 1918 H1N1 virus challenge.

Cross-protective efficacy of 2010-2011 seasonal TIV to 1918 pandemic H1N1 virus. We next assessed the degree of cross-protection against the 1918 H1N1 virus conferred by seasonal TIV. TIV-immunized and unimmunized control ferrets received a similar high-titer (10^6 PFU) challenge dose of the reconstructed 1918 virus. On day 1 p.c., all unimmunized control ferrets exhibited an early spike in body temperature, ranging from 1.3 to 1.6°C above the baseline (mean maximum = 1.4°C) (Table 2), and elevated temperatures (>0.8°C above the baseline) were maintained for 7 days p.c. (not shown). TIV-immunized animals also displayed an early spike in body temperature (mean maximum = 1.1°C) but had significantly lower ($P \leq 0.05$) body temperatures than controls on days 4 and 6 p.c. Significant differences in weight loss were also observed between the two groups on day 1 p.c. onwards ($P \leq 0.04$). Control animals exhibited 13.2% mean maximum weight loss compared with 2.8% weight loss for vaccinated ferrets (Fig. 3A and Table 2).

Prior seasonal-TIV administration also reduced shedding of 1918 virus in challenged ferrets. The viral titers measured in nasal washes were significantly reduced ($P \leq 0.04$) on days 2, 4, and 6, and vaccinated ferrets exhibited higher rates of virus clearance than controls (Fig. 3B). Taken together, these data suggest that TIV immunization offered significant protection against substantial weight loss and viral shedding following 1918 virus challenge.

The effect of 2010-2011 seasonal-TIV vaccination on virus replication in the respiratory tract and histopathology in lung tissue. In another experiment, the extent of TIV protection against the 1918 virus was measured in terms of (i) viral titers in the upper (nasal turbinate and trachea) and lower (lung) respiratory tract tissues of individual ferrets on days 3 and 5 p.c. and (ii) lesion formation in lung tissues. Vaccinated and control ferrets were similarly challenged with 10^6 PFU of the 1918 virus 5 weeks after the second vaccine boost (Table 1, experiment 2). On days 3 and 5 p.c., unimmunized control animals showed high titers ($\geq$4.7 $\log_{10}$ PFU/ml) of replicating virus in each of the three respiratory tract tissues (Fig. 3C). In contrast, the mean tissue titers among vaccinated animals were lower and significant reductions

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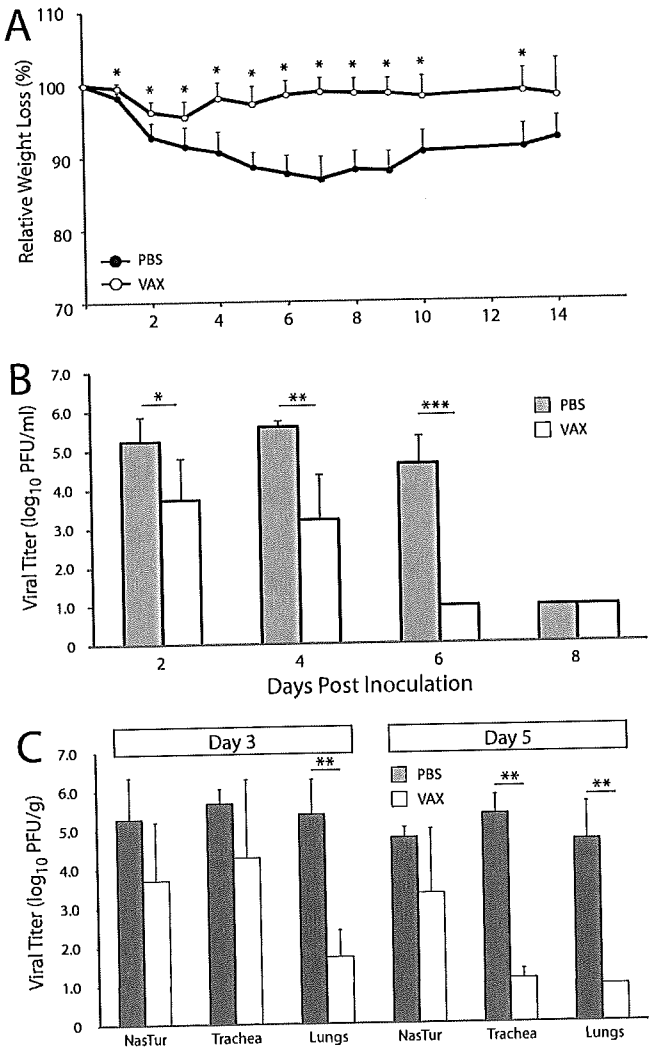

FIG 3 Clinical symptoms and viral shedding in TIV-immunized and control ferrets following 1918 virus challenge. (A) Mean weight loss observed among vaccinated (open circles) and PBS control (filled circles) ferrets following challenge with 10^6 PFU of the 1918 virus. Nasal washes (B) and tissues (C) at 3 (left) and 5 (right) days p.c. were collected and viruses titrated in a standard plaque assay. Gray-shaded bars represent results for unimmunized (PBS) control ferrets, and open bars represent results for TIV-immunized ferrets. The data shown are mean values plus standard deviations (error bars) for 5 ferrets per group (A and B) and for 3 ferrets per group for each time period (C). Differences in viral titers between groups were analyzed by Student's t test (*, $P \leq 0.04$; **, $P \leq 0.005$; ***, $P \leq$ 3E-06).

were noted in the tracheal (day 5 p.c., $P < 0.005$) and lung tissues (days 3 and 5 p.c., $P < 0.005$). The mean viral titers for tracheal samples on day 5 were 17,000-fold less than those in unimmunized controls, while the mean viral titers in lung tissue on days 3 and 5 p.c. were at least 4,600-fold less than those in unimmunized controls. On day 3 p.c., only trace amounts of infectious virus were found in lung tissue of one of three vaccinated ferrets, and on day 5 p.c., no virus was measured above the limit of detection (1.0 log_{10} PFU/g).

Lung tissue sections from TIV-immunized and unimmunized control ferrets were also examined for viral antigen and microscopic lesion formation. Following 1918 virus challenge, lung lesions from unimmunized controls resembled lesions previously reported in 1918 virus-infected ferrets (41). The damage affected multiple lung lobes and consisted of moderate to severe necrotizing bronchiolitis and alveolitis with focal areas of acute alveolar necrosis, edema, and hemorrhage. (Fig. 4A to D). At both 3 and 5 days p.c., control lungs displayed mild epithelial necrosis in bronchus submucosal serous glands and moderate to severe bronchitis and bronchiolitis accompanied by mild to moderate peribronchiolar accumulation of mixed inflammatory cells. The lumen of the bronchi and bronchioles contained cellular debris and various amounts of neutrophils and mononuclear cells. Fibrin and edema were also observed within the airways of unimmunized control ferrets. Additionally, mild to severe, focal to diffuse alveolitis and alveolar epithelial necrosis with hemosiderosis, intra-alveolar edema, and hemorrhage were observed, especially at 5 days p.c. Virus antigen was present in epithelial cells and in detached cells in the lumen of bronchi and bronchioles, in submucosal serous glands, in alveolar epithelium, and in alveolar macrophages (Fig. 4A to D).

Two of three TIV-immunized ferrets challenged with the 1918 influenza virus examined at day 3 p.c. displayed milder lung lesions than control lungs, characterized by mild peribronchial and perivascular interstitial pneumonia with focal areas of congestion and edema (Fig. 4E). Thickening of the alveolar epithelium and some hemosiderosis were also observed; however, no specific viral antigen staining was detected in these lung samples (Fig. 4E). The third ferret examined at this same time point displayed bronchopneumonia, with macrophages, neutrophils, and cellular debris present in the airways and multifocal edema and hemorrhage. Viral staining was infrequent in bronchiole and bronchus respiratory epithelium but common in alveolar epithelium and macrophages (not shown). In general, the three vaccinated ferrets examined at day 5 p.c. displayed milder lung disease; some sections showed mild to moderate bronchointerstitial pneumonia with edema and congestion (Fig. 4F). No viral antigen was detected in lung sections in any of the vaccinated animals at this time point (Fig. 4F).

DISCUSSION

Following multiple reassortment events, the A(H1N1)pdm09 virus emerged from North American swine to infect humans and possessed an HA gene belonging to the classical swine lineage (12). The ancestry of the classical swine influenza HA can be traced back to H1N1 viruses that circulated in pigs since 1918. The presence of a swine influenza-like HA in the genome of the A(H1N1)pdm09 virus which has largely remained antigenically intact suggested that seasonal influenza vaccination would provide some level of protection against the 1918 pandemic virus. Using the ferret model, regarded as the most relevant model for influenza vaccine efficacy assessments, we showed that the 2010-2011 seasonal TIV was able to induce neutralizing antibodies that reacted with 1918 virus and provided a significant reduction in morbidity and virus shedding compared to these parameters in 1918-challenged control ferrets. Seasonal TIV was also effective at reducing virus replication and pathology in the lung associated with 1918 virus infection.

Although the prechallenge cross-neutralizing titers against the 1918 virus were generally low (GMT of 23 and 28) relative to homologous titers to seasonal B/Brisbane/60 and Mex/4482 H1N1 viruses (GMT of 121 to 485), the antibody titers to one

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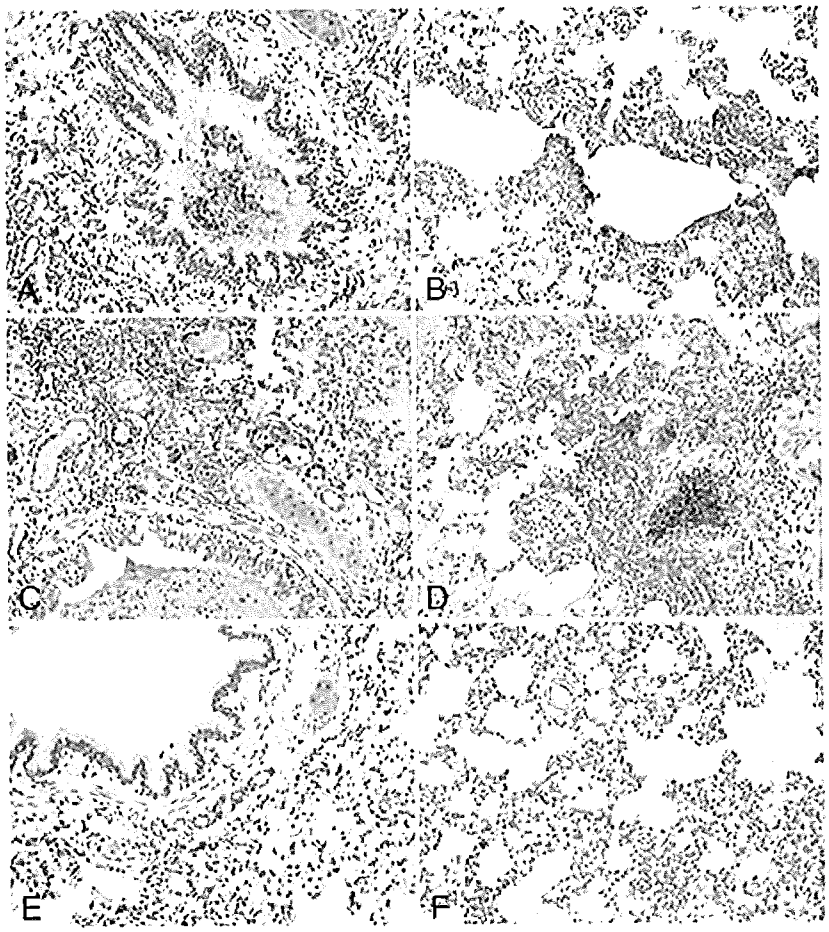

FIG 4 Immunohistochemistry of 1918 TIV-immunized and control ferrets following 1918 virus challenge. Photomicrographs of lung tissue sections from control (A to D) and TIV-immunized (E and F) ferrets stained (in red) for the presence of viral antigen 3 and 5 days p.c. Viral antigen was found in bronchiolar epithelial cells and in desquamated cells in the lumen of bronchioles on day 3 p.c. (A and B), in cells of submucosal serous glands of the bronchus on day 5 p.c. (C), and in alveolar cells and macrophages on day 5 p.c. (D). TIV-immunized ferrets challenged with the 1918 virus examined at day 3 p.c. displayed milder lung lesions than controls, with no specific viral antigen staining detected on day 3 p.c. (E) and day 5 p.c. (F).

homologous virus, Perth/16 (H3N2) were also low (GMT of 35 and 47) and in the same titer range as the neutralizing titers generated to the 1918 virus. In general, inactivated influenza vaccines are poorly immunogenic in immunologically naïve animals and appreciable antibody responses are induced only at high vaccine doses or when vaccines are adjuvanted (6, 28). In particular, purified surface antigen preparations of TIV (also referred to as "split" vaccines) are weakly immunogenic in naïve ferrets, often necessitating multiple rounds of vaccine boosting when adjuvants are not used (2, 3, 11). For the current study, ferrets were vaccinated with TIV manufacturer-filled syringes designed for single-dose administration. We chose not to use an adjuvant to vaccinate animals since the standard TIVs against influenza are mostly non-adjuvanted vaccines (21). It is worth noting that adjuvanted vaccines have demonstrated the ability, particularly in immunologically naïve animals, to significantly augment the cross-reactivity of antibodies induced by influenza virus vaccines (23, 37). Thus, it would be of interest to evaluate the potential for adjuvants in TIV formulations to induce greater cross-neutralizing antibody titers against the 1918 virus than were shown in this study for the group that received TIV alone. Although the precise immunological mechanism conferring cross-protection was not addressed in the current study, the generation of TIV-induced neutralizing antibodies against the 1918 virus most certainly played a prominent role in decreasing viral shedding and replication in trachea and lung tissues. TIV generally does not effectively induce mucosal immune responses or influenza-specific $CD8^+$ T cells, two immune effectors capable of protecting the host (10, 14, 16, 19).

The antigenic similarities between the 1918 and A(H1N1)pdm09 viruses are supported by the results of passive transfer experiments in which 1918 HA-specific monoclonal antibodies administered to mice protected them against the A(H1N1)pdm09 virus (25). Taking an opposite experimental approach, Medina and colleagues showed that human A(H1N1)pdm09-positive sera was sufficient to protect mice from 1918 lethal challenge (26). The same study demonstrated that a partially purified whole-virus vaccine preparation from a recombinant influenza virus carrying the HA gene from A/California/04/2009 virus provided protection against 1918 virus challenge in mice. Conversely, immunization with an inactivated 1918 virus vaccine but not a former seasonal H1N1 influenza vaccine conferred protection against the A(H1N1)pdm09 virus in mice, further demonstrating the antigenic similarity between these viruses (34, 42).

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The A(H1N1)pdm09 virus is now part of seasonal influenza virus circulation and is a component of the seasonal trivalent vaccine. The 2009 H1N1 antigen became one of three components in the 2010-2011 seasonal TIV and is the same vaccine virus as was used in the 2009 H1N1 monovalent vaccine. Moreover, there was no change in vaccine components for the 2011-2012 Northern Hemisphere TIV. The current worldwide production capacity for trivalent influenza vaccine is approximately 300 million doses per year (20, 21). Among persons 6 months of age or older, an estimated 130.9 million people, or 43% of the U.S. population, received a seasonal influenza vaccine during August 2010 through May 2011 (http://www.cdc.gov/flu/professionals/vaccination/vaccinecoverage.htm). The results of our study suggest that individuals who receive the contemporary seasonal influenza vaccine, which contains the A(H1N1)pdm09 virus component, may develop neutralizing antibodies that cross-react against the 1918 virus. Indeed, it was demonstrated that the 2009 H1N1 monovalent vaccine induced cross-reactive antibodies to the 1918 virus among adults ≥18 years enrolled in an A(H1N1)pdm09 influenza vaccine trial (26). Furthermore, human serology studies showed a high prevalence of A(H1N1)pdm09 cross-reactive antibodies in people born before 1930 due to probable exposure to 1918 or 1918-like H1N1 virus (15, 25). This correlated with a reduced frequency of hospitalization during the 2009 pandemic among individuals aged ≥65 years when compared with the risks for complications and hospitalizations from seasonal influenza virus infection (4, 22). A recent study by Reed et al. (C. Reed, J. M. Katz, K. Hancock, A. Balish, J. Singleton, and A. M. Fry for the H1N1 Serosurvey Working Group, unpublished data) estimated that by December 2009, approximately 20% of the U.S. population, including over half of school-aged children, were infected with A(H1N1)pdm09 virus. The extent to which human infection with A(H1N1)pdm09 virus elicits neutralizing antibody against the 1918 virus warrants further study.

Improving biosafety and assessing the dual-use potential and risks of working with highly pathogenic influenza viruses are integral components of public health research. This is the first report to demonstrate that the seasonal trivalent influenza vaccine for 2010-2011 offers protection against the reconstructed 1918 virus. Although more human serology data are needed to determine the frequency of cross-reactive antibodies to the 1918 H1N1 virus, these data (along with other data cited in the text) suggest that there is significant preexisting immunity to potential 1918 virus infection in the human population, which is likely to increase as more individuals acquire immunity through influenza vaccination or infection.

ACKNOWLEDGMENTS

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

We thank Eddie Jackson and his team for exceptional care of animals kept under biosafety level 3 containment (with appropriate enhancements).

REFERENCES

1. Anhlan D, Grundmann N, Makalowski W, Ludwig S, Scholtissek C. 2011. Origin of the 1918 pandemic H1N1 influenza A virus as studied by codon usage patterns and phylogenetic analysis. RNA 17:64–73.
2. Baras B, et al. 2011. Pandemic H1N1 vaccine requires the use of an adjuvant to protect against challenge in naive ferrets. Vaccine 29:2120–2126.
3. Baras B, et al. 2011. Longevity of the protective immune response induced after vaccination with one or two doses of AS03A-adjuvanted split H5N1 vaccine in ferrets. Vaccine 29:2092–2099.
4. Barker WH, Mullooly JP. 1980. Impact of epidemic type A influenza in a defined adult population. Am. J. Epidemiol. 112:798–811.
5. Belser JA, Szretter KJ, Katz JM, Tumpey TM. 2009. Use of animal models to understand the pandemic potential of highly pathogenic avian influenza viruses. Adv. Virus Res. 73:55–97.
6. Bresson JL, et al. 2006. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 367:1657–1664.
7. Brockwell-Staats C, Webster RG, Webby RJ. 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza Other Respi. Viruses 3:207–213.
8. Centers for Disease Control and Prevention. 2010. Update: influenza activity—United States, August 30, 2009–March 27, 2010, and composition of the 2010-11 influenza vaccine. MMWR Morb. Mortal. Wkly. Rep. 59:423–430.
9. Chosewood LC, Wilson DE. 2009. Biosafety in microbiological and biomedical laboratories, 5th ed. U. S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health, Washington, DC.
10. Forrest BD, et al. 2008. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. Clin. Vaccine Immunol. 15:1042–1053.
11. Galarza JM, Latham T, Cupo A. 2005. Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol. 18:365–372.
12. Garten RJ, et al. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201.
13. Glezen WP. 1996. Emerging infections: pandemic influenza. Epidemiol. Rev. 18:64–76.
14. Gorse GJ, et al. 1996. Induction of mucosal antibodies by live attenuated and inactivated influenza virus vaccines in the chronically ill elderly. J. Infect. Dis. 173:285–290.
15. Hancock K, et al. 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N. Engl. J. Med. 361:1945–1952.
16. He XS, et al. 2006. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. J. Virol. 80: 11756–11766.
17. Herlocher ML, et al. 2001. Ferrets as a transmission model for influenza: sequence changes in HA1 of type A (H3N2) virus. J. Infect. Dis. 184:542–546.
18. Itoh Y, et al. 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460:1021–1025.
19. Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. 1986. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. J. Infect. Dis. 154:121–127.
20. Kieny MP, et al. 2006. A global pandemic influenza vaccine action plan. Vaccine 24:6367–6370.
21. Lang PO, Govind S, Mitchell WA, Siegrist CA, Aspinall R. 2011. Vaccine effectiveness in older individuals: what has been learned from the influenza-vaccine experience. Ageing Res. Rev. 10:389–395.
22. Louie JK, et al. 2009. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. JAMA 302: 1896–1902.
23. Lu X, et al. 2006. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 24:6588–6593.
24. Maines TR, et al. 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J. Virol. 79:11788–11800.
25. Manicassamy B, et al. 2010. Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines. PLoS Pathog. 6:e1000745. doi:10.1371/journal.ppat.1000745.
26. Medina RA, et al. 2010. Pandemic 2009 H1N1 vaccine protects against 1918 Spanish influenza virus. Nat. Commun. 1:28.
27. Mozdzanowska K, Furchner M, Washko G, Mozdzanowski J, Gerhard W. 1997. A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-specific antibodies that dis-

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play very low virus-neutralizing activity in vitro. J. Virol. 71:4347–4355.
28. Nicholson KG, et al. 2001. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 357:1937–1943.
29. Olsen CW. 2002. The emergence of novel swine influenza viruses in North America. Virus Res. 85:199–210.
30. Palese P, Wang TT. 2011. Why do influenza virus subtypes die out? A hypothesis. mBio 2:e00150–11. doi:10.1128/mBio.00150-11.
31. Pearce MB, Belser JA, Houser KV, Katz JM, Tumpey TM. 2011. Efficacy of seasonal live attenuated influenza vaccine against virus replication and transmission of a pandemic 2009 H1N1 virus in ferrets. Vaccine 29:2887–2894.
32. Reid AH, et al. 2003. 1918 influenza pandemic caused by highly conserved viruses with two receptor-binding variants. Emerg. Infect. Dis. 9:1249–1253.
33. Reid AH, Taubenberger JK. 2003. The origin of the 1918 pandemic influenza virus: a continuing enigma. J. Gen. Virol. 84:2285–2292.
34. Skountzou I, et al. 2010. Immunity to pre-1950 H1N1 influenza viruses confers cross-protection against the pandemic swine-origin 2009 A (H1N1) influenza virus. J. Immunol. 185:1642–1649.
35. Slemons RD, Swayne DE. 1990. Replication of a waterfowl-origin influenza virus in the kidney and intestine of chickens. Avian Dis. 34:277–284.
36. Smith H, Sweet C. 1988. Lessons for human influenza from pathogenicity studies with ferrets. Rev. Infect. Dis. 10:56–75.
37. Stephenson I, et al. 2005. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J. Infect. Dis. 191:1210–1215.
38. Swayne DE, Beck JR, Perdue ML, Brugh M, Slemons RD. 1996. Assessment of the ability of ratite-origin influenza viruses to infect and produce disease in rheas and chickens. Avian Dis. 40:438–447.
39. Tumpey TM, et al. 2005. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 310:77–80.
40. Tumpey TM, et al. 2004. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc. Natl. Acad. Sci. U. S. A. 101:3166–3171.
41. Tumpey TM, et al. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315: 655–659.
42. Wei CJ, et al. 2010. Cross-neutralization of 1918 and 2009 influenza viruses: role of glycans in viral evolution and vaccine design. Sci. Transl. Med. 2:24ra21. doi:10.1126/scitranslmed.3000799.
43. Zeng H, et al. 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type I interferon response in polarized human bronchial epithelial cells. J. Virol. 81:12439–12449.

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DOI:10.1111/irv.12070
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Short Article

Evaluation of a commercial enzyme-linked immunosorbent assay for detection of antibodies against the H5 subtype of *Influenza A virus* in waterfowl

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Accepted 23 November 2012. Published Online 22 December 2012.

The ID Screen Influenza H5 Antibody Competition enzyme-linked immunosorbent assay was tested for the detection of antibodies to the H5 subtype of influenza A (IA) virus in waterfowl. Assays were conducted with sera obtained from Mallards (*Anas platyrhynchos*) and Pekin Ducks (*Anas platyrhynchos domestica*), experimentally infected with eight low pathogenic (LP) and nine highly pathogenic (HP) H5N1 IA viral strains. Three incubation periods (1, 4 and 18 hours) and two dilutions (1:2 and 1:5) were tested. All serum samples from LP H5-infected birds tested positive; however, improved detection rates were observed for viruses belonging to the HP H5N1 clade 2.2.1 as compared with those belonging to clade 2.1.3.

Keywords Avian influenza, enzyme-linked immunosorbent assay, H5, serology, waterfowl.

Please cite this paper as: Lebarbenchon *et al.* (2012) Evaluation of a commercial enzyme-linked immunosorbent assay for detection of antibodies against the H5 subtype of *Influenza A virus* in waterfowl. Influenza and Other Respiratory Viruses. DOI: 10·1111/irv.12070.

Testing for antibodies to Influenza A (IA) virus is a common diagnostic tool used in poultry[1] and also has recently been incorporated into wild bird surveillance efforts.[2–8] These assays usually are based on the detection of IA virus nucleoprotein antibodies using agar gel immunodiffusion or enzyme-linked immunosorbent assays (ELISA), and hemagglutinin (HA) and neuraminidase antibodies using hemagglutination inhibition (HI) and neuraminidase inhibition tests, respectively. Several commercial ELISA's have recently been developed and evaluated for use in both poultry and wild birds.[7,9,10]

In the current study, the ID Screen Influenza H5 Antibody Competition ELISA (IDVET, Montpellier, France) was tested. We investigated the ability of the assay to detect H5 antibodies in sera obtained from Mallards (*Anas platyrhynchos*) and Pekin ducks (*Anas platyrhynchos domestica*) experimentally infected with eight low pathogenic (LP) and nine highly pathogenic (HP) virus strains, respectively.

For LP IA viruses, serum samples were obtained from 43 one-month-old Mallards experimentally infected with eight different virus subtypes (Table 1) as well as from eight sham-inoculated birds.[10] Blood samples were collected at the end of the experiments (14 or 21 days post-infection) and sera stored at −20°C until tested. Virus isolation, PCR testing as well as NP ELISAs[10] verified infections of inoculated birds (see references 11–14 for details related to the experimental infection trials).

For HP H5N1 viruses, serum samples were obtained from 38 Pekin ducks inoculated with nine different viral strains; five and four viruses being identified as belonging to the HP H5N1 clade 2.1.3 and 2.2.1, respectively (Table 2). Blood samples were collected at the end of the experiments (10 days post-virus inoculation), and sera stored at −20°C until tested. Virus isolation, PCR testing as well as HI assays verified infections of inoculated birds (Pantin-Jackwood *et al.* in preparation).

The H5 IA virus-specific ELISA was performed using slightly modified protocols from the manufacturer's instructions: three incubation periods (1, 4 and 18 hours) and two dilutions factors (1:2 and 1:5) were evaluated. Briefly, serum samples were diluted with sample diluent provided by the manufacturer, and 100 μl of the diluted samples were dispensed into the antigen-coated test plates. Samples were incubated at 36°C at 1, 4 or 18 hours and manually washed three times with approximately 300 μl of wash solution (provided in the kit), per well. Next, 50 μl of conjugate were

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Table 1. Effect of the incubation period and sample dilution factor on the results obtained with the enzyme-linked immunosorbent assay for the detection of non-H5 Influenza A virus antibodies in experimentally infected Mallards

			1 hour				4 hours				18 hours			
			1:5		1:2		1:5		1:2		1:5		1:2	
Subtype	Strain name	N	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS
Sham-inoculated birds		8	0·94	0	0·89	0	0·89	0	0·89	0	1·00	0	0·85	0
H3N8	A/Mallard/MN/Sg-00169/2007	5	0·94	0	0·83	0	0·86	0	0·79	0	0·88	0	0·72	0
H4N6	A/Surface water/MN/NW1-T/2006	5	0·91	0	0·83	0	0·87	0	0·73	0	0·85	0	0·66	0
H4N8	A/Mallard/MN/Sg-00219/2007	5	0·96	0	0·79	0	0·89	0	0·78	0	0·84	0	0·70	0
H6N1	A/Mallard/MN/Sg-00170/2007	5	0·91	0	0·72	0	0·76	0	0·71	0	0·74	0	0·59	0
H6N2	A/Mallard duck/MN/Sg-00107/2007	5	0·86	0	0·71	0	0·74	0	0·65	0	0·67	0	0·58	0
H6N8	A/Green-winged teal/MN/Sg-00197/2007	5	0·89	0	0·82	0	0·87	0	0·66	0	0·76	0	0·58	0
H8N4	A/Mallard/MN/Sg-00570/2008	4	0·89	0	0·80	0	0·81	0	0·73	0	0·83	0	0·58	0 (+1)

N, Number of tested samples; S/N, sample-to-negative control ratio; POS, number of positive samples; Numbers in parenthesis represent samples considered doubtful (S/N:[0·35–0·40]); 1:5 and 1:2, sample dilution factor.

added to each well, and plates were incubated for 30 minutes at 23°C. Each well was washed again three times as described above. Finally, 50 µl of substrate solution was added to each well, and plates were incubated at 23°C in the dark for 10 minutes. The reactions were stopped by adding 50 µl of stop solution. Sample absorbance was measured at 450 nm with a BIO-RAD Benchmark microplate reader (Hercules, CA, USA). Serum samples with a sample-to-negative control (S/N) ratio values greater than or equal to 0·40 were considered negative. According to the manufacturer instructions, samples with S/N ratio values between 0·35 and 0·40 were considered to be suspect positives. Serum samples with S/N ratio values below 0·35 were considered positive for the presence of H5 antibodies. A single validation was performed for each sample.

Results obtained for non-H5 viruses are presented in Table 1. Although all sera tested positive for the presence of IA virus antibodies with two commercial ELISA,[10] H5-specific antibodies were not detected regardless of incubation time or sample dilution, suggesting that false-positive results were unlikely for the tested virus subtypes. A note of caution, however, is warranted as not all non-H5 subtypes were tested in this study (i.e. 16 different HA have been described in wild birds); in addition, one H8N4 serum sample was considered doubtful for the 18 hours incubation with a 1:2 dilution factor.

All low pathogenic H5N2 sera tested positive with the ELISA assay particularly when the duration of incubation was increased from 1 hour to 4 or 18 hours (Table 2). The same trend was observed for HP H5N1 sera, with slightly improved results obtained when the sample dilution was decreased to 1:2. Results obtained for the HP sera also were highly variable depending on the tested viral strain (Table 2), although all sera tested positive with a HI assay (Pantin-Jackwood *et al.* in preparation). Overall, an improved detection rate was obtained for viruses belonging to the H5N1 clade 2.2.1 as compared with those belonging to clade 2.1.3 (e.g. only 14% of tested samples yielded positive results for A/Chicken/West Java/SMI-PAT/2006). These results suggest that although the ELISA may be suitable for the detection of H5 IA virus-specific antibodies, the important antigenic diversity existing for viruses such as the HP H5N1 Asian lineage is likely to affect assay sensitivity and yield false-negative results.

Serology is an important diagnostic tool commonly used in poultry to detect anti-IA antibodies. When included in surveillance programmes, serology should be considered a flock-level test, as multiple variables relating to infection (unknown exposure date), host (antibody response), virus (virulence, antigenicity), or serologic assay (sensitivity) may influence results in an individual bird. Serology has historically been an underutilized diagnostic tool for IA in wild birds; however, the recent availability of accurate species-independent commercially available assays has initiated new research and surveillance efforts in a variety of wild

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Table 2. Effect of the incubation duration and sample dilution factor on the results obtained with the enzyme-linked immunosorbent assay for the detection of low- and highly pathogenic H5-specific Influenza A virus antibodies

Subtype	Strain name	Species	N	1 hour 1:5 Mean S/N	POS	1:2 Mean S/N	POS	4 hours 1:5 Mean S/N	POS	1:2 Mean S/N	POS	18 hours 1:5 Mean S/N	POS	1:2 Mean S/N	POS
H5N2 (LP)	A/Mallard/MN/355779/2000	Mallard	9	0·36	4 (+1)	0·20	8 (+1)	0·21	9	0·09	9	0·14	9	0·06	9
H5N1 (HP) Clade 2.1.3	A/Chicken/Garut/BBVW-223/2007	Pekin duck	3	0·71	0	0·61	0	0·50	1	0·48	1	0·43	1	0·39	1
	A/Chicken/Pekalongan/BBVW/2007	Pekin duck	2	0·51	0	0·33	2	0·44	1	0·29	2	0·27	1 (+1)	0·15	2
	A/Chicken/West Java/29/2007	Pekin duck	3	0·55	1	0·51	1	0·44	1	0·41	1	0·43	1	0·41	1
	A/Chicken/West Java/SMI-PAT/2006	Pekin duck	7	0·76	0	0·60	1	0·64	0	0·60	0 (+1)	0·63	0	0·53	1 (+1)
	A/Chicken/West Java/TASIKO B/2006	Pekin duck	5	0·55	0 (+1)	0·72	0	0·36	2 (+2)	0·26	4	0·30	4 (+1)	0·22	4 (+1)
H5N1 (HP) Clade 2.2.1	A/Chicken/Egypt/06207-NLQP/2006	Pekin duck	4	0·63	1	0·36	2 (+2)	0·56	3	0·28	4	0·44	2 (+1)	0·25	4
	A/Chicken/Egypt/07118-NLQP/2006	Pekin duck	6	0·42	2 (+1)	0·34	4	0·29	4	0·22	5	0·21	5	0·17	6
	A/Chicken/Egypt/0813-NLQP/2008	Pekin duck	6	0·47	1	0·34	4	0·27	5	0·22	6	0·28	5 (+1)	0·22	6
	A/Duck/Egypt/0923-NLQP/2009	Pekin duck	2	0·71	0	0·54	0	0·44	0	0·35	1 (+1)	0·30	1 (+1)	0·22	2

N, Number of tested samples; S/N, sample-to-negative control ratio; POS, number of positive samples; Numbers in parenthesis represent samples considered doubtful (S/N:[0·35–0·40]); 1:5 and 1:2, sample dilution factor; LP, low pathogenic; HP, high pathogenic.

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avian species throughout the world. As with poultry, these serosurveys have been performed on a population level and have complimented traditional isolation or molecular approaches to expand our understanding on IA natural history and epidemiology.

Rapid serological tools for subtype-specific IA antibody testing can greatly enhance our ability to evaluate both exposure and potential reservoir status of diverse wild bird populations. These tests also provide a means for detecting species involvement in IA virus epidemiology in situations where virus detection is difficult, for instance because of limited shedding. Although the results presented in this study present limitation as they were not compared with a reference test (e.g. H5 HI), our findings suggest that with slight and reasonable modifications to the manufacturer's protocols, the commercial ELISA may perform adequately enough to provide valuable LP H5 exposure data in waterfowl. We, however, warrant that the important antigenic diversity existing for viruses such as the Asian lineage of HP H5N1 IA virus is likely to affect the sensitivity of the assay and yield to false-negative results.

Acknowledgements

The authors thank Taiana Costa and Monique Franca for providing LP H5 serum samples from experimentally infected Mallards. This work was additionally supported by IDVET, which generously provided ELISA test kits and recommendations for protocol adjustments. The National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health and Human Services, under Contract No. HHSN266200700007C funded the current work. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

References

1 Spackman E, Suarez DL, Senne DA. Avian influenza diagnostic and surveillance methods; in Swayne DE (ed): Avian Influenza. Ames, IO: Blackwell Publishing, 2008.

2 Brown JD, Luttrell MP, Berghaus RD *et al.* Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. J Wildl Dis 2010; 46:896–911.

3 Brown JD, Luttrell MP, Uhart MM *et al.* Antibodies to type A influenza virus in wild waterbirds from Argentina. J Wildl Dis 2010; 46:1040–1045.

4 De Marco M, Campitelli L, Foni E *et al.* Influenza surveillance in birds in Italian wetlands (1992-1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots? Vet Microbiol 2004; 98:197–208.

5 Hammouda A, Pearce-Duvet J, Chokri MA *et al.* Prevalence of influenza A antibodies in Yellow-legged Gull (*Larus michahellis*) eggs and adults in Southern Tunisia. Vector Borne Zoonotic Dis 2011; 11:1583–1590.

6 Pearce-Duvet JMC, Gauthier-Clerc M, Jourdain E, Boulinier T. Maternal antibody transfer in Yellow-legged Gulls. Emerg Infect Dis 2009; 15:1147–1149.

7 Pérez-Ramírez E, Rodríguez V, Sommer D *et al.* Serologic testing for avian influenza viruses in wild birds: comparison of two commercial competition enzyme-linked immunosorbent assays. Avian Dis 2010; 54:729–733.

8 Toennessen R, Germundsson A, Jonassen CM *et al.* Virological and serological surveillance for type A influenza in the Black-legged Kittiwake (*Rissa tridactyla*). Virol J 2011; 8:21.

9 Brown JD, Stallknecht DE, Berghaus RD *et al.* Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clin Vaccine Immunol 2009; 16:824–829.

10 Lebarbenchon C, Brown JD, Luttrell MP, Stallknecht DE. Comparison of two commercial enzyme-linked immunosorbent assays for detection of influenza A virus antibodies. J Vet Diagn Invest 2012; 24:161–165.

11 Costa TP, Brown JD, Howerth EW, Stallknecht DE. Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. Avian Pathol 2011; 40:119–124.

12 Lebarbenchon C, Yang M, Keeler SP *et al.* Viral replication, persistence in water and genetic characterization of two influenza A viruses isolated from surface lake water. PLoS ONE 2011; 6:e26566.

13 Lebarbenchon C, Sreevatsan S, Lefèvre T *et al.* Reassortant influenza A viruses in wild duck populations: effects on viral shedding and persistence in water. Proc Biol Sci 2012b; 279:3967–3975.

14 Brown JD, Poulson R, Carter DL, Lebarbenchon C, Stallknecht DE. Infectivity of avian influenza virus-positive field samples for Mallards: what do our diagnostic results mean? J Wildl Dis; in press.

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Virus Research 167 (2012) 196–206

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses

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ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 11 April 2012
Accepted 27 April 2012
Available online 15 May 2012

Keywords:
Ducks
Highly pathogenic avian influenza
H5N1
Pathogenesis
Innate immune response
Host gene expression

ABSTRACT

The pathogenicity of H5N1 highly pathogenic avian influenza (HPAI) viruses in domestic ducks varies between different viruses and is affected by the age of the ducks, with younger ducks presenting a more severe disease. In order to better understand the pathobiology of H5N1 HPAI in ducks including the role of host responses, 2 and 5-week-old Pekin ducks were infected with three different H5N1 HPAI viruses. Virus-induced pathology ranged from no clinical signs to severe disease and mortality, with the 2-week-old ducks being more severely affected by the more virulent viruses. However, these more virulent viruses induced higher body temperatures in the 5-week-old ducks than in the 2-week-old ducks indicating possible differences in innate immune responses. To analyze the ducks host responses to H5N1 HPAI virus infection, expression of innate immune-related genes was measured in the spleens and lungs of infected ducks at the peak of virus infection. IFN-α, RIG-I, and IL-6 RNA levels were increased in spleens regardless of the virus given and the age of the ducks, however differences were observed in the levels of up-regulation of IFN-α and RIG-I between the 2 and the 5-week-old ducks with the more virulent virus. Differences in IL-2 gene expression were also observed. In the lungs, the levels of expression of innate immune-related genes were lower than in the spleen, with mostly up-regulation of RIG-I and IL-6 and down-regulation of IFN-α and IL-2; no significant difference in expression was found between the 2 and the 5-week-old ducks. The differences observed in the innate immune responses to infection with H5N1 HPAI viruses could explain in part the differences in pathogenicity found between the 2 and 5-week-old ducks, however earlier time points after infection and additional innate immune-related genes should be examined.

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1. Introduction

The H5N1 highly pathogenic avian influenza (HPAI) viruses continue circulating in poultry in Asia and have also spread to countries in the Middle East, Europe and Africa, causing great losses to the poultry industry. H5N1 HPAI virus infections are constantly monitored worldwide not only because of the high mortality they produce in poultry but also because of the spread to humans and fear of a pandemic. Both wild and domestic ducks have been implicated in the dissemination of H5N1 HPAI viruses (Chen et al., 2004; Gilbert et al., 2006; Hulse-Post et al., 2005; Li et al., 2004; Songserm et al., 2006; Sturm-Ramirez et al., 2005). Domestic ducks not only can be affected by H5N1 HPAI, but also serve as key intermediates in the transmission of the virus and therefore are included in control programs including vaccination (Li et al., 2004; Swayne, 2006).

Normally avian influenza (AI) viruses, including the highly pathogenic H5 and H7 strains, do not cause disease or death in ducks (Swayne and Halvorson, 2008). Mortality among ducks naturally infected with AI viruses was first reported in 2001 during an outbreak of H7N1 HPAI virus in Italy, and then in 2002, with Asian-origin H5N1 HPAI viruses causing mortality in wild birds and waterfowl (Capua and and Mutinelli, 2001; Ellis et al., 2004). Since then, a number of H5N1 HPAI viruses have been found to cause disease in ducks (reviewed in Pantin-Jackwood and Swayne, 2009). This pathogenicity is not consistently observed with all H5N1 HPAI viruses, and the age and species of the ducks appears to influence the outcome of the infection (Cagle et al., 2011; Londt et al., 2010; Pantin-Jackwood et al., 2007; Phuong et al., 2011; Wasilenko et al., 2011). Vaccination has proven effective in protecting ducks against H5N1 HPAI and is being used in several Asian countries to control the disease (Sims, 2007); however, virus circulation may still occur in clinically healthy vaccinated populations, which may contribute to endemic situations (Capua and Alexander, 2008).

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0168-1702/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.virusres.2012.04.015

In order to curtail the spreading of H5N1 HPAI, it's necessary to control AI in ducks, and this will be facilitated by a better understanding of viral pathogenesis including host–pathogen interactions and host immune responses to virus infection. Control of influenza virus infections in naive hosts is based on innate immunity and subsequent adaptive cytotoxic T cell and B cell immunity. Cytokine production appears to play an important role in the pathogenesis of H5N1 HPAI viruses in mammals (Basler and Aguilar, 2008; Cameron et al., 2008; de Jong et al., 2006; Hayden et al., 1998; Peiris et al., 2004; Szretter et al., 2007). High virulence in mammals has been associated with induction of high levels of pro-inflammatory cytokines, commonly referred to as "cytokine storms", and in humans, clinical features associated with H5N1 HPAI infections have been linked with cytokine dysregulation (Chan et al., 2005; To et al., 2001). The role of cytokines in the pathogenesis of H5N1 HPAI viruses in avian species is less well understood. Several studies have addressed individual innate immune gene expression in chickens, and chicken or duck-origin cells, infected with AI viruses (Adams et al., 2009; Burggraaf et al., 2011; Degen et al., 2006; Liang et al., 2011; Rebel et al., 2011; Reemers et al., 2009, 2010; Sarmento et al., 2008a,b; Suzuki et al., 2009; Wasilenko et al., 2008, 2009; Watanabe et al., 2011; Xing et al., 2009), but studies exploring gene expression *in vivo* in ducks infected with AI viruses are less common (Barber et al., 2010; Burggraaf et al., 2011; Cagle et al., 2011; Sarmento et al., 2010). Viral replication has been associated with increased pro-inflammatory responses in chickens and ducks infected with H5N1 HPAI viruses (Burggraaf et al., 2011; Cagle et al., 2011; Suzuki et al., 2009). Expression of iNOS in organs was also associated with a higher severity of H5N1 HPAI virus infection, with higher levels of expression in chickens compared to ducks (Burggraaf et al., 2011). It is still unclear whether infection with different AI virus strains results in host gene expression patterns that are specific to each virus or are representative of a common response to infection with HPAI viruses. In addition, the role of host factors, including species, physiologic status and age of the bird, needs to be elucidated.

Previous studies have shown differences in response to H5N1 HPAI virus infection between domestic ducks of different ages, with younger ducks presenting a more severe disease and higher mortality (Kishida et al., 2005; Londt et al., 2010; Pantin-Jackwood et al., 2007). A difference in age susceptibility was also reported in natural outbreaks of H5N1 HPAI in commercial ducks (Kwon et al., 2005). As for wild ducks, the age of mallard ducks infected with a low pathogenicity avian influenza (LPAI) virus did not affect susceptibility to infection but did affect the extent of virus shedding (Costa et al., 2010). Results of a study examining age-related susceptibility to H9N2 influenza infection in Japanese quail, indicated that adult birds had consistently stronger immune responses than young and aged birds, however, clinical signs and duration of viral infection was similar in the different age groups (Lavoie et al., 2007). In another study, differences between 1 and 4-week-old chickens infected with H9N2 virus were related to strength and timing of host responses, which could be a key factor in age-dependent susceptibility to infection (Reemers et al., 2009). Differences in the outcome of hepatitis B virus infection in ducks of different ages has been attributed to both increasing maturation of the immune system with age, and a link between host cell maturation and the capacity to support viral replication (Jilbert et al., 1998). Also, baby ducks were more vulnerable to persistence of hepatitis B virus infection than older ducks with evidence that the innate immune response is less effective in the young ducks (Tohidi-Esfahani et al., 2010).

The objective of this study was to investigate the effect of age on the pathogenesis of H5N1 HPAI viruses in domestic ducks and to examine the innate immune responses to infection. For this, three H5N1 HPAI viruses of different virulence in ducks were used to infect 2 and 5-week-old domestic Pekin ducks. Innate immune-related gene expression associated with virus infection was compared by conducting quantitative real-time RT-PCR for selected genes in spleens and lungs. The information generated provides insights into HPAI virus pathogenesis and host responses that can be used to develop improved strategies to control AI in avian species.

2. Materials and methods

2.1. Viruses

The following H5N1 HPAI viruses were used in this study: Chicken/Hong Kong/220/97 (Ck/97) (Perkins and Swayne, 2001), A/Egret/HK/757.2/02 (Egret/02) (Pantin-Jackwood et al., 2007), and Duck/Vietnam/218/05 (Dk/05) (Pfeiffer et al., 2009). Virus stocks were propagated in embryonating chicken eggs (ECE) as previously described (Pantin-Jackwood and Swayne, 2007). Serial titrations were performed in ECE and 50% egg infectious dose (EID_{50}) titers were determined by testing hemagglutination activity (Swayne et al., 1998). A sham inoculum was made using sterile allantoic fluid diluted 1:300 in brain heart infusion (BHI) medium. All experiments using H5N1 HPAI viruses, including work with animals, were performed in biosecurity level-3 enhanced (BSL-3E) 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).

2.2. Pathogenicity studies in Pekin ducks

Two identical experiments were conducted using domestic Pekin ducks (*Anas platyrhynchos*, var. *domestica*) obtained from a commercial hatchery. In the first experiment the ducks were two weeks of age and in the second experiment they were five weeks of age. The two experiments were run separately because of space limitations, however all possible measures were taken to minimize differences in experimental conditions. Serum samples were collected from fifteen ducks in each experiment prior to inoculation to ensure that the birds were serologically negative for AI virus as determined by an ELISA test using IDEXX FlockChek AI MultiS-Screen (Idexx Laboratories, ME). Ducks were housed in self-contained isolation units that were ventilated under negative pressure with HEPA-filtered air, and maintained under continuous lighting. Feed and water were provided with *ad libitum* access. General care was provided as required by the SEPRL Institutional Animal Care and Use Committee. The experimental design has been previously described (Pantin-Jackwood and Swayne, 2007). Briefly, ducks were separated into a control group and three virus-inoculated groups. All groups contained 14 ducks. Control birds were intranasally (IN) inoculated through the choanal slit with 0.1 ml of the sham inoculum. The three virus-inoculated groups were inoculated IN with 0.1 ml of inoculum containing 10^5 EID_{50} of the viruses. The virus dose used is above the median infectious dose (ID50) for H5N1 HPAI in ducks (Aldous et al., 2010). At 2 days post-inoculation (dpi) six ducks per group were bled for serum. In addition, four ducks from each group were euthanized and necropsied. Tissues from three of these ducks per group were collected in 10% neutral buffered formalin solution to determine microscopic lesions and the extent of virus replication in tissues as previously described (Pantin-Jackwood and Swayne, 2007). Portions of brain, lung, skeletal muscle, heart and spleen were also collected from three ducks per group in BHI with antibiotics for virus titration. In addition, portions of the spleen and lung from

all four euthanized ducks were collected for RNA isolation. Tissue samples were ground through 100 μm cell strainers (BD Falcon, Franklin Lakes, NJ) into 2 ml of Dulbecco's Modified Eagle Medium (DMEM). The cell suspension was then added to 6 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at −70 °C.

The remaining ducks (ten per group) were observed for signs of illness over a 9 day period during which time the clinical signs were recorded daily. Body temperatures were taken with a rectal digital thermometer from all ducks at 2 and 3 dpi. Body weights were taken from all ducks at the day of inoculation and at 3 dpi. Oropharyngeal and cloacal swabs from three ducks per group were also taken at 3 dpi to determine virus shedding. Sample birds, moribund birds (recumbent ducks that would not eat or drink, or had severe neurological signs), and all birds remaining at the end of the nine day period were euthanized by the intravenous (IV) administration of sodium pentobarbital (100 mg/kg body weight).

Tissues and swabs collected in BHI were stored frozen at −70 °C and titers of infectious virus were subsequently determined as previously described (Swayne et al., 1998). Briefly, tissues were normalized by weight and homogenized in BHI medium and clarified homogenates were titrated for virus infectivity in embryonating chicken eggs. The minimal detectable titer was $10^{0.9}$ EID_{50}/ml from swabs and $10^{1.97}$ EID_{50}/g from tissue.

One-way ANOVA with Tukey's post-test was used to analyze body temperature, body weight, and virus titers in swabs using Prism v.5.01 software (GraphPad Software Inc.). Statistical significance was set at $P < 0.05$. The survival rate data was analyzed using the Mantel–Cox Log-rank test.

2.3. RNA extraction

Spleen and lung cell suspensions stored in TRIzol were thawed at room temperature and 1.6 ml of chloroform was added. Samples were incubated for 10 min on ice and then spun at 2400 × g for 15 min. The aqueous phase was removed and an equal volume of 70% ethanol was added. The RNA was then extracted using the Qiagen RNeasy midi prep kit (Qiagen, Valencia, CA). RNA integrity (RIN) of the samples was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and confirmed to have values above 7. RNA concentrations from the four individual birds per group were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). To eliminate possible contamination with genomic DNA, 100 micrograms of RNA from each sample was treated with on column DNase digestion (RNase-Free DNase Set, Qiagen, Valencia, CA) in the Qiagen RNeasy mini prep kit (Qiagen, Valencia, CA) for 15 min at room temperature.

2.4. Quantitative real-time RT-PCR

The relative quantitation of IFN-α, RIG-I, and IL-6 gene expression following infection was performed using primers previously reported (Cagle et al., 2011). Primers for IL-2 gene were designed based on published GenBank sequences (AY821656) (forward primer: ctggcaaactctgcaatgttact; reverse primer: tgggggaattaggtccataagtt). Amplification and detection of specific products was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, CA). Total RNA extracted from spleen and lung homogenates from four individual ducks from the infected groups and from four ducks from the non-infected controls was used in the reactions. Reactions were done in triplicate using the Power SYBR® Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems, CA) and 50 ng of total RNA per reaction. Quantitative real-time RT-PCR (qRRT-PCR) conditions and cycling parameters were the same for all primer pairs used and were as follows: one cycle at 50 °C for 30 min, one cycle at 95 °C for 15 min, 40 cycles of 95 °C for 15 s and 58 °C for 32 s followed by dissociation curve determination of the products with one cycle at 95 °C for 15 s, 55 °C for 20 s, 99 °C for 15 s and 60 °C for 15 s. Expression fold change was determined by the $2^{-\Delta\Delta Ct}$ method using β-actin as the endogenous reference gene to normalize the level of target gene expression (Cagle et al., 2011). The Mann–Whitney test was used to determine the statistical significance between fold change values of the two age ducks, and one-way ANOVA with Tukey's post-test was used to determine the statistical difference between the fold change values obtained with the three viruses using Prism v.5.01 software (GraphPad Software Inc.). Statistical significance was set at $P < 0.05$.

Table 1
Morbidity, mortality, and mean death times resulting from intranasal inoculation of 2 and 5-week-old ducks with 10^5 EID_{50} of the H5N1 HPAI viruses.

Groups	Sick/dead/total (MDT)[a]	
	2-Week-old ducks	5-Week-old ducks
Controls	0/0/10	0/0/10
Ck/97	0/0/10	0/0/10
Egret/02	10/10/10 (5.5)	10/6/10 (6.5)
Dk/05	10/10/10 (3.9)	10/10/10 (4.4)

[a] MDT = mean death time (days).

An estimate of the virus titers in spleen and lung was also determined for infected ducks by real-time RT-PCR. This was done to examine if differences in virus replication could affect the cytokine gene expression results. RNA (50 ng/ul) from all duck samples previously used in the qRRT-PCR assay were run with a test targeting the influenza A matrix gene (Spackman et al., 2002). All reactions were run in triplicate with ABI PRISM 7500 using Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, CA) for 1 cycle at 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 57 °C for 30 s.

2.5. Nitric oxide assay

Total nitrate and nitrite content produced by nitric oxide synthase (NOS) was measured in serum samples collected at 2 dpi from six ducks from each virus-infected group and four ducks from the non-infected controls. Sera were passed through a 10 kDa ultrafilter (Millipore Corp., Billerica, MA) prior to analysis. The total protein in the filtered sera was measured using a DC Protein Assay (Bio-Rad, Hercules, CA). A Nitric Oxide Synthase Assay kit (Calbiochem, Gibbstown, NJ) was used according to the manufacturer's specifications on 40 μL of filtered serum. Values were normalized to the total protein in the filtered sera. One-way ANOVA with Tukey's post-test was used the results using Prism v.5.01 software (GraphPad Software Inc.). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Pathogenicity of H5N1 HPAI virus infections in ducks

Differences in pathogenicity between three H5N1 HPAI viruses were determined in 2 and 5-week-old Pekin ducks. These ages were chosen based on results of our previous study indicating differences in response to H5N1 HPAI virus infection in Pekin ducks of different ages (Pantin-Jackwood and Swayne, 2007). Serum samples collected from ducks prior to inoculation showed that the ducks were serologically negative for AIV.

3.1.1. Clinical signs and gross lesions

The morbidity and mortality induced by infection with the viruses in 2 and 5-week-old ducks is summarized in Table 1, and survival curves are shown in Fig. 1. Survival rates were significantly different ($P < 0.05$) upon infection with the three viruses in each age group. Survival of the 2 and 5-week-old ducks infected with

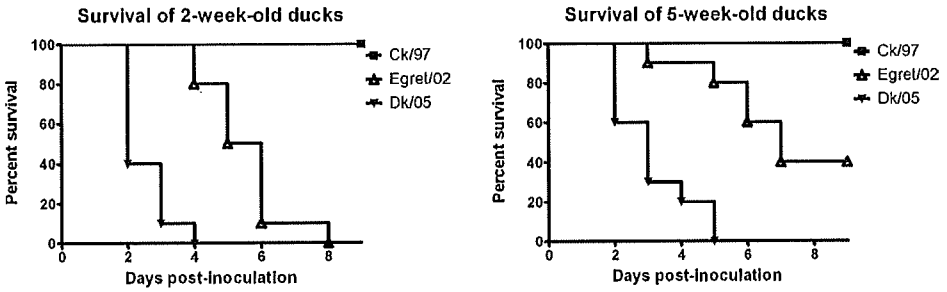

Fig. 1. Survival rate of ducks after inoculation with the H5N1 HPAI viruses. Two and 5-week-old ducks were inoculated with 10^5 EID_{50} of the viruses and mortality was monitored for 9 days. Survival rates were statistically different between the three virus-inoculated groups within each age, as determined by the log rank test ($P < 0.05$). Significant difference in survival rate was also observed between the 2 and 5-week-old ducks infected with Egret/02.

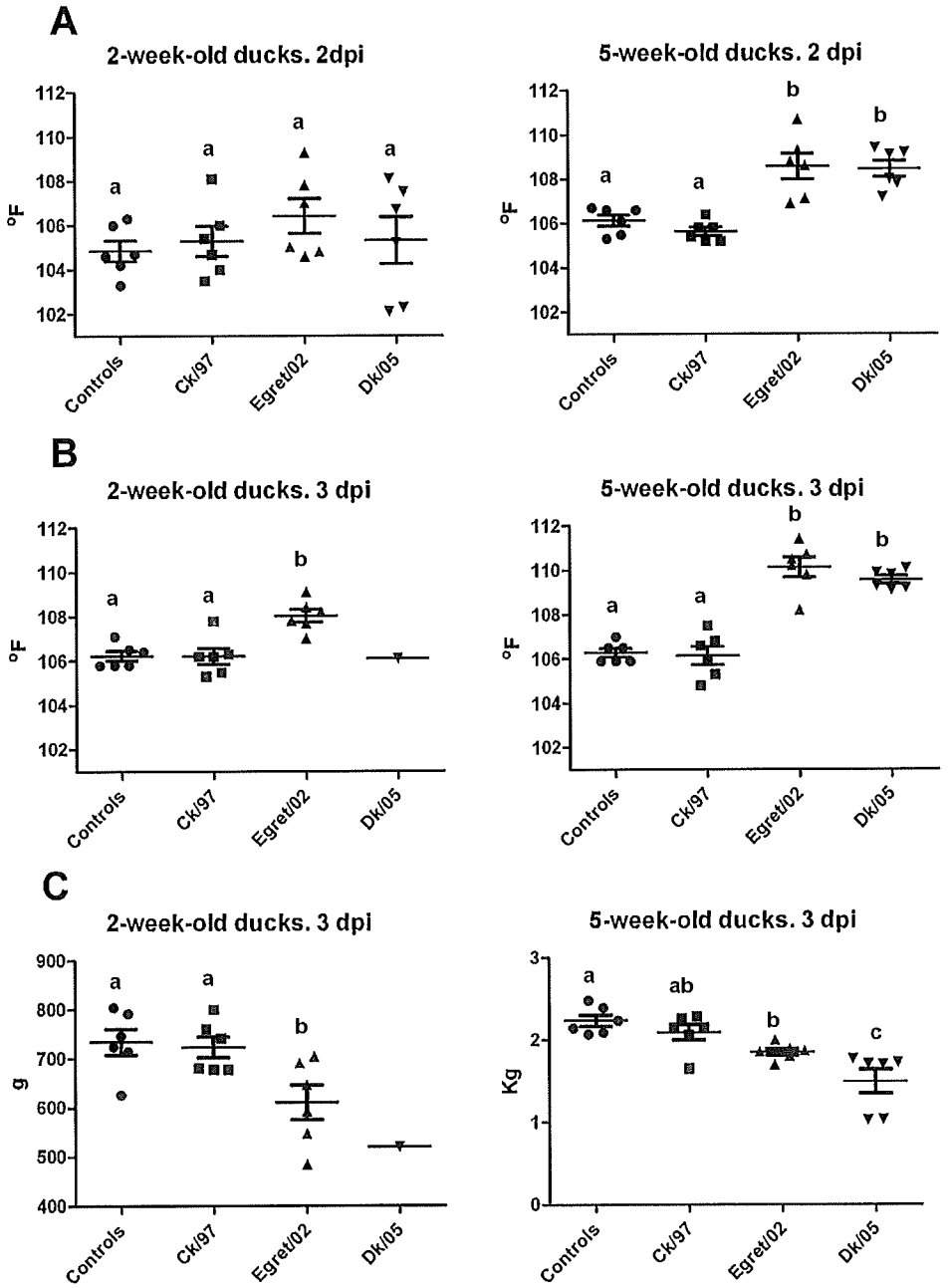

Fig. 2. Body temperatures of 2 and 5-week-old ducks infected with H5N1 HPAI viruses and compared to controls, 2 days post inoculation (dpi) (A) and 3 dpi (B). Body weights of 2 and 5-week-old ducks infected with H5N1 HPAI viruses and compared to controls, 3 dpi (C). Bars indicate the average ± SEM for each group. Groups with different lowercase letters within the respective age group are significantly different ($P < 0.05$).

Egret/02, but not with Dk/05, was also statistically different when compared to each other. No clinical signs or mortality was observed in the sham-inoculated control ducks or in the ducks inoculated with the Ck/97 virus in either of the two experiments. Ducks inoculated with the Egret/02 or Dk/05 viruses had clinical signs ranging from moderate to severe. Two-week-old ducks inoculated with the Egret/02 or Dk/05 viruses presented 100% mortality with lower mean death times when compared to the 5-week-old ducks. All ducks inoculated with Egret/02 and Dk/05 showed moderate to severe depression and anorexia as early as 2 dpi. Body temperatures taken at 2 and 3 dpi demonstrated that these ducks had fever, with higher temperatures observed in the 5 week-old ducks (Fig. 2). Significantly higher body temperatures were observed in the 5-week-old ducks inoculated with Egret/02 or Dk/05 when compared to the control ducks or the ducks inoculated with Ck/97, at 2 and 3 dpi. No significant difference in body temperature was observed between the 2-week-old ducks at 2 dpi, but ducks inoculated with Egret/02 had significantly higher body temperatures at 3 dpi. The body temperatures of two of the 2-week-old ducks inoculated with Dk/05 and examined at 2 dpi were very low indicating these ducks were probably in a moribund state. At the moment of infection there was no weight difference among the different experimental groups (data not shown). A clear effect on weight after infection was observed at 3 dpi (Fig. 2). In addition, all ducks infected with Egret/02 and Dk/05 displayed mild to severe neurological signs beginning at 2 dpi and characterized by tremors, lack of coordination, tilted head, seizures, and paralysis. Five-week-old ducks that survived from the Egret/02-inoculated group continued to have mild neurological signs until the end of the experiment.

At necropsy, control ducks and ducks inoculated with Ck/97 lacked lesions. Gross lesions were observed and were similar between the ducks inoculated with Egret/02 and Dk/05, and dehydration, empty intestines, splenomegaly, and thymus atrophy was present in most. Also commonly observed were nasal discharge, cyanotic bill and toes, dilated and flaccid heart with increased pericardial fluid, renomegaly and/or renal pallor and accentuated lobular surface architecture, congested, malacic brain, impacted proventriculus and gizzard with intense bile staining of the mucosa, and yellowish pancreas with petechia.

3.1.2. Microscopic lesions and viral antigen distribution

The distribution and severity of histologic lesions and avian influenza antigen staining in tissues collected from the 2 and 5-week-old ducks at 2 dpi are summarized in Table 2. The two week-old ducks had slightly more severe lesions and more widespread viral staining in tissues than the 5 week-old ducks. In ducks inoculated with Ck/97, mild to moderate tracheitis and bronchitis was observed, as well as mild focal degeneration of pancreatic acinar cells and splenic macrophages, mild lymphocyte depletion in the thymus and bursa, and mild lymphocyte infiltration in liver and kidneys. Mild to severe lesions in several organs were found in ducks inoculated with Egret/02 and Dk/05, however lesions were more severe and more organs were affected in ducks infected with Dk/05. The trachea presented mild to moderate degenerative changes of the overlying epithelium and mild lymphocytic infiltration in the submucosa. The lesions present in the lung were mild to severe consisting of congestion and interstitial inflammation with mixed mononuclear cells. Mild to moderate necrotizing bronchitis with lumen cellular debris, and mild airsacculitis was also observed. In the brain, randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema were observed. In the heart, random multifocal to confluent myocardial degeneration to necrosis and minimal to mild mononuclear cell inflammation was observed. Mild to severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium occurred in all of the sampled ducks. The corticotrophic cells, and less consistently the chromaffin cells, of the adrenal gland had mild to moderate multifocal to confluent areas of vacuolar degeneration to necrosis. Skeletal muscle sampled from the thigh presented degeneration to necrosis of individual myofibers. The intestinal epithelium was only minimally to mildly affected, with mild inflammatory changes in the lamina propria. In all groups, the proventriculus and gizzard occasionally contained small foci of superficial heterophilic infiltration that was accompanied by mild epithelial necrosis. Mild to moderate necrosis of hepatocytes with sinusoidal histiocytosis was observed in the liver. The spleen, thymus, bursa, and mucosa-associated lymphoid tissue had moderate to severe lymphoid depletion ranging from apoptosis to necrosis in remaining lymphocytes. Both cortical and medullary regions of bursa and thymus were affected. Renal changes indicative of dehydration, such as mild dilatation of the distal segments of nephrons, were observed in some of the ducks.

In ducks inoculated with Ck/97, viral antigen staining was less common than the observed with the other two viruses (Table 2). In the Egret/02 and Dk/05 virus-inoculated ducks, viral antigen staining was present in multiple organs and was more widespread in tissues from ducks infected with Dk/05. In lymphoid organs, viral antigen was only identified in resident and infiltrating phagocytes and not in apoptotic lymphocytes. Vascular endothelium was consistently negative for the presence of viral antigen. Viral antigen was detected in the epithelium of the trachea, phagocytes and alveolar epithelium of the lung, air sac epithelium, the neurons and glial cells of the brain, cardiac and skeletal myocytes, adrenal corticotrophic cells, bursal phagocytes, medullary epithelium and tingible body macrophages of the thymus, phagocytes of the spleen, and pancreatic acinar epithelium. Viral antigen staining was rare or infrequent in renal tubular epithelium and in the epithelium and autonomic ganglia of the enteric tract. In ducks infected with Dk/05, viral staining was also found in hepatocytes and Kupfer cells in the liver, and smooth muscle of the gizzard and epithelial cells of the proventriculus.

3.2. Replication of H5N1 HPAI viruses in ducks

Virus was recovered from the oropharyngeal route at 3 dpi in all three of the 2 week-old ducks and in two of the 5-week-old ducks infected with Ck/97 (Table 3). However virus was only detected in the cloacal swabs of two of the 2-week-old ducks inoculated with this virus. Infectious virus could be recovered from all the oropharyngeal and cloacal swabs collected from 2 and 5-week-old ducks inoculated with Egret/02 and Dk/05. Oropharyngeal virus titers were higher in the 2 week-old ducks than in the 5 week-old ducks infected with Egret/02 and Dk/05 viruses, but were not statistically significant. Cloacal shedding of these two viruses was statistically higher than for Ck/97 in both 2 and 5-week-old ducks.

Virus replication was also examined at 2 dpi in brain, lung, spleen, heart, and muscle tissue following infection with the HPAI H5N1 viruses (Fig. 3). Differences in virus titers were found between tissues of ducks infected with Ck/97, Egret/02 or Dk/05 when comparing within the age groups. Most of the tissues from ducks infected with Egret/02 or Dk/05 had significantly higher virus titers than tissues from ducks infected with Ck/97 (data not shown). Infection of 2-week-old ducks with the Ck/97 virus resulted in no detectable viral titers in heart and muscle, and low titers in brain, lung and spleen. In the 5-week-old ducks inoculated with Ck/97, virus was only detected in the lung and spleen in low titers. Infection of 2 and 5-week-old ducks with Egret/02 and Dk/05 resulted in detectable virus in all organs examined. A significant difference in tissue titers between the different age ducks was only found between brains of ducks infected with Ck/97.

Table 2
Histological lesions and distribution of viral antigen in tissues from 2 and 5-week-old ducks inoculated with H5N1 HPAI viruses. Tissues were taken from three ducks at 2 days post infection and were immunohistochemically stained with antibodies to avian influenza virus nucleoprotein to visualize the viral antigen (histological lesions/viral antigen staining).[a]

Tissue	2 Week-old ducks			5 Week-old ducks		
	Ck/97	Egret/02	Dk/05	Ck/97	Egret/02	Dk/05
Trachea	++/++	++/++	+++/++	++/+	+/+	++/++
Lung	++/++	+++/++	+++/+++	+/+	++/++	+++/+++
Heart	−/−	+/++	+++/+++	−/−	++/++	++/+++
Brain	−/−	++/++	+++/+++	−/−	++/++	++/+++
Adrenal	−/−	+/+	+++/+++	−/−	−/−	+/++
Enteric tract	−/−	+/−	++/+	−/−	−/−	+/++
Pancreas	+/+	++/+	+++/+++	+/+	+/+	++/++
Liver	+/+	++/−	++/++	+/+	++/+	+/+
Kidney	−/−	−/−	+/+	+/−	−/−	+/+
Spleen	++/++	+/+	++/+++	++/++	+/++	+++/+++
Bursa	+/−	+/++	++/++	+/−	++/+	++/++
Thymus	+/+	+/++	+++/+++	+/+	++/++	++/+++
Muscle	−/−	+/+	++/++	−/−	−/+	+/++
Gizzard	−/−	−/−	+/+	−/−	−/−	+/+
Proventriculus	+/−	−/−	+++/+++	+/−	−/−	+/++

[a] Lesions were scored as follows: − = no lesions; + = mild; ++ = moderate; +++ = severe. The intensity of viral antigen staining in each section was scored as follows: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

Table 3
Oropharyngeal and cloacal viral titers in ducks infected with H5N1 HPAI viruses, 3 days post inoculation. Virus titers were determined in eggs and are expressed as the $\log_{10}$ EID_{50}/ml. Data are presented as means ± standard deviation of titers of positive samples. The threshold of detection is 0.9 $\log_{10}$ EID_{50}/ml. The number of birds that shed virus is indicated in parentheses (number shedding/number tested).

H5N1 virus	Virus titer			
	2 Week-old ducks		5 Week-old ducks	
	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
Ck/97	2.2 ± 0.4 (3/3)[a]	0.6 ± 1.1 (2/3)[a]	1.5 ± 1.3 (2/3)[a]	(0/3)[a]
Egret/02	3.6 ± 0.6 (3/3)[a]	2.5 ± 0.5 (3/3)[b]	3.2 ± 1.3 (3/3)[a]	2.3 ± 0.3 (3/3)[b]
Dk/05	3.4 ± 0.9 (3/3)[a]	2.5 ± 0.3 (3/3)[b]	3.4 ± 0.6 (3/3)[a]	2.3 ± 0.3 (3/3)[b]

Different lowercase superscripts denote significance between treatment groups (within columns) ($P < 0.05$).

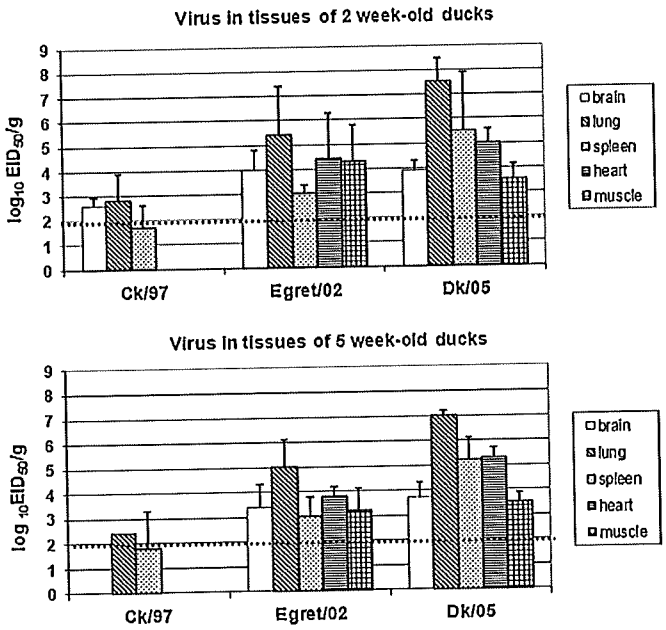

Fig. 3. Virus titers in the brain, lung, spleen, heart, and skeletal muscle of 2 and 5-week-old ducks after infection with H5N1 HPAI viruses. Tissues were collected at 2 dpi. Virus titers were determined in eggs and are expressed as the $\log_{10}$ EID_{50}/g. Values are the mean + standard error ($n = 3$). The threshold of detection is 1.97 $\log_{10}$ EID_{50}/gram of tissue. Titers from some tissues from ducks infected with Ck/97 were under the threshold of detection.

3.3. *Immune-related genes expression following H5N1 HPAI virus infection*

Relative quantitative real-time RT-PCR (qRRT-PCR) assays were conducted using RNA extracted from the spleens and lungs of the 2 and 5-week-old ducks infected with Ck/97, Egret/02 and Dk/05 and from sham-inoculated ducks. Gene expression of a type I interferon (IFN-α), a pro-inflammatory cytokine (IL-6), a RNA sensor present in duck cells (RIG-I), and a T cell proliferative interleukin (IL-2) was examined. Baseline levels of expression of these innate immune genes in the spleens and lungs of the non-infected ducks were present and similar when comparing expression between the two age groups. However, there was a difference in the baseline values of the tested genes when comparing organs, with similar levels of IFN-α and RIG-I but lower baseline levels of IL-6 and IL-2 in the lungs compared to the spleens. qRRT-PCR revealed increased levels of IFN-α, RIG-I, IL-6, and IL-2 RNA in most of the spleens from virus-infected 2 and 5-week-old ducks when compared to controls (Fig. 4). Significant higher levels of IFN-α, RIG-I and IL-2 gene expression were found in the 5-week-old ducks when compared to the 2-week-old ducks infected with the more virulent virus, Dk/05. All three viruses induced similar up-regulation of IFN-α, RIG-I and IL-6, but differences in expression of IL-2 were observed between the viruses.

The levels of expression of the innate immune-related genes in the lung of the virus-infected ducks were lower compared to the levels of expression for the same genes in the spleens, in some cases even down-regulated when compared to controls. No significant differences were observed in expression of any

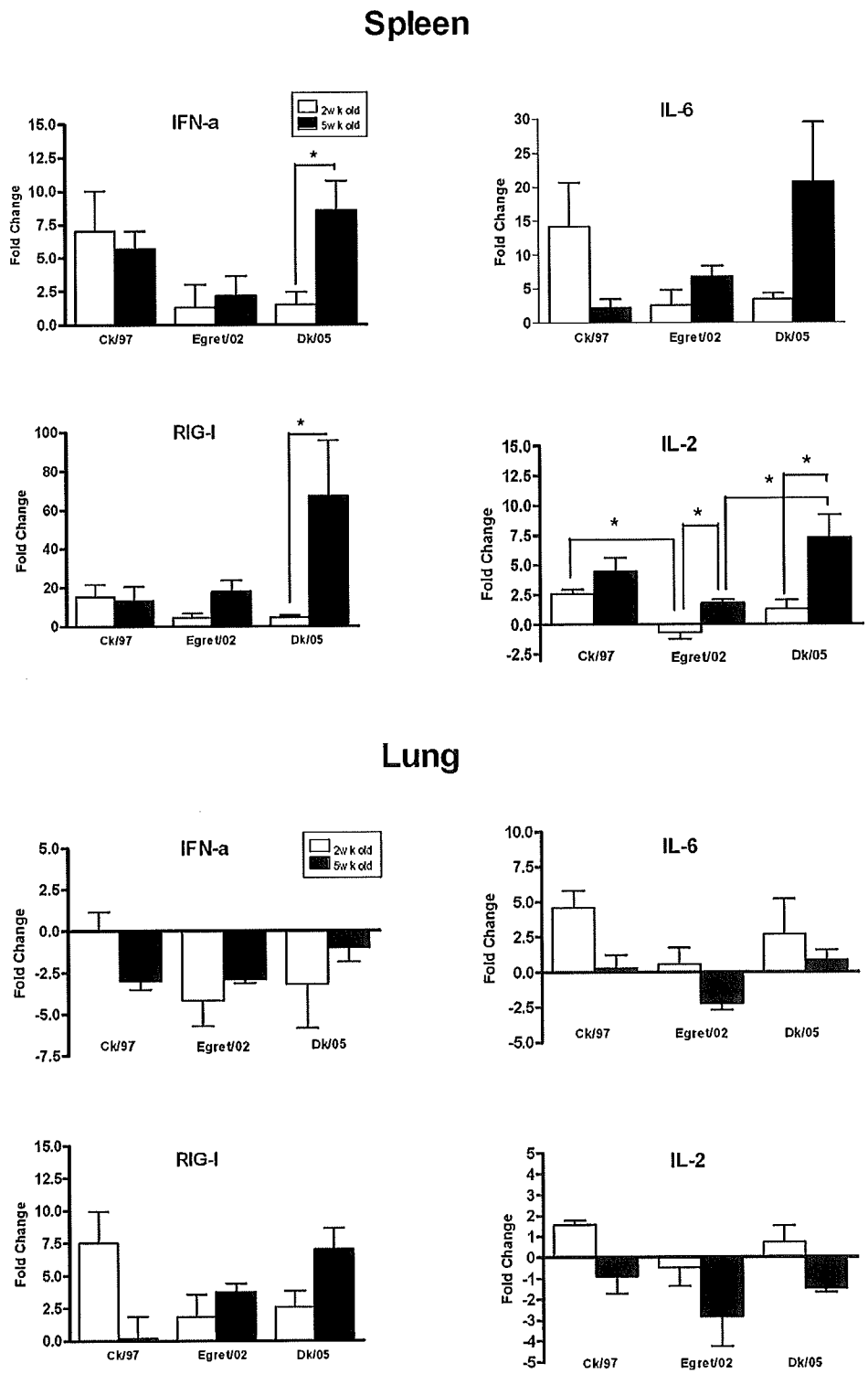

Fig. 4. Fold change expression of immune-related genes in spleens and lungs of 2 and 5-week-old ducks infected with Ck/97, Egret/02 and Dk/05 H5N1 HPAI viruses and compared to controls, 2 days post inoculation. Changes were quantified by qRRT-PCR and expressed as fold change in the birds infected ($n=4$) compared with non-infected birds ($n=4$). Bars represent standard error of the mean. Presence of the asterisk indicates significant difference in cytokine expression between groups ($P<0.05$).

of the genes examined, when comparing the 2 and 5-week-old ducks or the viruses. RIG-I and IL-6 genes expression were mostly up-regulated, with the exception of IL-6 in the 5-week-old ducks. Levels of IFN-α RNA were decreased in the lungs of all ducks except in the lungs of the 2-week-old ducks that received Ck/97. IL-6 gene expression was up-regulated in the lungs of all ducks except in the 5-week-old ducks infected with Egret/02. Expression of IL-2 was variable, and in most cases down-regulated.

The relative amounts of viral RNA in spleen and lung were also determined by real-time RT-PCR using RNA from the same samples

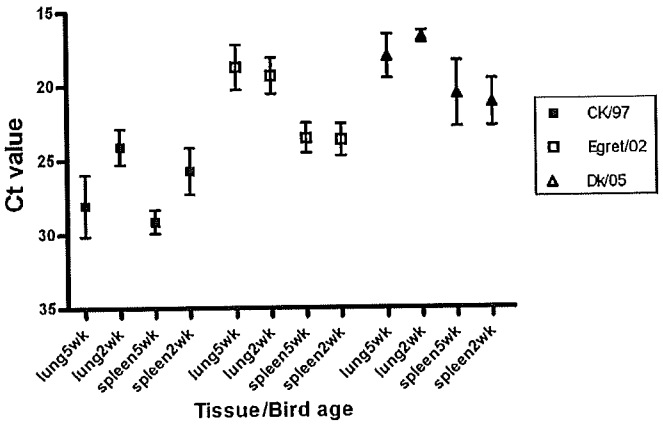

Fig. 5. Cycle threshold values representing titers of H5N1 HPAI viruses in spleen and lungs of infected ducks.

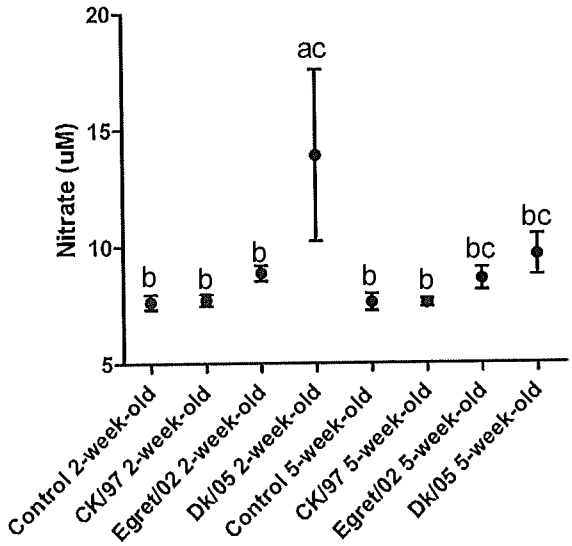

Fig. 6. Total nitrate and nitrite content produced by NO in serum samples taken at 2 dpi from H5N1 HPAI virus-infected ducks and non-infected controls and normalized to the total protein in the filtered sera. Values are the mean ± standard error ($n = 6$). Different lowercase letters indicate significant differences of the means ($P < 0.05$).

(Fig. 5). Similar levels of viral RNA were present in the lungs and spleens of the 2 and 5-week old ducks infected with Egret/02 and Dk/05, with higher titers present in lung. The 2-week-old ducks inoculated with Ck/97 had higher titers in lung and spleen the 5-week-old ducks.

3.4. Nitric oxide levels in serum

Total nitrate and nitrite content produced by NOS measured in serum samples from infected ducks when compared to non-infected controls showed a significant increase in ducks infected with Dk/05 when compared to the control ducks and ducks infected with Ck/97 (Fig. 6). A mild increase was also observed in ducks infected with Egret/02.

4. Discussion

In the present study we examined the pathobiology and host gene expression responses in 2 and 5-week-old ducks infected with H5N1 HPAI viruses. Clear differences in pathogenicity were observed between the three viruses studied and also between the younger and older ducks, similar to what we have previously described (Pantin-Jackwood and Swayne, 2007; Pantin-Jackwood et al., 2007; Pfeiffer et al., 2009). The loss of body weight correlated with the severity of the disease produced by the viruses, and interestingly, body temperatures measured after infection were higher in the 5-week-old ducks inoculated with the more virulent viruses, Egret/02 and Dk/05, than in the 2-week-old ducks inoculated with these same viruses, indicating possible differences in the innate immune responses.

Previous studies have demonstrated that the pathogenicity of H5N1 HPAI viruses in ducks directly correlates with levels of virus replication in tissues, with the more pathogenic viruses replicating to higher titers and in more tissues (Pantin-Jackwood and Swayne, 2007; Pfeiffer et al., 2009; Wasilenko et al., 2011). Ducks that died after infection with these pathogenic viruses presented neurological signs and heart damage, either lesions being possible contributors to death. In the present study, and as previously observed, viral titers in tissues were also elevated in ducks infected with the two more virulent viruses, Egret/02 and Dk/05, and antigen staining in tissues from these ducks indicated replication of the viruses in several organs and in association with the histological lesions observed. However, clear differences in viral titer in tissues between the 2-week-old and 5-week-old infected ducks was only observed in the ducks infected with the less pathogenic Ck/97 virus, with higher or detectable titers found in the younger ducks. This was also seen with the RT-PCR results when examining for presence of viral RNA in lungs and spleens. Nevertheless, the higher titers of Ck/97 in the younger ducks were not reflected in increased clinical signs or the outcome of infection. Contrary to this, in a similar study, 2 and 5-week-old ducks infected with two different H5N1 HPAI viruses showed that one virus grew to similar high titers in tissues in both age groups, and the other virus grew to higher titers in the younger ducks (Pantin-Jackwood et al., 2007); but higher mortality was observed in the younger ducks infected with either virus. Although showing similar patterns, minor incongruence was observed between the immunohistochemistry (IHC) results and the virus titers in tissues and swabs (Tables 2 and 3 and Fig. 3). Virus staining in tissues can help determine in which cells the virus is replicating in and how widespread is this replication; however, IHC is not a very sensitive assay and not the most adequate method to quantify virus replication in tissues. This is in part because of the high virus titers needed for virus visualization and the possible loss of infected cells during the IHC procedure. In addition, and compared to virus isolation, during microscopic examination only a small portion of the tissue is examined. Similar viral titers and viral distribution in tissues at 2 dpi in 2 and 5-week-old ducks infected with Egret/02 and Dk/05 viruses, suggests that other factors might be contributing to the differences observed in clinical presentation and survival. Since little difference in viral RNA expression in lungs and spleens was found between the 2 and 5-week-old ducks infected with these two viruses, the differences observed in innate immune-related gene expression are probably caused by differences in host responses between age groups.

Influenza virus infection triggers a cascade of host defenses that are responsible for virus control and clearance, but also for many of the symptoms associated with clinical disease. In mammals, high virulence is also linked with substantial levels of viral replication *in vivo* (Basler and Aguilar, 2008); but the manner with which viral replication contributes to virulence remains to be determined. High virulence in mammals has also been associated with potent, perhaps excessive, inflammatory responses, and it is possible that these responses may prove to be the direct cause of the damage seen in severe disease (Basler and Aguilar, 2008; de Jong et al., 2006; Kash et al., 2004). Clinical features that are prominent in human H5N1 infections, such as acute respiratory

distress syndrome (ARDS) and multiple organ dysfunctions have been associated with cytokine dysregulation (Chan et al., 2005; Peiris et al., 2004; To et al., 2001). Studies to define which aspects of the pro-inflammatory immune response are beneficial and which are detrimental have only begun, sometimes giving contradictory results (Basler and Aguilar, 2008). Not much is known on the role of the innate immune responses to AI infection in avian species and how much of the clinical signs observed after infection are consequence of these responses.

Upon viral infection, production of type-I interferon (IFN) and IFN-induced genes is a typical innate defense response resulting in an antiviral state of non-infected neighboring target cells. Type I IFN is also known to act as an important immune inducer and immunoregulatory factor. In this study, type I (IFN-α) interferon gene expression was induced in the spleens of ducks following H5N1 HPAI virus infection and varied depending on the virus and the age of the ducks with significant differences in expression only observed with the Dk/05 virus. Up-regulation of IFN-α was also observed in spleens of Pekin and Muscovy ducks infected with another virulent H5N1 HPAI virus (Cagle et al., 2011). In both cases, levels of viral RNA in the spleen did not always correlate with IFN-α gene expression, suggesting differences between viruses in the induction of innate responses but also, like in the case of the Dk/05 virus, an effect of age on host responses to infection. Future studies will need to address the fluctuation of gene expression at different times after viral infection. This fluctuation could in part explain the down-regulation of IFN-α observed in the lungs of most virus-infected ducks in our study. However, differences in types of cells found in spleens and lungs likely play a role in the differences observed in the innate immune responses to virus infection between spleens and lung. The results obtained are the average gene expression of cells in the entire tissue including infected and uninfected cells.

Viral replication efficiencies and host immune responses against H5N1 HPAI virus infection has been shown to vary among established chicken cell lines (Watanabe et al., 2011). In the host, macrophages constitute a major component of the innate immune response against virus infections. At the local sites of infection, activated macrophages induce pro-inflammatory responses aimed at the elimination of invading pathogens and infected cells. Our immunohistochemistry data on tissues from the infected ducks showed the presence of the virus in resident and infiltrating macrophages in several tissues. It is not clear to what degree the virus replicates in macrophages *in vivo*, but the role of these cells in clearing virus and releasing immune mediators likely has a very important effect on AI pathogenicity. The presence of circulating or resident macrophages in tissues would surely affect the magnitude of the innate immune responses.

Increase of pro-inflammatory cytokine IL-6 gene expression in spleens was previously reported in Pekin and Muscovy ducks infected with a H5N1 HPAI virus (Cagle et al., 2011). Up-regulation of IL-6 was also found in tissues of ducks infected with the three H5N1 HPAI viruses used in this study. However no significant differences in gene expression were observed between the 2 and 5 week-old ducks. Differences in IL-2 gene expression were observed in the spleens and lungs of the infected ducks, indicating that modulation of the T-helper cytokine response might be differently induced by these viruses or affected by the age of the ducks.

Up-regulation of RIG-I in ducks after infection with H5N1 HPAI viruses has been reported previously (Barber et al., 2010; Cagle et al., 2011). RIG-I is present in ducks and plays a critical role in the innate immune response to influenza (Barber et al., 2010). RIG-I initiates interferon pathways which initiate antiviral defenses (Magor, 2011). In this study, the higher levels of expression of RIG-I in the spleens of the 5-week-old ducks infected with Dk/05when compared to the 2-week-old ducks, could indicate a more efficient innate immune response to infection in the older ducks.

To date there is little information available about fever in birds, although there is evidence that suggests that functional characteristics of avian fever are similar to those found in mammals (Gray et al., 2005). Very little is known about the mediators and modulators of the febrile response in avian species. There is some evidence that suggests that prostaglandins (PGs), as in mammalian fevers, play a role in the generation of fever in birds (Baert et al., 2005; Gray et al., 2005; Macari et al., 1993). A role for cytokines, including IL-1 β and IL-6 has been suggested in the signaling pathways that lead to prostaglandin synthesis (Macari et al., 1993). Elevated levels of IL-6 gene expression were found in the spleens of the 5-week-old ducks infected with the most virulent virus. These ducks also had high fever, but the levels of IL-6 did not seem to correlate with the differences observed in body temperature. Nitric oxide (NO) has also been postulated to play a role in fever genesis (De Paula et al., 2000). Currently, little is known about the role of NO in avian fever. Studies in Pekin ducks suggested that lipopolysaccharide (LPS)-induced fever is mediated by prostaglandins and nitric oxide and is modulated by adrenocorticoid hormones (Gray et al., 2005). Levels of NO in serum were elevated in the ducks infected with the two more virulent viruses when compared to the non-infected ducks, but the highest levels were observed in the 2-week-old ducks infected with Dk/05 and did not correlate with increased body temperature.

The inflammatory response observed after infection with H5N1 HPAI virus infection is compounded by significant necrosis and increased oxidative stress in tissues. The activation of these pathology-associated pathways in AI virus infection results in the development of severe disease and consequently death. In mature ducks these responses could have curtailed and eventually controlled virus infection as seen after infection with Egret/02 in the 5-week-old ducks. However, with the more pathogenic Dk/05 virus, the host responses were not sufficient to control the virus and all infected ducks died.

The data presented indicates that differences in the severity of HPAI virus infections in ducks were associated with differences in viral replication and, to certain extent, host gene expression. The more virulent viruses replicated to high titers in tissues of both the 2 and 5-week-old ducks, however, some of the older ducks survived infection, and the ones that died lived longer than the younger ducks. The inability to fight off infection observed in younger ducks could be explained in part by a less efficient innate immune response. The weaker expression of some immune-related genes in the younger ducks suggests that the immune system in younger ducks is still not completely developed and consequently is not as efficient in fighting infection as in more mature ducks. More studies examining innate immune responses at earlier time points after virus infection would help to elucidate this.

Acknowledgements

The authors appreciate the assistance provided by Tracy Smith-Faulkner, Ronald Graham and Roger Brock in conducting these studies. The authors also thank the FSIS histopathology laboratory at Russell Research Center-USDA for technical assistance. This research was supported by USDA/ARS CRIS project #6612-32000-048. Mention of trade names or commercial products in this presentation is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Aldous, E.W., Seekings, J.M., McNally, A., Nili, H., Fuller, C.M., Irvine, R.M., Alexander, D.J., Brown, I.H., 2010. Infection dynamics of highly pathogenic avian influenza and virulent avian paramyxovirus type 1 viruses in chickens, turkeys and ducks. Avian Pathology 39 (4), 265–273.

Adams, S.C., Xing, Z., Li, J., Cardona, C.J., 2009. Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Molecular Immunology 46 (8–9), 1744–1749.

Baert, K., Duchateau, L., De Boever, S., Cherlet, M., De Backer, P., 2005. Antipyretic effect of oral sodium salicylate after an intravenous *E. coli* LPS injection in broiler chickens. British Poultry Science 46 (2), 137–143.

Barber, M.R., Aldridge Jr., J.R., Webster, R.G., Magor, K.E., 2010. Association of RIG-I with innate immunity of ducks to influenza. Proceedings of the National Academy of Sciences of the United States of America 107 (13), 5913–5918.

Basler, C.F., Aguilar, P.V., 2008. Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses. Antiviral Research 79 (3), 166–178.

Burggraaf, S., Bingham, J., Payne, J., Kimpton, W.G., Lowenthal, J.W., Bean, A.G., 2011. Increased inducible nitric oxide synthase expression in organs is associated with a higher severity of H5N1 influenza virus infection. PLoS One 6 (1), e14561.

Cagle, C., To, T.L., Nguyen, T., Wasilenko, J., Adams, S.C., Cardona, C.J., Spackman, E., Suarez, D.L., Pantin-Jackwood, M.J., 2011. Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. Vaccine 29, 6549–6557.

Cameron, C.M., Cameron, M.J., Bermejo-Martin, J.F., Ran, L., Xu, L., Turner, P.V., Ran, R., Danesh, A., Fang, Y., Chan, P.K., Mytle, N., Sullivan, T.J., Collins, T.L., Johnson, M.G., Medina, J.C., Rowe, T., Kelvin, D.J., 2008. Gene expression analysis of host innate immune responses during Lethal H5N1 infection in ferrets. Journal of Virology 82 (22), 11308–11317.

Capua, I., Mutinelli, F., 2001. Mortality in Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser* var. *domestica*) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathology 30, 179–183.

Capua, I., Alexander, D.J., 2008. Avian influenza vaccines and vaccination in birds. Vaccine 26 (Suppl. 4), D70–D73.

Chan, M.C., Cheung, C.Y., Chui, W.H., Tsao, S.W., Nicholls, J.M., Chan, Y.O., Chan, R.W., Long, H.T., Poon, L.L., Guan, Y., Peiris, J.S., 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respiratory Research 6, 135.

Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., Zhang, L., Liu, Z., Webster, R.G., Yu, K., 2004. The evolution of H5N1 influenza viruses in ducks in southern China. Proceedings of the National Academy of Sciences of the United States of America 101 (28), 10452–10457.

Costa, T.P., Brown, J.D., Howerth, E.W., Stallknecht, D.E., 2010. The effect of age on avian influenza viral shedding in mallards (*Anas platyrhynchos*). Avian Diseases 54 (Suppl. 1), 581–585.

de Jong, M.D., Simmons, C.P., Thanh, T.T., Hien, V.M., Smith, G.J., Chau, T.N., Hoang, D.M., Chau, N.V., Khanh, T.H., Dong, V.C., Qui, P.T., Cam, B.V., Ha do, Q., Guan, Y., Peiris, J.S., Chinh, N.T., Hien, T.T., Farrar, J., 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nature Medicine 12 (10), 1203–1207.

De Paula, D., Steiner, A.A., Branco, L.G., 2000. The nitric oxide pathway is an important modulator of stress-induced fever in rats. Physiology and Behavior 70 (5), 505–511.

Degen, W.G., Smith, J., Simmelink, B., Glass, E.J., Burt, D.W., Schijns, V.E., 2006. Molecular immunophenotyping of lungs and spleens in naive and vaccinated chickens early after pulmonary avian influenza A (H9N2) virus infection. Vaccine 24 (35–36), 6096–6109.

Ellis, T.M., Bousfield, R.B., Bissett, L.A., Dyrting, K.C., Luk, G.S., Tsim, S.T., Sturm-Ramirez, K., Webster, R.G., Guan, Y., Malik Peiris, J.S., 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathology 33 (5), 492–505.

Gilbert, M., Chaitaweesub, P., Parakamawongsa, T., Premashthira, S., Tiensin, T., Kalpravidh, W., Wagner, H., Slingenbergh, J., 2006. Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerging Infectious Diseases 12 (2), 227–234.

Gray, D.A., Maloney, S.K., Kamerman, P.R., 2005. Lipopolysaccharide-induced fever in Pekin ducks is mediated by prostaglandins and nitric oxide and modulated by adrenocortical hormones. American Journal of Physiology: Regulatory Integrative and Comparative Physiology 289 (5), R1258–R1264.

Hayden, F.G., Fritz, R., Lobo, M.C., Alvord, W., Strober, W., Straus, S.E., 1998. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. Journal of Clinical Investigation 101 (3), 643–649.

Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., Scholtissek, C., Puthavathana, P., Buranathai, C., Nguyen, T.D., Long, H.T., Naipospos, T.S., Chen, H., Ellis, T.M., Guan, Y., Peiris, J.S., Webster, R.G., 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences of the United States of America 102 (30), 10682–10687.

Jilbert, A.R., Botten, J.A., Miller, D.S., Bertram, E.M., Hall, P.M., Kotlarski, J., Burrell, C.J., 1998. Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. Virology 244 (2), 273–282.

Kash, J.C., Basler, C.F., Garcia-Sastre, A., Carter, V., Billharz, R., Swayne, D.E., Przygodzki, R.M., Taubenberger, J.K., Katze, M.G., Tumpey, T.M., 2004. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. Journal of Virology 78 (17), 9499–9511.

Kishida, N., Sakoda, Y., Isoda, N., Matsuda, K., Eto, M., Sunaga, Y., Umemura, T., Kida, H., 2005. Pathogenicity of H5 influenza viruses for ducks. Archives of Virology 150 (7), 1383–1392.

Kwon, Y.-K., Joh, S.-J., Kim, M.-C., Sung, H.-W., Lee, Y.-J., Choi, J.-G., Lee, E.-K., Kim, J.-H., 2005. Highly pathogenic avian influenza (H5N1) in the commercial domestic ducks of South Korea. Avian Pathology 34, 367–370.

Lavoie, E.T., Sorrell, E.M., Perez, D.R., Ottinger, M.A., 2007. Immunosenescence and age-related susceptibility to influenza virus in Japanese quail. Developmental and Comparative Immunology 31 (4), 407–414.

Li, K.S., Guan, Y., Wang, J., Smith, G.J., Xu, K.M., Duan, L., Rahardjo, A.P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Estoepangestie, A.T., Chaisingh, A., Auewarakul, P., Long, H.T., Hanh, N.T., Webby, R.J., Poon, L.L., Chen, H., Shortridge, K.F., Yuen, K.Y., Webster, R.G., Peiris, J.S., 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430 (6996), 209–213.

Liang, Q.L., Luo, J., Zhou, K., Dong, J.X., He, H.X., 2011. Immune-related gene expression in response to H5N1 avian influenza virus infection in chicken and duck embryonic fibroblasts. Molecular Immunology 48 (6–7), 924–930.

Londt, B.Z., Nunez, A., Banks, J., Alexander, D.J., Russell, C., Richard-Londt, A.C., Brown, I.H., 2010. The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks (*Anas platyrhynchos*) infected experimentally. Influenza and Other Respiratory Viruses 4 (1), 17–25.

Macari, M., Furlan, R.L., Gregorut, F.P., Secato, E.R., Guerreiro, J.R., 1993. Effects of endotoxin, interleukin-1 beta and prostaglandin injections on fever response in broilers. British Poultry Science 34 (5), 1035–1042.

Magor, K.E., 2011. Immunoglobulin genetics and antibody responses to influenza in ducks. Developmental and Comparative Immunology 35 (9), 1008–1016.

Pantin-Jackwood, M.J., Swayne, D.E., 2007. Pathobiology of Asian Highly Pathogenic Avian Influenza H5N1 virus infections in ducks. Avian Diseases 51, 250–259.

Pantin-Jackwood, M.J., Suarez, D.L., Spackman, E., Swayne, D.E., 2007. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. Virus Research 130 (1–2), 151–161.

Pantin-Jackwood, M.J., Swayne, D.E., 2009. Pathogenesis and pathobiology of avian influenza virus infection in birds. Revue Scientifique et Technique 28 (1), 113–136.

Peiris, J.S., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y., Guan, Y., 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 363 (9409), 617–619.

Perkins, L.E., Swayne, D.E., 2001. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Veterinary Pathology 38 (2), 149–164.

Pfeiffer, J., Pantin-Jackwood, M., To, T.L., Nguyen, T., Suarez, D.L., 2009. Phylogenetic and biological characterization of highly pathogenic H5N1 avian influenza viruses (Vietnam 2005) in chickens and ducks. Virus Research 142 (1–2), 108–120.

Phuong, D.Q., Dung, N.T., Jorgensen, P.H., Handberg, K.J., Vinh, N.T., Christensen, J.P., 2011. Susceptibility of Muscovy (*Cairina moschata*) and mallard ducks (*Anas platyrhynchos*) to experimental infections by different genotypes of H5N1 avian influenza viruses. Veterinary Microbiology 148 (2–4), 168–174.

Rebel, J.M., Peeters, B., Fijten, H., Post, J., Cornelissen, J., Vervelde, L., 2011. Highly pathogenic or low pathogenic avian influenza virus subtype H7N1 infection in chicken lungs: small differences in general acute responses. Veterinary Research 42 (1), 10.

Reemers, S.S., Groot Koerkamp, M.J., Holstege, F.C., van Eden, W., Vervelde, L., 2009. Cellular host transcriptional responses to influenza A virus in chicken tracheal organ cultures differ from responses in in vivo infected trachea. Veterinary Immunology and Immunopathology 132 (2–4), 91–100.

Reemers, S.S., van Leenen, D., Koerkamp, M.J., van Haarlem, D., van de Haar, P., van Eden, W., Vervelde, L., 2010. Early host responses to avian influenza A virus are prolonged and enhanced at transcriptional level depending on maturation of the immune system. Molecular Immunology 47 (9), 1675–1685.

Sarmento, L., Afonso, C.L., Estevez, C., Wasilenko, J., Pantin-Jackwood, M., 2008a. Differential host gene expression in cells infected with highly pathogenic H5N1 avian influenza viruses. Veterinary Immunology and Immunopathology 125 (3–4), 291–302.

Sarmento, L., Pantin-Jackwood, M., Kapczynski, D.R., Swayne, D.E., Afonso, C.L., 2008b. Immediate early responses of avian tracheal epithelial cells to infection with highly pathogenic avian influenza virus. Developmental Biology (Basel) 132, 175–183.

Sarmento, L., Wasilenko, J., Pantin-Jackwood, M., 2010. The effects of NS gene exchange on the pathogenicity of H5N1 HPAI viruses in ducks. Avian Diseases 54 (Suppl. 1), 532–537.

Sims, L.D., 2007. Experience in control of avian influenza in Asia. Developmental Biology (Basel) 130, 39–43.

Songserm, T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., Webster, R.G., 2006. Domestic ducks and H5N1 Influenza Epidemic, Thailand. Emerging Infectious Diseases 12 (4), 575–581.

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., Suarez, D.L., 2002. Development of a real-time reverse

transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40 (9), 3256–3260.

Sturm-Ramirez, K.M., Hulse-Post, D.J., Govorkova, E.A., Humberd, J., Seiler, P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Chaisingh, A., Long, H.T., Naipospos, T.S., Chen, H., Ellis, T.M., Guan, Y., Peiris, J.S., Webster, R.G., 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of Virology 79 (17), 11269–11279.

Suzuki, K., Okada, H., Itoh, T., Tada, T., Mase, M., Nakamura, K., Kubo, M., Tsukamoto, K., 2009. Association of increased pathogenicity of Asian H5N1 highly pathogenic avian influenza viruses in chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. Journal of Virology 83 (15), 7475–7486.

Swayne, D.E., 2006. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Annals of the New York Academy of Sciences 1081, 174–181.

Swayne, D.E., Halvorson, D.A., 2008. Influenza. In: Glisson, J.R., Saif, Y.M., Fadly, A.M., McDougald, L.R., Nolan, L. (Eds.), Diseases of Poultry. , 12th ed. Blackwell, Ames, IA.

Swayne, D.E., Senna, D.A., Beard, C.W., 1998. Avian influenza. In: Swayne, J.R.G.D.E., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens. , 4th ed. American Association of Avian Pathologists, Kennett Square, PA, pp. 150–155.

Szretter, K.J., Gangappa, S., Lu, X., Smith, C., Shieh, W.J., Zaki, S.R., Sambhara, S., Tumpey, T.M., Katz, J.M., 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. Journal of Virology 81 (6), 2736–2744.

To, K.F., Chan, P.K., Chan, K.F., Lee, W.K., Lam, W.Y., Wong, K.F., Tang, N.L., Tsang, D.N., Sung, R.Y., Buckley, T.A., Tam, J.S., Cheng, A.F., 2001. Pathology of fatal human infection associated with avian influenza A H5N1 virus. Journal of Medical Virology 63 (3), 242–246.

Tohidi-Esfahani, R., Vickery, K., Cossart, Y., 2010. The early host innate immune response to duck hepatitis B virus infection. Journal of General Virology 91 (Pt 2), 509–520.

Wasilenko, J.L., Arafa, A.M., Selim, A.A., Hassan, M.K., Aly, M.M., Ali, A., Nassif, S., Elebiary, E., Balish, A., Klimov, A., Suarez, D.L., Swayne, D.E., Pantin-Jackwood, M.J., 2011. Pathogenicity of two Egyptian H5N1 highly pathogenic avian influenza viruses in domestic ducks. Archives of Virology 156 (1), 37–51.

Wasilenko, J.L., Lee, C.W., Sarmento, L., Spackman, E., Kapczynski, D.R., Suarez, D.L., Pantin-Jackwood, M.J., 2008. NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens. Journal of Virology 82 (9), 4544–4553.

Wasilenko, J.L., Sarmento, L., Pantin-Jackwood, M.J., 2009. A single substitution in amino acid 184 of the NP protein alters the replication and pathogenicity of H5N1 avian influenza viruses in chickens. Archives of Virology 154 (6), 969–979.

Watanabe, C., Uchida, Y., Ito, H., Ito, T., Saito, T., 2011. Host immune-related gene responses against highly pathogenic avian influenza virus infection in vitro differ among chicken cell lines established from different organs. Veterinary Immunology and Immunopathology 144 (3–4), 187–199.

Xing, Z., Cardona, C.J., Adams, S., Yang, Z., Li, J., Perez, D., Woolcock, P.R., 2009. Differential regulation of antiviral and proinflammatory cytokines and suppression of Fas-mediated apoptosis by NS1 of H9N2 avian influenza virus in chicken macrophages. Journal of General Virology 90 (Pt 5), 1109–1118.

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Implication of Inflammatory Macrophages, Nuclear Receptors, and Interferon Regulatory Factors in Increased Virulence of Pandemic 2009 H1N1 Influenza A Virus after Host Adaptation

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***J. Virol.* 2012, 86(13):7192. DOI: 10.1128/JVI.00563-12.**
Published Ahead of Print 24 April 2012.

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Implication of Inflammatory Macrophages, Nuclear Receptors, and Interferon Regulatory Factors in Increased Virulence of Pandemic 2009 H1N1 Influenza A Virus after Host Adaptation

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While pandemic 2009 H1N1 influenza A viruses were responsible for numerous severe infections in humans, these viruses do not typically cause corresponding severe disease in mammalian models. However, the generation of a virulent 2009 H1N1 virus following serial lung passage in mice has allowed for the modeling of human lung pathology in this species. Genetic determinants of mouse-adapted 2009 H1N1 viral pathogenicity have been identified, but the molecular and signaling characteristics of the host response following infection with this adapted virus have not been described. Here we compared the gene expression response following infection of mice with A/CA/04/2009 (CA/04) or the virulent mouse-adapted strain (MA-CA/04). Microarray analysis revealed that increased pathogenicity of MA-CA/04 was associated with the following: (i) an early and sustained inflammatory and interferon response that could be driven in part by interferon regulatory factors (IRFs) and increased NF-κB activation, as well as inhibition of the negative regulator TRIM24, (ii) early and persistent infiltration of immune cells, including inflammatory macrophages, and (iii) the absence of activation of lipid metabolism later in infection, which may be mediated by inhibition of nuclear receptors, including PPARG and HNF1A and -4A, with proinflammatory consequences. Further investigation of these signatures in the host response to other H1N1 viruses of various pathogenicities confirmed their general relevance for virulence of influenza virus and suggested that lung response to MA-CA/04 virus was similar to that following infection with lethal H1N1 r1918 influenza virus. This study links differential activation of IRFs, nuclear receptors, and macrophage infiltration with influenza virulence *in vivo*.

The 2009 H1N1 influenza virus was responsible for the first pandemic of the 21st century and caused more than 18,000 deaths worldwide between April 2009 and August 2010 (50). People infected during the pandemic experienced mostly mild symptoms, but there were reports of severe primary viral pneumonia in young and healthy people, which is not typically seen during seasonal influenza epidemics. The 2009 H1N1 virus is highly transmissible among humans and is now circulating as a seasonal influenza virus strain (51), and thus there is a threat of emergence of more virulent strains in the future resulting from continuous mutation of the virus. Therefore, it is crucial to improve our understanding of 2009 H1N1 pathogenicity.

The mouse model has been used to study the pathogenic potential of 2009 H1N1 virus and characterize viral factors critical for virulence. Most 2009 H1N1 isolates replicate efficiently in the lungs of BALB/c mice but are almost avirulent for this animal (50% mouse lethal dose [MLD_{50}] $\geq 10^6$ PFU [18, 5, 30] for A/California/04/09 and A/Mexico/4482/09). However, they can gain virulence and become lethal at a lower inoculum after serial lung passages in mice (16, 34, 39, 53, 56). Virulence determinants of mouse-adapted H1N1 virus have been identified in the ribonucleoprotein (RNP) complex (16, 34, 39, 53, 56) and hemagglutinin (HA) (16, 34, 39, 53). Several mutations have been reported, with independent and synergistic effects on virulence (39, 53). Mutations in HA have been associated with a change in receptor binding specificity (16, 39, 53), and mutations in PA, PB1, PB2, and NP have been associated with enhanced polymerase activity (16, 39, 53, 56). These studies have characterized the mouse-adapted virus pathogenic determinants; however, the corresponding effects on host response following infection have not been described.

To gain a better understanding of virulence and pathogenic potential following adaptation of 2009 H1N1 virus, we performed a time course study in BALB/c mice to compare the prototypic 2009 H1N1 strain A/California/04/09 (CA/04) and a corresponding mouse-adapted variant, MA1-A/California/04/09 (MA-CA/04) (16). MA-CA/04 contains five genetic mutations (in HA, G155E, S183P, and D222G; in PB2, E158G; and in NP, D101G) and is associated with a dramatic increase in virulence in mice (16). Using global gene expression profiling in tandem with bioinformatics methods, we characterized the differences in the host response to both viruses and found that the increased pathogenicity of MA-CA/04 was associated with an early and sustained inflammatory response, infiltration of inflammatory macrophages, and perturbation of lipid metabolism. Our data suggest several transcription factors (TFs) could mediate these responses. To put our findings into a larger context of influenza virulence, we compared these signatures to gene expression in the lungs of mice

Received 2 March 2012 Accepted 13 April 2012
Published ahead of print 24 April 2012
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Supplemental material for this article may be found at http://jvi.asm.org/.
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doi:10.1128/JVI.00563-12

infected with either lethal r1918 or other nonlethal H1N1 influenza A viruses. Similar expression pattern of these biological signatures among viruses with similar pathogenicities suggests their importance in differential responses to lethal and nonlethal influenza viruses.

MATERIALS AND METHODS

Viruses. Viral stocks for A/California/04/2009, mouse-adapted MA1-A/California/04/2009 (generously provided by Richard Webby, St. Jude Children's Hospital, Memphis, TN [16]), A/Mexico/4482/09 (Mex/4482), and A/Brisbane/59/07 (Brisbane/59/07) were propagated in Madin-Darby canine kidney (MDCK) cells for 48 h at 37°C. A/New Jersey/8/76 (NJ/8/76) was propagated in the allantoic cavity of 10-day-old embryonated hens' eggs for stock preparation. The supernatants were clarified by centrifugation, aliquoted, and stored at −70°C. Virus titers were determined by standard plaque assay on MDCK cells (55). The identities of virus genes were confirmed by sequence analysis to verify that no inadvertent mutations were present during the generation of virus stocks.

Mouse experiments. Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). All 2009 H1N1 *in vivo* experiments were performed under biosafety level 3 enhanced (BSL3+) containment. Mice were anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin; Sigma-Aldrich, St. Louis, MO) and intranasally (i.n.) inoculated with 50 μl of phosphate-buffered saline (PBS; mock) or with 10^6 PFU of virus in a 50-μl volume. Six animals in each group were monitored for morbidity and mortality as measured by weight loss and survival over a period of 16 days. Viral titers were determined in the lungs, spleen, and brain on days 1, 3, and 5 postinoculation in nine animals per group (three animals for each time point). An additional nine animals were used for transcriptional profiling, again three animals per time point. Histopathology was performed on another batch of nine mice for each virus, with three animals used per time point. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Histopathology and immunohistochemistry. Lung sections were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Histopathological lesions were examined after staining with hematoxylin and eosin (HE) following standard protocols and scored as follows: 1, no lesion; 2, mild lesion; 3, moderate lesion; 4, severe lesion. Immunohistochemical viral antigen detection was performed using a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein as previously described (4), and the following scores were used: 0, no antigen; 1, rare positive cells; 2, infrequent positive cells; 3, common positive cells; and 4, widespread positive cells.

Microarray experiments. Transcriptional profiles were determined by microarray analysis of RNA isolated from lung tissue from three individual animals per group at days 1, 3, and 5 postinfection (p.i.). Total RNA extraction from lung samples was performed as previously described (4). Probe labeling and microarray slide hybridization for each biological replicate was performed using a whole mouse genome microarray kit (G4122-60520; Agilent Technologies) according to the manufacturer's instructions.

Data analysis. Extracted raw data were background corrected using the "norm-exp" method with an offset of 1 and quantile normalized using Agi4x44PreProcess (28) in the R software environment. Replicate probes were mean summarized, and all probes were required to pass Agilent QC flags for 75% replicates of at least one infected time point (30,827 probes passed).

Mean expression values were calculated by determining the arithmetic mean for the individual replicates. From this, ratios of values for the infected samples to the mean values of time-matched mock samples were calculated and logarithm base 2 transformed. These values are referred to as "$\log_2$ ratios."

A Welch *t* test on $\log_2$-scaled intensity values was performed to determine the probes that were differentially expressed (DE) between each virus and time-matched mock and between two different viruses at each time point. Criteria for DE were an absolute $\log_2$ ratio of >1 and a Benjamini-Hochberg adjusted q value of <0.05.

Genes discriminating CA/04 and MA-CA/04 were grouped in three sets based on their expression levels. Set 1 (upregulated lethal signature) was defined as genes whose expression conformed, for at least 1 day p.i., to the following rules: $\log_2$ ratio(CA/04) > −1 and $\log_2$ ratio(MA-CA/04) > 0 and $\log_2$ ratio(MA-CA/04) > $\log_2$ ratio(CA/04); set 2 (survival/recovery signature) was defined as genes with $\log_2$ ratio(CA/04) >1 and $\log_2$ ratio(CA/04) > $\log_2$ ratio(MA-CA/04) for at least 1 day p.i.; and set 3 (downregulated lethal signature) was defined as genes with $\log_2$ ratio(CA/04) < 1 and $\log_2$ ratio(MA-CA/04) < −1 and $\log_2$ ratio(MA-CA/04) < $\log_2$ ratio(CA/04) for at least 1 day p.i.

Functional analysis of statistically significant gene expression changes was performed using the Ingenuity Pathways Knowledge Base (IPA; Ingenuity Systems) as previously described (4). All enrichment scores were calculated in IPA using all probes that passed our QC filter as the background data set.

TF analysis. We used two different methods to identify potential transcriptional regulators that could control the gene expression response to infection. Transcription factor (TF) binding motif enrichment was performed with the PSCAN software program (54) using the JASPAR database and promoter definition from −450 to +50 nucleotides (relative to the transcription start site). PSCAN computes a *z*-test *P* value for each TF, which assesses whether its binding motif is significantly over- or underrepresented in promoters of the query genes. We also used a second complementary method, based on prior knowledge of expected effects between transcriptional regulators and their known target genes according to the IPA database, to predict regulators and infer their activation state. The IPA TF analytic tool calculates a *z* score that determines whether gene expression changes for known targets of each TF are consistent with what is expected from the literature ($z > 0$, TF predicted to be "activated") or if the changes are anticorrelated with the literature ($z < 0$, TF predicted to be "inhibited"). *z* scores greater than 2 or smaller than −2 are considered significant. Using both JASPAR and IPA complementary approaches in our study, we may reduce the likelihood of missing significant enrichment of TFs that might be absent in one database or the other.

Meta-analysis of immune cells. A meta-analysis was performed using publicly available microarray data from Mouse GeneAtlas v3 of major types of immune cells and lung tissue in mice. Raw *CEL* files were downloaded from the NCBI GEO website (GSE10246) (24, 42), preprocessed in the R program using the affy package, and normalized using the robust multiarray analysis (RMA) method (17). For comparison with our data set, Unigene ID was chosen as the common gene identifier. A gene was defined as highly expressed in a cell subset if its expression was 20-fold greater in this subset than in the lung samples (from Mouse GeneAtlas v3). Fisher's exact test was used to determine whether the proportion of highly expressed immune genes in each viral signature was significantly greater than the proportion of immune genes in the background (30,827 probes passing quality control [QC] assessment). Enrichment scores were calculated as $-\log_{10}(P \text{ value})$.

Meta-analysis of other H1N1 influenza A viruses. Data for meta-analysis of the host transcriptional response to other H1N1 viruses was obtained from mouse infection experiments using Mex/4482, Brisbane/59/07, and NJ/8/76, performed as described above. We also used microarray data derived from stored mRNA isolated from lungs of BALB/c mice infected with r1918 virus in the study by Kash et al. (21). For comparison with CA/04 and MA-CA/04 expression profiles, all data were quantile normalized together and the COMBAT algorithm in R was used to correct batch effects (19).

To visualize transcriptional dissimilarity between samples in a 2-di-

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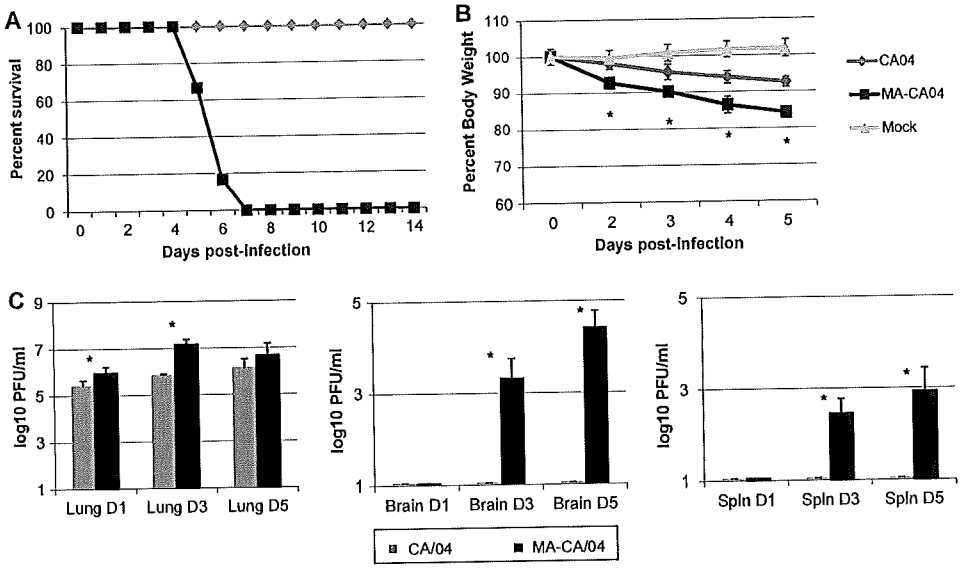

FIG 1 Characterization of mortality, weight loss, and viral titers in MA-CA/04- and CA/04-infected mice. Mice were inoculated with 10^6 PFU of each virus or PBS as a mock control. (A) Mortality data from mice inoculated with influenza virus or mock inoculated (6 mice/group). (B) Average weight of surviving animals at each time point (expressed as mean ± standard deviation) from a total of 18 animals (6 mice/group). (C) Influenza virus titers measured in lungs, brain, and spleen at 1, 3, and 5 day p.i. (3 mice/virus/time point). Differences between MA-CA/04 and CA/04 for weight and titer were determined by a Student *t* test ($P < 0.05$). Statistical significance is denoted with an asterisk.

mensional (2D) space, Euclidian distances were calculated for transcriptomic data expressed in intensity scale, and nonmetric multidimensional scaling (MDS) was performed with the MASS package in the Bioconductor software program (48) to map the distances into the 2-dimensional space with minimal loss of information (evaluated by Kruskal's stress).

Microarray data accession number. Raw microarray data have been deposited in NCBI's Gene Expression Omnibus under GEO Series accession number GSE36328 and are also accessible through the Systems Virology website (http://www.systemsvirology.org).

RESULTS

Mouse adaptation results in increased virulence and lung pathology. BALB/c mice were infected with 10^6 PFU of either CA/04 or MA-CA/04 virus; weight loss, survival, and viral titers were determined at different days p.i. While mice infected with CA/04 lost minimal weight and recovered by day 8 p.i., infection with MA-CA/04 resulted in continuous weight loss and 100% lethality by day 7 p.i. (Fig. 1A and B). This increase in virulence was accompanied by increased replication of MA-CA/04 virus in lungs, as quantified by plaque assay (Fig. 1C). Since extrapulmonary replication was previously reported for MA-CA/04 (16) but not for other 2009 H1N1 mouse-adapted viruses, we verified that under our experimental conditions, MA-CA/04 disseminated to the brain and spleen on days 3 and 5 p.i. but CA/04 did not.

Microscopic lesions were examined by hematoxylin-and-eosin staining of lung tissue sections from infected mice, and viral antigen was detected by immunohistochemistry. Infection with either CA/04 or MA-CA/04 virus produced lesions typical of influenza A infections: bronchiolitis and bronchitis with accompanying necrosis of respiratory epithelium and associated histiocytic alveolitis (Fig. 2; see also Table S1 in the supplemental material). In general, the lesions produced by MA-CA/04 were more severe than the lesions observed with CA/04. At 1 day p.i., both viruses produced mild to moderate bronchiolitis and bronchitis with degenerative changes, including vacuolation, of the respiratory epithelium. Viral staining was commonly present in bronchioles and bronchial respiratory epithelium (Fig. 2B). The MA-CA/04 virus also produced mild histiocytic alveolitis at this time point, and infrequent viral staining was present in alveolar epithelium and macrophages. At 3 days p.i., mild to moderate bronchiolitis and bronchitis with necrosis and loss of the respiratory epithelium, accompanied by mild to moderate peribronchiolar histiocytic to lymphocytic alveolitis, was observed with both viruses (Fig. 2C and D). Alveolar necrosis with congestion and edema was more evident in mice infected with the MA-CA/04 virus. Infrequent viral staining in bronchioles and bronchial respiratory epithelium was seen with both viruses, but viral staining in alveolar epithelium and macrophages was more common in tissues from mice infected with MA-CA/04 (Fig. 2C and D, inserts). At 5 days p.i., mice infected with CA/04 had mild lymphocytic to histiocytic peribronchiolar to diffuse alveolitis, as well as bronchiolitis and bronchitis with necrosis and loss of the respiratory epithelium. In some cases neutrophils were also present (Fig. 2E). Rare to common viral staining was present in alveolar epithelium, in macrophages, and in bronchioles and bronchial respiratory epithelium. At this same time point, mice infected with MA-CA/04 had moderate to severe peribronchiolar to diffuse alveolitis, with histiocytes, lymphocytes and uncommon neutrophils present (Fig. 2F). Moderate to severe bronchiolitis and bronchitis with necrosis and loss of the respiratory epithelium were also observed. Viral staining was infrequent in bronchioles and bronchial respiratory epithelium but common in alveolar epithelium and macrophages.

In summary, increased virulence of MA-CA/04 was associated with more severe lung lesions, more evident infection of alveolar epithelium and macrophages, and indications of a greater number of histiocytes, lymphocytes, and neutrophils infiltrating the lung than with wild-type CA/04 virus.

MA-CA/04 induces a strong host transcriptional response after infection. To characterize the impact of mouse adaptation on

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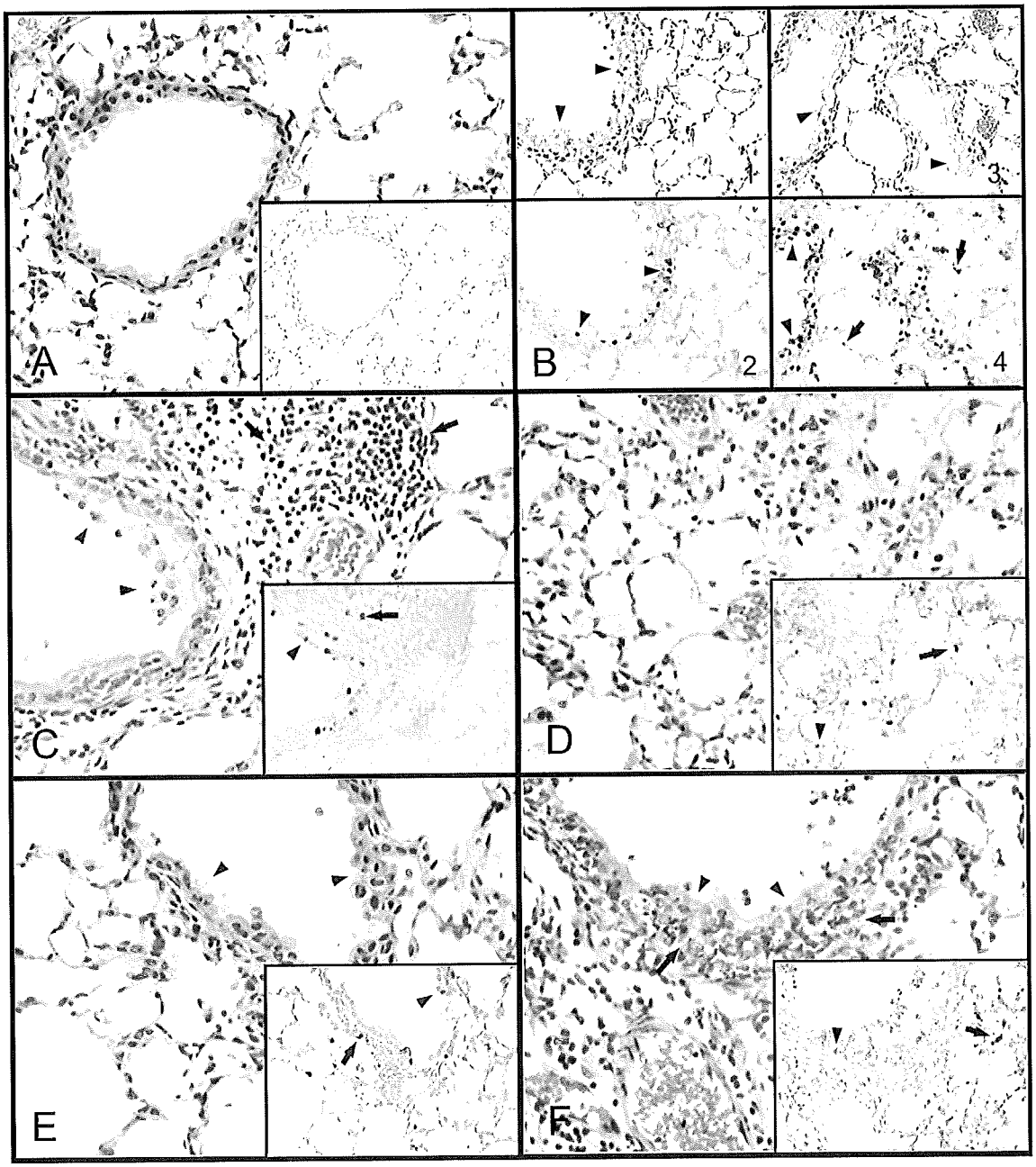

FIG 2 Histopathologic changes and immunostaining of tissues from H1N1 virus-inoculated mice. Photomicrographs of lung tissue sections stained with hematoxylin and eosin and for the presence of viral antigen (×400). (A) Lung tissue from mock-infected mouse; lack of viral antigen staining in lung (insert). (B to E) Lung tissues from mice infected with the H1N1 viruses. (B) Moderate bronchiolitis with vacuolation of the respiratory epithelium (arrowheads) in mice infected with CA/04 (B-1 and B-2) or MA-CA/04 (B-3 and B-4) at 1 day p.i.. Viral antigen staining (in brown) found in cells of the bronchiole respiratory epithelium (arrowheads) (B-2 and -4) and in adjacent alveolar epithelial cells (arrows) (B4). (C) Moderate bronchiolitis with necrosis and loss of the respiratory epithelium (arrowheads) and associated moderate peribronchiolar lymphocytic alveolitis (arrows) in mouse infected with CA/04 at 3 days p.i. Viral antigen (in brown) found in bronchiolar epithelial cells (arrowheads) and macrophages (arrows) (insert). (D) Moderate interstitial pneumonia in mouse infected with MA-CA/04 at 3 days p.i. Viral antigen (in brown) found in alveolar epithelium (arrowheads) and macrophages (arrows) (insert). (E) Mild bronchiolitis (arrowheads) with mild peribronchial alveolitis in mouse infected with CA/04 at 5 days p.i. Viral staining (in brown) in bronchiole respiratory epithelium (arrowheads) and alveolar epithelium and macrophages (arrows) (insert). (F) Severe bronchiolitis (arrowheads) and peribronchiolar alveolitis (arrows), mainly histiocytic but also with lymphocytes and some neutrophils, in mouse infected with MA-CA/04 at 5 days p.i. Viral staining (in brown) found in bronchiole respiratory epithelium (arrowheads), desquamated cells, and alveolar epithelium and macrophages (arrows) (insert).

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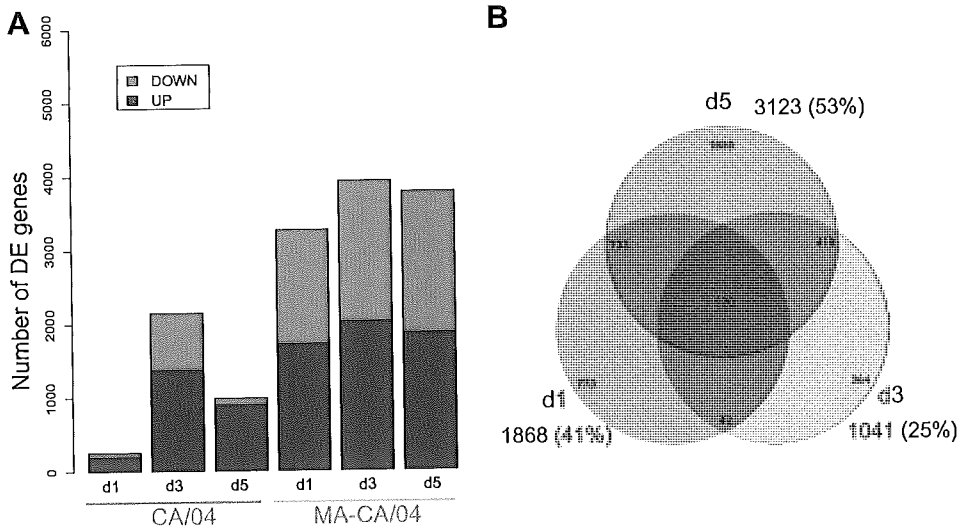

FIG 3 Number of genes differentially expressed (DE) after infection for each virus compared to results for time-matched mocks (A) or overlap between genes differentially regulated between MA-CA/04 and CA/04 at each time point (B). (A) Numbers of upregulated (red) and downregulated (green) DE probes after MA-CA/04 and CA/04 infections on days 1, 3, and 5. DE genes were defined with a Welch *t* test between the time-matched mock and each infected group (q value < 0.05; absolute $\log_2$ ratio > 1). (B) Venn diagram representing relative overlap between genes differentially regulated between MA-CA/04 and CA/04 on day 1 p.i. (blue), day 3 p.i. (gray), and day 5 p.i. (red). The total number of genes differentiating MA-CA/04 and CA/04 infection is indicated for each time point along, with the percentage of those genes unique to the time point. These genes were defined with a Welch *t* test between MA-CA/04 and CA/04 for each time point (q value < 0.05; absolute $\log_2$ ratio [CA/04 to MA-CA/04] >1).

host responses, pulmonary gene expression was compared between time matched mock-infected mice and mice infected with either CA/04 or MA-CA/04 virus at 1, 3, and 5 days p.i. We observed that MA-CA/04 infection resulted in more differentially expressed (DE) genes at each time point than did infection with CA/04 (Fig. 3A). The number of DE genes was greatest on day 3 p.i. for both viruses; however, importantly, transcriptional responses to CA/04 infection decreased dramatically on day 5, whereas for MA-CA/04, the number of DE genes decreased only marginally. Functional analysis of upregulated genes showed that MA-CA/04 infection resulted in a sustained increase in the expression of genes associated with the inflammatory response, immune cell trafficking, cell death, and cellular growth and proliferation (see Fig. S1 in the supplemental material). After CA/04 infection, only 195 transcripts were significantly upregulated on day 1 p.i., and they were not strongly associated with any biological function (see Fig. S1). In contrast, on day 3 p.i., 1,374 genes were upregulated after CA/04 infection, and their functional enrichment profile was very similar to that of genes induced by MA-CA/04 at the same time. At day 5 p.i., lipid and amino acid metabolism processes were upregulated in CA/04-infected mice but not in mice infected with MA-CA/04. Genes downregulated following MA-CA/04 and CA/04 virus infection were enriched to a limited extent in different general biological functions, such as tissue development, genetic disorder, cellular function, and maintenance (see Fig. S1). On day 3 p.i., 60% of the functions associated with downregulated genes after CA/04 or MA-CA/04 infection were in common (see Fig. S1).

The enhanced virulence of MA-CA/04 is therefore characterized by an increased host response, with upregulated genes strongly associated with immune/inflammatory processes across all time points. In contrast, whereas CA/04 infection also induced a strong immune response, the response was limited at day 3, with metabolism functions being induced later in infection.

MA-CA/04 and CA/04 elicit different transcriptional programs. To directly examine differences in the host response to CA/04 and MA-CA/04, we determined the genes that were DE between CA/04 and MA-CA/04 at each day p.i. (Welch *t* test, absolute $\log_2$ ratio > 1 and q value < 0.05). A total of 4,202 unique DE transcripts were identified, with an overlap between time points of 36%. At day 3, only 25% of the transcripts discriminating CA/04 and MA-CA/04 were not DE at the other time points, whereas at days 1 and 5 p.i., there were 41% and 53% unique genes, respectively (Fig. 3B).

Genes that were DE between MA-CA/04 and CA/04 were categorized into three sets based on their general expression patterns (Fig. 4; see also Data Set S1 in the supplemental material). Genes in set 1 (upregulated lethal signature; 2,107 genes) were more highly expressed during MA-CA/04 infection at at least one time point, and many of these genes remained highly expressed throughout infection. These genes were only transiently upregulated, and to a lesser extent, on day 3 p.i. in CA/04-infected animals. Genes in set 2 (survival/recovery signature; 968 genes) were upregulated in CA/04-infected animals at at least one time point while unchanged or downregulated during MA-CA/04 infection. The largest differential within this group was found on day 5 p.i. Finally, genes in set 3 (downregulated lethal signature; 1,126 genes) were more downregulated in MA-CA/04-infected mice at at least one time point and showed a trend opposite from that of genes in set 1. Many of the genes in set 3 were highly downregulated early after MA-CA/04 infection and remained so throughout infection, while they were only transiently downregulated, and to a lesser extent, on day 3 p.i. in CA/04-infected animals.

Genes highly induced after MA-CA/04 infection are associated with the immune response and enriched with interferon regulatory factor (IRF) binding motifs. Functional analysis was performed using IPA to determine the canonical pathways and biological functions (Table 1) that were most significantly associ-

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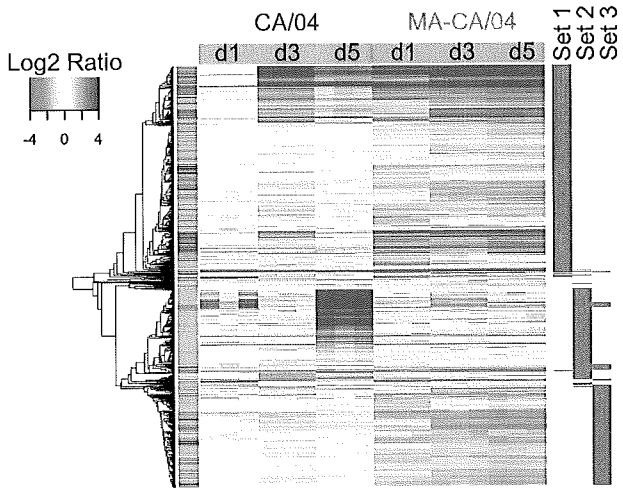

FIG 4 Genes DE between MA-CA/04 and CA/04 infection formed 3 main transcriptional programs. The heat map represents expression levels (in $\log_2$ ratios) of 4,202 transcripts that were DE between MA-CA/04 and CA/04 infection at at least one time point. Genes were clustered based on their expression values across samples using Pearson correlation and average linkage function. Row colors on the left correspond to the Venn diagram of Fig. 3B and indicate on which day p.i. each gene was found DE between MA-CA/04 and CA/04 infection. Three sets of genes were determined based on gene expression levels: set 1 represents the upregulated lethal signature (2,107 probes), set 2 is the survival/recovery signature (968 probes), and set 3 is the downregulated lethal signature (1,126 probes). There was a minimal overlap between each set. Only 2 genes (*Ear5* and *Chi3l4*) were found more downregulated for CA/04 than for MA-CA/04 infection at at least one time point, and set 4 is therefore not depicted.

ated with differences in gene expression between the two viruses (Fig. 4). Set 1 was strongly enriched in pathways related to innate immunity and relationships between innate and adaptive immunity (Table 1). Approximately 14% of the transcripts (291 probes) in set 1 encoded factors involved in immune cell trafficking (Table 1; see also Data Set S1 in the supplemental material), including a large number of CCL and CXCL cytokines and their receptors. We also noted an increase in the expression of genes coding for inflammatory molecules, such as the classical proinflammatory cytokines IL-1b, IL-6, and TNF-α, and molecules involved in tissue destruction and cell death, including matrix metallopeptidases, those encoded by FAS, TNF, and the TNF receptor, TNFRSF1B, and several receptors of Fc fragments. We also compared the list of genes in set 1 with the list of genes that were DE following intranasal treatment of mice with alpha/beta interferon (IFN-α/β) (see Data Set S2) (9). Among the 2,107 transcripts in set 1, 39% (825 probes) were thus defined as IFN-regulated genes (Fisher's exact P value $< 2.2e-16$), suggesting that a large part of the set 1 transcriptional program was driven by an upregulation of the innate immune response.

To identify potential transcriptional regulators of the genes within set 1, we used PSCAN to analyze the promoter regions of these genes for binding motifs of 130 transcription factors (TFs) (Table 2; see also Data Set S3 in the supplemental material). We found a strong enrichment for IRF1 and -2 binding sites, as well as NF-κB and STAT3. IRF3 and NF-κB have been previously described as being highly activated during pathogenic influenza virus infection of cell lines (36, 49), and STAT TFs are extensively involved in the antiviral response *in vivo* (26). It may be interesting to note that among ISGF3 components (STAT1-STAT2-IRF9), only STAT1 is present in PSCAN. STAT1 binding motif was enriched for set 1 (P value $= 3.6e-4$) (see Data Set S3) but not for the other sets. The sequence of the ISGF3 binding site partially overlaps the IRF binding element, and we found significant enrichment for IRF binding element in set 1. This may suggest that ISGF3 also plays a role in regulating the transcriptional program associated with lethality. Besides, since IRF1 and -2 were the only

TABLE 1 IPA canonical pathways and biological functions enriched in each transcriptional program discriminating MA-CA/04 and CA/04[a]

Canonical pathway	Canonical Pathway q value	Category	Function annotation	Function q value
Set 1: upregulated genes associated with lethality				
Communication between innate and adaptive immune cells	3.E−17	Inflammatory response	Immune response	2.E−80
Role of pattern recognition receptors in recognition of bacteria and viruses	5.E−13	Cell-to-cell signaling and interaction	Activation of cells	3.E−49
Dendritic cell maturation	3.E−12	Immune cell trafficking	Leukocyte migration	6.E−47
Role of hypercytokinemia/hyperchemokinemia in the pathogenesis of influenza	3.E−12	Cell-to-cell signaling and interaction	Activation of blood cells	8.E−46
Cross talk between dendritic cells and natural killer cells	4.E−11	Inflammatory response	Inflammatory response	4.E−45
Set 2: transcriptional program associated with survival/recovery				
FXR/RXR activation	2.E−28	Lipid metabolism	Metabolism of terpenoid	4.E−30
Metabolism of xenobiotics by cytochrome P450	4.E−28	Lipid metabolism	Steroid metabolism	2.E−27
LPS/IL-1-mediated inhibition of RXR function	4.E−27	Lipid metabolism	Fatty acid metabolism	7.E−25
LXR/RXR activation	1.E−25	Amino acid metabolism	Metabolism of amino acids	1.E−21
Fatty acid metabolism	1.E−21	Lipid metabolism	Transport of lipid	7.E−21
Set 3: downregulated genes associated with lethality				
LXR/RXR activation	4.E−02	Cardiovascular disease	Venous thrombosis	4.E−03
Metabolism of xenobiotics by cytochrome P450	4.E−02	Cancer	Metastatic colorectal cancer	7.E−03
Glycolysis/gluconeogenesis	4.E−02	Lipid metabolism	Release of cholesterol	8.E−03
		Lipid metabolism	Transport of lipid	1.E−02
		Lipid metabolism	Oxidation of lipid	1.E−02

[a] The top-ranked significant pathways and functions are represented (cutoff for significance, $q < 0.05$).

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TABLE 2 TF binding motifs enriched in each transcriptional program discriminating MA-CA/04 and CA/04[a]

TF	*P* value (PSCAN)
Set 1: upregulated genes associated with lethality	
NF-κB	1.38E−18
NFKB1	1.27E−15
IRF2	2.82E−15
IRF1	1.24E−14
RELA	2.19E−12
MZF1_1-4	8.38E−12
Set 2: transcriptional programs associated with recovery	
HNF4A	9.34E−46
NR2F1	6.05E−31
HNF1A	2.05E−24
NR1H2::RXRA	1.42E−21
HNF1B	4.90E−21
PPARG::RXRA	8.20E−17
Set 3: downregulated genes associated with lethality	
ZEB1	4.73E−08
INSM1	1.08E−07
SP1	1.57E−07
Myf	1.34E−06
PLAG1	1.24E−05
PPARG::RXRA	2.86E−05

[a] Results for all 130 TFs analyzed in PSCAN are provided in Data Set S3 in the supplemental material.

IRFs in the JASPAR database (see Data Set S3), and since IRFs bind similar DNA motifs (though with some specificities [38]), it is likely that other IRFs could also control the expression of genes that were upregulated after MA-CA/04 infection. Other novel TFs potentially regulating the set 1 transcriptional response based on binding motif enrichment (Table 2; see also Data Set S3) were myeloid zinc finger protein 1 (MZF-1) and SPI1 (also known as PU-1), TFs that are both involved in myeloid cell development.

Together, these results suggest that MA-CA/04 may trigger a greater activation of pattern recognition receptors (PRRs) resulting in IRF and NF-κB activation, leading subsequently to a high IFN and proinflammatory response, mediated by STAT TFs. This cytokine environment could affect the functions of infiltrating innate (dendritic cells and macrophages) and adaptive immune cells and also be responsible for lung lesions. In contrast, in CA/04 virus-infected animals, this activation was more limited and restrained to day 3 p.i.

Survival is associated with increased expression of lipid metabolism genes and genes enriched with nuclear receptor binding sites. Survival of infection with CA/04 was associated with the induction of 968 genes that were significantly more upregulated in CA/04-infected mice than in MA-CA/04 infected mice (Fig. 4). Functional analysis of these genes found that 268 transcripts (28%) encoded molecules involved in lipid metabolism (see Data Set S1 in the supplemental material), including several ATP-binding cassette (ABC) transporters, apolipoprotein, diverse enzymes involved in fatty acid metabolism, vitamin D, arachidonic acid, lipid synthesis and uptake, several solute carrier family members (SLC), cytochrome P450 members, and surfactant proteins (SP-D and A1). Induction of these genes in CA/04-infected mice could be involved in viral clearance, as with SP-D (25), and in limiting the inflammatory response, as with CYP2C and -2J synthesizing anti-inflammatory metabolites (44), and enzymes involved in arachidonic acid metabolism. In addition, functional analysis showed a significant enrichment in genes related to amino acid metabolism. The upregulation of amino acid and lipid metabolism genes may be required to build cellular components essential for recovery from lung tissue damage.

To find potential regulators of the survival/recovery signature, we looked for TF binding motifs in promoters of genes from set 2. The top-ranked TFs were nuclear receptors acting in a heterodimer with RXR (NR1H2::RXRA and PPARG::RXRA) or as a homodimer (NR2F1 and several hepatocyte nuclear factors). These results were consistent with canonical pathway analysis, because three out of the five most enriched pathways involved RXR and one pathway (FXR/RXR activation) involved hepatocyte nuclear factor TFs (Table 1). Overall, these data indicate that nuclear receptors may be involved in lipid and amino acid metabolism induction potentially required for recovery from influenza infection.

Lethal infection is associated with suppression of coagulation genes. The last set of genes DE between MA-CA/04 and CA/04 included genes that were more downregulated in MA-CA/04 mice at at least one time point (Fig. 4). Most of these genes were consistently more downregulated throughout infection with MA-CA/04 while being unchanged in CA/04-infected animals, but a small fraction were upregulated in these animals on days 1 and 5 p.i. and therefore also belonged to set 2. Overall enrichment scores were less significant for this set than for the other two sets (Table 1). However, we found a significant representation of lipid metabolism and coagulation-related genes in this set (Table 1; see also Data Set S1 in the supplemental material). Lipid enrichment was only partially explained by overlap with set 2, since several genes belonging only to set 3 were also associated with lipid metabolism. Procoagulant (*F2, fibrinogen*) and anticoagulant (*Serpin C1 = Antithrombin III*) factors, as well as fibrinolytic (*Plasminogen*) and antifibrinolytic (*Serpin F2*) factors, were more downregulated in MA-CA/04 mice on day 3 p.i. On day 5 p.i., several anticoagulants (encoded by *Protein C, Serpin D1, C1,* and *A1*) and profibrinolytic factors (encoded by *Plasminogen, F12* and *Kallikrein B*) were more upregulated in CA/04-infected than in MA-CA/04-infected mice (see Data Set S1) and could limit fibrin deposition involved in acute lung injury.

Transcription factor analysis using PSCAN found significant enrichment for PPARG::RXRA, which could therefore also be implicated in downregulation of set 3 genes, as well as several transcriptional repressors (ZEB1, INSM1, SP1, and PLAG1). In conclusion, MA-CA/04 infection induced a consistent downregulation of lipid metabolism and coagulation-associated genes, which could be due to regulation of PPARG and/or transcriptional repressors.

Prediction of TF activation state supports IRF and nuclear receptor regulation of transcription. Because we found several TF binding motifs that were highly enriched in each set of genes discriminating CA/04 and MA-CA/04, we were interested in determining their activation state following infection. For this purpose, we used the IPA TF analytic tool, which can predict the activation of TFs based on the expression levels of their known targets. We performed this analysis using $\log_2$ ratio expression values for the 4,203 genes discriminating CA/04- and MA-CA/04-infected animals. Figure 5A represents *z* scores for 16 TFs that were found to be very likely ($|z| > 4$) to be involved in the tran-

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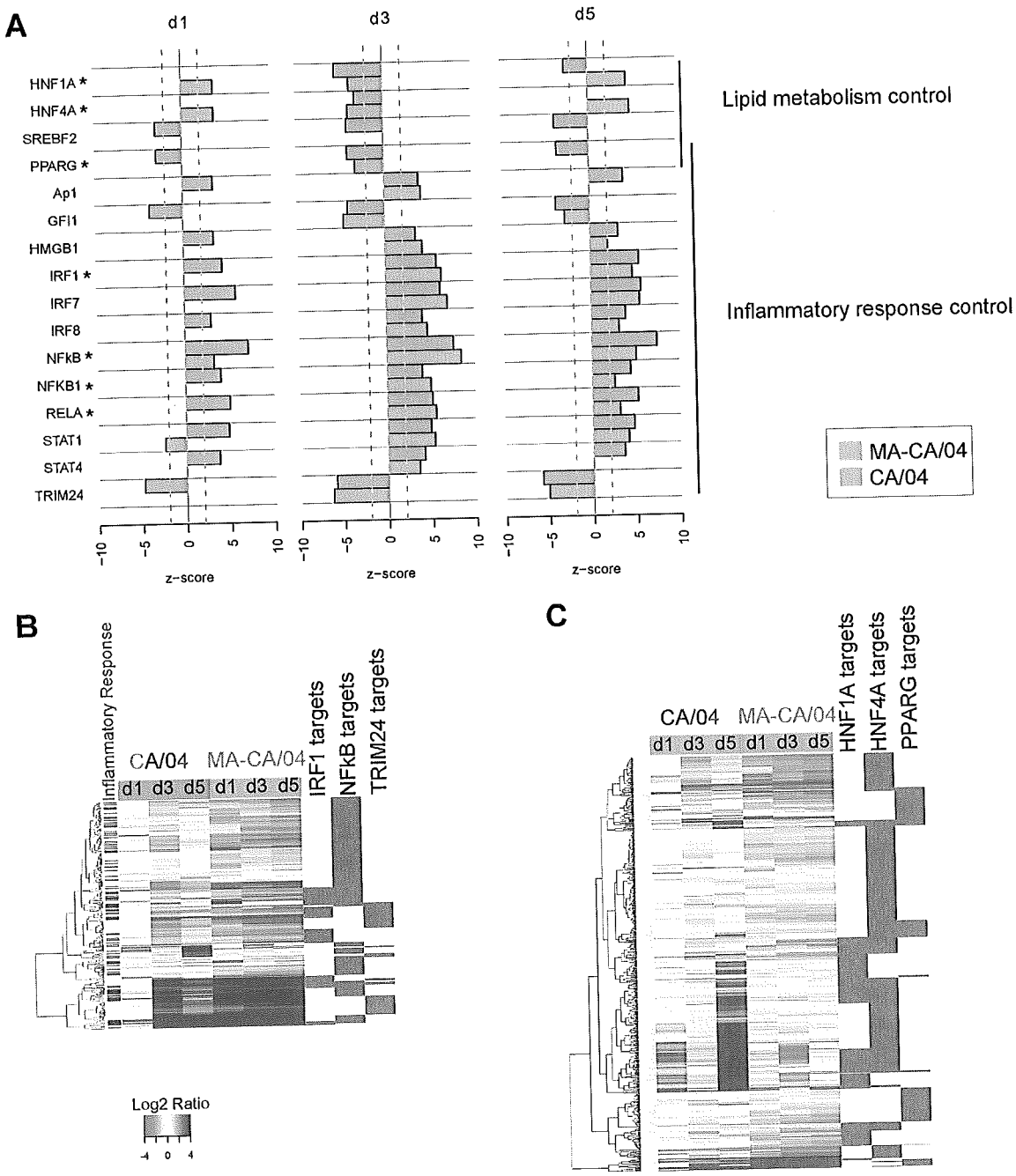

FIG 5 TF activation state prediction in MA-CA/04- and CA/04-infected mice. (A) z scores predicting activation state of TFs ($|z| > 4$) based on expression values of genes discriminating CA/04 and MA-CA/04. Dashed lines depict $|z| = 2$, which is considered to be the limit of significance. $z > 2$ predicts an activation of the regulator. $z < -2$ predicts an inhibition. "*" denotes TFs that were found among the top 5 ranked TFs in PSCAN analysis (Table 2). (B) Expression levels (in $\log_2$ ratio) for 353 targets of the 3 most significant TFs controlling inflammatory response. Genes that belong to the "inflammatory response" category in IPA are indicated in purple. (C) Expression levels (in $\log_2$ ratio) for 597 targets of the 3 most significant TFs controlling lipid metabolism. Genes that belong to the "lipid metabolism" category in IPA are indicated in yellow.

scriptional response to infection at at least one time point and in one group. Based on the annotation of their target genes, we found that these TFs were involved either in lipid metabolism control or in the inflammatory response.

Binding motifs for several TFs likely to be involved in inflammatory response control were also found in our previous PSCAN analysis to be present in promoters of genes from the upregulated lethal signature (Table 2; see also Data Set S3 in the supplemental material). These TFs were predicted to be activated throughout infection with MA-CA/04 virus, while in CA/04-infected mice they would be activated only by day 3 p.i., with a possible decrease in their activation on day 5 p.i. (Fig. 5A). Consistently, most of

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their target genes belonged to the upregulated lethal signature (set 1) and were more expressed in MA-CA/04-infected mice than in CA/04-infected mice (Fig. 5B). Two additional IRFs (IRF7 and -8) were also predicted to be more activated in MA-CA/04-infected mice, which supports the general implication of IRFs in controlling the transcriptional program associated with lethality. Additionally, two negative regulators, GFI1 and TRIM24, were found to be constantly inactivated in MA-CA/04 samples, and we verified that their targets were also more upregulated in MA-CA/04-infected mice (Fig. 5B).

Among the four TFs that could control lipid metabolism during infection, three were previously found in PSCAN (HNF1A, HNF4A, and PPARG). The hepatocyte nuclear factors (HNFs) were predicted to be activated in CA/04-infected lungs on days 1 and 5 p.i., while they would be repressed or unchanged at the same time points in MA-CA/04-infected animals (Fig. 5A). Consistently, most of their target genes were more expressed in CA/04 animals than in MA-CA/04 animals on days 1 and 5 p.i. (Fig. 5C). PPARG was another nuclear receptor found by the two complementary TF analyses. It was predicted to be inhibited throughout infection with MA-CA/04 virus, while not with infection with CA/04, on days 1 and 5 p.i. (Fig. 5A). Most of its targets were more downregulated in MA-CA/04 samples and were associated with lipid metabolism. Notably, the small subset of PPARG targets that were found to be more upregulated in MA-CA/04-infected mice are genes that are known to be repressed by PPARG, including those encoding several proinflammatory cytokines (*IL-1*, *IL-6*, *IFNG*, and *TNF*) and several chemokines (*CXCL-1*, *CXCL-3*, *CXCL-6*, and *CXCL-14* and *CCL-13*, *CCL-17*, and *CCL-22*). This was consistent with an inhibition of PPARG activity in MA-CA/04-infected animals and indicates that this regulation could be involved in both the absence of lipid metabolism upregulation and increased inflammatory response observed in MA-CA/04-infected animals.

These results overall support the implication of several nuclear receptors (HNF1A, HNF4A, and PPARG), IRFs, and the NF-κB complex in the differential host response to MA-CA/04 or CA/04 infection. We also found novel transcription regulators not present in the JASPAR database, notably TRIM24, whose repression could play a role in increasing the inflammation in MA-CA/04 virus-infected animals.

Genes associated with inflammatory macrophages and granulocytes are highly induced during MA-CA/04 infection. Since several pathways differentially induced between MA-CA/04 and CA/04 were related to specific immune cells or included chemoattractants, we hypothesized that observed transcriptional differences elicited by MA-CA/04 and CA/04 infection could be partially due to differences in immune cell populations and/or their activation state in the lungs. To further explore this possibility, we compared our gene expression profiles to microarray data for each immune cell population present in the GNF Mouse Gene Atlas v3 database (42). We defined, for each immune cell population present in the database, a list of genes that were expressed 20-fold more in a given cell subset than in whole lung. We then analyzed whether there was significant enrichment in genes that were more upregulated in response to infection with either MA-CA/04 (Fig. 6, orange lines) or CA/04 (Fig. 6, blue lines). We could therefore evaluate whether differences in gene expression profiles between MA-CA/04 and CA/04 infection were associated with immune cell infiltration.

The list of genes that exhibited no change in expression after CA/04 infection but which were strongly induced by MA-CA/04 at days 1 and 5 p.i. was highly enriched in genes associated with lipopolysaccharide (LPS)-stimulated macrophages (from both bone marrow-derived macrophages [BMM] and thioglycolate-elicited peritoneal macrophages [TEPM]) (Fig. 6A). LPS was used as an *in vitro* stimulus in the Mouse Gene Atlas study to trigger proinflammatory pathways activating macrophages (24). Higher infiltration of inflammatory macrophages in MA-CA/04-infected lungs may therefore contribute to the transcriptional program associated with lethality. Similarly, analysis of the other immune cell-associated genes (Fig. 6B) suggested a higher infiltration of NK cells at day 1 p.i. and of granulocytes and mast cells for all time points after MA-CA/04 infection. In contrast, on day 3 p.i., there were fewer genes more highly expressed in MA-CA/04-infected mice, and there were fewer associated with macrophages, suggesting a similar proportion of macrophages in CA/04- and MA-CA/04-infected lungs on day 3 p.i. Macrophage infiltration after CA/04 infection may therefore be transient, while such infiltration is sustained in MA-CA/04-infected animals.

Closer examination of enrichment scores at day 3 p.i. revealed that genes highly induced by CA/04 included genes that were highly expressed in unstimulated bone marrow-derived macrophages (Fig. 6A, "BMM_0h," enrichment score of 11.1,), B cells, and FoxP3$^+$ T cells (Fig. 6B). However, most of the genes from the transcriptional program associated with survival/recovery (set 2) that were highly expressed on day 5 p.i. with CA/04 infection were not associated with immune cells (Fig. 6).

Overall, these results suggest that macrophages present in lungs after infection with MA-CA/04 or CA/04 could be functionally different, with noninflammatory macrophages contributing to the gene expression response to CA/04, while inflammatory macrophage, granulocyte, and mast cell infiltration contributes to the transcriptional response measured in lung samples from MA-CA/04-infected animals.

Transcriptional programs associated with lethality and recovery in the context of other H1N1 influenza virus infections. We next examined whether the transcriptional signatures associated with lethality and recovery that were derived from the comparison of CA/04- and MA-CA/04-infected mice corresponded to the expression patterns elicited by other lethal and nonlethal H1N1 influenza viruses. Several nonlethal H1N1 viruses were selected for comparison. These included Mex/4482 and NJ/8/76, both of which were isolated from patients with severe respiratory illness, and Brisbane/59/07, a seasonal H1N1 virus. Intranasal infection by these viruses in BALB/c mice resulted in a range of virulence, in agreement with a previous report of H1N1 murine virulence (5). Brisbane/59/07 and NJ/8/76 caused minimal weight loss (see Fig. S2A in the supplemental material) and were classified as low pathogenicity. Mex/4482 caused significant weight loss, which was intermediate between those of CA/04 and MA-CA/04 infection (Student's *t* test, $P < 0.05$ from days 4 and 2 p.i., respectively) (see Fig. S2A). Viral titers in the lungs were much lower in Brisbane/59/07-infected animals than in all other groups ($P < 0.05$), while titers were similar in mice infected with Mex/4482, NJ/8/76, or CA/04 (see Fig. S2B). Stored RNA isolated from the lungs of mice infected with the reconstructed 1918 pandemic influenza virus (r1918) was used as an additional lethal H1N1 comparator (see Materials and Methods). As described previously, mice infected with r1918 had 75% weight loss at day 4 p.i., and

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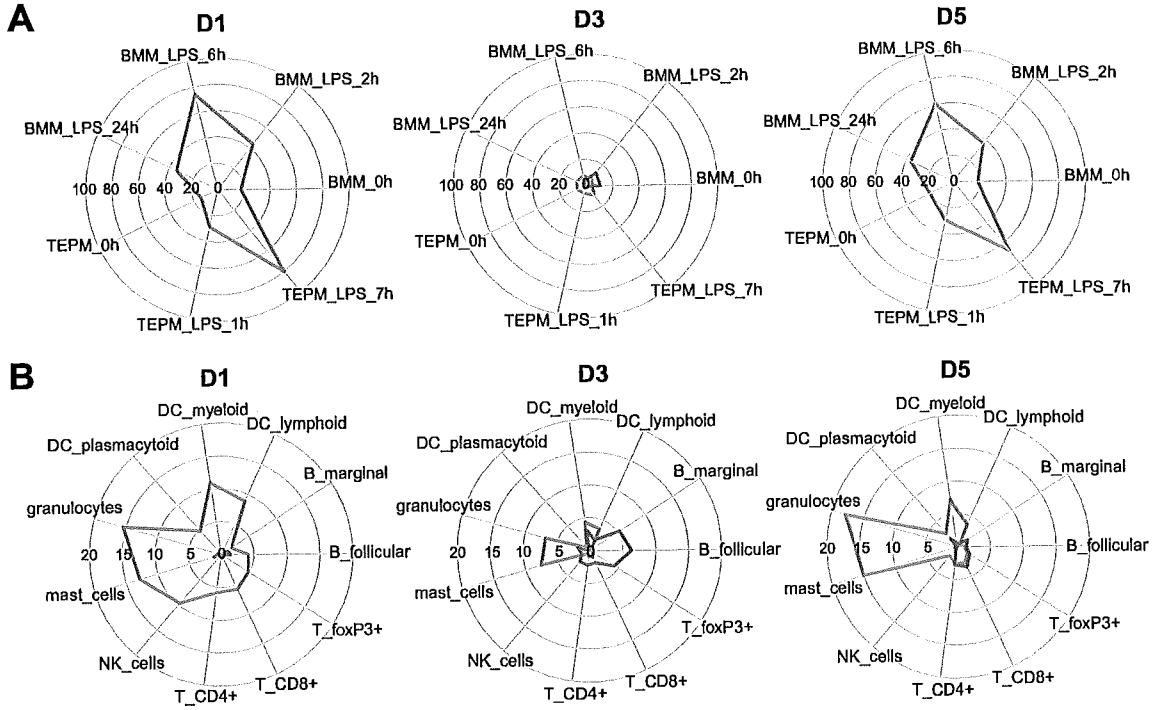

FIG 6 Enrichment in immune cell highly expressed genes for genes that were differentially changed between MA-CA/04 and CA/04 infection. Genes specific to each immune cell population were determined using the Gene Atlas v3 data set to be genes that were 20-fold more increased in each immune population than for whole lung tissue. Genes upregulated in MA-CA/04 infection are shown in orange, and genes upregulated in CA/04 infection are shown by blue lines. On each day p.i., we calculated the proportion of immune cell-related genes in 2 lists of genes DE between MA-CA/04 and CA/04 infection: (i) genes with a $\log_2$ ratio in MA-CA/04 infection > that in CA/04 infection > 0 (orange); (ii) genes with a $\log_2$ ratio in CA/04 infection > that in MA-CA/04 infection > 0 (blue). Enrichment scores (ES) were calculated as a $-\log_{10} P$ value, using a right-tailed Fisher exact test. Because of differences of scale between ES in macrophages and in other immune cells, they were depicted in 2 different radial plots for each day p.i. (A) ES for bone marrow-derived macrophages (BMM) and thioglycolate-elicited peritoneal macrophages (TEPM). (B) ES for other immune cells: lymphoid ($CD8a^+$) dendritic cells (DC), myeloid ($CD8a^-$) DC, plasmacytoid ($B220^+$) DC, B cells from follicular and marginal zones, different T cell populations ($CD4^+$, $CD8^+$, and $FoxP3^+$), natural killer (NK) cells, mast cells, and granulocytes ($mac1^+$ $gr1^+$).

there was 100% lethality by day 6 p.i. (21). Viral titers in the spleen and brain of animals infected with these lethal and nonlethal viruses indicated that none of these viruses disseminated systemically in this mouse model.

To determine whether genes discriminating the transcriptional response to CA/04 and MA-CA/04 on each day p.i. were also able to discriminate low-pathogenicity H1N1 (Brisbane/59/07 and NJ/8/76) from the intermediate-pathogenicity Mex/4482 and from the high-pathogenicity r1918, we used nonparametric multidimensional scaling (MDS) to visualize global gene expression concordance between all samples and for each signature (Fig. 7A). Kruskal's stress was used to measure the information loss during dimensionality reduction. We had very small stress values (<5%), indicating a good fit of the MDS representations. On day 1 p.i., and using the genes that were DE between mice infected with CA/04 and those infected with MA-CA/04 on that time point (1,868 genes), MA-CA/04- and r1918-infected samples were located in the same 2D space, indicating that these genes were expressed at similar levels for both lethal viruses. Low and medium pathogens were clustered closer to the mock inoculation. This confirmed that, as with CA/04, less pathogenic viruses induced limited changes in the expression of these genes on day 1 p.i., compared to results for the highly pathogenic viruses MA-CA/04 and r1918.

Likewise, MDS analysis showed that genes DE between mice infected with CA/04 or MA-CA/04 on day 3 p.i. (1,041 genes) also differentiated highly pathogenic (r1918 and MA-CA/04) from less pathogenic viruses (CA/04, Brisbane/59/07, and NJ/8/76). Interestingly, Mex/4482, which induced significant weight loss at this time point, elicited a host response similar to that for the lethal viruses on day 3 p.i. Finally, on day 5 p.i., we observed three different clusters of samples based on expression of 3,123 genes DE between mice infected with MA-CA/04 or CA/04. Mice infected with MA-CA/04 or r1918 expressed these genes similarly, since Mex/4482 elicited a response intermediate between those for low- and high-pathogenicity viruses, and a third cluster was formed by the less pathogenic viruses, with results for Brisbane/59/07 and NJ/8/76 being more similar to those for the mock samples than those for CA/04. These results indicate that genes DE between mice infected with CA/04 and MA-CA/04 discriminate different levels of pathogenicity of other H1N1 viruses.

We next compared these DE gene signatures across viruses by examining the distribution and correlation of gene expression across groups (Fig. 7B). We observed that the upregulated lethal signature was expressed to similar levels between MA-CA/04 and r1918 (Pearson correlation, $r = 0.81$), with a higher median of expression than that for the other viruses on each day p.i. In addition, these genes were overall slightly more expressed in re-

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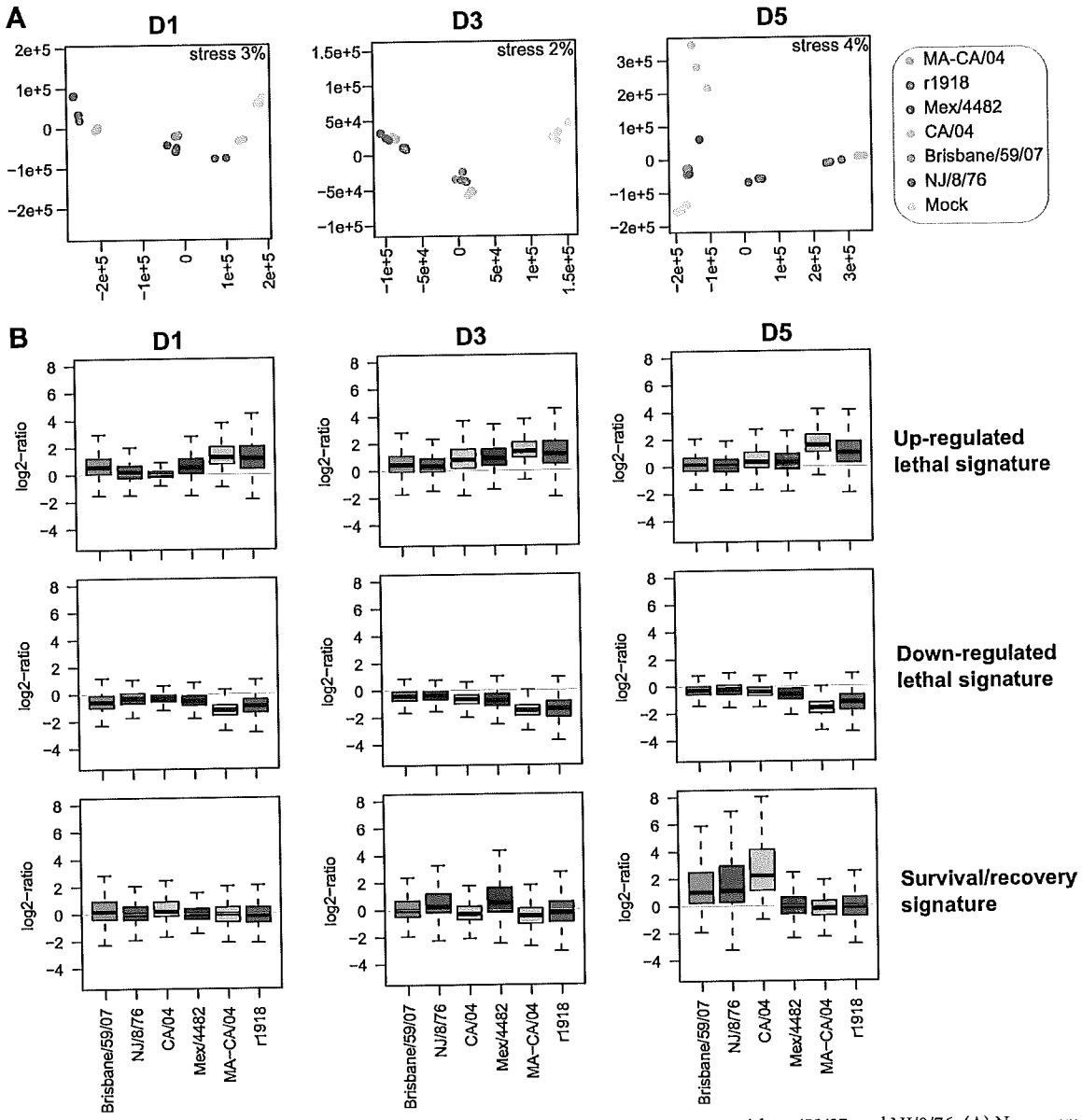

FIG 7 Comparison of MA-CA/04 and CA/04 transcriptional profiles with those of r1918, Mex/4482, Brisbane/59/07, and NJ/8/76. (A) Nonparametric multidimensional scaling (MDS) representing the Euclidian distances between samples on each day p.i. The matrix distance was calculated using genes that were found DE between MA-CA/04 and CA/04 on each day p.i. Kruskal's stress evaluates the loss of information in the representation. (B) Box plot of each transcriptional signature discriminating MA-CA/04 and CA/04 for days 1, 3, and 5 p.i. data.

sponse to Mex/4482 than to CA/04. Downregulated genes from the lethal transcriptional signatures were also more downregulated by the lethal viruses on each day p.i. (Pearson correlation between MA-CA/04 and r1918, $r = 0.51$). Finally, the recovery signature on day 5 p.i. was upregulated only by less pathogenic viruses, with the highest upregulation for CA/04-infected mice but good correlation with results for Brisbane/59/07 ($r = 0.89$) and NJ/8/76 ($r = 0.9$). The intermediate-pathogenicity virus, Mex/4482, which induced weight loss continuously until day 8 p.i., did not upregulate the recovery-associated genes on day 5 p.i., confirming that these genes were associated with recovery and that their expression might be delayed during Mex/4482 virus infection. This analysis confirmed the general relevance of the transcriptional programs associated with lethality and survival/recovery for H1N1 pathogenicity.

Overall, using global gene expression profiling, we found that the increased virulence of the mouse-adapted CA/04 virus was associated with higher and earlier expression of inflammatory and immune cell-related genes following activation of IRF, NB-κB, and STAT TFs. Comparison of our data to metadata collected in other studies from multiple immune cell populations suggested that these differences may be due in part to increases in inflammatory macrophage infiltration in the lungs of mice infected with MA-CA/04. Lethality was also associated with the lack of upregulation of lipid and amino acid metabolism genes, which were upregulated in CA/04-infected mice. Transcription factor analysis

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suggested that this absence of signaling during lethal infection may be mediated in part by nuclear receptors. Finally, comparison of the gene expression patterns associated with lethality and recovery suggests similar patterns in the expression of these genes between CA/04 and other nonlethal H1N1 influenza viruses and between the lethal viruses MA-CA/04 and r1918.

DISCUSSION

The 2009 pandemic H1N1 virus requires only a few mutations following limited passage in mice to increase its virulence extraordinarily in this animal model (16, 34, 39, 53, 56). As 2009 H1N1 continued circulating in humans in 2010-2011 and in the current influenza season, concern remains about its pathogenic potential. The mouse-adapted virus therefore provides an important opportunity to identify host response correlates to a more virulent form of the 2009 H1N1 virus.

In the current study, we compared the host response to the wild-type prototypic 2009 H1N1 viral CA/04 and its mouse-adapted variant, MA-CA/04. These strains have only 5 amino acid differences, but while the wild-type strain was a mild pathogen in BALB/c mice, MA-CA/04 induced 100% lethality by day 7. Histopathological examination revealed differences in lung lesions associated with lethality. Additionally, for MA-CA/04-infected mice, some virus was detected in brain and spleen at later time points of infection. CA/04 and MA-CA/04 viruses both lack viral determinants that were previously reported to be responsible for systemic spread (HA polybasic site [13] and lysine at position 627 of PB2 [10]). Moreover, only one mutation in MA-CA/04, HA G155E, has not been found in the other nondisseminating mouse-adapted 2009 H1N1 virus. This specific mutation could be involved in systemic spread, but dissemination could also result from the unique combination of mutations present in MA-CA/04. Extrapulmonary dissemination could also be explained by the high efficiency of viral replication overwhelming the host response, as was shown for H5N1 HK483 (41).

To our knowledge, this is the first use of global gene expression profiling to characterize the host response to a lethal 2009 H1N1 virus. Several transcriptomic studies have compared the host response to 2009 H1N1 viruses and other seasonal H1N1 viruses in macaques (33), mice (22), and pigs (29). These studies described a higher upregulation of genes related to immune and inflammatory responses following infection with pandemic strains compared than with seasonal virus. Our microarray analysis also revealed a strong immune response in CA/04-infected mice at day 3, but this response was delayed and transient compared to that elicited by MA-CA/04. These findings are consistent with those of previous studies (10, 21) showing that early and sustained activation of the inflammatory response is associated with lethal influenza virus infection. In addition to higher expression of inflammatory and immune cell-related genes, lethality following MA-CA/04 infection was also associated with the lack of upregulation of lipid metabolism genes. Genes discriminating MA-CA/04 and CA/04 were differentiating other lethal and nonlethal H1N1 viruses, confirming their general relevance for virulence of influenza virus. Distribution of expression for signatures associated with lethality and recovery was not contingent on differences in virus levels. Therefore, although we cannot exclude the possibility that the level of viral replication impacts pathogenesis and the host response, our data suggest that identified signatures were more related to virulence than viral replication.

Previous studies have shown that immune cell infiltration in the lungs is an important pathological feature of lethal influenza viruses. Patients who died from 2009 H1N1 virus infection showed profound inflammatory infiltration in the lungs and diffuse alveolar damage similar to that seen in past pandemics (11). Enrichment analysis leveraging immune cell transcription information accessed through the Mouse Gene Atlas suggested differences in kinetics and in activation of immune cell subsets infiltrating the lungs of virus-infected mice. While CA/04-induced genes were transiently associated with noninflammatory macrophage and B and T-regulatory cell ($FoxP3^+$) infiltration on day 3 p.i., genes that were more upregulated in MA-CA/04 mice were associated with inflammatory macrophages, granulocytes, and mast cells for all time points. The relevance of these findings is supported by histopathological examination of infected lungs, pointing out histiocyte and lymphocyte infiltration in the peribronchiolar area of CA/04-infected mice from day 3 only. In contrast, these cells were present in the lungs of MA-CA/04-infected mice from day 1 and increased during infection in the peribronchiolar and alveolar compartments.

Infiltrating macrophages and neutrophils are essential to control viral replication at early time points (46), but excessive inflammatory cell recruitment contributes to lung injury (1, 12, 27, 35). Similarly, several studies suggest that CD4 and CD8 T cells also contribute to both influenza virus clearance and immunopathology (reviewed in reference 32). Our results suggest that a transient and strong immune infiltration could be important for the protective response to CA/04, with certain cell types being associated with survival. For example, regulatory T cells could play a role in moderating the inflammatory response on day 3 p.i. in CA/04-infected mice. In contrast, persistent and strong lung infiltration with inflammatory macrophages, granulocytes, and mast cells in response to lethal viruses was associated with hypercytokinemia (Table 1) and was likely to induce airway epithelium destruction (12, 14). Beyond immune cell types, our results suggest that specific signaling pathways activated in infiltrating cells, such as the LPS-stimulated pathway in macrophages, were associated with influenza pathogenesis. It is possible that MA-CA/04 infection of macrophages plays a role in this pathway induction. Indeed, we detected viral antigen more frequently in macrophages for MA-CA/04-infected animals, which suggests a change in viral tropism that could be mediated by HA mutations. In addition, LPS activation and antiviral response result in activation of common signaling pathways, such as the activation of IRF3.

Our data support the role for the IFN regulatory factor (IRF) family in influenza virulence. Binding motifs for IRF1 and IRF2 were found to be enriched in the promoters of genes that were induced more highly by MA-CA/04 than by CA/04. IRF1 was also predicted to be activated early after infection with MA-CA/04 but not CA/04 based on the expression of its target genes. Since the upregulated lethal signature was also strongly associated with immune cells, especially macrophages, it is possible that IRFs were activated in immune cells infiltrating the lung during infection, with possible consequences for their functions (reviewed in reference 2). In particular, IRF1 and IRF8 are involved in macrophage activation and inhibit generation of regulatory T cells, which could have an important impact in influenza pathogenesis. IRFs may be activated in immune and epithelial cells by cytokines secreted during infection and by direct infection (47). A recent *in vitro* study concluded that IRFs were equally involved in the re-

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sponse of primary endothelial cells to infection with low- or high-pathogenicity influenza viruses (49). In contrast, our results suggest that IRFs were more activated in mice infected with lethal viruses, being in part responsible for a sustained inflammatory response and macrophage activation.

In addition to IRFs, sustained activation of NF-κB and STATs was implicated in the inflammatory response following MA-CA/04 infection. These TFs have been described previously as activated in response to highly pathogenic influenza virus infection (26, 36). Since NF-κB and STAT signaling pathways are involved in immune cell activation following different stimuli, their higher activation in the lungs of mice infected with MA-CA/04 may also reflect a difference in the activation state of infiltrating immune cells. Finally, we report that some TFs, which have not previously been associated with influenza pathogenesis to our knowledge, could potentially regulate the transcriptional program associated with lethal infection. These transcription regulators include MZF1, whose binding motif was found to be enriched in these genes (Table 2), and TRIM24 and GFI1, whose regulation was predicted to be significant based on the expression of their known targets (Fig. 5A). TRIM24 and GFI1 are two transcriptional repressors that were predicted to be inhibited during MA-CA/04 infection. Interestingly, GFI1 suppression in macrophages results in an exaggerated production of inflammatory cytokines following LPS stimulation (20). In addition, TRIM24 knockout (KO) mice have an overactivation of the IFN/STAT pathway (45). Inhibition of both TRIM24 and GFI1 could therefore have important inflammatory consequences in MA-CA/04-infected mice.

Transcription factors involved in the survival/transcriptional program associated with recovery include members of the nuclear receptor ligand-dependent family. HNF1A and -B, HNF4A, and PPARG were predicted to be activated in CA/04-infected mice and/or repressed in MA-CA/04-infected mice based on expression values of their target genes. To our knowledge, this is the first report associating HNF TFs with influenza virus pathogenesis. HNF1A and -4A are two major liver TFs that control diverse metabolic functions. These TFs are also expressed to a lesser extent in pathological or normal lung (3, 23) and were predicted to be activated in CA/04-infected mice on days 1 and 5 p.i. and either unchanged or repressed for HNF1A in MA-CA/04-infected animals. Known targets of HNF1A and -4A include many genes involved in lipid and amino acid metabolism, as well as several blood coagulation factors (15, 40). Pulmonary and systemic activation of coagulation has been implicated in a fatal human case of 2009 H1N1 infection (11) and in lethal H1N1 influenza virus infection in mice (37). We found different changes in coagulation factor synthesis in the lungs of mice infected with MA-CA/04 or CA/04 viruses. Absence of fibrinolytic and anticoagulant factor induction on day 5, in combination with plasma protein leakage and induction of tissue factor and plasminogen activator inhibitor (PAI)-1 by activated macrophages (52), could contribute to intra-alveolar fibrin deposition in the lungs of mice infected with lethal influenza viruses. In addition to coagulation, activation of lipid and amino acid metabolism may be required to build cellular membrane components for cell proliferation during recovery from influenza virus infection. Similar to our results, microarray analysis of lungs from pigs infected with CA/04 also showed that recovery from infection is associated with an overall increase in lipid metabolism at day 5 (29). The NS1 segment of r1918 has been previously implicated in lipid metabolism and CYP repression in cell culture (7). However, there was no difference in NS1 sequence between MA-CA/04 and CA/04, suggesting that other viral properties could be implicated in this regulation.

Our results suggest that lipid metabolism inhibition after MA-CA/04 infection could also involve PPARG repression. This nuclear receptor is active as a heterodimer with retinoid X receptor (RXR). Repression of PPARG activity could involve suppression of RXRα-dependent gene expression due to Jun N-terminal protein kinase (JNK) phosphorylation, itself activated by tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) signaling (57), or RXRα repression mediated by IRF3 in hepatocytes and BMMs (8). Known targets of PPARG that were DE between MA-CA/04 and CA/04 infection were associated with lipid metabolism and the inflammatory response. This TF has been extensively studied for its role in bridging lipid metabolism and inflammation in several diseases, including atherosclerosis (reviewed in references 6 and 43). Considering their functions in transrepressing inflammatory TF target genes, such as those encoding NF-κB, NFAT, STAT, and AP-1, PPARG activation could be crucial to limiting inflammation in CA/04-infected mice. Besides, PPARG activation potentiates polarization of circulating monocytes into macrophages of the M2 phenotype, which generate anti-inflammatory products and mediate tissue repair. This suggests that macrophages present in CA/04-infected lung may have a protective M2 phenotype, compared to the sustained infiltration with inflammatory M1 macrophages in MA-CA/04-infected mice. Based on their anti-inflammatory role, PPARG agonists have been used as immune modulators, providing protection in mice infected with highly pathogenic and pandemic strains of influenza virus (1, 31). We provide for the first time evidence that PPARG, together with other nuclear receptors, is involved in inducing genes associated with recovery from influenza virus infection.

In conclusion, the virulence of CA/04 was dramatically increased following host adaptation. Enhanced virulence was associated with an increased and sustained inflammatory response, immune cell infiltration, and no increase in lipid metabolism later in infection. Our results suggest that regulation of several key transcription factors may be involved in these processes, including induction of IRF1 and -2 and lack of activation of HNF4A, PPARG, and LXR-β. These TFs may also regulate the function of infiltrating immune cells, including macrophages. Identifying specific genes involved in deleterious infiltrating cell subsets may point to new therapies to prevent severe disease associated with influenza virus infection.

ACKNOWLEDGMENTS

We thank Richard Webby of St. Jude Children's Research Hospital for use of the MA1-CA/04 virus. We also thank Lynn Law, Sean Proll, and Marcus Korth for valuable feedback on the manuscript.

This project was funded in part by federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract HHSN272200800060C (to M.G.K.).

The findings and conclusions in this report are those of the authors and do not necessarily reflect the views of the funding agency.

REFERENCES

1. Aldridge JR, Jr, et al. 2009. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. Proc. Natl. Acad. Sci. U. S. A. 106:5306–5311.

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2. Battistini A. 2009. Interferon regulatory factors in hematopoietic cell differentiation and immune regulation. J. Interferon Cytokine Res. 29: 765–780.
3. Baumhueter S, Courtois G, Crabtree GR. 1988. A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the beta fibrinogen gene promoter as HNF-1. EMBO J. 7:2485–2493.
4. Belisle SE, et al. 2010. Genomic profiling of tumor necrosis factor alpha (TNF-alpha) receptor and interleukin-1 receptor knockout mice reveals a link between TNF-alpha signaling and increased severity of 1918 pandemic influenza virus infection. J. Virol. 84:12576–12588.
5. Belser JA, et al. 2010. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. J. Virol. 84: 4194–4203.
6. Bensinger SJ, Tontonoz P. 2008. Integration of metabolism and inflammation by lipid-activated nuclear receptors. Nature 454:470–477.
7. Billharz R, et al. 2009. The NS1 protein of the 1918 pandemic influenza virus blocks host interferon and lipid metabolism pathways. J. Virol. 83: 10557–10570.
8. Chow EK, et al. 2006. A role for IRF3-dependent RXRalpha repression in hepatotoxicity associated with viral infections. J. Exp. Med. 203:2589–2602.
9. Cilloniz C, et al. 2012. Molecular signatures associated with Mx1-mediated resistance to highly pathogenic influenza virus infection: mechanisms of survival. J. Virol. 86:2437–2446.
10. Fornek JL, et al. 2009. A single-amino-acid substitution in a polymerase protein of an H5N1 influenza virus is associated with systemic infection and impaired T-cell activation in mice. J. Virol. 83:11102–11115.
11. Gill JR, et al. 2010. Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections. Arch. Pathol. Lab. Med. 134:235–243.
12. Herold S, et al. 2008. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. J. Exp. Med. 205:3065–3077.
13. Horimoto T, Kawaoka Y. 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68:3120–3128.
14. Hu Y, et al. 2012. Mast cell-induced lung injury in mice infected with H5N1 influenza virus. J. Virol. 86:3347–3356.
15. Hwang-Verslues WW, Sladek FM. 2010. HNF4alpha—role in drug metabolism and potential drug target? Curr. Opin. Pharmacol. 10:698–705.
16. Ilyushina NA, et al. 2010. Adaptation of pandemic H1N1 influenza viruses in mice. J. Virol. 84:8607–8616.
17. Irizarry RA, et al. 2003. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31:e15.
18. Itoh Y, et al. 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460:1021–1025.
19. Johnson WE, Li C, Rabinovic A. 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8:118–127.
20. Karsunky H, et al. 2002. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nat. Genet. 30:295–300.
21. Kash JC, et al. 2006. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature 443:578–581.
22. Kash JC, et al. 2011. Lethal synergism of 2009 pandemic H1N1 influenza virus and Streptococcus pneumoniae coinfection is associated with loss of murine lung repair responses. mBio 2(5):e00172–11. doi:10.1128/mBio.00172-11.
23. Kunii R, et al. 2011. The predominant expression of hepatocyte nuclear factor 4alpha (HNF4alpha) in thyroid transcription factor-1 (TTF-1)-negative pulmonary adenocarcinoma. Histopathology 58:467–476.
24. Lattin JE, et al. 2008. Expression analysis of G protein-coupled receptors in mouse macrophages. Immunome Res. 4:5. doi:10.1186/1745-7580-4-5.
25. LeVine AM, Whitsett JA, Hartshorn KL, Cronch EC, Korfhagen TR. 2001. Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. J. Immunol. 167:5868–5873.
26. Levy DE, Garcia-Sastre A. 2001. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. Cytokine Growth Factor Rev. 12:143–156.
27. Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD. 2008. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. J. Immunol. 180:2562–2572.
28. Lopez-Romero P. 2012. Agi4x44PreProcess: PreProcessing of Agilent 4x44 array data. R package version 1.8.0. ed.
29. Ma W, et al. 2011. 2009 pandemic H1N1 influenza virus causes disease and upregulation of genes related to inflammatory and immune responses, cell death, and lipid metabolism in pigs. J. Virol. 85:11626–11637.
30. Maines TR, et al. 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science 325:484–487.
31. Moseley CE, Webster RG, Aldridge JR. 2010. Peroxisome proliferator-activated receptor and AMP-activated protein kinase agonists protect against lethal influenza virus challenge in mice. Influenza Other Respi. Viruses 4:307–311.
32. Peiris JS, Hui KP, Yen HL. 2010. Host response to influenza virus: protection versus immunopathology. Curr. Opin. Immunol. 22:475–481.
33. Safronetz D, et al. 2011. Pandemic swine-origin H1N1 influenza A virus isolates show heterogeneous virulence in macaques. J. Virol. 85:1214–1223.
34. Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. 2011. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. Virus Res. 158:124–129.
35. Sakai S, et al. 2000. Therapeutic effect of anti-macrophage inflammatory protein 2 antibody on influenza virus-induced pneumonia in mice. J. Virol. 74:2472–2476.
36. Schmolke M, Viemann D, Roth J, Ludwig S. 2009. Essential impact of NF-kappaB signaling on the H5N1 influenza A virus-induced transcriptome. J. Immunol. 183:5180–5189.
37. Schouten M, et al. 2010. Activated protein C ameliorates coagulopathy but does not influence outcome in lethal H1N1 influenza: a controlled laboratory study. Crit. Care 14:R65. doi:10.1186/cc8964.
38. Servant MJ, Tenoever B, Lin R. 2002. Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. J. Interferon Cytokine Res. 22:49–58.
39. Seyer R, et al. 2012. Synergistic adaptive mutations in the hemagglutinin and polymerase acidic protein lead to increased virulence of pandemic 2009 H1N1 influenza A virus in mice. J. Infect. Dis. 205:262–271.
40. Shih DQ, et al. 2001. Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism. Nat. Genet. 27:375–382.
41. Shinya K, et al. 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320:258–266.
42. Su AI, et al. 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc. Natl. Acad. Sci. U. S. A. 101:6062–6067.
43. Szanto A, Roszer T. 2008. Nuclear receptors in macrophages: a link between metabolism and inflammation. FEBS Lett. 582:106–116.
44. Theken KN, et al. 2011. Activation of the acute inflammatory response alters cytochrome P450 expression and eicosanoid metabolism. Drug Metab. Dispos. 39:22–29.
45. Tisserand J, et al. 2011. Tripartite motif 24 (Trim24/Tif1alpha) tumor suppressor protein is a novel negative regulator of interferon (IFN)/signal transducers and activators of transcription (STAT) signaling pathway acting through retinoic acid receptor alpha (Raralpha) inhibition. J. Biol. Chem. 286:33369–33379.
46. Tumpey TM, et al. 2005. Pathogenicity of influenza virnses with genes from the 1918 pandemic virns: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J. Virol. 79:14933–14944.
47. Veckman V, et al. 2006. TNF-alpha and IFN-alpha enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells. Virology 345:96–104.
48. Venables WN, Ripley BD (ed). 2002. Modern applied statistics with S, 4th ed. Springer, Berlin, Germany.
49. Viemann D, et al. 2011. H5N1 virus activates signaling pathways in human endothelial cells resulting in a specific imbalanced inflammatory response. J. Immunol. 186:164–173.
50. World Health Organization. 2010. Pandemic (H1N1) 2009, update 112. World Health Organization, Geneva, Switzerland.
51. World Health Organization. 2011. Review of the 2011 winter influenza season, southern hemisphere. Wkly. Epidemiol. Rec. 86:480–496.
52. Wygrecka M, et al. 2007. Cellular origin of pro-coagulant and (anti)-

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fibrinolytic factors in bleomycin-injured lungs. Eur. Respir. J. 29:1105–1114.
53. Ye J, et al. 2010. Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype? PLoS Pathog. 6:e1001145. doi:10.1371/journal.ppat.1001145.
54. Zambelli F, Pesole G, Pavesi G. 2009. Pscan: finding over-represented transcription factor binding site motifs in sequences from co-regulated or co-expressed genes. Nucleic Acids Res. 37:W247–W252.
55. Zeng H, et al. 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type i interferon response in polarized human bronchial epithelial cells. J. Virol. 81:12439–12449.
56. Zhou B, et al. 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza A viruses in mice. J. Virol. 85:357–365.
57. Zimmerman TL, Thevananther S, Ghose R, Burns AR, Karpen SJ. 2006. Nuclear export of retinoid X receptor alpha in response to interleukin-1beta-mediated cell signaling: roles for JNK and SER260. J. Biol. Chem. 281:15434–15440.

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Journal of Virology

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***J. Virol.* 2012, 86(5):2437. DOI: 10.1128/JVI.06156-11.**
Published Ahead of Print 21 December 2011.

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Molecular Signatures Associated with Mx1-Mediated Resistance to Highly Pathogenic Influenza Virus Infection: Mechanisms of Survival

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Understanding the role of host factors during lethal influenza virus infection is critical to deciphering the events that determine the fate of the host. One such factor is encoded by the Mx1 gene, which confers resistance to influenza virus infection. Here, we compared pathology and global gene expression profiles in lung tissue from BALB/c ($Mx1^{-}$) and BALB · A2G-Mx1 mice ($Mx1^{+/+}$) infected with the fully reconstructed 1918 pandemic influenza virus. $Mx1^{+/+}$ mice showed less tissue damage than Mx^{-} animals, and pathology and mortality were further reduced by treating the mice with interferon prior to infection. Using global transcriptional profiling, we identified distinct molecular signatures associated with partial protection, complete protection, and the contribution of interferon to the host response. In the absence of interferon treatment, partial protection was characterized by the generation of an acute response with the upregulation of genes associated with apoptosis, reactive oxygen species, and cell migration. Complete protection was characterized by the downregulation of cytokine and chemokine genes previously associated with influenza virus pathogenesis. The contribution of interferon treatment to total protection in virus-infected $Mx1^{+/+}$ mice was characterized by the altered regulation of cell cycle genes. These genes were upregulated in $Mx1^{+/+}$ mice treated with interferon but downregulated in the absence of interferon treatment. Our results suggest that $Mx1^{+/+}$ mice generate a protective antiviral response by controlling the expression of key modulator molecules associated with influenza virus lethality.

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 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 (11). In addition, highly pathogenic avian H5N1 influenza viruses continue to circulate in diverse parts of the world (21, 37), 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 in $Mx1^{+/+}$ mice with the goal of gaining insights into the underlying protective mechanisms that make these animals resistant to lethal influenza virus infection. The expression of the Mx1 gene is under tight transcriptional control of type I and III interferon (26) and codes for a nuclear 72-kDa protein with antiviral properties that controls influenza A virus infection in mice (9, 30).

A study comparing the virulence of influenza A virus in a mouse model of infection (6) found that an unusual influenza virus strain was virulent in $Mx1^{+/+}$ mice. Interestingly, this virus was well controlled by $Mx1^{+/+}$ mice treated with interferon before infection, suggesting that rapid virus growth can outcompete the antiviral response. The results from an independent study using mouse infection models suggest that the Mx1 gene protects mice against highly lethal influenza viruses (33). In that study, Mx1 was reported to be a key component of the innate immune system that mediates protection against both zoonotic and human pandemic strains of influenza viruses of high virulence. Another study using a mouse infection model and *in vitro* experiments showed that the origin of the viral NP gene determines Mx sensitivity and that human influenza viruses acquired adaptive mutations to evade MxA restriction (40).

In the present study, we examined the host response to the 1918 virus in wild-type BALB/c and $Mx1^{+/+}$ mice; additional findings were obtained from animals treated with interferon before infection. The $Mx1^{+/+}$ mice were partially protected from lethal 1918 virus infection; however, complete protection was achieved only upon interferon treatment before infection. In contrast, untreated and interferon-treated BALB/c mice succumbed to 1918 virus infection. Global gene expression profiling data revealed distinct molecular signatures associated with partial protection (untreated mice), complete protection (interferon-treated $Mx1^{+/+}$ mice), and the specific contribution of interferon to complete protection of $Mx1^{+/+}$ mice. In summary, these findings suggest that mice exhibiting a fully functional Mx1 phenotype are able to generate a strong antiviral state by downregulating key modulator molecules associated with influenza virus lethality.

Received 26 August 2011 Accepted 9 December 2011
Published ahead of print 21 December 2011
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doi:10.1128/JVI.06156-11

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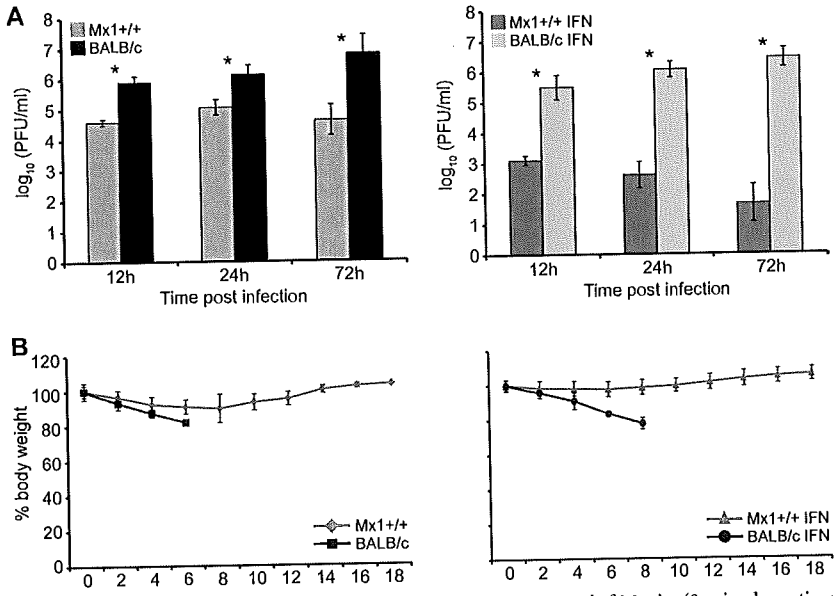

FIG 1 Mx1 competent mice control lethal influenza virus infection. (A) Lung virus titer data from a total of 36 mice (3 animals per time point/treatment/mouse strain). The limit of detection of the assay was $10^{0.95}$ PFU/ml. (B) Morbidity data from a total of 36 mice (9 mice per group). The error bars indicate standard deviations.

MATERIALS AND METHODS

Viruses. The reconstructed 1918 H1N1 virus (32), possessing the A/South Carolina/1/18 hemagglutinin (HA), was previously shown to be highly virulent for both mice and ferrets (15, 31, 32). 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 (31). 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 virus was grown as previously described (22). 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 the CDC's Institutional Animal Care and Use Committee and in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. BALB/c mice carrying a defective allele of the Mx1 resistance gene (29) or congenic BALB · A2G-Mx1 mice carrying the functional Mx1 allele (28) (kindly provided by Peter Staeheli, University of Freiburg, Freiburg, Germany), were anesthetized by intraperitoneal injection of 0.2 ml of 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Sigma-Aldrich, Milwaukee, WI). One hundred times the 50% lethal dose (LD_{50}), 3.2×10^5 PFU of the reconstructed 1918 virus in 50 μl of infectious virus diluted in phosphate-buffered saline (PBS), was inoculated intranasally. LD_{50} titers were calculated by the method of Reed and Muench (25).

The replication of the reconstructed 1918 virus in mice (3 animals/group/time point) was examined by determining the virus titers in the lung (12, 24, and 72 h), brain, and spleen (24 and 72 h) following intranasal inoculation of virus. The clarified homogenates were titrated for virus infectivity in eggs from an initial dilution of 1:10 (lung). Additional inoculated mice (6 animals/group, making a total of 24 animals) were followed for morbidity and mortality. In addition, half of the animals were intranasally treated with 10,000 units of recombinant human alpha interferon (IFN-α) A/D (R&D Systems, Minneapolis, MN) before infection with the reconstructed 1918 virus and euthanized 12, 24, and 72 h post-treatment.

Histopathology. Collected lung sections were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were cut to 5 μm, stained with hematoxylin and eosin (HE), and examined microscopically for histopathological alterations.

RNA preparation and oligonucleotide microarray processing. For RNA isolation, lungs (3 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 (2). RNA was extracted from mouse lungs using a Qiagen RNeasy Minikit with RNA Later solution (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was further purified using RNeasy columns (Qiagen, Valencia, CA). RNA samples were spectroscopically verified for purity, and the quality of the intact RNA was assessed using an Agilent 2100 Bioanalyzer. For each set of treatment conditions, three of the five RNA samples exhibiting the highest RNA integrity number (RIN) determined using the Bioanalyzer were used for microarray analysis. cRNA probes were generated from each sample by the use of an Agilent one-color LowInput Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA). Individual cRNA samples were hybridized to Agilent mouse whole-genome oligonucleotide 4 by 44 microarrays (approximately 39,000 unique mouse genes) according to the manufacturer's instructions. Samples from individual animals were not pooled to enable examination of animal-to-animal variation as part of the data analysis: they included three animals per time point for each virus (60 animals in total). Select samples were hybridized a second time ($n = 2$ technical replicates) to verify the quality of the process.

Slides were scanned with an Agilent DNA microarray scanner, and the resulting images were analyzed using Agilent Feature Extractor version 8.1.1.1. This software was used to perform image analysis, including the significance of signal and spatial detrending and to apply a universal error model. For these hybridizations, the most conservative error model was applied. Quality control (QC) reports generated from Agilent Feature Extraction software were used to assess data quality for each microarray

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and to identify outliers. The raw data were then loaded into a custom-designed laboratory information management system.

Microarray analysis and bioinformatics. Global gene expression in infected lungs was analyzed using GeneData Expressionist, Analyst Module (version 2.2.1); Spotfire DecisionSite for Functional Genomics version 9; and Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Inc., Redwood City, CA). Raw intensity data from all microarrays were normalized by applying the central tendency algorithm with the standard reference set at the 75th percentile of each microarray. One- or two-way analysis of variance (ANOVA) was carried out for various groups of samples stratified by mouse strains and/or time points (see Results for more grouping details). Fixed-effect models were built to identify gene signatures associated with protection phenotypes against 1918 virus infection (see Fig. 3, 5, and 7). The Benjamini-Hochberg algorithm was used to control the false discovery rate (FDR) of multiple testing (1). Primary data are available (http://viromics.washington.edu) in accordance with proposed Minimum Information about a Microarray Experiment (MIAME) standards. Functional and network analysis of statistically significant gene expression changes was performed using IPA. The Benjamini-Hochberg algorithm was applied to correct for multiple testing in determining the probability that each biological function assigned to the genes within each statistical analysis result was due to chance alone.

RESULTS

Mx1$^{+/+}$ mice partially control 1918 virus infection. Previous studies by our group and others (6, 27, 33) have shown that knock-in mice carrying a fully functional Mx1 gene are resistant to lethal influenza virus infection; however, those studies did not investigate the host response elicited under such experimental conditions. In the present study, our main goal was to understand the contribution of the elicited host response to survival. To achieve this goal, we performed global gene expression analyses on lung tissue from mice infected with the fully reconstructed 1918 virus.

Our initial experiments showed that Mx1$^{+/+}$ mice were able to reduce the growth of the 1918 virus by 1 log unit at 12 and 24 h postinfection (p.i.) with a reduction of 2 log units by 72 h p.i. compared to the virus growth observed in Mx1^{-} mice (Fig. 1A, left). Administration of interferon to the animals prior to infection resulted in an even greater decrease of 1918 virus growth in Mx1$^{+/+}$ mice, with 2.5-, 3.5-, and 5-log-unit reductions at 12, 24, and 72 h p.i., respectively. However, there was no significant change in virus growth in BALB/c (Mx1^{-}) mice (Fig. 1A, right); these observations were in agreement with previous studies (33). Although there was a large amount of virus detected in the lungs of infected animals, especially in BALB/c mice, we did not detect viral dissemination to the brain or spleen (data not shown). However, morbidity data showed dramatic differences between the two mouse strains. While BALB/c mice were highly susceptible to 1918 virus infection, as shown by the rapid and steady decrease in body weight, interferon-treated Mx1$^{+/+}$ mice were insensitive to 1918 infection (as measured by weight loss) throughout the experiment. In the absence of interferon treatment, an initial slight decline followed by a recovery in body weight in Mx1$^{+/+}$ mice may be observed as a characteristic of interferon treatment (Fig. 1B).

1918 virus infection generates less tissue damage in Mx1$^{+/+}$ mice. In the absence of interferon treatment, infection with the 1918 virus resulted in lung and airway lesions typical of influenza A virus infection in both Mx1$^{+/+}$ and BALB/c mice. The lesions varied depending on the individual animals and the area of the lung examined, but in general, the lesions in BALB/c mice were more severe than the lesions observed in the Mx1$^{+/+}$ mice (Fig. 2).

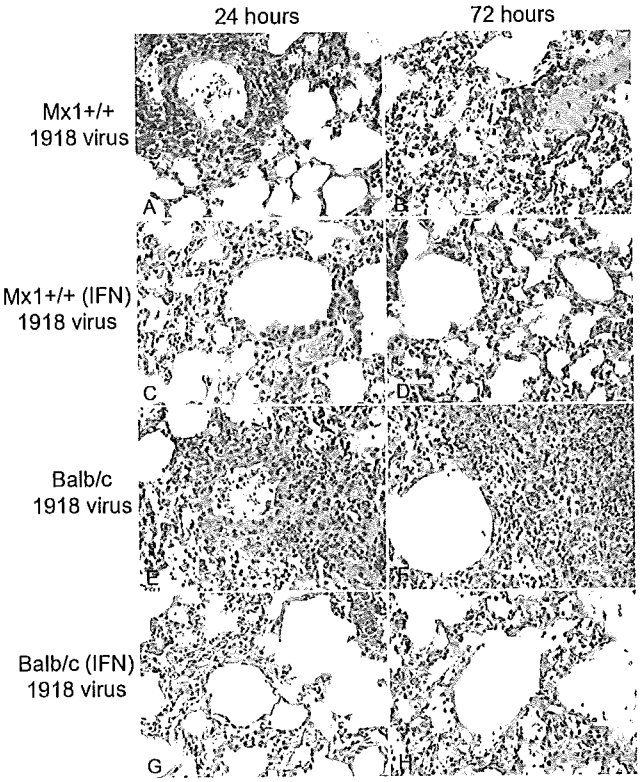

FIG 2 Histopathological changes in tissues from 1918 virus-infected mice. Shown are photomicrographs of lung tissue sections stained with hematoxylin and eosin. (A) Severe necrotizing bronchiolitis and associated peribronchiolar lymphocytic inflammation and congestion in a 1918 virus-infected Mx1$^{+/+}$ mouse at 24 h p.i. (B) Severe histiocytic-to-lymphocytic alveolitis with some neutrophils, congestion, and edema in a 1918 virus-infected Mx1$^{+/+}$ mouse at 72 h p.i. (C) Loss of bronchiolar epithelium with minimal peribronchiolar inflammation and moderate histiocytic alveolitis in an interferon-treated Mx1$^{+/+}$ mouse at 24 h p.i. (D) Moderate histiocytic alveolitis with congestion of the alveolar epithelium in an interferon-treated Mx1$^{+/+}$ mouse at 72 h p.i. (E) Severe necrotizing bronchiolitis and associated peribronchiolar histiocytic inflammation and congestion in a 1918 virus-infected BALB/c mouse at 24 h p.i. (F) Severe diffuse histiocytic alveolitis with presence of lymphocytes, neutrophils, and plasma cells in a 1918 virus-infected BALB/c mouse 72 h p.i. (G) Moderate histiocytic alveolitis with loss of the bronchial epithelium and congestion in an interferon-treated 1918 virus-infected BALB/c mouse 24 h p.i. (H) Alveolar and bronchial epithelial necrosis with edema and congestion in an interferon-treated 1918 virus-infected BALB/c mouse 72 h p.i.

At 24 h p.i., BALB/c mice exhibited moderate to severe necrotizing bronchiolitis and bronchitis. The bronchiolar walls showed vacuolation and loss of epithelium and were thickened by the presence of edema fluid, fibrin, neutrophils, eosinophils, and mononuclear cells. There was perivascular and peribronchiolar infiltration of macrophages, lymphocytes, and plasma cells. At this time point, Mx1$^{+/+}$ mice exhibited only mild diffuse histiocytic alveolitis with congestion and edema, whereas BALB/c mice had moderate interstitial pneumonia. At 72 h p.i., loss of the bronchial and bronchiolar epithelium was evident, accompanied by severe perivascular and peribronchiolar inflammation, mostly composed of macrophages, but also lymphocytes, plasma cells, and neutrophils. Mild to severe diffuse interstitial pneumonia was present, with thickening of the alveolar walls; presence of inflammatory cells; congestion; and areas of alveolar necrosis, atelectasia, hemorrhage, or consolidation.

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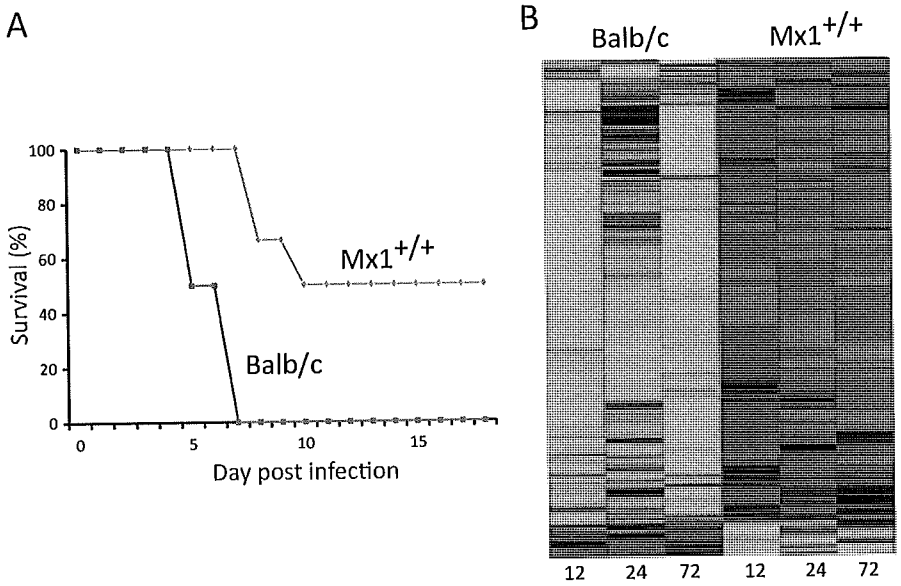

C

Biological function	P-value
Cellular movement	7.89E-02 – 2.2E-01
Cellular growth and proliferation	7.89E-02 – 2.2E-01
Cellular development	7.89E-02 – 2.33E-01
Cell cycle	2.2E-02 – 2.2E-01
Cell death	2.2E-02 – 2.34E-01
Cell morphology	2.2E-02 – 2.33E-01
Amino acid metabolism	2.2E-01 – 2.2E-01
Cellular assembly and organization	2.2E-01 – 2.2E-01
DNA replication, recombination and repair	2.2E-01 – 2.2E-01
Small molecule biochemistry	2.2E-01 – 2.2E01

FIG 3 $Mx1^{+/+}$ mice are partially protected against 1918 virus infection. (A) Survival plot of a total of 18 mice (9 animals/mouse strain). (B) Heat map illustrating 547 differentially transcribed genes associated with 50% protection of animals compared to the wild-type animals in the absence of interferon treatment; cutoff values were ≥2-fold change and a *P* value of ≤0.01 (ANOVA), FDR corrected. Blue indicates downregulation. Red indicates upregulation in the activation of genes. (C) Top 10 biological functions assigned to the 547 differentially regulated genes determined by IPA. The *P* values of the functional categories are shown on the right.

The character of the lesions varied in both the virus-infected $Mx1^{+/+}$ mice and BALB/c mice treated with interferon compared to the mice not treated with interferon. At 24 h p.i., lesions to the bronchiolar and bronchial epithelium were moderate in all mice with, in some cases, loss of the respiratory epithelium, but with minimal or no peribronchial or peribronchiolar inflammation. Alveolar lesions were moderate to severe at this time point, with diffuse alveolar necrosis, hemorrhage, and edema. At 72 h p.i., $Mx1^{+/+}$ mice had mild to moderate diffuse interstitial pneumonia with alveolar necrosis, and the inflammatory cells were mostly macrophages. In contrast, the BALB/c mice exhibited more severe pathology, with mild to severe necrotizing bronchiolitis and moderate to severe alveolitis with alveolar necrosis, congestion, and edema.

$Mx1^{+/+}$ mice exhibit increased survival of 1918 virus infection. Our results indicated that when infected with the 1918 virus, $Mx1^{+/+}$ mice had lower viral titers and less severe lung lesions than BALB/c mice. In addition, whereas the 1918 virus was uniformly lethal in BALB/c mice, 50% of the $Mx1^{+/+}$ mice survived infection (Fig. 3A). In order to assess the effects of both genetic background ($MX1^{+/+}$ and BALB/c) and time points (12, 24, and 72 h) on gene expression, two-way ANOVA was performed using genetic backgrounds and time points as two independent variables. We identified 547 genes that were differentially expressed (Fig. 3B). A large proportion of these genes were downregulated in the BALB/c animals, whereas the same genes were strongly upregulated in the $Mx1^{+/+}$ mice. The top 10 biological categories in which these genes are involved are shown in Fig. 3C.

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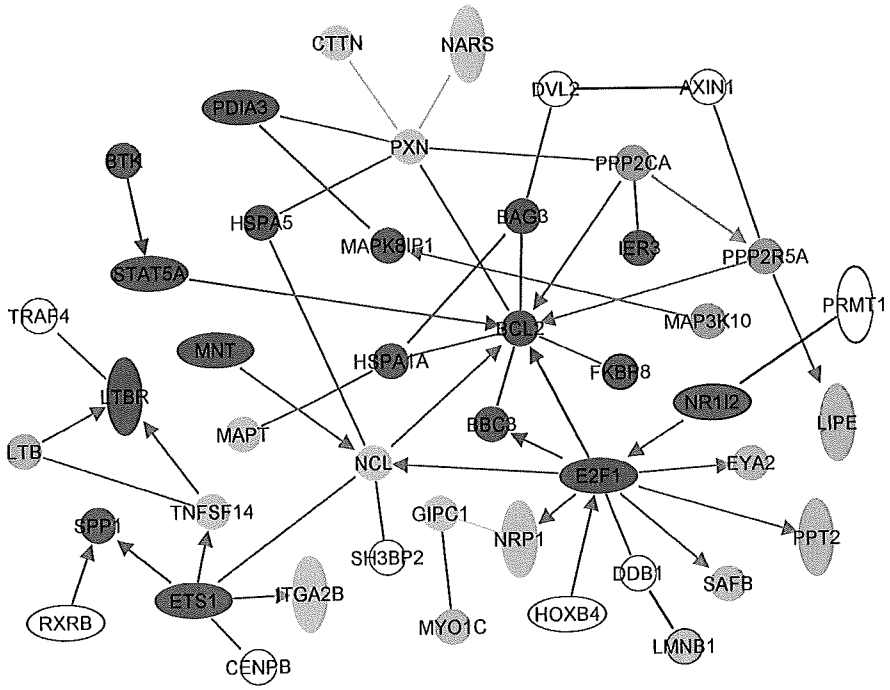

FIG 4 Molecular signature associated with partial protection of $Mx1^{+/+}$ mice. The biological network shows the direct functional relationship of genes associated with partial protection determined by IPA identified from the 547 differentially regulated genes. This network highlights upregulated and downregulated (SPP1 and PPP2R5A only) genes in $Mx1^{+/+}$ mice in the absence of interferon treatment. Purple, apoptosis; orange, cell migration; light blue, connective tissue disorder; white, production of reactive oxygen species.

We used IPA to generate a network of genes functionally associated with survival. As annotated in IPA, the resulting network included genes associated with apoptosis, cell migration, connective tissue disorders, and the production of reactive oxygen species (Fig. 4). All of these genes, with the exception of spp1 and Ppp2r5a, were upregulated in the $Mx1^{+/+}$ mice but were downregulated in BALB/c mice. These results suggest that increased survival of $Mx1^{+/+}$ mice is associated with the upregulation of these pathways.

Interferon-dependent host response associated with complete protection. Previous studies by our group and others (6, 33) showed that pretreatment of $Mx1^{+/+}$ mice with interferon results in increased resistance to influenza virus infection. In the present study, we observed similar results, with all $Mx1^{+/+}$ mice pretreated with interferon surviving a challenge with a high dose of 1918 virus (Fig. 5A). In this context, we wanted to gain insights into the specific host response required for complete protection. Using a two-way ANOVA approach, we identified 2,071 differentially expressed genes between the $Mx1^{+/+}$ and BALB/c interferon-treated mice (Fig. 5B). There was a clear anticorrelation in gene expression between the two mouse strains; however, the $Mx1^{+/+}$ mice showed a strong upregulation of these genes by 12 h p.i. that continued, although to a lesser extent, at later time points. Functional analysis of these genes using IPA showed that the majority of the genes were associated with host physiological functions, such as cell-to-cell signaling and interaction or cellular movement (Fig. 5C). Network analysis of these genes allowed us to identify a set of physically interacting genes that might be responsible for survival (Fig. 6). The most striking feature of this molecular signature was that the $Mx1^{+/+}$ mice were able to downregulate the expression of inflammatory cytokine and chemokine genes, as well as acute-phase genes. These results suggest that complete survival of mice is linked to the downregulation of key modulator molecules. We also note that these changes in gene expression were not due to differences in the response to interferon, since in the absence of infection, the gene expression signatures of interferon-treated $Mx1^{+/+}$ and BALB/c mice were similar (see Table S1 in the supplemental material for a complete list of differentially expressed genes).

Specific contribution of interferon to survival. Because treatment of $Mx1^{+/+}$ mice with interferon prior to 1918 virus infection increased survival from 50% to 100% (Fig. 7A), we next investigated the contribution of interferon to differences in the host transcriptional response to infection. Using two-way ANOVA, we identified 234 differentially expressed genes between these two experimental conditions (Fig. 7B), and the top 10 functional categories assigned to these genes are indicated in Fig. 7C.

Network analysis identified functionally related genes associated with survival. All of the genes depicted in this specific network were related to cell cycle regulation, including cyclins (CCNA1, CCNA2, CCNB1, and CCNB2), cell cycle DNA damage check point regulation (BRCA1, CDK1, CKS2, TOP2A), and cell division cycle-associated genes (CDCA5, CDCA8) (Fig. 8). A large proportion of these genes were upregulated in the interferon-treated animals but were downregulated in the absence of interferon, suggesting that tight control of this specific set of genes involved in cell cycle regulation is important to elicit a response capable of providing complete protection. Although during our previous studies we found that the expression of some cell cycle-related genes was differentially regulated upon influenza virus infection, the extent and functional network of cell cycle genes as-

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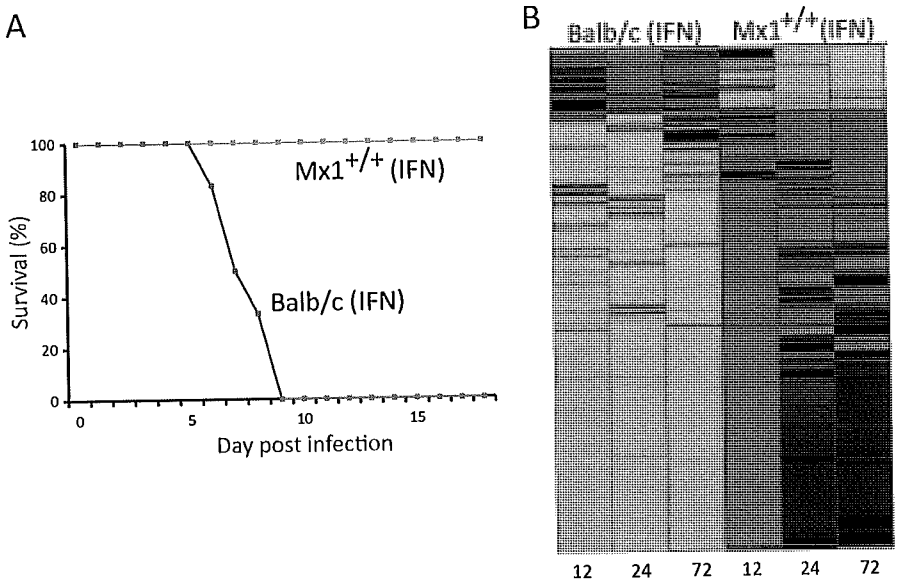

C

Biological function	P-value
Cell-to-cell signaling and interaction	1.91E-06 – 7.17E-02
Cellular movement	1.17E-05 – 7.17E-02
Antigen presentation	3.5E-04 – 7.09E-02
Cellular growth and proliferation	2.90E-03 – 7.17E-02
Cellular development	5.56E-03 – 7.06E-02
Cell signaling	5.79E-03 – 6.09E-02
Molecular transport	5.79E-03 - 6.67E-02
Vitamin and mineral metabolism	5.79E-03 - 6.52E-02
Amino acid metabolism	1.05E-02 – 3.42E-02
Gene expression	1.05E-02 – 6.52E-02

FIG 5 Interferon-treated $Mx1^{+/+}$ mice are resistant to lethal 1918 virus infection. (A) Survival plot of a total of 18 mice (9 animals/mouse strain). (B) Heat map illustrating 2,071 differentially transcribed genes associated with complete protection of $Mx1^{+/+}$ animals compared to the wild-type animals during interferon treatment; cutoff values were ≥2-fold change and a *P* value of ≤0.01 (ANOVA), FDR corrected. Blue indicates downregulation. Red indecates upregulation in the activation of genes. (C) Top 10 biological functions assigned to the 2,071 differentially regulated genes determined by IPA. The *P* values of the functional categories are shown on the right.

sociated with IFN pretreatment of $Mx1^{+/+}$ mice challenged with the 1918 virus is a novel finding.

DISCUSSION

In this study, we observed that $Mx1^{+/+}$ mice were able to partially control 1918 virus growth and that this control was highly enhanced by pretreatment of the animals with interferon. In contrast, BALB/c mice were not able to control virus growth in the presence or absence of interferon treatment. We were not able to detect viral dissemination to brain or spleen in either mouse strain. Histopathological analysis indicated that $Mx1^{+/+}$ animals had less severe lung damage than was present in BALB/c mice, and pathology was further reduced by interferon pretreatment. These results indicate that the ability of $Mx1^{+/+}$ mice to control virus growth is linked to reduced disease severity and lethality. This is consistent with earlier studies using a highly pathogenic mouse-adapted virus (6) or the 1918 pandemic strain (33). Nevertheless, because pretreatment of $Mx1^{+/+}$ mice with interferon was required for full protection, additional elements of the host response are required. We therefore used gene expression profiling to obtain a picture of the global host transcriptional response to infection under each experimental condition.

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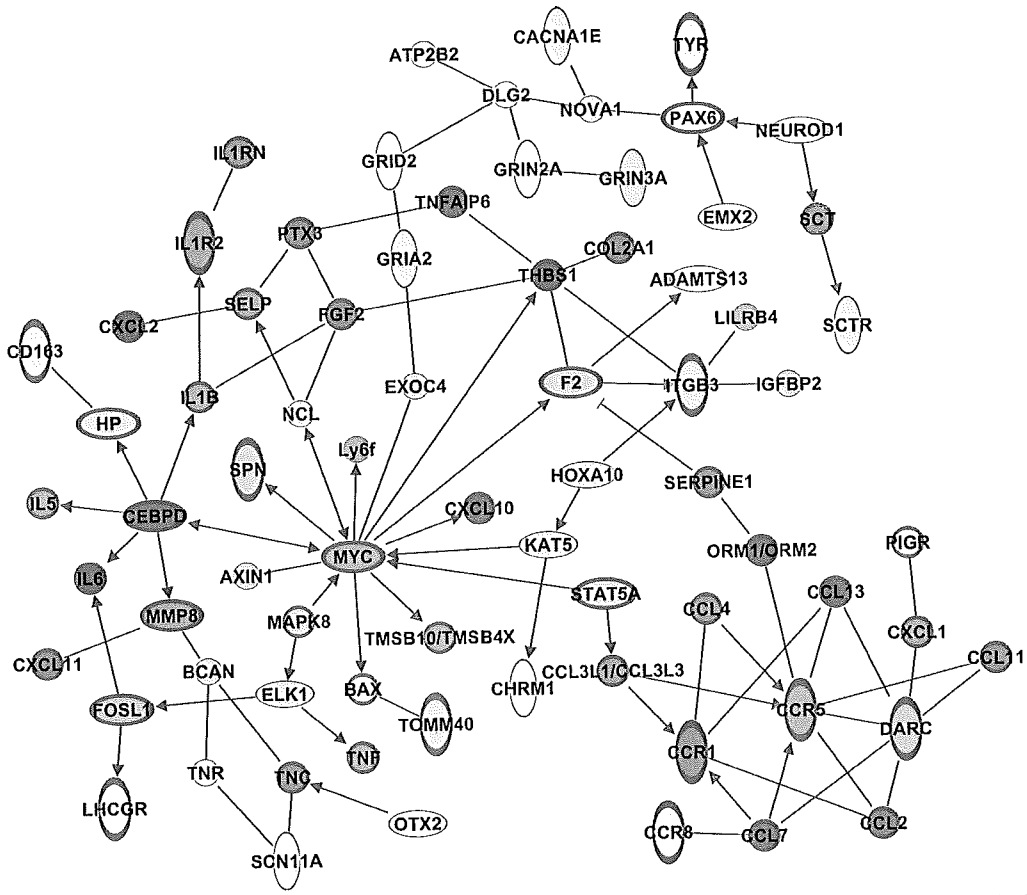

FIG 6 Molecular signature associated with complete protection of $Mx1^{+/+}$ mice. The biological network shows the direct functional relationship of genes associated with complete protection determined by IPA identified from the 2,071 differentially regulated genes. This network highlights the upregulation (yellow) and downregulation (blue) of genes in $Mx1^{+/+}$ interferon-treated mice. Genes associated with the inflammatory response are outlined in pink.

Elicited host response in untreated mice. Comparison of the transcriptional responses of $Mx1^{+/+}$ and BALB/c mice showed a striking anticorrelation in the response to 1918 virus infection. Our results suggest that under these experimental conditions, the normal interferon response is not enough to fully potentiate the protective nature of the Mx1 protein. One of the initial consequences during influenza virus infection is the rapid virus-mediated antagonism of the host innate response to avoid the generation of a strong antiviral state (8). This is accomplished by several tactics, such as inhibition of the RIG-I signaling pathway (7, 17, 19), dysregulation of gene expression (18, 23), downregulation of type I and II interferon receptor signaling (20, 24, 34), inhibition of PKR (12, 14) and OAS (14), and modulation of apoptosis (5, 13, 16, 36, 38, 39). Consequently, on one hand, the 1918 virus antagonizes the innate response, but on the other hand, Mx1 competent animals are able to partially control virus growth. This decrease in virus growth, however, was not enough to fully protect all $Mx1^{+/+}$ animals.

Our functional genomics analysis performed on lungs from 1918 virus-infected $Mx1^{+/+}$ mice allowed us to identify a specific molecular signature associated with partial protection of mice. The novelty of this finding is the altered regulation of specific acute-response components, such as genes associated with apoptosis (BCL2, BAG3, BBC3, HSPA1A, SPP1, ETS1, BTK, STAT5A, LTBR, PDIA3, HSPA5, IER3, MAPK8IP1, MNT, FKBP8, and NR1I2) and cell migration (TNFSF14, ITGA2B, MAPT, NCL, GIPC1, NRP1, PXN, CTTN, and NARS). We also observed the upregulation and direct functional relationship of genes associated with connective tissue disorders (LTB, MYO1C, SAFB, EYA2, PPT2, LIPE, and LMNB1) and genes involved in the production of reactive oxygen species in macrophages (PPP2CA, PPP2R5A, and MAP3K10). This functional signature illustrates a striking dichotomy in the gene expression profiles during the course of the experimental infection, where $Mx1^{+/+}$ mice elicited a steady upregulation of an acute-response environment that likely generated an antiviral state that partially protected the 1918 virus-infected animals.

Interferon-dependent host response is associated with complete protection. Analysis of the global transcriptional responses of interferon-treated $Mx1^{+/+}$and BALB/c mice challenged with the 1918 virus revealed a striking difference in the expression of 2,071 genes. We found that the majority of these genes were downregulated in BALB/c mice but upregulated in the $Mx1^{+/+}$ animals. Using a functional analysis approach, we identified a molecular signature associated with complete protection of $Mx1^{+/+}$ mice. The most relevant feature of this signature was the downregulation of genes involved in hypercytokinemia and hyperchemokinemia, including interleukin 6 (IL-6), IL-1β, tumor necrosis factor (TNF), CCL4, CCL12, CCR1, CCR5, CXCL10, and IL-1RN genes. These results are particularly interesting in the context of our pre-

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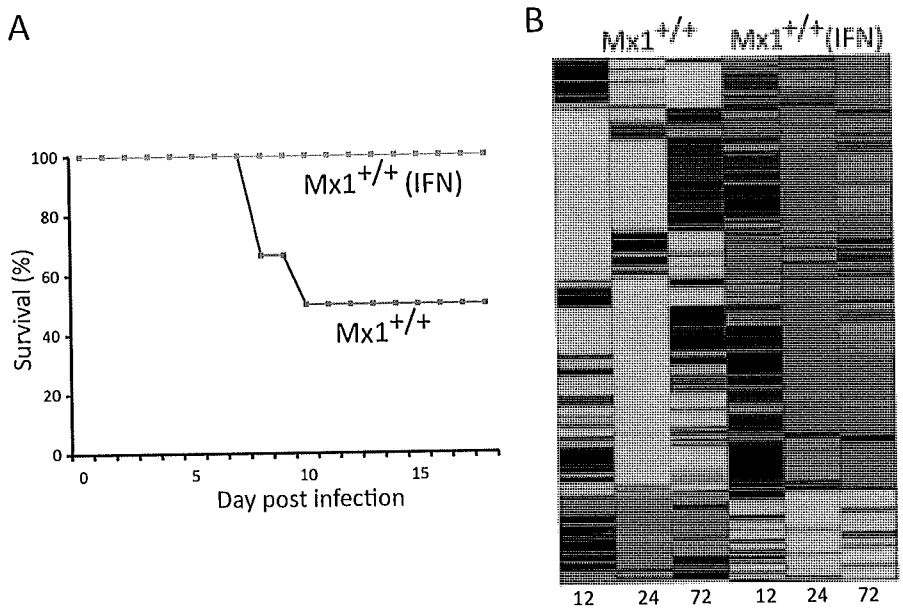

C

Biological function	P-value
Cell cycle	2.45E-14 – 6.46E-02
Cellular assembly and organization	3.29E-10 - 5.81E-02
DNA replication, recombination and repair	3.29E-10 - 5.81E-02
Cellular movement	1.15E-07 – 5.81E-02
Cellular growth and proliferation	8.43E-04 – 6.35E-02
Cellular function and maintenance	1.58E-03 - 5.81E-02
Cellular compromise	3.48E-03 - 5.81E-02
Cellular development	8.30E-03 – 6.35E-02
Cell death	1.17E-02 – 6.29E-02
Gene expression	2.44E-02 – 5.81E-02

FIG 7 Contribution of interferon to complete protection. (A) Survival plot of a total of 18 mice (9 animals/treatment). (B) Heat map illustrating 234 differentially transcribed genes that may contribute to the additional 50% difference to achieve complete protection of $Mx1^{+/+}$ treated versus $Mx1^{+/+}$ untreated animals; cutoff values were ≥2-fold change and a *P* value of ≤0.01 (ANOVA), FDR corrected. Blue indicates downregulation. Red indicates upregulation in the activation of genes. (C) Top 10 biological functions assigned to the 234 differentially regulated genes determined by IPA. The *P* values of the functional categories are shown on the right.

vious studies, where we have shown that lethal influenza virus infection of mice is associated with strong upregulation of the inflammatory response, which includes multiple cytokines and chemokines (3). These results suggest that Mx1 exerts its strongest role against influenza virus infection by targeting and downregulating an excessive cytokine response, which in mice lacking a functional Mx1 is highly upregulated to the detriment of the host.

Specific contribution of interferon treatment to survival. Our experimental approach allowed us to identify the genes responsible for complete protection that were not activated in the absence of interferon pretreatment of $Mx1^{+/+}$ mice. Functional analysis of the 234 differentially regulated genes between the treated and untreated $Mx1^{+/+}$ animals allowed us to identify a molecular signature associated with complete protection upon interferon treatment. At first glance, this functional network illustrates the upregulation of a large proportion of genes related to cell cycle regulation (CCNA1, CCNA2, CCNB1, CDK1, CKAP2, CKS2, CDC20, CDCA8, CDCA5, BIRC5, PBK, AURKB, SPAG5, MKI67, TK1, UBE2C, MYB, and BRCA1). These results suggest that complete protection of mice required the activation of a specific set of cell cycle-related genes that were downregulated in the untreated $Mx1^{+/+}$ animals, which elicited only partial protection. Our previous studies of nonhuman primates and

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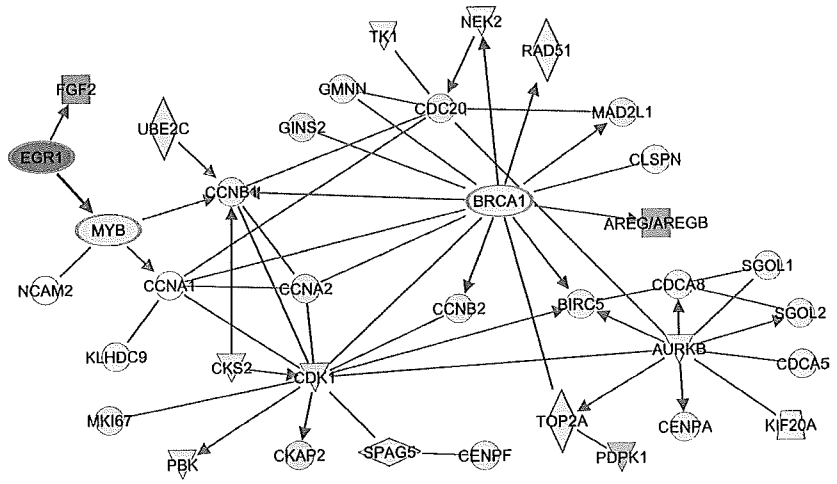

FIG 8 Molecular signature of the contribution of interferon to complete protection. The biological network shows the direct functional relationship of cell cycle regulation genes associated with the specific contribution of interferon to survival determined by PA identified from the 234 differentially regulated genes. This network highlights the upregulation (yellow) and downregulation (blue) of genes in the $Mx1^{+/+}$ interferon-treated mice.

mice infected with highly pathogenic viruses have also shown the differential regulation of cell cycle-related genes (3, 4), suggesting that this is an important mechanism for host survival during lethal influenza virus infection. This is interesting, because it has been shown that influenza virus infection induces cell cycle arrest, which promotes a favorable environment for influenza virus protein expression (10). Consequently, the activation of cell cycle regulatory genes counters this mechanism by promoting host cell proliferation. Our results depict a scenario where complete protection of the $Mx1^{+/+}$ animals was achieved only when there was sustained upregulation of genes related to apoptosis, reactive oxygen species, migration of cells, cytokines, chemokines, and the cell cycle, suggesting that modulation of

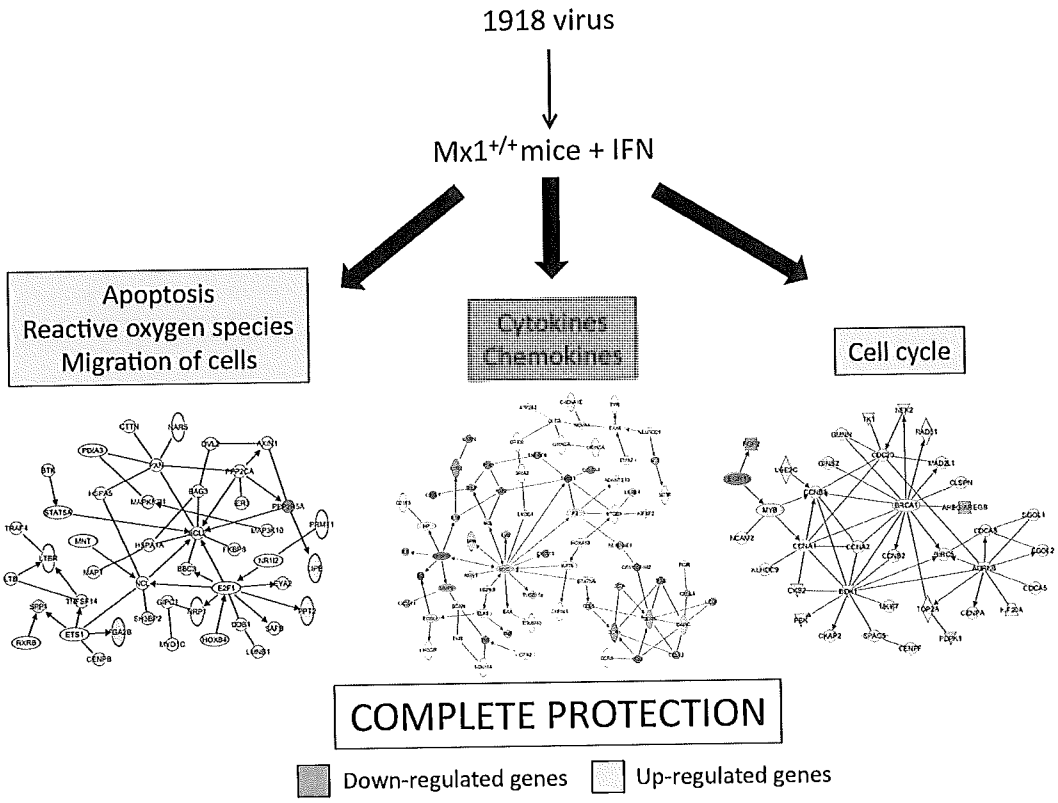

FIG 9 Model illustrating our understanding of the host response necessary for complete protection against 1918 virus infection in Mx1 competent mice pretreated with interferon. The graph depicts the events leading to complete protection of $Mx1^{+/+}$ animals under the described experimental conditions. Yellow indicates the upregulation of genes. Blue indicates the downregulation of genes.

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the activities of these genes is essential for the generation of a strong antiviral state that protects all interferon-treated $Mx1^{+/+}$ mice from 1918 virus infection (Fig. 9).

In summary, our results suggest that a functional Mx1 is not sufficient to protect all mice from challenge with the fully reconstructed 1918 virus. We identified molecular signatures associated with partial protection ($Mx1^{+/+}$ mice), complete protection ($Mx1^{+/+}$ mice pretreated with interferon), and the specific contribution of interferon treatment to the host response. Partial protection of untreated mice was characterized by the generation of an acute-response environment, whereas complete protection was characterized by the downregulation of inflammatory cytokines and chemokines. The contribution of interferon to total protection in the 1918 virus-infected $Mx1^{+/+}$ mice was characterized by the differential upregulation of cell cycle-related genes in the $Mx1^{+/+}$ treated mice, which were downregulated in the absence of interferon treatment. Thus, novel therapies aimed at regulating the cytokine and chemokine environment may be of particular benefit in treating highly pathogenic influenza virus infection.

ACKNOWLEDGMENTS

This study was funded in part by Public Health Service grant P01AI058113 from the National Institute of Allergy and Infectious Diseases.

We thank Pavel Sova (Department of Microbiology, University of Washington) for helpful discussions and reviewing the manuscript.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

REFERENCES

1. Benjamini Y, Hochberg Yosef. 1995. Controlling the false discovery rate—a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57:289–300.
2. Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.
3. Cilloniz C, et al. 2010. Lethal dissemination of H5N1 influenza virus is associated with dysregulation of inflammation and lipoxin signaling in a mouse model of infection. J. Virol. 84:7613–7624.
4. Cilloniz C, et al. 2009. Lethal influenza virus infection in macaques is associated with early dysregulation of inflammatory related genes. PLoS Pathog. 5:e1000604.
5. Ehrhardt C, et al. 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J. Virol. 81:3058–3067.
6. Grimm D, et al. 2007. Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. Proc. Natl. Acad. Sci. U. S. A. 104:6806–6811.
7. Guo Z, et al. 2007. NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am. J. Respir. Cell Mol. Biol. 36:263–269.
8. Hale BG, Albrecht RA, Garcia-Sastre A. 2010. Innate immune evasion strategies of influenza viruses. Future Microbiol. 5:23–41.
9. Haller O, Frese M, Kochs G. 1998. Mx proteins: mediators of innate resistance to RNA viruses. Rev. Sci. Tech. 17:220–230.
10. He Y, et al. 2010. Influenza A virus replication induces cell cycle arrest in G0/G1 phase. J. Virol. 84:12832–12840.
11. Johnson NP, Mueller J. 2002. Updating the accounts: global mortality of the 1918–1920 "Spanish" influenza pandemic. Bull. Hist. Med. 76:105–115.
12. Katze MG, et al. 1988. Influenza virus regulates protein synthesis during infection by repressing autophosphorylation and activity of the cellular 68,000-Mr protein kinase. J. Virol. 62:3710–3717.
13. Kurokawa M, Koyama AH, Yasuoka S, Adachi A. 1999. Influenza virus overcomes apoptosis by rapid multiplication. Int. J. Mol. Med. 3:527–530.
14. Li S, Min JY, Krug RM, Sen GC. 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology 349:13–21.
15. Maines TR, et al. 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J. Virol. 79:11788–11800.
16. Mao H, et al. 2009. Influenza virus directly infects human natural killer cells and induces cell apoptosis. J. Virol. 83:9215–9222.
17. Mibayashi M, et al. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81:514–524.
18. Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM. 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′ end formation of cellular pre-mRNAs. Mol. Cell 1:991–1000.
19. Opitz B, et al. 2007. IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell. Microbiol. 9:930–938.
20. Pauli EK, et al. 2008. Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. PLoS Pathog. 4:e1000196.
21. Peiris JS, et al. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 363:617–619.
22. Perrone LA, et al. 2009. Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. J. Virol. 83:5726–5734.
23. Plotch SJ, Bouloy M, Ulmanen I, Krug RM. 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23:847–858.
24. Pothlichet J, Chignard M, Si-Tahar M. 2008. Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 tbrough a RIG-I/IFNAR1-dependent pathway. J. Immunol. 180:2034–2038.
25. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497.
26. Sadler AJ, Williams BR. 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8:559–568.
27. Salomon R, et al. 2007. Mx1 gene protects mice against the highly lethal human H5N1 influenza virus. Cell Cycle 6:2417–2421.
28. Staeheli P, Dreiding P, Haller O, Lindenmann J. 1985. Polyclonal and monoclonal antibodies to the interferon-inducible protein Mx of influenza virus-resistant mice. J. Biol. Chem. 260:1821–1825.
29. Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O. 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol. Cell. Biol. 8:4518–4523.
30. Staeheli P, Haller O, Boll W, Lindenmann J, Weissmann C. 1986. Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell 44:147–158.
31. Tumpey TM, et al. 2005. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 310:77–80.
32. Tumpey TM, et al. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315:655–659.
33. Tumpey TM, et al. 2007. The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. J. Virol. 81:10818–10821.
34. Uetani K, et al. 2008. Influenza A virus abrogates IFN-gamma response in respiratory epithelial cells by disruption of the Jak/Stat pathway. Eur. J. Immunol. 38:1559–1573.
35. Wilson DE, Chosewood LC. 2009. Laboratory biosafety level criteria, p 30–59. *In* Wilson DE, Chosewood LC (ed), Biosafety in microbiological and biomedical laboratories, 5th ed. Centers for Disease Control and Prevention, Atlanta, GA.
36. Xing Z, et al. 2009. Differential regulation of antiviral and proinflammatory cytokines and suppression of Fas-mediated apoptosis by NS1 of H9N2 avian influenza virus in chicken macrophages. J. Gen. Virol. 90:1109–1118.
37. Yuen KY, et al. 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 351:467–471.
38. Zhirnov OP, Klenk HD. 2007. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. Apoptosis 12: 1419–1432.
39. Zhirnov OP, Konakova TE, Wolff T, Klenk HD. 2002. NS1 protein of influenza A virus down-regulates apoptosis. J. Virol. 76:1617–1625.
40. Zimmermann P, Manz B, Haller O, Schwemmle M, Kochs G. 2011. The viral nucleoprotein determines Mx sensitivity of influenza A viruses. J. Virol. 85:8133–8140.

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Influenza Virus Respiratory Infection and Transmission Following Ocular Inoculation in Ferrets

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Abstract

While influenza viruses are a common respiratory pathogen, sporadic reports of conjunctivitis following human infection demonstrates the ability of this virus to cause disease outside of the respiratory tract. The ocular surface represents both a potential site of virus replication and a portal of entry for establishment of a respiratory infection. However, the properties which govern ocular tropism of influenza viruses, the mechanisms of virus spread from ocular to respiratory tissue, and the potential differences in respiratory disease initiated from different exposure routes are poorly understood. Here, we established a ferret model of ocular inoculation to explore the development of virus pathogenicity and transmissibility following influenza virus exposure by the ocular route. We found that multiple subtypes of human and avian influenza viruses mounted a productive virus infection in the upper respiratory tract of ferrets following ocular inoculation, and were additionally detected in ocular tissue during the acute phase of infection. H5N1 viruses maintained their ability for systemic spread and lethal infection following inoculation by the ocular route. Replication-independent deposition of virus inoculum from ocular to respiratory tissue was limited to the nares and upper trachea, unlike traditional intranasal inoculation which results in virus deposition in both upper and lower respiratory tract tissues. Despite high titers of replicating transmissible seasonal viruses in the upper respiratory tract of ferrets inoculated by the ocular route, virus transmissibility to naïve contacts by respiratory droplets was reduced following ocular inoculation. These data improve our understanding of the mechanisms of virus spread following ocular exposure and highlight differences in the establishment of respiratory disease and virus transmissibility following use of different inoculation volumes and routes.

Citation: Belser JA, Gustin KM, Maines TR, Pantin-Jackwood MJ, Katz JM, et al. (2012) Influenza Virus Respiratory Infection and Transmission Following Ocular Inoculation in Ferrets. PLoS Pathog 8(3): e1002569. doi:10.1371/journal.ppat.1002569

Editor: Ron A. M. Fouchier, Erasmus Medical Center, Netherlands

Received October 14, 2011; **Accepted** January 24, 2012; **Published** March 1, 2012

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Funding: The source of funding for this work was the Centers for Disease Control and Prevention. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Despite reports of conjunctivitis following infection with numerous respiratory pathogens (including influenza, adenovirus, respiratory syncytial virus, and others), research investigating the role of ocular infection in virus pathogenicity and transmissibility has been underrepresented [1–4]. Influenza virus represents a highly transmissible respiratory pathogen, resulting in >200,000 hospitalizations in the United States annually [5]. While ocular disease is generally rare following influenza virus infection in humans, viruses within the H7 subtype have demonstrated an apparent ocular tropism, with the majority of human infections with H7 influenza viruses associated with conjunctivitis [6]. Moreover, ocular complications have been sporadically documented following seasonal, 2009 H1N1 pandemic, and avian H5N1 virus infections in humans [7–13].

Numerous properties allow the eye to serve as both a potential site of influenza virus replication as well as a gateway for the establishment of a respiratory infection. Similar to epithelial cells within the human respiratory tract, human ocular tissue and secreted mucins express sialic acids, the cellular receptor for influenza viruses [14–16]. The anatomical proximity between the eye and nasal passages, notably the linkage of both systems via the nasolacrimal duct, facilitates aqueous exchange and provides shared lymphoid tissue between these sites [17,18]. Influenza virus can rapidly spread between ocular and respiratory tissues, as was demonstrated in a recent study which detected by RT-PCR live attenuated influenza vaccine (LAIV) in nasal washes in humans within 30 minutes of experimental ocular exposure to LAIV-containing aerosols [19].

Well-characterized mammalian models to study extraocular spread following ocular inoculation with influenza virus have been limited to the mouse [20]. The ferret, widely used to study influenza pathogenesis and transmission following intranasal inoculation, has also been recognized as an appropriate experimental model for studies involving the visual system [21–23]. A previous study demonstrated H7N3 virus replication in nasal, ocular, and rectal tissue following ocular inoculation in ferrets, but did not comprehensively examine the ability of multiple subtypes to use the eye as a portal of entry or examine virus transmissibility following inoculation by this route [24].

It is clear from epidemiological and laboratory data that ocular exposure to influenza virus can manifest in both ocular and respiratory disease. However, the properties that contribute

Author Summary

Most infections with influenza virus result in respiratory disease. However, influenza viruses of the H7 subtype frequently cause ocular and not respiratory symptoms during human infection, demonstrating that the eye represents an alternate location for influenza viruses to infect humans. Using a ferret model, we studied the ability of influenza viruses to cause disease following ocular inoculation. We found that both human and avian influenza viruses could use the eye as a portal of entry to establish a respiratory infection in ferrets. Influenza viruses were also detected in ocular samples taken from ferrets during virus infection. We identified that influenza viruses spread to different tissues in ferrets when inoculated by ocular or respiratory routes, and that these differences affected the transmissibility of influenza viruses in this model. This study is the first to confirm that virus can spread from the eye to the respiratory tract in a replication-independent manner, and offers greater insight in understanding the ability of influenza viruses of all subtypes to cause human infection by the ocular route.

towards the ocular tropism of select influenza virus subtypes, and the mechanisms of virus spread from ocular to respiratory tract tissue following ocular exposure to influenza virus, are not well understood. Here, we present a ferret model where influenza virus in a liquid suspension is placed on the surface of the eye and massaged across the surface of the eye within the conjunctival sac (ocular inoculation) to study the ability of human and avian influenza viruses to cause disease and transmit to naïve animals. We found that both human and avian influenza viruses can mount a productive virus infection following ocular inoculation, attributable to replication-independent drainage of virus inoculum from the site of inoculation to respiratory tract tissue. The viral infection following ocular inoculation was capable of causing severe and fatal disease (in the case of H5N1 virus), but was less transmissible by respiratory droplets (in the case of seasonal influenza viruses) compared with infection following inoculation by the traditional intranasal route.

Results

Human and avian influenza viruses are capable of mounting a productive infection following ocular inoculation in ferrets

Due to a high degree of similarity in lung physiology and distribution of sialic acids in respiratory tract tissues, the ferret model is frequently utilized to model the kinetics and severity of respiratory disease following administration of human and avian influenza viruses by the intranasal route [23,25]. To determine if this homology extends to ocular tissue, we examined the composition of sialic acids on the ferret cornea, which represents a potential site of influenza virus replication following ocular inoculation. Staining of ferret corneal epithelial sheets with biotinylated lectins specific for α2–3 and α2–6 sialic acids revealed a predominance of α2–3-linked sialic acids with relatively weak expression of α2–6 sialic acids on the epithelial surface, an expression pattern similar to human corneal and conjunctival tissue (data not shown) [2,20].

To assess the pathogenicity of influenza viruses of multiple subtypes following ocular inoculation (i.o.) in ferrets, we administered 10^6 EID_{50} of each indicated virus in a volume in 100 µl on the corneal epithelial surface of the right eye of an anesthetized ferret and massaged the inoculum across the surface of the eye with the eyelid (Table 1). Ferrets were observed daily for two weeks for clinical signs of illness (including fever, weight loss, nasal or ocular discharge). Nasal wash (NW) and rectal swab (RS) samples were collected on alternate days post-inoculation (p.i.) and were titered for infectious virus, while conjunctival wash (CW) samples were collected alternate days p.i. to measure the incidence and kinetics of infectious virus replication and levels of viral RNA from inoculated eyes (Tables 1 and 2, Figures 1 and 2).

All virus subtypes tested replicated in ferrets following ocular inoculation, as measured by detectable virus in NW samples as early as day 1 p.i. (Table 2 and Figure 1). The duration of virus shedding from NW samples and transient fever and weight loss generally mirrored that seen following intranasal (i.n.) inoculation for each virus [26–29]. However, in comparison to i.n. inoculation, the incidence of nasal discharge was reduced following i.o. inoculation with all influenza viruses tested, and

Table 1. Summary of virus pathotyping in ferrets following 10^6 EID_{50} ocular inoculation.

			Following 10^6 EID_{50} ocular inoculation in ferrets					
Virus	Name in study	Subtype[a]	# ferrets infected[b]	# ferrets survived	% mean max wt loss[c]	mean max temp change[c]	# ferrets with sneezing	# ferrets with nasal discharge
A/Netherlands/219/03	NL/219	HPAI H7N7	3/3	3/3	9.8	1.6	0/3	0/3
A/Netherlands/230/03	NL/230	HPAI H7N7	3/3	3/3	4.5	1.7	1/3	0/3
A/Canada/504/04	Can/504	HPAI H7N3	2/3	3/3	4.7	1.7	1/3	0/3
A/New York/107/03	NY/107	LPAI H7N2	3/3	3/3	2.6	1.7	0/3	0/3
A/Vietnam/1203/04	VN/1203	HPAI H5N1	2/3	2/3	25.0	2.6	2/3	2/3
A/Thailand/16/04	Thai/16	HPAI H5N1	3/3	0/3	20.1	2.4	0/3	3/3
A/Mexico/4108/09	Mex/4108	H1N1	3/3	3/3	7.4	2.2	2/3	0/3
A/Brisbane/59/07	Brisbane	H1N1	3/3	3/3	6.0	2.2	1/3	0/3
A/Panama/2007/99	Panama	H3N2	3/3	3/3	6.9	1.2	2/3	0/3

[a]Pathogenicity phenotype using the Intravenous Pathogenicity Index (IVPI) [60]. HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza.
[b]As determined by isolation of virus from samples collected during observation and seroconversion at the end of the experiment.
[c]Average of peak mean change among ferrets from which virus was isolated from samples collected during observation.
doi:10.1371/journal.ppat.1002569.t001

Table 2. Incidence of viral replication in ferrets following 10^6 EID_{50} ocular inoculation.

Virus	Virus in NW		Virus in CW		Virus in RS	
	# ferrets with virus in NW[a]	Peak mean titer (day)[b]	# ferrets with virus in CW	Peak mean titer (day)	# ferrets with virus in RS	Peak mean titer (day)
NL/219	3/3	5.2±1.3 (5)	3/3	1.8±0.8 (5)	3/3	2.6±1.5 (5)
NL/230	3/3	6.8±1.4 (3)	3/3	3.1±2.3 (5)	3/3	1.8±0.3 (3)
Can/504	2/3	6.5±0.4 (3)	2/3	1.4±0.5 (5)	2/3	2.0±0.7 (5)
NY/107	3/3	6.8±0.4 (3)	2/3	1.4±0.5 (3)	2/3	2.5±1.4 (5)
VN/1203	2/3	4.8±0.0 (3)	1/3	2.2 (7)	2/3	2.1±0.9 (7)
Thai/16	3/3	3.3±1.6 (7)	1/3	1.5 (7)	0/3	ND[c]
Mex/4108	3/3	7.6±1.6 (3)	3/3	3.9±1.5 (3)	3/3	2.1±0.6 (5)
Brisbane	3/3	6.6±1.6 (1)	3/3	3.6±1.6 (5)	3/3	2.3±1.3 (7)
Panama	3/3	6.6±0.3 (5)	3/3	4.6±0.9 (3)	1/3	2.25 (7)

[a]Limit of virus detection in nasal wash (NW) and rectal swab (RS) was $10^{1.5}$ EID_{50}/ml, conjunctival wash (CW) was $10^{0.8}$ EID_{50}/ml.
[b]Titer of ferrets with positive virus isolation expressed as $\log_{10}$ EID_{50}/ml ± standard deviation.
[c]ND, not detected.
doi:10.1371/journal.ppat.1002569.t002

sneezing was less frequent in ferrets following infection with human influenza viruses (Table 1) [30,31]. Infectious virus was detected in CW samples from all influenza virus subtypes tested, with levels of viral RNA generally correlating with virus titers (Figure 3).

Ocular inoculation with all H7 influenza viruses tested resulted in elevated peak mean viral titers in NW samples and a higher incidence of detection in CW samples compared with H5N1 viruses (45% positive among H7 virus samples compared with 25% of H5 virus samples) (Table 2, Figure 2). However, despite reduced viral replication in NW samples, H5N1 viruses were capable of causing >20% weight loss and lethal disease in ferrets following i.o. inoculation (Table 1). Interestingly, all seasonal H1N1 and H3N2 viruses evaluated were detected at high titer in both NW and CW samples; i.o. inoculation with Mex/4108, Brisbane, and Panama viruses, along with the H7N7 NL/230 virus, resulted in the highest peak mean titers in CW samples ($>10^3$ EID_{50}/ml) compared with all viruses examined (Table 2, Figure 2). All viruses with the exception of Thai/16 virus were also detected at low titer in RS samples, with peak titers $10^{1.8}$–$10^{2.7}$ EID_{50}/ml observed days 3–7 p.i. (Table 2).

In summary, we found that both avian and human influenza viruses were capable of mounting a productive infection in ferrets following i.o. inoculation, with virus replication observed in samples collected from both ocular and respiratory tract locations regardless of virus subtype. H7 influenza viruses replicated to peak titers >2 logs higher compared with H5 influenza viruses in NW samples following i.o. inoculation, yet H5N1 influenza viruses were capable of maintaining a lethal phenotype following introduction by the ocular route. Seasonal and 2009 H1N1 pandemic influenza viruses efficiently used the eye as a portal of entry to replicate efficiently in the upper respiratory tract as well as ocular tissue.

Intranasal and ocular inoculation routes result in differential patterns of systemic spread of virus in ferrets

To examine the capacity of influenza viruses to cause severe disease following ocular inoculation, and to better identify those features specific to ocular inoculation, we inoculated ferrets by either the traditional i.n. route (using a 1 mL inoculation volume) or the i.o. route (using a 100 µl inoculation volume) with 10^6 EID_{50} of NL/219, NL/230, or Brisbane virus and collected systemic tissues on day 3 p.i. (Table 3 and 4). While i.n. inoculation with the H7N7 viruses tested in this study results in high virus titers throughout the respiratory tract of ferrets, H7N7 virus dissemination following i.o. inoculation was generally restricted to the upper respiratory tract, with a >3 log reduction in titers in nasal turbinates ($p<0.05$) and only sporadic virus isolation in trachea and lung samples compared with intranasal inoculation ($p<0.005$) (Table 3). A similar pattern of virus dissemination following H7N7 i.o. virus infection was observed when ferrets were inoculated by the i.n. route using a 100 µl and not 1 mL inoculation volume (Table 3). The H1N1 virus Brisbane replicated with comparable efficiency in nasal turbinate samples regardless of the inoculation route or volume chosen, but similar to H7N7 virus ocular infections, did not consistently replicate to high titers in lower respiratory tract tissues. Unlike virus dissemination to the respiratory tract, virus spread to the intestinal tract was not contingent on the route or volume of inoculation.

Despite restriction of virus following i.o. inoculation to upper respiratory tract tissues compared with traditional 1 mL intranasal inoculation through day 3 p.i., virus introduced by the ocular route was still capable of causing lethal disease, as ferrets inoculated with the HPAI H5N1 virus Thai/16 by the ocular route required euthanasia days 7–8 p.i. due to development of neurological signs (Table 1). Ferrets which succumbed to Thai/16 virus infection following ocular inoculation exhibited pronounced lymphopenia in peripheral blood and systemic spread of virus to all regions of the respiratory tract and brain comparable to 1 mL intranasal inoculation, albeit with reduced lethargy and a delayed time-to-death ([32] and data not shown). These data suggest that ferrets inoculated by the ocular route succumb to a similar course of disease as intranasally inoculated ferrets, however following i.o. inoculation there is a delay in both the kinetics of virus dissemination and the development of neurological signs and severe disease, potentially owing to differences in virus inoculum reaching lower respiratory tract tissues at the time of ocular inoculation.

Ocular tissue is not routinely titrated following i.n. inoculation of influenza virus in ferrets, making it difficult to elucidate if viral titers in ocular tissue are a function of i.o. inoculation or are detected regardless of the inoculation route. Therefore, we

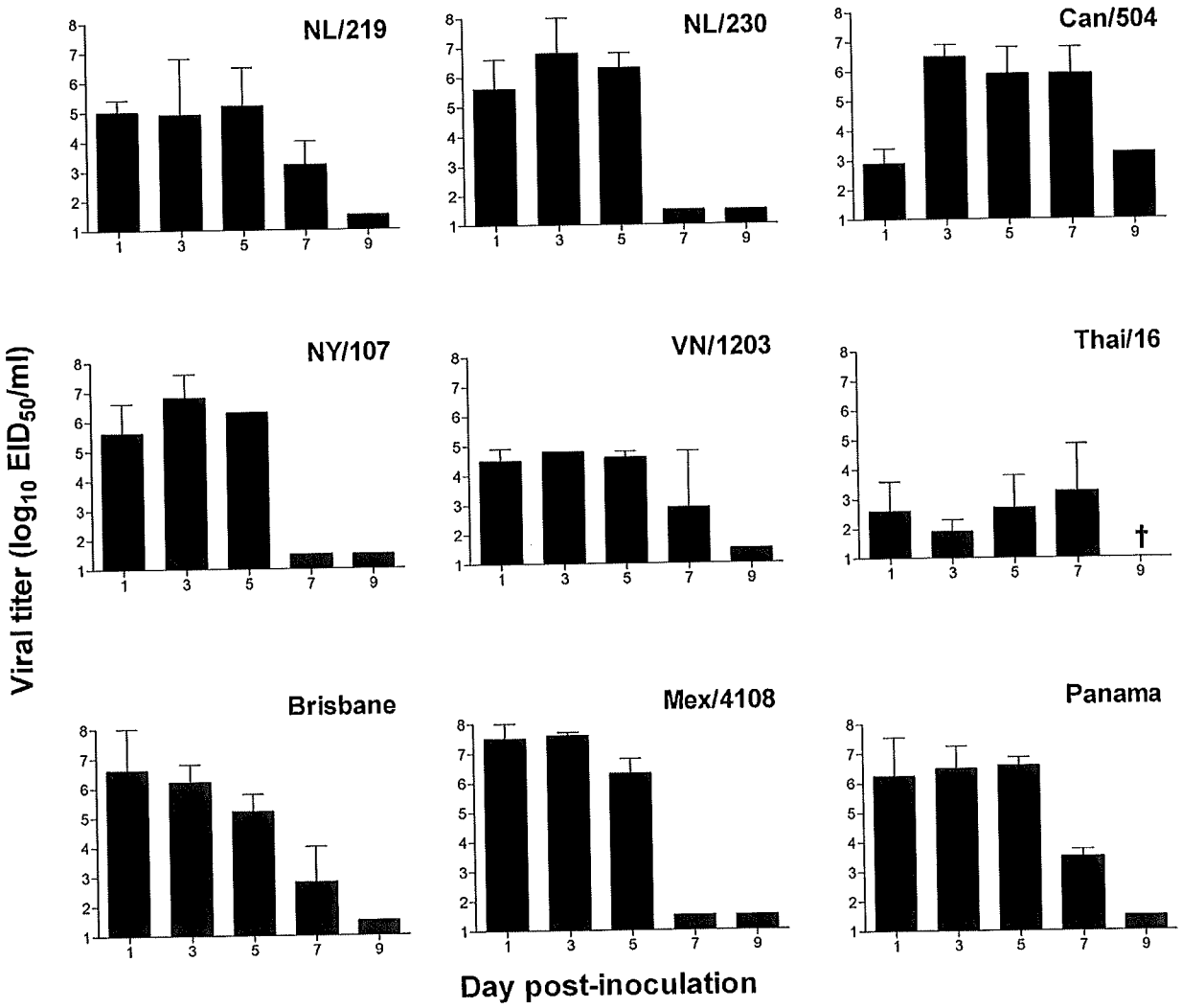

Figure 1. Comparison of mean titers of influenza viruses recovered from nasal wash following ocular inoculation of ferrets. Ferrets were inoculated by the ocular route with 10^6 EID_{50}/ml of each virus shown. Viral titers were measured in nasal washes collected on indicated days following serial titration in eggs; endpoint titers are expressed as mean log_{10} EID_{50}/ml plus standard deviation. The limit of virus detection was $10^{1.5}$ EID_{50}/ml. †, ferrets did not survive to day 9 p.i.
doi:10.1371/journal.ppat.1002569.g001

collected both left and right whole ferret eyes and all surrounding conjunctiva/eyelid for virus titration from ferrets inoculated by the intranasal or ocular route with NL/219, NL/230, or Brisbane viruses (Table 4). Surprisingly, sporadic viral titers from both left and right eye and conjunctival tissue were detected following HPAI H7N7 virus infection by both i.n. (using either a 1 mL or 100 µl inoculation volume) or i.o. inoculation routes (Table 4). While the magnitude of viral titers and viral RNA was generally similar between intranasal and ocular routes of inoculation, real-time RT-PCR detected CW-positive samples with a greater sensitivity compared with viral culture. Isolation of virus from ocular tissue may be a reflection of the ability of these HPAI viruses to spread to extra-pulmonary tissues post-inoculation as previously described [26]. However, virus was also detected in left and right conjunctival tissue following i.n. or i.o. inoculation of the H1N1 virus Brisbane, a virus which lacks a high capacity for systemic spread [28]. Comparable levels of viral RNA were isolated from CW samples from ferrets inoculated with Brisbane virus by either intranasal or ocular routes, although infectious virus was only detected in CW samples collected from the eyes of ferrets inoculated by the ocular route. To confirm that virus detected in the eye and conjunctiva was associated with tissue-specific virus replication, immunohistochemistry (IHC) was performed to visualize the presence of influenza A nucleoprotein (NP) in ferret ocular tissues. As shown in Figure 3, influenza virus antigen was detected in epithelial cells from both the lacrimal glands in the conjunctiva and the ciliary processes in the eye collected day 3 p.i. from ferrets inoculated by the ocular route. These results indicate that the route of virus inoculation in ferrets can affect the extent of virus dissemination in respiratory tract tissue, but extra-pulmonary spread, notably to ocular tissue, is present regardless of the point of entry once an infection is established.

Visualization of replication-independent spread of virus following ocular inoculation

The detection of high viral titers in NW samples as early as day 1 p.i. following i.o. inoculation suggests replication-independent

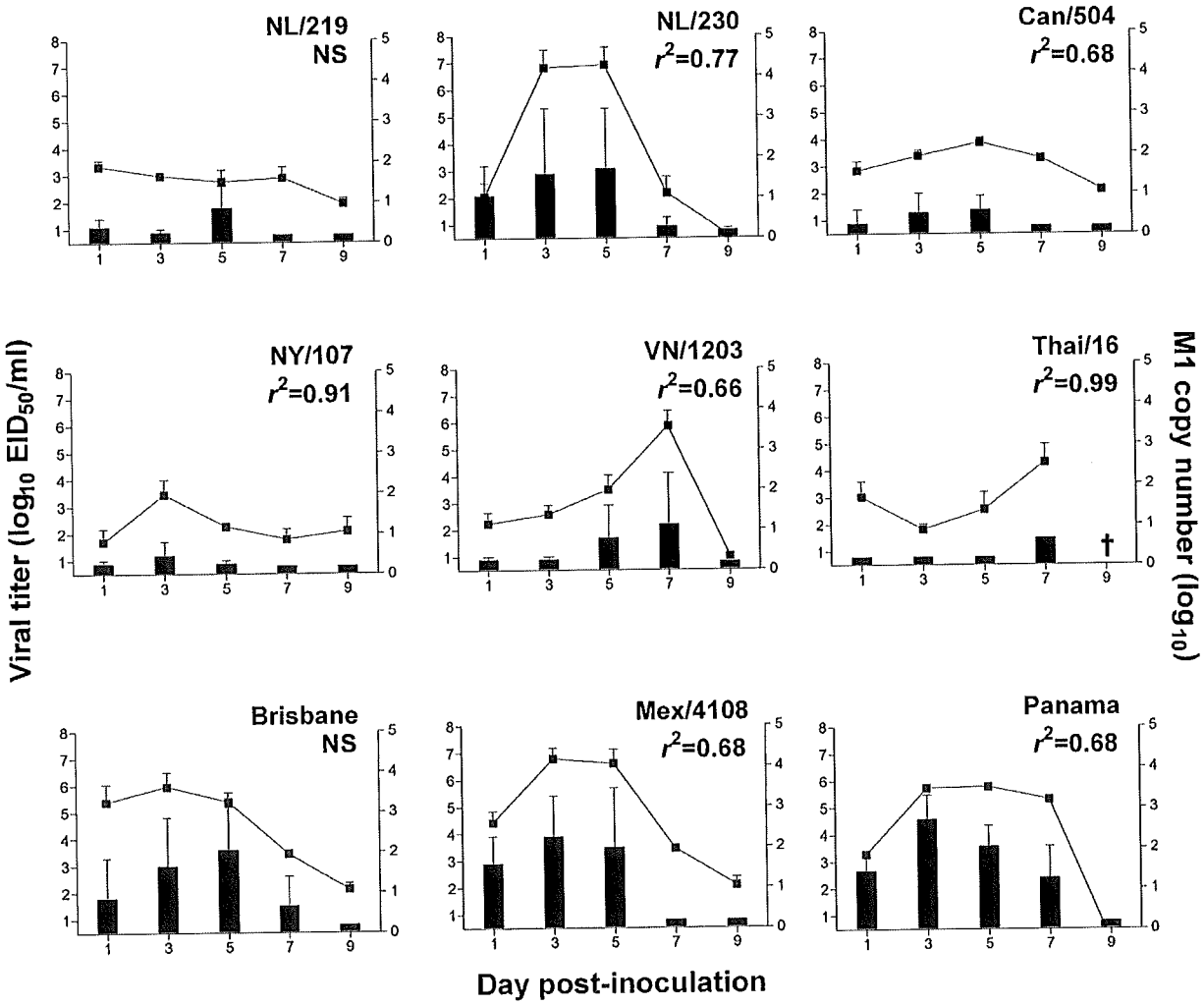

Figure 2. Comparison of influenza virus recovery in conjunctival wash samples following ocular inoculation of ferrets. Ferrets were inoculated by the ocular route with 10^6 EID_{50}/ml of each virus shown. Viral titers were measured in conjunctival washes (CW) collected on indicated days following serial titration in eggs; endpoint titers are expressed as mean log_{10} EID_{50}/ml plus standard deviation (left y-axis and bars). Relative viral RNA copy number in conjunctival washes was determined by real-time PCR using a universal M1 primer and extrapolated using a standard curve based on samples of known virus (right y-axis and lines). The limit of virus detection was $10^{1.5}$ EID_{50}/ml. †, no ferrets survived until day 9 p.i. R-squared values are shown for those viruses where a statistically significant ($p<0.05$) correlation between viral titer and viral RNA copy number exists. NS, not significant.
doi:10.1371/journal.ppat.1002569.g002

spread of virus from the eye to the respiratory tract (Figure 1); this has been similarly hypothesized in previous studies, but has yet to be proven experimentally [20,33,34]. Reduced viral titers in the lungs of ferrets on day 3 p.i. following ocular compared with intranasal administration further indicates differential patterns of virus spread following inoculation (Table 3). To visualize the deposition of virus immediately following different routes of inoculation, we labeled NL/219 virus with an AF680 fluorescent tag (NL/219-FL) and inoculated ferrets with equal quantities of NL/219-FL virus by the ocular (100 µl total volume) or intranasal (1 ml total volume diluted in PBS) route (Figure 4). Ferrets were euthanized 15 minutes following virus inoculation for *ex vivo* imaging. In ferrets inoculated by the traditional intranasal route, the majority of virus was deposited in the nasal turbinates and lungs, consistent with a previous study demonstrating virus dissemination throughout upper and lower respiratory tract tissue following this route of inoculation [32]. In contrast, virus deposition in ferrets inoculated by the ocular route (right side only) was primarily localized in the nasal turbinates and right conjunctiva. Lower relative quantities of virus inoculum were present in the upper trachea and esophagus following either intranasal or ocular inoculation. These findings demonstrate that, following i.o. inoculation in ferrets, influenza virus rapidly spreads to the nasal turbinates and upper trachea in a replication-independent manner, but in contrast to i.n. inoculation, does not immediately deposit in peripheral lung tissue. Furthermore, initial deposition of virus inoculum following ocular inoculation occurs not on the corneal surface of the eye but is rather concentrated in the surrounding conjunctival tissue.

Influenza virus transmissibility following ocular inoculation

To determine if ocular exposure to influenza virus results in a transmissible respiratory infection, we inoculated ferrets by the

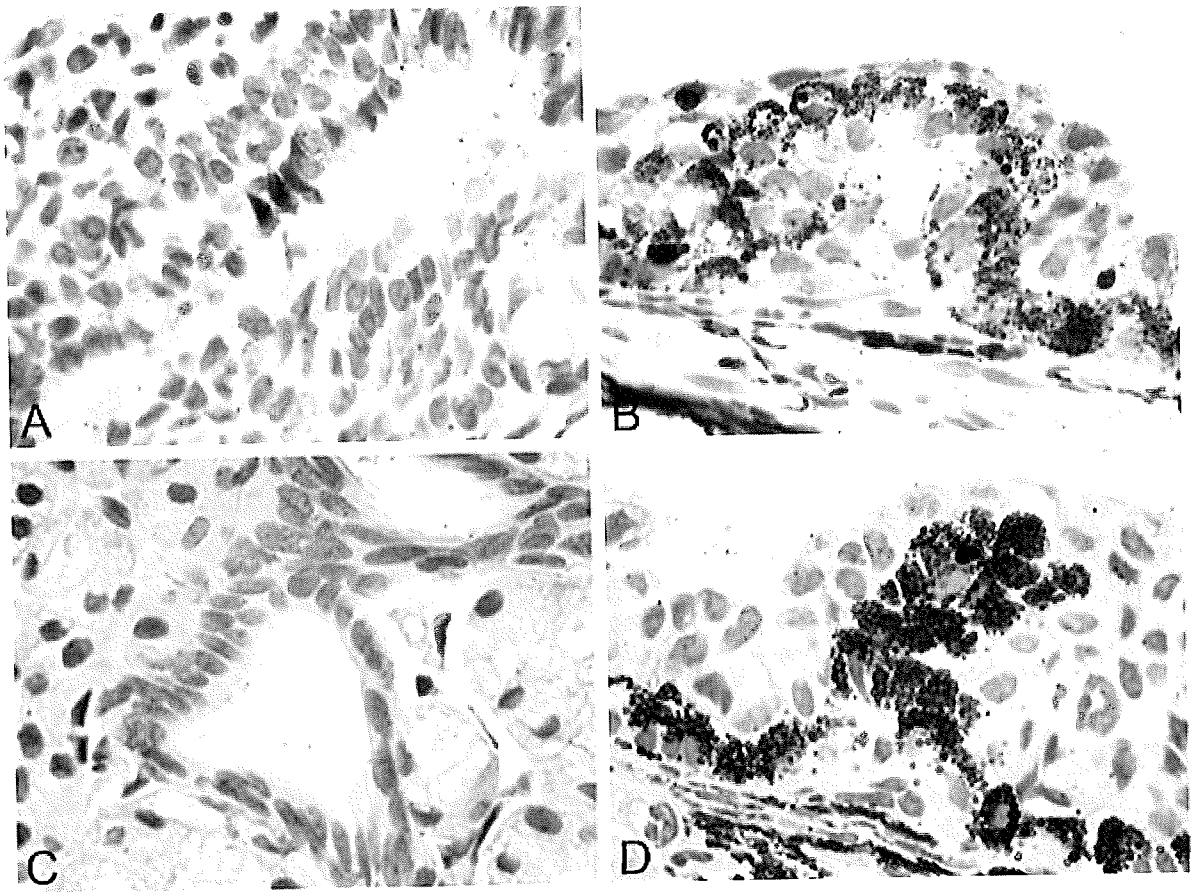

Figure 3. Photomicrographs of ferret tissue sections stained for the presence of influenza viral antigen following ocular inoculation. Ferrets were inoculated by the ocular route with 10^6 EID_{50}/ml of NL/230 or Brisbane virus, and tissues were collected day 3 p.i. for analysis. Viral antigen (staining in red) found in epithelial cells of lacrimal glands in the conjunctiva of a ferret inoculated with Brisbane virus (A) and epithelial cells of the ciliary processes in the eye of a ferret infected with NL/230 virus (B). No virus staining was present in the conjunctiva (C) or eye (D) of control ferrets.
doi:10.1371/journal.ppat.1002569.g003

Table 3. Comparative viral pathogenesis between intranasal and ocular inoculation day 3 p.i. in extra-ocular tissue.

		Viral titer (log_{10} EID_{50}/ml or g ± SD)[a]						
Virus	Route[b]	NW[c]	NT	Trachea	Lung	RS[c]	Intestine[d]	Bn OB
NL/219	i.n.	6.3±0.5 (3/3)	8.1±0.3 (3/3)	6.7±0.1 (3/3)	7.2±1.0 (3/3)	2.9±1.3 (2/3)	3.7±0.9 (3/3)	4.2 (1/3)
NL/219	i.n.[e]	5.8± 0.6 (3/3)	6.9±0.3 (3/3)	2.9 (1/3)	2.2 (1/3)	ND	4.3±1.3 (3/3)	ND
NL/219	i.o.	5.0±1.6 (4/6)	4.9±0.5 (2/2)	3.4 (1/2)	3.2 (1/2)	2.1±0.8 (4/6)	4.0±1.0 (2/2)	ND
NL/230	i.n.	5.9±0.7 (3/3)	7.4±0.8 (3/3)	6.2±1.0 (3/3)	6.8±1.0 (3/3)	2.5±0.4 (2/3)	2.4±0.4 (3/3)	3.3±0.1 (2/3)
NL/230	i.o.	6.2±1.7 (6/6)	3.6±1.6 (3/3)	ND[f]	ND	2.2±0.3 (3/6)	3.1±1.1 (2/3)	2.3 (1/3)
Brisbane	i.n.	6.1±0.5 (3/3)	5.9±0.3 (3/3)	3.7±0.6 (3/3)	ND	2.0 (1/3)	2.5 (1/3)	Not tested
Brisbane	i.n.[e]	5.2±0.4 (3/3)	6.0±0.4 (3/3)	ND	ND	ND	ND	Not tested
Brisbane	i.o.	6.7±1.1 (6/6)	6.5±1.4 (3/3)	ND	2.75 (1/3)	2.8 (1/6)	2.25 (1/3)	Not tested

[a]All viral titers expressed per gram of tissue except NW, NT, and RS samples which are expressed per ml. Limit of detection is 1.5 log_{10} EID_{50}. The mean viral titer of all ferrets with positive virus isolation (denoted in parentheses) is shown.
[b]Route of inoculation. i.n., intranasal (10^6 EID_{50}/ml) unless otherwise specified; i.o.; ocular inoculation (10^6 EID_{50}/100 µl).
[c]i.o. NW and RS samples are inclusive of 6 ferrets tested.
[d]Viral titers represent a pooled intestinal sample consisting of the duodenum, jejuno-ileal loop, and descending colon.
[e]Intranasal inoculation performed using 10^6 EID_{50}/100 µl virus dose.
[f]ND, not detected.
doi:10.1371/journal.ppat.1002569.t003

Table 4. Comparative viral pathogenesis between intranasal and ocular inoculation day 3 p.i. in ocular tissue.

		Viral titer (log_{10} EID_{50}/ml ± SD)[a]					
Virus	Route[b]	R eye	R conj	L eye	L conj	CW[c]	CW RNA[c]
NL/219	i.n.	3.5 (1/3)	4.1±0.5 (2/3)	3.1±0.6 (3/3)	Not tested	1.8 (1/3)	1.3±0.3 (3/3)
NL/219	i.n.[d]	ND[e]	1.0 (1/3)	ND	ND	3.5 (1/3)	0.5±0.3 (3/3)
NL/219	i.o.	2.8 (1/2)	4.3 (1/2)	ND	Not tested	1.0 (1/6)	1.5±0.5 (6/6)
NL/230	i.n.	ND	2.6±0.2 (2/3)	2.3 (1/3)	3.0±0. 4 (2/3)	ND	0.6±0.4 (3/3)
NL/230	i.o.	ND	2.75 (1/3)	ND	1.9 (1/3)	3.9±2.3 (2/6)	3.9±1.9 (6/6)
Brisbane	i.n.	ND	3.75 (1/3)	ND	2.1±0.2 (3/3)	ND	3.5±0.5 (3/3)
Brisbane	i.n.[d]	ND	1.1±0.2 (2/3)	1.3 (1/3)	1.75 (1/3)	1.25 (1/3)	2.8±0.5 (5/6)
Brisbane	i.o.	2.6±0.2 (2/3)	3.9±1.0 (3/3)	ND	2.3 (1/3)	2.9±1.7 (6/6)	3.6±0.6 (6/6)

[a]Limit of detection is 1.5 log_{10} EID_{50} (eye and conj) or 0.8 log_{10} EID_{50} (CW, all 100 µl i.n. samples). The mean viral titer of all ferrets with positive virus isolation (denoted in parentheses) is shown.
[b]Route of inoculation. i.n., intranasal (10^6 EID_{50}/ml) unless otherwise specified; i.o.; ocular inoculation (10^6 EID_{50}/100 µl).
[c]Relative viral RNA copy number in CW samples determined by RT-PCR and extrapolated using a standard curve of known virus. i.o. CW samples are inclusive of 6 ferrets tested.
[d]Intranasal inoculation performed using 10^6 EID_{50}/100 µl virus dose.
[e]ND, not detected.
doi:10.1371/journal.ppat.1002569.t004

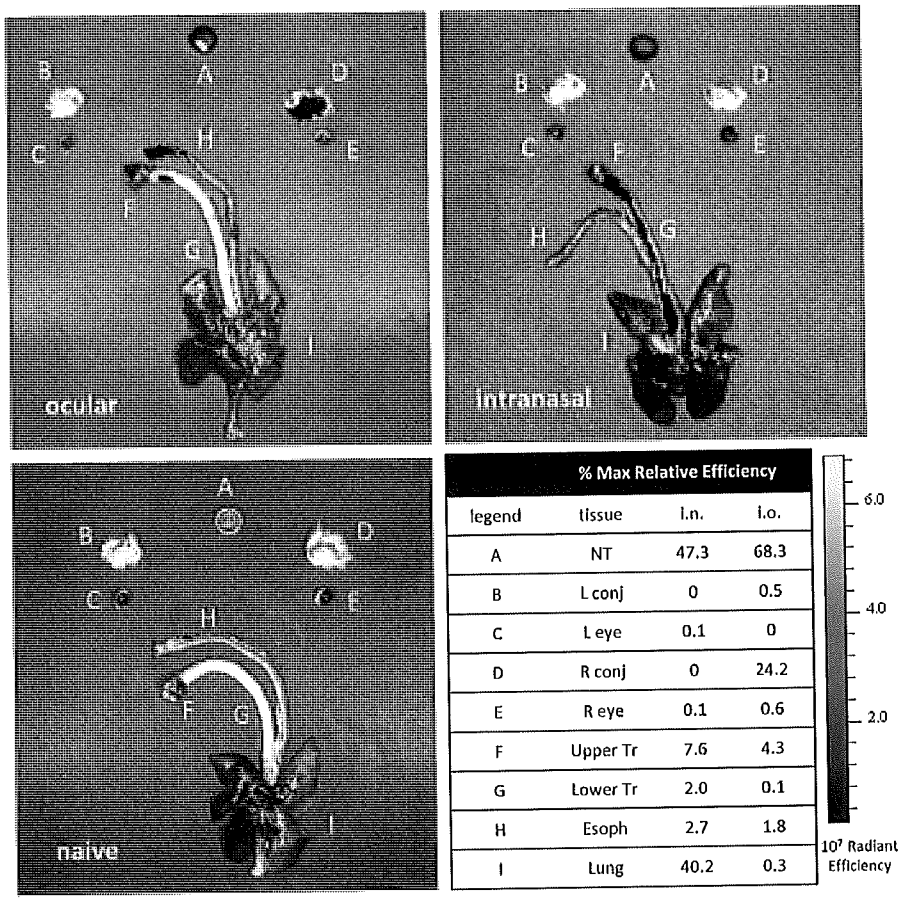

Figure 4. Virus deposition in ferrets following different routes of inoculation. Fluorescent-labeled A/NL/219/03 virus (NL219-FL) was administered to ferrets by the intranasal or ocular route. Each ferret was euthanized 15 min following virus administration and organs were collected for *ex vivo* imaging. Nasal turbinates are contained within the cap; left and right conjunctiva and eyes are below, respectively. An increasing fluorescence signal is indicated by brightness from red to yellow. Images are representative of triplicate independent inoculations for each route. Percentages represent the mean maximum relative efficiency for each tissue (n = 3) above levels in corresponding naïve tissue for each route of inoculation.
doi:10.1371/journal.ppat.1002569.g004

ocular route with selected influenza viruses known to transmit following traditional i.n. inoculation to naïve contacts in the presence of direct contact or by respiratory droplets (Figure 5). Transmission was assessed by the detection of virus in NW samples and seroconversion of contact ferrets. To assess virus transmission in the presence of direct contact, ferrets were inoculated by the ocular route with the H7N2 virus NY/107 or the H7N7 virus NL/230, both viruses which transmit efficiently by this route following i.n. inoculation in ferrets [27]. Twenty-four hours later, a naïve ferret was placed in the same cage as each inoculated ferret to assess transmission. Both NY/107 and NL/230 viruses replicated efficiently in the inoculated ferrets following ocular inoculation as expected, and spread to 2/3 and 3/3 contact ferrets by day 7 post-contact (p.c.), respectively (Figure 5A). In addition to high titers of virus in the NW of contact ferrets, NY/107 contact ferrets with detectable virus in NW samples also had detectable infectious virus in CW samples (2/3 ferrets, peak titers $10^{0.98-2.25}$ EID_{50}), and NL/230 contact ferrets had detectable infectious virus in CW (3/3 ferrets, peak titers $10^{0.98-2.25}$ EID_{50}) and RS (2/3 ferrets, peak titers $10^{1.98-2.75}$ EID_{50}) samples. All NL/230 and NY/107 DC contact ferrets seroconverted by the end of the observation period (data not shown). These results indicate that virus transmission in the presence of direct contact can occur following exposure to ferrets which exhibit a respiratory infection generated by i.o. inoculation, with virus recovery from contact ferrets in both respiratory and ocular samples.

To assess virus transmissibility by respiratory droplets in the absence of direct contact, ferrets were inoculated by the ocular route with the H1N1 virus Brisbane or the H3N2 virus Panama, both viruses which transmit efficiently by this route following traditional i.n. inoculation [28,30]. Twenty-four hours following i.o. inoculation, a naïve ferret was placed in an adjacent cage with modified side walls, so that air exchange was permitted between inoculated and contact ferrets in the absence of direct or indirect contact. Unlike the efficient transmission observed with these viruses following traditional i.n. inoculation, ferrets inoculated by the ocular route with either Brisbane or Panama only transmitted virus by respiratory droplets to 1/3 contact ferrets (Figure 5B).

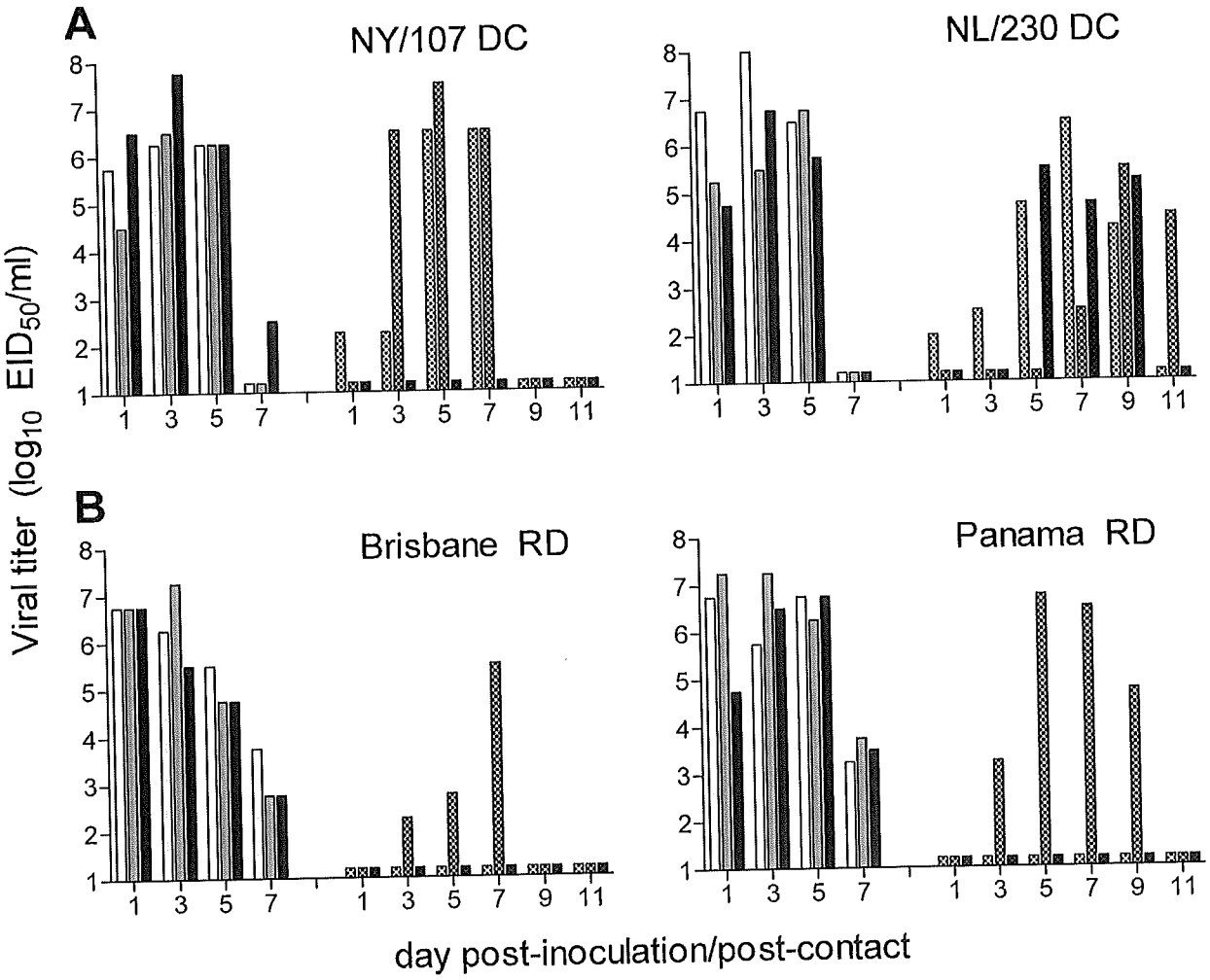

Figure 5. Transmissibility of influenza viruses in ferrets following ocular inoculation. Three ferrets were inoculated by the ocular route with 10^6 EID_{50} of NY/107, NL/230, Brisbane or Panama virus, and nasal washes were collected from each ferret on the indicated day p.i. (solid bars). A naïve ferret was placed either in the same cage (A) or in an adjacent cage with perforated side walls (B) as each inoculated ferret 24 hrs p.i., and nasal washes were collected from each contact ferret on indicated days p.c. (hatched bars) to assess virus transmission in the presence of direct contact or respiratory droplets, respectively. The limit of virus detection was $10^{1.5}$ EID_{50}/ml.
doi:10.1371/journal.ppat.1002569.g005

Virus was not detected in CW or RS samples from the infected Brisbane contact ferret; the infected Panama contact ferret had a peak CW titer of $10^{2.75}$ EID_{50} day 5 p.c. and peak RS titer of $10^{2.5}$ EID_{50} day 7 p.c. While RD contacts with detectable virus in NW samples seroconverted to homologous virus at the end of the experimental period, contact ferrets which did not have detectable virus in NW samples did not exhibit seroconversion (data not shown). Ferrets inoculated intranasally with 10^6 EID_{50} of Brisbane virus in a reduced 100 µl volume and tested for their ability to transmit virus by respiratory droplets exhibited a similar pattern of virus transmissibility as ferrets inoculated by the ocular route, with virus shedding and seroconversion detected in only 1/3 contact ferrets (data not shown). These findings suggest that despite high titers of virus in NW samples, the respiratory infection resulting from inoculation of ferrets with a reduced volume, by either ocular or intranasal inoculation routes, is distinct from that following traditional i.n. inoculation, characterized by a diminished incidence of sneezing and nasal discharge and resulting in reduced transmission of virus by respiratory droplets.

Discussion

While the ferret has proved essential for the study of influenza virus pathogenesis and transmission, the use of this species to examine alternate inoculation methods has been limited [32,35–38]. Characterizing the progression of disease following alternate routes of inoculation with influenza virus will assist in the better understanding of unique features and the relative severity and risk associated with different exposure routes. In this study, we established an *in vivo* model using the ferret to assess the ability of influenza viruses of multiple subtypes to use the eye as a gateway to establish a productive infection. Both human and avian influenza viruses were capable of mounting a respiratory virus infection in ferrets following i.o. inoculation. The detection of virus in ocular samples collected from ferrets inoculated by either ocular or intranasal routes demonstrates the importance of studying ocular involvement in respiratory virus infection. Divergent patterns of virus transmissibility by respiratory droplets following use of different inoculum volumes and routes of inoculation highlights the complexity of properties which govern virus transmission.

The high similarity of respiratory tract tissue between humans and ferrets makes the ferret model an attractive one for modeling human respiratory disease and investigating the role of receptor specificity in influenza virus pathogenesis, providing an advantage over murine models [25]. We found that the sialic acid composition of ferret corneal epithelial sheets more closely mimics that of humans compared with a mouse model, demonstrating another physiological parallel between ferrets and humans [20]. Bridging the α2–3 rich corneal and conjunctival epithelial surfaces with α2–6 rich upper respiratory tract tissue is the lacrimal duct, which expresses both α2–3 and α2–6 linked sialic acids [14,34]. Characterization of the distribution of sialic acids in the ferret conjunctiva and lacrimal duct, in addition to the composition of ferret ocular mucins, will allow for a better understanding of virus attachment and replication in these locations. However, in a previous study, we demonstrated that the ability of influenza viruses to bind to or replicate in ocular tissue cannot be explained by sialic acid binding specificity alone [20]. Our detection in ferret ocular tissue of both human and avian influenza viruses with distinct binding specificities further underscores this point (Table 4, Figure 3).

Macroscopic signs of ocular disease in ferrets were not observed during the course of infection with any virus tested, similar to prior observations in mouse and rabbit models following deposition of influenza virus on the corneal surface [20,39]. A previous study in ferrets reported mild conjunctival inflammation following i.o. inoculation with an H7N3 virus, however this may be attributable to strain-specific differences or the use of younger (3–5 month old) ferrets [24]. Despite the absence of visible ocular complications, virus was consistently detected in CW samples from ferrets inoculated by the intranasal or ocular route (Table 2, Figure 2). Levels of viral M1 RNA generally correlated with the magnitude of virus isolation, and were a more sensitive detection method compared with virus isolation in CW samples, similar to that observed in human eye swabs (Table 4, Figure 2) [40]. The presence of virus in RS samples following both i.n. and i.o. inoculation with influenza virus has been previously reported and likely originates from virus swallowed during inoculation [24,32,41], as indicated by deposition of virus in the esophagus following initial virus inoculation by both inoculation routes (Table 3, Figure 3).

Unlike in a murine model, the ferret model supported virus replication of both human and avian influenza viruses following i.o. inoculation [20]. In this ferret model, H7 viruses were detected at higher titer in NW samples and with a higher frequency in ocular CW samples compared with H5N1 viruses, suggesting a recapitulation of the tropism of the H7 virus subtype observed in humans (Table 2). However, the permissiveness of multiple virus subtypes to cause a productive infection following i.o. inoculation in the ferret points to a greater capacity of influenza viruses to use the eye as a portal of entry in an experimental *in vivo* setting, just as previous *in vitro* studies have demonstrated that numerous human ocular cell types distributed throughout the ocular area support infection and replication with both avian and human influenza viruses [42–44]. Cumulatively, these previous *in vitro* studies suggest that the apparent ocular tropism associated with viruses of the H7 subtype is not due to a superior ability to replicate in ocular cells compared with other virus subtypes. Future studies evaluating potential fine receptor specificity differences on the ocular surface and the composition of ocular mucins which may restrict exposure to the ocular epithelial surface to selected virus subtypes may provide a greater understanding of this property.

Consistently high titers of human influenza viruses in ocular samples following both i.n. and i.o. inoculation indicates that these H1N1 and H3N2 viruses are not exhibiting a preferential tropism for ocular infection but more likely are a reflection of the high titers observed in the upper respiratory tract in these tissues independent of the initial inoculation route. Specifically, the nasolacrimal duct which links the ocular lacrimal sac to the nasal meatus could serve as a conduit for virus-containing fluid exchange between ocular and respiratory tract tissue [18]. Numerous reports have documented drainage of vaccine or immunizing agents to nasal tissue following topical ocular administration as well as the spread of intranasally administered solutions to the conjunctival mucosal surface [18,45]. However, spread of virus from respiratory tract tissue to ocular tissue following i.n. inoculation with human or avian influenza viruses has not been observed previously in the ferret and only sporadically reported in the mouse, possibly due to relatively low titers of virus in nasal tissue or other anatomical differences [20,24,26,46]. Detection of both human and avian influenza viruses in ferret eyes and conjunctival tissue following i.n. inoculation indicates that virus can circulate proximal to the nasal cavity and nasolacrimal canal more readily than previously considered and that more routine collection of ocular tissue during standard virus pathotyping in mammalian models is warranted to better understand the extent of viral ocular

dissemination (Table 4). While it is unlikely that ferret grooming practices are solely responsible for virus spread between these locations, human studies with numerous respiratory viruses have demonstrated the ability to self-inoculate between ocular and respiratory sites, and it is possible that self-inoculation could be further contributing to virus spread in this model [47,48]. Inoculation of ferrets by the ocular route with 10^6 EID_{50} of selected human and avian influenza viruses in a 20 µl volume resulted in comparable results to those obtained employing a 100 µl inoculation volume, indicating that replication-independent drainage of virus to respiratory tract tissue and subsequent virus detection in NW and CW samples reported in this study was not contingent on the inoculum volume (data not shown).

Visualization of virus deposition using fluorescently-tagged virus has allowed for a new understanding of dissemination patterns following *in vivo* inoculation. Using this technique, we confirmed previously hypothesized reports of replication-independent drainage from ocular to respiratory tract tissue [20,33]. Viral load measured in respiratory tract tissues day 3 p.i. following i.n. or i.o. inoculation largely mirrored initial virus deposition patterns, with tissues possessing the greatest quantity of virus reflecting those sites of greatest initial virus deposition during virus inoculation (Table 3–4, Figure 4). Both i.o. and i.n. inoculation using a 100 µl volume resulted in detection of virus at high titers in upper and not lower respiratory tract tissues day 3 p.i., indicating that inoculation with a reduced volume leads to limited initial virus deposition in respiratory tract tissues regardless of inoculation route. The delay in high virus titer recovery from lower respiratory tract tissues during HPAI virus infection after i.o. inoculation and the delay in onset of severe disease and lethal outcome with Thai/16 virus is likely due to replication-dependent spread (and not deposition) of virus to the lower respiratory tract, suggesting that during inoculation, the majority of virus was retained in conjunctival tissues, drained to the nasal turbinates, or swallowed and diverted away from the lower respiratory tract; future study of ferret lacrimal tissue in this role is warranted. Comparable delays in onset of severe disease compared with traditional i.n. inoculation were observed in a murine model of i.o. inoculation and a ferret model of aerosol inoculation, despite ultimately similar lethal outcomes [20,32]. The finding here of H5N1 subtype viruses using the eye as a portal of entry to initiate a lethal infection, shown previously in a mouse model, underscores the risk of ocular exposure to influenza viruses, even those subtypes not typically considered to have a tropism for this tissue [20].

Despite efficient replication of seasonal H1N1 and H3N2 viruses in the upper respiratory tract of ferrets following i.o. inoculation, these viruses did not result in frequent detection of sneezing and nasal discharge, and did not transmit efficiently to naïve contacts by respiratory droplets (Table 1, Figure 5). Infrequent sneezing is commonly observed among influenza viruses which do not transmit efficiently by respiratory droplets and could be contributing to the reduced transmissibility seen here [28,30,31]. Further research is needed to better understand the virologic and immunologic properties which confer the incidence of sneezing and nasal discharge and the role of these properties in virus transmissibility [49]. Additionally, the efficiency of virus transmission by respiratory droplets following i.o. inoculation was likely influenced by the reduced initial virus deposition and subsequent limited replication in the ferret trachea, as reduced virus transmissibility by respiratory droplets was observed following i.n. inoculation when using a 100 µl but not 1 mL volume (Table 3, Figure 4, and data not shown). Despite similarly high virus titers and duration of virus shedding in NW samples, the presence of expelled virus particles originating from tracheal replication which was present during traditional i.n., but not i.o. or i.n. inoculation using a reduced volume, may have contributed to differing transmission efficiencies between inoculation routes and may represent a previously unrecognized role for virus replication in tracheal tissue in virus transmissibility by respiratory droplets. In contrast, virus transmission in the presence of direct contact did not differ between inoculation routes. The reduced transmissibility of virus following i.o. inoculation is in agreement with epidemiological studies which demonstrate that the majority of human cases of conjunctivitis following H7 influenza virus exposure are self-limiting [50]. Further study evaluating the shedding of virus into the environment among persons infected with influenza viruses which cause respiratory or ocular disease will shed light on potential differences in transmission dynamics independent of virus subtype.

The diversity of potential exposures to influenza virus underscores the importance of studying the development of respiratory disease resulting from alternate exposure routes. This knowledge is critical for both a greater understanding of the establishment of influenza virus respiratory disease as well as differences in virus transmission dynamics following differing exposure routes. The facile dissemination of virus inoculum from ocular to nasal tissue, and the detection of virus in both NW and CW samples throughout the acute phase of ferret infection, highlights the ability for concurrent ocular and respiratory disease following influenza virus infection; not surprisingly, reports of conjunctivitis and influenza-like illness in the same individual have been documented during H7 outbreaks resulting in human infection [40,51]. While much regarding the properties which regulate the ocular tropism of influenza viruses remains to be determined, our results highlight the potential for a range of influenza A subtypes to initiate infection through the eye and support the use of eye protection during occupational exposure to aerosols containing influenza viruses [19,52,53].

Materials and Methods

Ethics statement

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All ferret procedures were approved by Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention and in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. Animal studies were performed in accordance with the IACUC guidelines under protocol #2195TUMFERC-A3: "Studies on the Pathogenesis and Transmission of Recombinant Influenza Viruses in Ferrets".

Viruses

Influenza A viruses of the H7, H5, and H1 subtype used in this study are shown in Table 1. Virus stocks were propagated in the allantoic fluid cavity of 10 day old embryonated hens' eggs as previously described [26]; virus stocks were confirmed by sequencing to be free of mutations. The 50% egg infectious dose (EID_{50}) for each virus stock was calculated by the method of Reed and Muench [54] following serial titration in eggs. Fluorescent-tagged virus (NL/219-FL) was generated using formalin-inactivated NL/219 virus and a SAIVI Antibody Alexa Fluor 680 Labeling kit (Invitrogen) per manufacturer's instructions as previously described [32]. All experiments with HPAI viruses were conducted under biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agent Program [55].

Ferret experiments

Male Fitch ferrets (Triple F Farms), 7 to 10 months old and serologically negative by hemagglutination inhibition to currently circulating influenza viruses, were used in this study. Ferrets were housed in a Duo-Flow Bioclean mobile clean room (Lab Products) for the duration of each experiment. Intranasal (i.n.) inoculations were performed under anesthesia as previously described using 10^6 EID_{50} of virus diluted in PBS in a 1 ml or 100 µl volume [29]. Ocular (i.o.) inoculations were performed under anesthesia using 10^6 EID_{50} of virus diluted in PBS in a 100 µl or 20 µl volume. Virus inoculum was administered dropwise to the surface of the right eye of each ferret and massaged over the surface of the eye by the eyelid.

Ferrets were monitored daily post-inoculation for morbidity and clinical signs of infection as previously described [29]. Any ferret which lost >25% of its pre-inoculation body weight or exhibited neurological dysfunction was euthanized. Virus shedding was measured on alternate days post-inoculation (p.i.) in nasal washes (NW), conjunctival washes (CW), and rectal swabs (RS). NW and RS samples were collected as previously described [28,29]. CW were obtained by bathing the inoculated (right) ferret eye three times with 500 µl wash solution (PBS containing Pen/Strep, Gentamycin, and BSA) and collecting the run-off in a small petri dish, then swabbing the surface and surrounding conjunctival tissue of the right eye with a pre-wettened cotton swab for 5 seconds, and placing the swab in a collection tube containing the run-off liquid. All samples were immediately frozen on dry ice and stored at −70°C until processed.

To assess virus dissemination following i.n. or i.o. inoculation, three ferrets per group were inoculated with indicated viruses and euthanized 3 days p.i. for postmortem examination and collection of tissues for virus titration as previously described [29]. Tissue specimens were collected for virus titration were immediately frozen on dry ice and stored at −70°C until processed. Blood samples collected during necropsy were subjected to complete blood counts (CBCs) and serum chemistry analyses performed per manufacturer's instructions as previously described [56].

Virus transmissibility following i.o. inoculation was assessed by inoculating ferrets by the ocular route with indicated viruses and, 24 hrs p.i., placing a naïve ferret in the same cage as an inoculated ferret [to assess transmission in the presence of direct contact (DC)] or in an adjacent cage with modified side-walls to allow air exchange between inoculated and contact animals via perforations but inhibiting direct or indirect contact between animals [to assess transmission by respiratory droplets (RD)] as previously described [30]. For each i.o. transmission experiment, an aliquot of each virus stock used to characterize transmissibility in previous publications by the i.n. route was tested. NW, CW, and RS samples were collected on alternate days p.i./post-contact (p.c.) to assess kinetics of virus shedding. Serum was collected days 17–21 p.i./p.c. to measure seroconverison. Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Sample titration and processing

NW, CW, and RS samples were serially titrated in eggs, starting at a 1:10 dilution (NW, RS; limit of detection, $10^{1.5}$ EID_{50}/ml) or 1:2 dilution (CW; limit of detection, $10^{0.8}$ EID_{50}/ml). Virus infectivity for all samples was calculated by the method of Reed and Muench [54]. At the time samples were thawed for virus titration, RNA was extracted from CW samples using a QIAamp Viral RNA kit (Qiagen). Real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR kit (Qiagen) using an influenza A virus M1 gene primer set to determine viral load [32]. Viral RNA copy numbers were extrapolated using a standard curve based on samples of known virus as previously described [32,57]. Baseline levels were determined by collecting CW samples from uninfected ferrets.

Tissue specimens were homogenized in 1 ml cold PBS using disposable sterile tissue grinders and clarified by centrifugation before serial titration in eggs, starting at a 1:10 dilution. Ferret eye and conjunctival tissues were rinsed with PBS prior to virus titration. Eye, conjunctival, and nasal turbinate tissues are expressed as EID_{50}/ml, while all other tissues are expressed as EID_{50}/g.

Immunofluorescence staining and microscopy

Uninfected ferret corneal epithelial sheets were dissociated from excised whole ferret eyes following incubation in tetrasodium EDTA for determination of expression of surface sialoligosaccharides as previously described [20,58]. To assess virus dissemination, ferrets were inoculated with NL/219-FL virus either i.o. (100 µl) or i.n. (1 ml) as previously described [32]. Fifteen minutes p.i., ferrets were euthanized and respiratory and ocular tissues were excised for ex vivo imaging using a Spectrum in vivo imaging system and Living Image 4.0 Software (Caliper Life Sciences). All *ex vivo* imaging was performed in triplicate. To quantify the presence of NL/219-FL virus in excised tissues, regions of interest were drawn around each tissue using Living Image 4.0 Software to obtain maximum relative efficiency values for each tissue, expressed as photons/second/cm^2/steradian, the mean of which was generated from three ferrets per tissue as expressed in Figure 3.

Tissues for immunohistochemistry (IHC) were collected day 3 p.i with the viruses indicated. or from uninfected ferrets, fixed by submersion in 10% neutral buffered formalin for 3 days, routinely processed, and embedded in paraffin. Immunohistochemical detection of influenza A virus nucleoprotein was performed as described previously [59].

Statistics

The Pearson product-moment correlation coefficient was generated to measure the correlation between viral titer and viral RNA copy number in CW sample using GraphPad Prism 5.0 (GraphPad Software, Inc.). Statistical significance for all other experiments was determined using Student's *t* test.

Acknowledgments

We thank Yan Li (Canadian Center for Human and Animal Health) and the Vietnam and Thailand Ministries of Health for providing viruses used in this study.

Author Contributions

Conceived and designed the experiments: JAB KMG TRM JMK MJPJ TMT. Performed the experiments: JAB KMG TRM MJPJ. Analyzed the data: JAB KMG TRM MJPJ JMK TMT. Contributed reagents/materials/analysis tools: JAB KMG TRM MJPJ. Wrote the paper: JAB KMG TRM JMK TMT.

References

1. Kemp MC, Hierholzer JC, Cabradilla CP, Obijeski JF (1983) The changing etiology of epidemic keratoconjunctivitis: antigenic and restriction enzyme analyses of adenovirus types 19 and 37 isolated over a 10-year period. J Infect Dis 148: 24–33.

2. Olofsson S, Kumlin U, Dimock K, Arnberg N (2005) Avian influenza and sialic acid receptors: more than meets the eye? Lancet Infect Dis 5: 184–188.
3. Fujishima H, Okamoto Y, Saito I, Tsubota K (1995) Respiratory syncytial virus and allergic conjunctivitis. J Allergy Clin Immunol 95: 663–667.
4. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, et al. (2004) Identification of a new human coronavirus. Nat Med 10: 368–373.
5. Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, et al. (2004) Influenza-associated hospitalizations in the United States. JAMA 292: 1333–1340.
6. Belser JA, Bridges CB, Katz JM, Tumpey TM (2009) Past, present, and possible future human infection with influenza virus A subtype H7. Emerg Infect Dis 15: 859–865.
7. Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, et al. (2008) Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 358: 261–273.
8. Chan PK (2002) Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. Clin Infect Dis 34 Suppl 2: S58–64.
9. Dubnov-Raz G, Somech R, Warschawski Y, Eisenberg G, Bujanover Y (2010) Clinical Characteristics of Children with 2009 Pandemic H1N1 Influenza Virus Infections. Pediatr Int 53: 426–430.
10. Lopez-Prats MJ, Sanz Marco E, Hidalgo-Mora JJ, Garcia-Delpech S, Diaz-Llopis M (2010) Bleeding Follicular Conjunctivitis due to Influenza H1N1 Virus. J Ophthalmol 2010: 423672.
11. Stuart-Harris CH (1961) Twenty years of influenza epidemics. Am Rev Respir Dis 83: 54–75.
12. Tam JS (2002) Influenza A (H5N1) in Hong Kong: an overview. Vaccine 20 Suppl 2: S77–81.
13. Lai CC, Chang YS, Li ML, Chang CM, Huang FC, et al. (2011) Acute anterior uveitis and optic neuritis as ocular complications of influenza a infection in an 11-year-old boy. J Pediatr Ophthalmol Strabismus 48: e30–33.
14. Paulsen F, Thale A, Kohla G, Schauer R, Rochels R, et al. (1998) Functional anatomy of human lacrimal duct epithelium. Anat Embryol (Berl) 198: 1–12.
15. Terraciano AJ, Wang N, Schuman JS, Haffner G, Panjwani N, et al. (1999) Sialyl Lewis X, Lewis X, and N-acetyllactosamine expression on normal and glaucomatous eyes. Curr Eye Res 18: 73–78.
16. Berry M, Ellingham RB, Corfield AP (1996) Polydispersity of normal human conjunctival mucins. Invest Ophthalmol Vis Sci 37: 2559–2571.
17. Knop E, Knop N (2001) Lacrimal drainage-associated lymphoid tissue (LDALT): a part of the human mucosal immune system. Invest Ophthalmol Vis Sci 42: 566–574.
18. Chentoufi AA, Dasgupta G, Nesburn AB, Bettahi I, Binder NR, et al. (2010) Nasolacrimal duct closure modulates ocular mucosal and systemic CD4(+) T-cell responses induced following topical ocular or intranasal immunization. Clin Vaccine Immunol 17: 342–353.
19. Bischoff WE, Reid T, Russell GB, Peters TR (2011) Transocular entry of seasonal influenza-attenuated virus aerosols and the efficacy of n95 respirators, surgical masks, and eye protection in humans. J Infect Dis 204: 193–199.
20. Belser JA, Wadford DA, Xu J, Katz JM, Tumpey TM (2009) Ocular infection of mice with influenza A (H7) viruses: a site of primary replication and spread to the respiratory tract. J Virol 83: 7075–7084.
21. Jackson CA, Hickey TL (1985) Use of ferrets in studies of the visual system. Lab Anim Sci 35: 211–215.
22. Hernandez-Guerra AM, Rodilla V, Lopez-Murcia MM (2007) Ocular biometry in the adult anesthetized ferret (Mustela putorius furo). Vet Ophthalmol 10: 50–52.
23. Belser JA, Katz JM, Tumpey TM (2011) The ferret as a model organism to study influenza A virus infection. Dis Models Mech 4: 575–579.
24. Aamir UB, Naeem K, Ahmed Z, Obert CA, Franks J, et al. (2009) Zoonotic potential of highly pathogenic avian H7N3 influenza viruses from Pakistan. Virology 390: 212–220.
25. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, et al. (2007) Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 171: 1215–1223.
26. Belser JA, Lu X, Maines TR, Smith C, Li Y, et al. (2007) Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. J Virol 81: 11139–11147.
27. Belser JA, Blixt O, Chen LM, Pappas C, Maines TR, et al. (2008) Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. Proc Natl Acad Sci U S A 105: 7558–7563.
28. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, et al. (2009) Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science 325: 484–487.
29. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, et al. (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol 79: 11788–11800.
30. Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, et al. (2006) Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A 103: 12121–12126.
31. Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, et al. (2009) Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A 106: 3366–3371.
32. Gustin KM, Belser JA, Wadford DA, Pearce MB, Katz JM, et al. (2011) Influenza virus aerosol exposure and analytical system for ferrets. Proc Natl Acad Sci U S A 108: 8432–8437.
33. Bitko V, Musiyenko A, Barik S (2007) Viral infection of the lungs through the eye. J Virol 81: 783–790.
34. Kumlin U, Olofsson S, Dimock K, Arnberg N (2008) Sialic acid tissue distribution and influenza virus tropism. Influenza Other Respi Viruses 2: 147–154.
35. Bodewes R, Kreijtz JH, van Amerongen G, Fouchier RA, Osterhaus AD, et al. (2011) Pathogenesis of Influenza A/H5N1 Virus Infection in Ferrets Differs between Intranasal and Intratracheal Routes of Inoculation. Am J Pathol 179: 30–36.
36. Lipatov AS, Kwon YK, Pantin-Jackwood MJ, Swayne DE (2009) Pathogenesis of H5N1 influenza virus infections in mice and ferret models differs according to respiratory tract or digestive system exposure. J Infect Dis 199: 717–725.
37. Basarab O, Smith H (1969) Quantitative studies on the tissue localization of influenza virus in ferrets after intranasal and intravenous or intracardial inoculation. Br J Exp Pathol 50: 612–618.
38. Shinya K, Makino A, Tanaka H, Hatta M, Watanabe T, et al. (2011) Systemic dissemination of H5N1 influenza A viruses in ferrets and hamsters after direct intragastric inoculation. J Virol 85: 4673–4678.
39. Zaitseva NS, Vinogradova VL, Krichevskaya GI (1978) Influence of experimental influenza infection of the eye on the course of herpetic keratitis in rabbits. Acta Virol 22: 470–476.
40. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, et al. (2004) Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363: 587–593.
41. Yen HL, Lipatov AS, Ilyushina NA, Govorkova EA, Franks J, et al. (2007) Inefficient transmission of H5N1 influenza viruses in a ferret contact model. J Virol 81: 6890–6898.
42. Michaelis M, Geiler J, Klassert D, Doerr HW, Cinatl J, Jr. (2009) Infection of human retinal pigment epithelial cells with influenza A viruses. Invest Ophthalmol Vis Sci 50: 5419–5425.
43. Chan MC, Chan RW, Yu WC, Ho CC, Yuen KM, et al. (2010) Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in ex vivo and in vitro cultures of human conjunctiva and respiratory tract. Am J Pathol 176: 1828–1840.
44. Belser JA, Zeng H, Katz JM, Tumpey TM (2011) Ocular tropism of influenza A viruses: Identification of H7 subtype-specific host responses in human respiratory and ocular cells. J Virol 85: 10117–10125.
45. Saitoh-Inagawa W, Hiroi T, Yanagita M, Iijima H, Uchio E, et al. (2000) Unique characteristics of lacrimal glands as a part of mucosal immune network: high frequency of IgA-committed B-1 cells and NK1.1+ alphabeta T cells. Invest Ophthalmol Vis Sci 41: 138–144.
46. Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, et al. (2010) Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J Virol 84: 11802–11813.
47. Azar MJ, Dhaliwal DK, Bower KS, Kowalski RP, Gordon YJ (1996) Possible consequences of shaking hands with your patients with epidemic keratoconjunctivitis. Am J Ophthalmol 121: 711–712.
48. Hendley JO, Wenzel RP, Gwaltney JM, Jr. (1973) Transmission of rhinovirus colds by self-inoculation. N Engl J Med 288: 1361–1364.
49. Maines TR, Belser JA, Gustin KM, van Hoeven N, Zeng H, et al. (2012) Local innate immune responses and influenza virus transmission and virulence in ferrets. J Infect Dis 205: 474–485.
50. Jonges M, Bataille A, Enserink R, Meijer A, Fouchier RA, et al. (2011) Comparative analysis of avian influenza virus diversity in poultry and humans during a highly pathogenic avian influenza A(H7N7) outbreak. J Virol 85: 10598–10604.
51. Tweed SA, Skowronski DM, David ST, Larder A, Petric M, et al. (2004) Human illness from avian influenza H7N3, British Columbia. Emerg Infect Dis 10: 2196–2199.
52. CDC (2006) Interim Guidance for Protection of Persons Involved in U.S. Avian Influenza Outbreak Disease Control and Eradication Activities. In: Services HaH, ed. U.S. Dept. of Health and Human Services, Centers for Disease Control and Prevention.
53. CDC (2010) Guidelines and recommendations: Prevention strategies for seasonal influenza in healthcare settings. U.S. Dept. of Health and Human Services, Centers for Disease Control and Prevention.
54. Reed LJ, Muench HA (1938) A simple method of estimating fifty per cent endpoints. Am J Hyg 27: 493–497.
55. Chosewood LC, Wilson DE, Centers for Disease Control and Prevention (U.S.), National Institutes of Health (U.S.) (2009) Biosafety in microbiological and biomedical laboratories. Washington, D.C.: U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health. pp xxii415.
56. Belser JA, Gustin KM, Maines TR, Blau DM, Zaki SR, et al. (2011) Pathogenesis and transmission of triple-reassortant swine H1N1 influenza viruses isolated before the 2009 H1N1 pandemic. J Virol 85: 1563–1572.
57. Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, et al. (2007) Highly Pathogenic Avian Influenza H5N1 Viruses Elicit an Attenuated Type I Interferon Response in Polarized Human Bronchial Epithelial Cells. J Virol 81: 12439–12449.

58. Gillette TE, Chandler JW (1981) Immunofluorescence and histochemistry of corneal epithelial flat mounts: use of EDTA. Curr Eye Res 1: 249–253.
59. Perkins LE, Swayne DE (2001) Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet Pathol 38: 149–164.
60. OIE (2011) Avian Influenza. In: OIE, editor Terrestrial Animal Health Code 2011.

AVIAN DISEASES 56:464–470, 2012

Characterization of Live LaSota Vaccine Strain–Induced Protection in Chickens upon Early Challenge with a Virulent Newcastle Disease Virus of Heterologous Genotype

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Received 27 December 2011; Accepted 26 February 2012; Published ahead of print 27 February 2012

SUMMARY. Newcastle disease (ND) is a major threat to the international poultry industry, causing bird mortality, reduction in growth and egg production, and trade restrictions. The primary strategy available to the poultry industry to control virulent Newcastle disease virus (NDV), the causative agent of ND, is vaccination. LaSota and other commonly used live-virus NDV vaccine strains were developed in the 1950s and 1960s and show a great degree of genetic divergence from currently circulating NDV strains. In order to characterize protective immunity induced by LaSota against a heterologous NDV strain, we vaccinated groups of specific-pathogen-free (SPF) chickens with LaSota (virus titers ranging from 10^2 to 10^8 egg infective dose 50 [EID_{50}] in 10-fold increments) and challenged the birds 14 days later with ZJ1 strain, an NDV strain that was isolated in the year 2000 from geese in China. We monitored multiple parameters of immunity, including serum antibody titers, antigen-specific lymphocyte proliferation, and splenic cytokine expression and determined that SPF birds vaccinated with an adequate titer of LaSota strain live vaccine are fully protected from morbidity and mortality due to challenge with ZJ1 strain NDV, and we concluded that in the absence of interfering maternal antibody, protection due to vaccination increases with vaccine titer until a threshold titer is reached, beyond which, little or no further benefit can be elucidated.

RESUMEN. Caracterización de la protección inducida por la cepa vacunal viva LaSota en pollos durante un desafío temprano con un virus virulento de la enfermedad de Newcastle de genotipo heterólogo.

La enfermedad de Newcastle es una gran amenaza para la industria avícola internacional, ya que causa mortalidad de aves, reducción en el crecimiento y en la producción de huevos y restricciones comerciales. La estrategia primaria disponible para la industria avícola para controlar el virus virulento de la enfermedad de Newcastle, que es el agente causante de la enfermedad de Newcastle, es la vacunación. La cepa LaSota y otras cepas vacunales del virus de Newcastle de uso común se desarrollaron en los años 1950 y 1960 y muestran un alto grado de divergencia genética con relación a las cepas circulantes del virus de Newcastle. A fin de caracterizar la inmunidad protectora inducida por la cepa LaSota en contra de una cepa heteróloga del virus de Newcastle, se vacunaron grupos de aves pollos libres de patógenos específicos con LaSota (con títulos virales que van desde 10^2 hasta 10^8 dosis infectantes en embrión de pollo [EID50] con incrementos de 10 veces) y las aves se desafiaron 14 días después la cepa ZJ1 cepa, una cepa de Newcastle que se aisló en el año 2000 de gansos en China. Se hizo un seguimiento de varios parámetros de la inmunidad, incluyendo los títulos de anticuerpos séricos, la proliferación de linfocitos específica de antígeno y la expresión de citoquinas por los esplenocitos. Se determinó que las aves libres de patógenos específicos vacunados con un título adecuado de la vacuna LaSota están totalmente protegidas contra la morbilidad y la mortalidad debidas al desafío con la cepa ZJ1 y se llegó a la conclusión de que en ausencia de anticuerpos maternos que puedan interferir, la protección inducida por la vacunación puede aumentar con el título de la vacuna hasta que se alcanza un título límite, más allá del cual, no se observa un beneficio adicional.

Key words: Newcastle disease virus, NDV, LaSota, live-virus vaccination, ZJ1

Abbreviations: Ct = cycle threshold; EID = egg infective dose; F = fusion; HN = hemagglutinin-neuraminidase; M = matrix; ND = Newcastle disease; NDV = Newcastle disease virus; NP = nucleoprotein; nvNDV = neurotropic velogenic Newcastle disease virus; P = phosphoprotein; SPF = specific pathogen free; vvNDV = viscerotropic velogenic Newcastle disease virus

Newcastle disease (ND) is a continuing threat and challenge to the international poultry industry. ND causes major losses acutely by causing high levels of bird mortality, decreased growth, and reduced egg production, and chronically by restricting trade and by forcing producers to implement expensive measures to prevent disease spread. Since there is no effective treatment for ND, the poultry industry relies primarily on vaccination and biosecurity to control the disease (3,19,22). The causative agent of ND, virulent Newcastle disease virus (NDV), also known as avian paramyxovirus type 1, belongs to the *Avulavirus* genus within the *Paramyxoviridae* family. Virions have a single-stranded, negative-sense, nonsegmented RNA genome that codes for six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA polymerase (L) (3). The HN and F glycoproteins allow NDV to attach and fuse to the host cell membranes, thereby initiating infection. Antibodies against HN and F neutralize the virus and are a primary component of vaccine-induced protection versus NDV (21).

Newcastle disease virus strains have been categorized into different pathotypes based on the disease they produce in chickens. The most commonly used vaccine strains: LaSota, B1, and Ulster, are lentogenic strains that produce no or only mild clinical signs in adult birds. Lentogenic-strain live-virus vaccines have been used for more than 50 yr to provide protection from disease caused by mesogenic and velogenic strains (3,11,13). Mesogenic strains cause neurologic and respiratory disease but very little mortality. Viscerotropic velogenic strains (vvNDV) are characterized by acutely fatal disease, usually with hemorrhagic lesions in the digestive tract of infected birds; neurotropic velogenic strains (nvNDV) cause high mortality after severe respiratory and neurologic disease, generally in the absence of gut lesions (13). Both mesogenic and velogenic isolates are defined by the World Organisation for Animal Health as being virulent for the purposes of trade (15).

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Table 1. Primer sequences for quantitative reverse transcription PCR; previously published (7).

Gene	Primer sequences (5′-3′)	Product size (bp)
IFN γ	Forward: gtgaagaaggtgaaagatatcatgga Reverse: gctttgcgctggattctca	71
IL-6	Forward: gctcgccggcttcga Reverse: ggtaggtctgaaaggcgaacag	71
IL-2	Forward: ttggaaaatatcaagaacaagattcatc Reverse: tcccaggtaacactgcagagttt	93
IFN β	Forward: gcccacacactccaaaacactg Reverse: ttgatgctgaggtgagcgttg	150
B-actin	Forward: agaggctcccctgaaccccaaagc Reverse: ctggatggctacatacatggctgg	94

Based on whole genome sequence analysis, NDV shows two major class divisions (class I and II), and class II, the major class of interest to the poultry industry, is further divided into at least 10 genotypes (I–X) (13). The most widely used NDV vaccine strain, LaSota, belongs to genotype II, while the majority of circulating velogenic viruses in the world belong to genotypes V–VII (5,13). Although all NDV strains belong to the same serotype, the protection provided by genotype II vaccines against heterologous challenge has been subject to some controversy. Yu *et al.* (23) and Qin *et al.* (17) reported the presence of genotype VII antigenic variants that could produce disease in birds vaccinated with LaSota. Unfortunately, these cross-protection experiments lacked corroborating data about the immune status of the birds prior to and after challenge. Two other groups, Jeon *et al.* (8) and Liu *et al.* (12), demonstrated that commercial genotype II vaccine strains could provide complete protection against morbidity and mortality and reduce shedding of heterologous challenge virus strains (genotypes III, VIg, VIb, VIId, and IX). In order to characterize the immune response induced by LaSota vaccination and to examine how LaSota strain can protect against heterologous challenge, we vaccinated groups of specific-pathogen-free (SPF) chickens with different titers of LaSota strain virus and challenged them with ZJ1 (Goose/China/ZJ1/2000) strain NDV, a genotype VII strain of vvNDV isolated from geese in China (12). We monitored morbidity and mortality, measured NDV specific cell-mediated and humoral immune responses due to LaSota vaccination, and measured relative changes in splenic cytokine expression in response to challenge.

MATERIALS AND METHODS

Chickens. Mixed-sex, specific-pathogen-free (SPF) white Leghorn chickens were acquired from the Southeast Poultry Research Laboratory flocks. Prevaccination sera samples were taken from 10% of the birds prior to group randomization. Birds were housed, vaccinated, and challenged in Biological Safety Level (BSL) 3 Enhanced (E) facilities. All birds were housed in Horsfall isolation units with feed and water *ad libitum.*

Viruses. LaSota NDV strain Chicken/New Jersey (LaSota)/1946, of low virulence and commonly used as a vaccine, and the recombinant virulent NDV constructed in 2007 (rZJ1/2007) of the wild type NDV ZJ1 strain, originally isolated in 2000 (Goose/China/ZJ1/2000), were propagated and titrated in 9–11-day-old SPF chicken embryos via the chorioallantoic sac route.

Experimental design. Four-week-old SPF white Leghorn chickens were arbitrarily divided into nine treatment groups: 1) brain heart infusion–NC; 2) brain heart infusion–ZJ1; 3) 10^2 LaSota; 4) 10^3 LaSota; 5) 10^4 LaSota; 6) 10^5 LaSota; 7) 10^6 LaSota; 8) 10^7 LaSota; and 9) 10^8 LaSota. Groups 1 and 2 contained 10 birds each, and groups 3–9 had 20 birds each. Birds were vaccinated 3 days after group randomization. Groups 1 and 2 received 100 µl of brain heart infusion (BHI) via intranasal (50 µl IN) and eye drop (50 µl ED) routes. Birds in groups 3–9 received live LaSota virus suspended in BHI (50 µl IN and 50 µl ED), with group 3 receiving a target titer of 10^2 egg-infectious dose 50 (EID_{50}) per bird, and each group thereafter receiving a 10-fold higher dose. Birds were monitored daily for clinical signs of vaccine reaction (conjunctivitis, lethargy, and snicking). Four days after vaccination, oropharyngeal swabs were taken for virus isolation. Seven days after vaccination, whole blood was collected via wing-vein into ethylenediaminetetraacetic acid (EDTA)-containing syringes from four birds per group for lymphocyte proliferation assay.

At day 14 postvaccination, blood was collected via wing-vein for serology, and the birds in groups 2–9 were challenged with live ZJ1 strain NDV suspended in BHI with a target titer of $10^{6.5}$ EID_{50} per bird split evenly between oral and eye-drop routes. Birds in group 1 were given BHI without ZJ1 strain NDV (BHI NC) in the same amount and via the same route. Birds were monitored daily for overt clinical signs of ND (conjunctivitis, depression, edema, muscle tremors, torticollis, paralysis of wings and legs) and mortality. Birds displaying severe signs of disease, unable to eat and drink, were euthanized by cervical dislocation. Oropharyngeal swabs were taken on days 2 and 4 postchallenge, and cloacal swabs were taken on day 4 postchallenge for virus isolation. On day 3 postchallenge, two birds each from groups 1 and 2 and four birds each from groups 3–9 were euthanized by cervical dislocation, and their spleens were collected for RNA isolation. These birds were randomly chosen before challenge to prevent interference with morbidity/mortality and virus isolation data analysis. On day 14 postchallenge, all surviving birds were anesthetized with ketamine/xylazine solution, and blood was collected for serology via cardiac puncture just prior to euthanasia by cervical dislocation.

NDV enzyme-linked immunosorbent assay (ELISA). Serum was tested for antibodies against NDV with a commercial ELISA test (Flockcheck™ IDEXX Laboratories Inc., Westbrook, ME). Chicken serum samples were diluted 1:500 and incubated in kit-provided 96-well microtiter plates coated with NDV-antigen. The ELISA was performed according to manufacturer's instructions.

Virus isolation. Swabs were collected into 2 ml of BHI broth containing antibiotics (1000 units/ml penicillin G, 200 µg/ml gentamicin sulfate, and 4 µg/ml amphotericin B; Sigma-Aldrich, St. Louis, MO) and stored at −80 C. Virus isolation was performed in SPF embryonated chicken eggs following standard procedures (2).

Splenic cytokine expression. Changes in the splenic expression of interferon γ (IFNγ), interleukin 6 (IL-6), interleukin 2 (IL-2), and interferon β (IFNβ) were determined by quantitative reverse transcription PCR (qRT-PCR). Genes were chosen based on previous research showing these genes to be the major cytokines involved in the host immune response to NDV (7,20).

RNA isolation. Spleen cell suspensions were generated by pushing freshly isolated tissue through a 70-mM-pore-size mesh cup, immediately into Trizol (Invitrogen, Carlsbad, CA), and then samples were 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 (Qiagen, Valencia, CA). 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, Santa Clara, CA). All RNA samples had an RNA integrity number greater than 8.6, with a mean of 9.4.

Quantitative RT-PCR. Relative changes in splenic RNA expression of IFNγ, IL-6, IL-2, IFNβ, and β-actin were measured as changes in SYBR green fluorescence after amplification with specific primer sets, as in Ecco *et al.* (7) (Table 1). Reactions were done in triplicate on 20-µl reaction mixtures containing 60 ng of total RNA, 500 nM of each primer, 10 µl of 2X master mix SYBR, and 0.16 µl of enzyme mix (125X) (PowerSYBR® Green RNA-to-CT™ 1-Step Kit; Applied Biosystems, Carlsbad, CA). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following cycle profile: one cycle at 48 C for 30 min and 95 C for 15 min, followed by 40 cycles at 95 C for 15 sec

Table 2. Vaccine and challenge-virus back-titrations (EID_{50}/100 µl).

Target titer	Actual titer
Vaccine	
BHI[A]	0
10^2 LaSota	$10^{2.3}$
10^3 LaSota	$10^{3.1}$
10^4 LaSota	$10^{4.1}$
10^5 LaSota	$10^{5.5}$
10^6 LaSota	$10^{6.5}$
10^7 LaSota	$10^{7.1}$
10^8 LaSota	$10^{8.5}$
Challenge	
BHI	0
$10^{6.5}$ ZJ1	$10^{6.75}$

[A]BHI = brain heart infusion.

and at 58 C for 32 sec. A dissociation curve was added to the end of all reactions, and the melting curve was analyzed.

Quantification of changes in relative cytokine RNA expression. Relative changes in gene expression were calculated according to the Pflaffl method (16) using LinReg version 12 (18) to calculate individual primer efficiency by linear regression. All cycle threshold fluorescence (Ct) values were corrected to BHI-NC for each gene of interest and the endogenous control (β-actin).

Lymphocyte proliferation assay. Lymphocyte proliferation in response to ultraviolet (UV)-inactivated ZJ1 strain NDV, measured by AlamarBlue reduction, was used as an indicator of cell-mediated immunity.

Lymphocyte preparation. Whole blood samples (5 ml of blood anticoagulated with EDTA) were gently layered onto 5 ml of 63% Percoll gradient (3.15 ml of Percoll [Sigma-Aldrich]; 0.5 ml of 1.5 M NaCl solution, and 1.35 ml sterile distilled water) in a 15-ml conical tube. The conical tubes were centrifuged at 1250 × *g* for 20 min. The cells at the interface were collected and suspended in 5 ml of RPMI 1640 (Invitrogen) supplemented with 8% fetal bovine serum (Invitrogen), 2% chicken serum (Sigma-Aldrich), and 1% PenStrep (Invitrogen). The cell suspension was centrifuged at 400 × *g* for 4 min, and the supernatant was removed. The cells were resuspended at 1×10^6 cells/ml, seeded into six-well cell culture plates and incubated for 60 min at 41 C in a 5% CO_2 incubator. Nonadherent cells were removed, resuspended at 1×10^6/ml (viability by Trypan blue exclusion was 90–95%), aliquoted into 96-well cell culture plates (100 µl per well in nine wells), and incubated overnight.

Proliferation assay. After 24 hr, 100-µl aliquots of proliferation media, including either Concanavalin A (ConA, 12.5 µg/ml; Sigma-Aldrich) as a positive control, media as a negative control, or UV-inactivated ZJ1 strain NDV (titer prior to inactivation = $10^{6.5}$ EID_{50}/100 µl), were added in triplicate wells. Twenty-two microliters of AlamarBlue (Biosource International, Inc., Camarillo, CA) were added to all wells and three blank wells containing 200 µl of media alone. AlamarBlue reduction was measured after 48 hr of incubation as described (10).

Statistical analysis. All data analysis was performed using JMP version 9 software (SAS Institute, Raleigh, NC). Frequencies of virus isolation for treatment groups were compared by contingency analysis of means for proportions. One-way analysis of variance with the Tukey-Kramer method of means comparisons was used to compare group serum NDV antibody ELISA titers, relative changes in splenic cytokine expression, and AlamarBlue reduction. Differences in prechallenge and postchallenge serum ELISA titers were analyzed by Student *t*-test. Bivariate fit analysis was used to determine the correlation between relative changes in splenic cytokine expression and group serum NDV antibody ELISA titer. All tests were performed with a 5% level of significance.

RESULTS

To determine the titer of LaSota that prevents mortality and NDV shedding after early challenge with a heterogenic NDV strain,

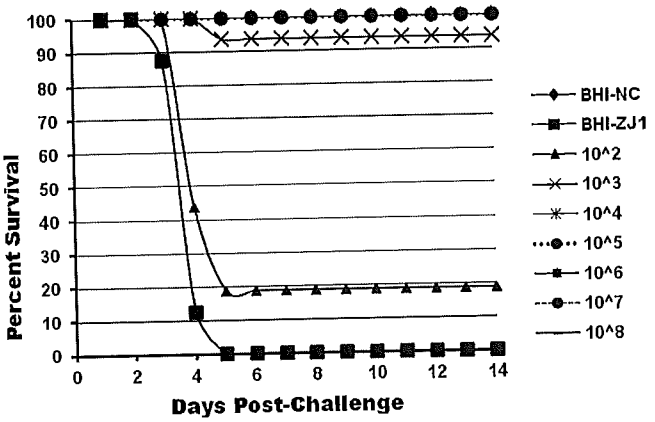

Fig. 1. Survival post-ZJ1 challenge. Chickens in each vaccine group were challenged at day 14 postvaccination with $10^{6.5}$ EID_{50} ZJ1 strain NDV or sham-challenged with brain heart infusion. Mortality was monitored for 14 days after challenge. BHI-NC indicates brain heart infusion sham-vaccinated, and sham-challenged (n = 8); BHI-ZJ1 indicates brain heart infusion, sham-vaccinated, and ZJ1 challenged (n = 8); all other groups were vaccinated with LaSota at indicated titer (EID_{50}) and challenged with ZJ1 (n = 16).

groups of 4-wk-old SPF white Leghorn chickens were inoculated with increasing doses of live LaSota NDV (virus titers ranging from 10^2 to 10^8 EID_{50}/100 µl) or BHI-control and challenged at day 14 postinoculation with $10^{6.5}$ EID_{50}/100 µl of wild-type ZJ1 (Table 2).

Morbidity and mortality. Birds were observed throughout the trial period for clinical signs of ND. No clinical signs of ND were observed in any birds prior to vaccination or challenge; however, one bird in group 3 died prior to vaccination. There were no clinical signs of vaccine reaction in any group (data not shown). After challenge, all birds in the ZJ1-challenged BHI-control (BHI-ZJ1) group displayed moderate to severe conjunctivitis and severe depression 2–4 days after ZJ1-inoculation, and 100% mortality was seen at day 5 postchallenge (Fig. 1). In the 10^2 LaSota group, all birds displayed moderate to severe conjunctivitis and severe depression at days 2–4 postchallenge, and 81.75% mortality was seen at day 5 postchallenge. Birds vaccinated with 10^3 LaSota virus displayed mild to moderate conjunctivitis and mild depression at days 3–4 postchallenge. At day 4 postchallenge, one bird in the 10^3 group was moderately depressed and recumbent and died on day 5 postchallenge. Birds in all other groups showed no clinical signs of ND after challenge.

Serum NDV antibody titers. Blood was collected for measurement of serum NDV antibody titer at day 14 postvaccination (prechallenge) and day 14 postchallenge (Table 3). Prechallenge, all birds inoculated with a LaSota titer of 10^3 EID_{50}/100 µl or less had negative serum NDV antibody ELISA titers (less than 300). Birds in all other groups had titers of 1500 or greater, with increases in prechallenge titer occurring in conjunction with increased vaccine virus titer. After challenge, birds in the 10^2, 10^3, and 10^4 LaSota groups seroconverted with statistically significant increases in serum titer ($p \leq 0.01$) of 7863 ± 450 (mean ± standard error of the mean), 5882 ± 1013, and 1732 ± 609, respectively. Birds in the remaining groups showed no significant increase in serum ELISA titer after challenge and had significantly lower postchallenge titers than birds in the 10^2 and 10^3 LaSota groups (all p-values < 0.05).

Virus shedding. Oral swabs were taken from all birds at day 4 postinoculation with LaSota and days 2 and 4 post–ZJ1-challenge (Table 4). Cloacal swabs were taken from all birds at day 4 postchallenge. All birds vaccinated with a LaSota titer of 10^4 EID_{50}/

Table 3. Serum NDV antibody ELISA titer.

Treatment group	No. pre/postchallenge	Prechallenge[A]	Postchallenge
BHI-no challenge	8/8	29.49 ± 12.52[E]	25.94 ± 8.08[D]
BHI-ZJ1 challenge	8/0	30.04 ± 10.10[E]	NS
10^2 LaSota	16/4	82.72 ± 38.76[E]	8069.44 ± 536.17[B]
10^3 LaSota	16/15	233.08 ± 62.76[E]	6492.83 ± 873.86[B]
10^4 LaSota	16/16	1713.55 ± 265.22[DE]	3446.22 ± 548.71[C]
10^5 LaSota	16/16	1528.63 ± 162.50[D]	2477.17 ± 428.05[C]
10^6 LaSota	16/16	3074.76 ± 531.19[C]	3188.83 ± 317.64[C]
10^7 LaSota	16/16	3031.59 ± 381.63[C]	4248.60 ± 604.77[BC]
10^8 LaSota	16/16	4167.05 ± 625.93[B]	3977.67 ± 571.37[C]

[A]Results are expressed as group mean serum ELISA titer ± standard error of the mean. Titers of 300 or greater are considered positive. Means within the same column with different superscripts (B–E) are significantly different by Tukey-Kramer ($P \leq 0.05$). BHI = brain heart infusion. NS = no survivors at time of blood collection.

100 µl or greater were shedding virus at day 4 postvaccination. Thirteen of 15 birds in the 10^3 LaSota and 1 of 16 birds in the 10^2 LaSota group were shedding virus at day 4 postvaccination. At day 2 postchallenge, all BHI-ZJ1 birds and 10^2 LaSota group birds were shedding virus. Significant numbers of 10^3 LaSota birds (13/15) were also shedding virus postchallenge. Large numbers of birds in groups vaccinated with a LaSota titer of 10^4 EID_{50}/100 µl or greater were shedding virus (range 3/16 to 8/16 birds); however, this was a statistically significantly lower incidence of shedding than was seen in the BHI-ZJ1, 10^2, and 10^3 LaSota birds. As on day 2 postchallenge, at day 4 postchallenge, significantly more birds in the BHI-ZJ1, 10^2, and 10^3 groups were shedding virus orally and cloacally than birds in groups vaccinated with 10^4 EID_{50}/100 µl LaSota or greater.

Splenic cytokine expression. At day 3 postchallenge, 4 birds per treatment group were euthanized, and their spleens were collected to measure changes in mRNA expression of IFNγ, IL-6, IL-2, and IFNβ (Table 5). The quality of RNA of spleens collected from birds vaccinated with 10^5 LaSota virus was too low to allow for analysis by qRT-PCR. IFNγ expression in the spleen was significantly up-regulated in the BHI-ZJ1 and the 10^2 LaSota groups (23.09- to 32.61-fold). IL-6 expression was also up-regulated in the BHI-ZJ1 and 10^2 LaSota groups, but to lesser extent than that seen with IFNγ (6.47- to 8.15-fold). There were significant inverse correlations between IFNγ expression and prechallenge serum NDV antibody titer ($R^2 = 0.652$, $P < 0.001$) and also between IL-6 expression and prechallenge ELISA titers ($R^2 = 0.596$, $P < 0.001$) (Fig. 2). Birds displaying low serum NDV antibody titers expressed higher levels of IFNγ and IL-6 mRNA in the spleen. There were mild increases (2.93- and 2.58-fold, respectively) in IL-2 expression in the 10^2 and the 10^3 LaSota groups postchallenge. IFNβ expression appeared to be up-regulated in all challenged groups. The 10^3 and 10^7 LaSota groups showed significant increases over BHI-NC (8.19- and 7.95-fold, respectively); the other groups showed very similar up-regulation (ranging from 3.87- to 7.23-fold), which was not statistically significant.

NDV-antigen-specific lymphocyte proliferation. To measure the cell-mediated immune response to vaccination, blood lymphocytes were isolated from four birds per treatment at day 7 postvaccination. Lymphocyte proliferation in response to ConA or UV-inactivated ZJ1 virus was measured by AlamarBlue reduction. Lymphocytes from all groups reduced AlamarBlue in response to ConA stimulation (range 4–6%, data not shown). There were no statistically significant differences in AlamarBlue reduced between groups; however, lymphocytes from birds that had been vaccinated with 10^4 EID_{50}/100 µl or greater tended to reduce less AlamarBlue in response to UV-inactivated ZJ1 than lymphocytes from birds vaccinated with less than 10^5 EID_{50}/100 µl (Fig. 3).

DISCUSSION

The major strategy employed by the poultry industry to control ND, along with stringent biosecurity, is vaccination. However, creating an effective vaccine strategy poses many challenges. For example, although all strains of NDV belong to the same serogroup, they display a great amount of genetic variability. Commercially available vaccine strains, such as LaSota, were derived from genotypes that were actively circulating during the 1940s and 1960s and are not closely related to the virulent NDV strains that are now in existence and currently in global circulation. Due to this large gap in amino acid homology between vaccine strain and

Table 4. Virus isolation in SPF embryonated chicken eggs.[A]

Treatment group	4 days after vaccination, oral swabs (positive/total)	2 days after ZJ1 challenge, oral swabs (positive/total)	4 days after ZJ1 challenge, oral swabs (positive/total)	4 days after ZJ1 challenge, cloacal swabs (positive/total)
BHI-no challenge	0/8[B]	0/8[B]	0/8[B]	0/8[B]
BHI-ZJ1 challenge	0/8[B]	8/8[C]	8/8[C]	7/8[C]
10^2 LaSota	1/16[B]	16/16[C]	16/16[C]	16/16[C]
10^3 LaSota	13/15[C]	13/15[C]	9/15[C]	9/15[C]
10^4 LaSota	16/16[C]	5/16[B]	3/16[B]	2/16[B]
10^5 LaSota	16/16[C]	5/16[B]	1/16[B]	0/16[B]
10^6 LaSota	16/16[C]	8/16[B]	0/16[B]	1/16[B]
10^7 LaSota	16/16[C]	3/16[B]	0/16[B]	0/16[B]
10^8 LaSota	16/16[C]	4/16[B]	0/16[B]	0/16[B]

[A]Proportions within the same column with different superscripts (B–C) are significantly different by analysis of means for proportions (chi-square, $P \leq 0.05$). BHI = brain heart infusion.

Table 5. Splenic cytokine expression 3 days after ZJ1 challenge.

Group	Fold-change splenic mRNA Expression			
	IFNγ[A]	IL-6	IL-2	IFNβ
BHI-ZJ1	32.61 ± 2.94[B]	8.15 ± 0.45[B]	2.38 ± 0.10[BCD]	6.61 ± 0.37[BC]
10^2 LaSota	23.09 ± 1.04[B]	6.47 ± 0.17[B]	2.93 ± 0.42[B]	5.37 ± 0.28[BC]
10^3 LaSota	11.28 ± 4.47[C]	3.05 ± 1.24[C]	2.58 ± 0.42[BC]	8.19 ± 1.34[B]
10^4 LaSota	2.64 ± 0.69[C]	1.43 ± 0.43[C]	0.97 ± 0.21[D]	6.25 ± 2.48[BC]
10^6 LaSota	8.47 ± 2.24[C]	2.85 ± 0.86[C]	2.08 ± 0.18[BCD]	7.23 ± 1.43[BC]
10^7 LaSota	4.96 ± 0.24[C]	1.66 ± 0.12[C]	1.14 ± 0.29[D]	7.95 ± 0.78[B]
10^8 LaSota	3.14 ± 0.84[C]	1.42 ± 0.06[C]	1.49 ± 0.08[CD]	3.87 ± 0.39[C]

[A]Data are presented as mean fold-change ± standard error of the mean. Fold-change was calculated relative to unchallenged BHI control using the Pflaffl method (16). Means within the same column with different superscripts (B–D) are significantly different by Tukey-Kramer ($P \leq 0.05$). BHI-ZJ1 = brain heart infusion control challenged with ZJ1 strain of NDV.

circulating virulent strains (12% to 20%), there are valid concerns about the ability of vaccine strains to provide adequate protection against challenge with novel variants of NDV.

In order to identify potential differences against heterologous challenge in protective immunity induced by LaSota, a commonly used NDV vaccine strain from genotype II, we vaccinated groups of SPF chickens with LaSota (virus titers ranging from 10^2 to 10^8 EID_{50} in 10-fold increments) and challenged the birds 14 days later with the genotype VII strain ZJ1. As was seen in a previous study (12), we determined that SPF birds properly vaccinated in a laboratory setting with an adequate titer of LaSota strain live-vaccine were fully protected from morbidity and mortality due to challenge with ZJ1 strain NDV, and we posit that in the absence of interfering maternal antibody, protection due to vaccination increases with vaccine titer until a threshold is reached, beyond which, little or no further benefit can be elucidated.

Birds vaccinated with titers higher than 10^4 EID_{50} LaSota were fully protected from morbidity and mortality, had a significantly lower incidence of virus shedding postchallenge, and had significantly higher prechallenge serum NDV antibody ELISA titers than unvaccinated birds and birds vaccinated with lower LaSota titers. Vaccine doses below 10^4 EID_{50} showed partial protection against ZJ1-challenge, with most birds in the 10^2 LaSota group and one of the birds in the 10^3 LaSota group dying postchallenge, and all birds in both groups displaying overt clinical signs of ND. Serum NDV antibody ELISA titers were also significantly lower in the 10^2 and 10^3 LaSota groups. ZJ1-specific cell-mediated immunity, as measured by antigen-specific *in vitro* lymphocyte proliferation, visibly increased in groups vaccinated with 10^5 EID_{50} LaSota or greater as compared to the birds in the 10^2, 10^3, and 10^4 LaSota groups. This disparity between humoral and cell-mediated immune data may indicate that humoral immunity due to neutralizing antibody is more important in the development of ND immunity, or it could be a function of differences in assay sensitivity (19). Surprisingly, hemagglutination-inhibition assays are typically performed to analyze antibody production after NDV infection or vaccination, even though they are only able to detect antibodies to the hemagglutinin-neuraminidase protein (3). We chose to use an ELISA system able to detect antibodies to the entire virus to evaluate the immune response, as they have a high degree of correlation with the HI results (4,6). Another method occasionally employed by researchers to measure humoral immunity is the serum neutralization assay. We were unable to perform a serum neutralization assay within the scope of this paper; however, the strong correlation between serum ELISA antibody titer and protection from challenge

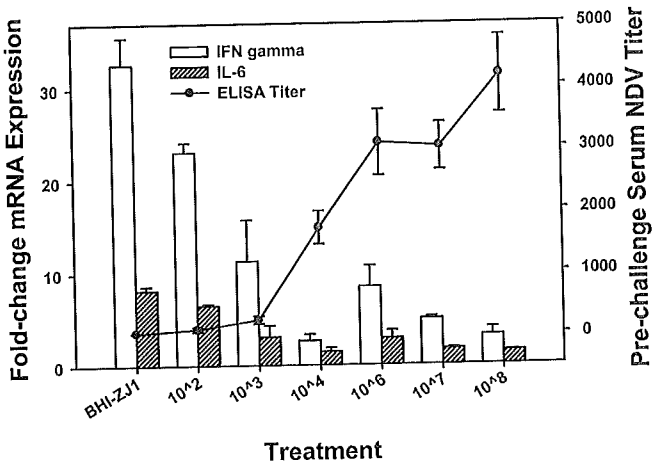

Fig. 2. Splenic interferon gamma (IFNγ) and interleukin 6 (IL-6) expression *vs.* serum NDV antibody ELISA titer. Relative changes in splenic cytokine expression (bars) were measured by quantitative reverse transcription PCR at day 3 post-ZJ1 challenge, and group serum NDV antibody titers (line) were measured by ELISA at day of challenge. Data are represented as group means ± standard error of the mean ($n = 4$).

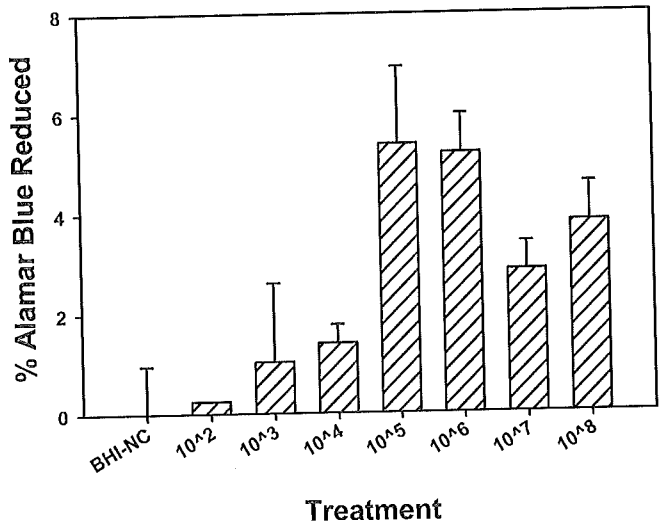

Fig. 3. Antigen-specific lymphocyte AlamarBlue reduction. Lymphocytes were isolated from EDTA-coagulated whole blood at day 7 postvaccination and stimulated to proliferate *in vitro* with UV-inactivated ZJ1-strain NDV (pre-inactivation titer = $10^{6.5}$ EID_{50}/100 μl). Proliferation data are represented as group mean percent AlamarBlue reduced ± standard error of the mean ($n = 4$).

suggests that serum from well-vaccinated chickens was able to neutralize invading virions.

Cytokines are produced by immune cells in order to coordinate the appropriate immune response to a pathogen. Traditionally, cytokines are separated into multiple categories: pro-inflammatory/innate cytokines (IFNα, IFNβ, IL-1β, and IL-6), T-helper-cell type 1 cytokines (IFNγ and IL-2), and T-helper-cell type 2 cytokines (IL-4 and IL-10). Pro-inflammatory cytokines, such as IL-1β and IL-6, are a major part of the innate immune response and are known to induce sickness behavior: anorexia, lethargy, and fever. IFNα and IFNβ are type I interferons and are the major mediators of the innate antiviral response within the host. The antiviral response occurs as a nonspecific reaction to virus infection and is characterized by decreased cell proliferation and increased display of intracellular antigens by all host cells in order to decrease virus spread and increase antiviral surveillance by the immune system. IL-2 is responsible for inducing the cell-mediated arm of the adaptive immune system, which aims to eliminate intracellular pathogens via the proliferation and activation of cytotoxic T cells. IFNγ also aids in cell-mediated immunity by activating macrophages and stimulating the innate antiviral response and by serving as a bridge between the innate and adaptive immune systems. T-helper-cell type 2 cytokines are responsible inducing a humoral immune response by inducing antigen-specific B cells to proliferate and secrete antibody (1,9).

Groups displaying overt clinical signs postchallenge (BHI-ZJ1-challenge control, 10^2, and 10^3) had up-regulated splenic expression of IFNγ and IL-6, while birds that were well-protected from challenge expressed relatively lower levels of IL-6 and IFNγ mRNA in the spleen. Furthermore, there was a significant negative correlation between serum NDV antibody ELISA titer and IFNγ/IL-6 expression, with a nearly linear decline in relative IL-6/IFNγ expression relating to a nearly linear increase in NDV antibody titers with increasing LaSota vaccine titers. This relationship between protection and IL-6/IFNγ expression is especially interesting since IL-6 and IFNγ have been implicated as key mediators of tissue damage, sickness behavior, and mortality due to "inflammatory cytokine storm" induction (7). Decreased expression in protected birds could indicate that the challenge virus could not penetrate the mucosa in a high enough amount to cause a systemic immune response. In other words, the local mucosal immune system has been sufficiently primed by live LaSota vaccine titers of at least 10^4 EID_{50} to allow for efficient clearance of the heterologous challenge virus and prevention of systemic disease. As opposed to IL-6/IFNγ, splenic expression of IL-2 and IFNβ did not show any clear pattern related to vaccine titer. However, the mild changes in splenic IL-2 expression tended to be in the less vaccinated groups. These results are similar to those seen in previous studies measuring splenic cytokine expression at 3 days postchallenge with virulent strains of NDV (7,20). It is possible that at a different postchallenge time point or in a different organ, such as the Harderian gland, a clearer pattern of IL-2 and IFNβ expression would be evident.

By using a range of LaSota virus titers to vaccinate SPF chickens against NDV challenge, we were able to elucidate a clear pattern of protection that related directly to the ability of the vaccine virus to proliferate at the site of infection. At titers below 10^4 EID_{50}, less than 100% of the vaccinated birds shed virus at the oral mucosa; this lower incidence of shedding was directly related to decreased protection from disease in these vaccinated groups, with 10^3 EID_{50} LaSota vaccinated birds having a higher incidence of shedding and a higher level of protection than the 10^2 EID_{50} LaSota group. As vaccine titer increased from 10^2 EID_{50} to 10^4 EID_{50}, there was a clear increase in vaccine-induced protection that is evident as a nearly linear increase in serum NDV antibody ELISA titers, a nearly linear decrease in splenic IL-6 and IFNγ expression, and an increase in antigen-specific lymphocyte proliferation.

At vaccine titers beyond 10^5 EID_{50}, serum antibody titers continued to rise; however, there was no change in the incidence of virus shedding, the lymphocyte proliferative response, or splenic cytokine expression. These data could suggest that serum antibody titer is the most sensitive method for measuring protection against NDV challenge, or they could suggest that beyond a certain serum antibody titer, no additional protection can be elucidated. This is in agreement with earlier studies that found neutralizing antibody principally functions in the prevention of infection during virus challenge, whereas cell-mediated immunity functions in the clearance of active infection (19,22). This may indicate that beyond a certain serum antibody titer, the mucosal surfaces become saturated and can no longer bind extra antibodies to prevent infection. If this is truly the case, then finding this "threshold" serum antibody titer that identifies protection would be of great value in the field, since unnecessarily high vaccine titers have been associated with high costs due to vaccine reaction (drop in growth and egg production) and vaccine costs. In this study, with SPF birds under experimental conditions, once an antibody titer of about 1500 was reached, the birds were completely protected from morbidity and mortality and exhibited little systemic immune response to challenge. Unfortunately, this pattern will not be as clear in the field, where problems due to vaccine mass-application techniques, comorbidity, and maternal antibody reduce the uniformity of a group immune response. It is also possible that changing the challenge dose could alter this minimum "threshold." A lower challenge dose could be more easily neutralized with fewer antibodies, and at a sufficiently high dose, no amount of antibody can truly prevent infection. Ideally, future experiments should test these interactions by using birds with different levels of maternal antibody crossed by different levels of live vaccine and challenge virus titer crossed by different methods of application.

As was found in previous studies, LaSota can protect against heterologous challenge (8,12). However, this study only examined LaSota-induced protection against one genetically divergent strain, and the possibility that new strains have evolved or will evolve antigenic divergence allowing for vaccine escape cannot be ignored. Nevertheless, disease due to the vast majority of NDV strains can be prevented by effective delivery of live LaSota vaccine; therefore, in addition to developing new strain-matched NDV vaccines to provide better protection and prevent shedding of challenge viruses (14), great effort should be focused on improving vaccine delivery, determining ideal levels of maternal and postvaccinal serum antibody titer, and determining causes of vaccine failure not related to genetic divergence in the field.

REFERENCES

1. Abbas, A. K., and A. H. Lichtman. Introduction to immunology. In: Cellular and molecular immunology, 5th ed. Elsevier Saunders, Philadelphia. pp. 1–40. 2005.

2. Alexander, D. Newcastle disease virus and other avian paramyxoviruses. In: Isolation and identification of avian pathogens. D. Swayne, J. Glisson, M. Jackwood, J. Pearson, and W. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 156–163. 1998.

3. Alexander, D. J., and D. A. Senne. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: Diseases of poultry, 12th ed. Y. M. Saif, ed. Blackwell, Ames, IA. pp. 75–115. 2008.

4. Brown, J., R. Resurreccion, and T. Dickson. The relationship between the hemagglutination-inhibition test and the enzyme-linked

immunosorbent assay for the detection of antibody to Newcastle disease. Avian Dis. 34:585–587. 1990.

5. Chong, Y. L., A. Padhi, P. J. Hudson, and M. Poss. The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. PLoS Pathog. 6:e1000872. 2010.

6. Cvelic-Cabrilo, V., H. Mazija, Z. Bidin, and W. Ragland. Correlation of hemagglutination inhibition and enzyme-linked immunosorbent assays for antibodies to Newcastle disease virus. Avian Pathol. 21:509–512. 1992.

7. Ecco, R., C. Brown, L. Susta, C. Cagle, I. Cornax, M. Pantin-Jackwood, P. J. Miller, and C. L. Afonso. *In vivo* transcriptional cytokine responses and association with clinical and pathological outcomes in chickens infected with different Newcastle disease virus isolates using formalin-fixed paraffin-embedded samples. Vet. Immunol. Immunopathol. 141:221–229. 2011.

8. Jeon, W.-J., E.-K. Lee, Y.-J. Lee, O.-M. Jeong, Y.-J. Kim, J.-H. Kwon, and K.-S. Choi. Protective efficacy of commercial inactivated Newcastle disease virus vaccines in chickens against a recent Korean epizootic strain. J. Vet. Sci. 9:295–300. 2008.

9. Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella gallinarum*. Microbiology 146:3217–3226. 2000.

10. Kapczynski, D. R. Evaluating the cell-mediated immune response of avian species to avian influenza viruses. Meth. Mol. Biol. 436:113–126. 2008.

11. Kapczynski, D. R., and D. J. King. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 23:3424–3433. 2005.

12. Liu, X. F., H. Q. Wan, X. X. Ni, Y. T. Wu, and W. B. Liu. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Arch. Virol. 148:1387–1403. 2003.

13. Miller, P. J., E. L. Decanini, and C. L. Afonso. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect. Gen. Evol. 10:26–35. 2010.

14. Miller, P. J., C. Estevez, Q. Yu, D. L. Suarez, and D. J. King. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 53:39–49. 2009.

15. [OIE] World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. In: Biological Standards Commission. World Organisation for Animal Health, Paris. pp. 576–589. 2009.

16. Pflaffl, M. A new mathematical model for relative quantification in real-time PCR. Nucl. Acids Res. 29:2002–2007. 2001.

17. Qin, Z.-M., L.-T. Tan, H.-Y. Xu, B.-C. Ma, Y.-L. Wang, X.-Y. Yuan, and W.-J. Liu. Pathotypical characterization and molecular epidemiology of Newcastle disease virus isolates from different hosts in China from 1996 to 2005. J. Clin. Microbiol. 46:601–611. 2008.

18. Ramakers, C., J. M. Ruijter, R. H. L. Deprez, and A. F. M. Moorman. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339:62–66. 2003.

19. Reynolds, D., and A. Maraqa. Protective immunity against Newcastle disease: the role of cell-mediated immunity. Avian Dis. 44:145–154. 2000.

20. Rue, C. A., L. Susta, I. Cornax, C. C. Brown, D. R. Kapczynski, D. L. Suarez, D. J. King, P. J. Miller, and C. L. Afonso. Virulent Newcastle disease virus elicits a strong innate immune response in chickens. J. Gen. Virol. 92:931–939. 2011.

21. Seal, B. S., D. J. King, and H. S. Sellers. The avian response to Newcastle disease virus. Develop. Comp. Immunol. 24:257–268. 2000.

22. Young, D., R. Randall, J. Hoyle, and B. Souberbielle. Clearance of persistent paramyxovirus infection is mediated by cellular immune responses but not serum neutralizing antibody. J. Virol. 64:5403–5411. 1990.

23. Yu, L., Z. Wang, Y. Jiang, L. Chang, and J. Kwang. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. J. Clin. Microbiol. 39:3512–3519. 2001.

ACKNOWLEDGMENTS

We wish to thank Timothy Olivier and Dawn Williams-Coplin for their excellent technical assistance and Roger Brock for his animal care assistance. This study was funded by USDA ARS CRIS number 6612-32000-049.

AVIAN DISEASES 56:128–133, 2012

Characterization of Newcastle Disease Viruses Isolated from Cormorant and Gull Species in the United States in 2010

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Received 23 August 2011; Accepted and published ahead of print 6 October 2011

SUMMARY. Newcastle disease virus (NDV), a member of the genus *Avulavirus* of the family *Paramyxoviridae*, is the causative agent of Newcastle disease (ND), a highly contagious disease that affects many species of birds and which frequently causes significant economic losses to the poultry industry worldwide. Virulent NDV (vNDV) is exotic in poultry in the United States; however, the virus has been frequently associated with outbreaks of ND in cormorants, which poses a significant threat to poultry species. Here, we present the characterization of 13 NDV isolates obtained from outbreaks of ND affecting cormorants and gulls in the states of Minnesota, Massachusetts, Maine, New Hampshire, and Maryland in 2010. All 2010 isolates are closely related to the viruses that caused the ND outbreaks in Minnesota in 2008, following the new evolutionary trend observed in cormorant NDV isolates since 2005. Similar to the results obtained with the 2008 isolates, the standard United States Department of Agriculture F-gene real-time reverse-transcription PCR (RRT-PCR) assay failed to detect the 2010 cormorant viruses, whereas all viruses were detected by a cormorant-specific F-gene RRT-PCR assay. Notably, NDV-positive gulls were captured on the eastern shore of Maryland, which represents a significant geographic expansion of the virus since its emergence in North America. This is the first report of vNDV originating from cormorants isolated from wild birds in Maryland and, notably, the first time that genotype V vNDV has been isolated from multiple wild bird species in the United States. These findings highlight the need for constant epidemiologic surveillance for NDV in wild bird populations and for consistent biosecurity measures to prevent the introduction of the agent into domestic poultry flocks.

RESUMEN. Caracterización de los virus de la Enfermedad de Newcastle aislados de Cormoranes y de gaviotas en los Estados Unidos en el 2010.

La enfermedad de Newcastle virus (NDV), un miembro del género *Avulavirus* de la familia *Paramyxoviridae*, es el agente causal de la enfermedad de Newcastle (ND), una enfermedad altamente contagiosa que afecta a muchas especies aviares y que con frecuencia causa importantes pérdidas económicas para la industria avícola en todo el mundo. La enfermedad de Newcastle virulenta (vNDV) es exótica para las aves comerciales en los Estados Unidos, sin embargo, el virus ha sido asociado con frecuencia a los brotes de enfermedad de Newcastle en cormoranes, lo que representa una amenaza significativa para las aves comerciales. En este trabajo se presenta la caracterización de 13 cepas del virus de Newcastle obtenidas de los brotes de la enfermedad de Newcastle que afectaron a los cormoranes y gaviotas en los estados de Minnesota, Massachusetts, Maine, New Hampshire, y Maryland en el año 2010. Todos los aislamientos del 2010 están estrechamente relacionados con el virus que causó los brotes de Newcastle en Minnesota en el año 2008, siguiendo la nueva tendencia de evolución observada en los aislamientos de Newcastle de cormoranes desde el 2005. De manera similar con los resultados obtenidos de los aislamientos del 2008, la técnica estándar de transcripción reversa y reacción en cadena de la polimerasa en tiempo real (RRT-PCR) para el gene de la proteína F, desarrollada por el Departamento de Agricultura de los Estados Unidos, no pudo detectar los virus de cormoranes del año 2010, mientras que todos los virus fueron detectados por un ensayo RRT-PCR para la proteína F específica de los virus de cormoranes. De manera sobresaliente, gaviotas positivas al virus de Newcastle fueron capturadas en la costa oriental de Maryland, lo que representa una expansión geográfica significativa del virus desde su aparición en América del Norte. Este es el primer repotte del aislamiento del virus de Newcastle de aves silvestres en Maryland y, sobre todo, es la primera vez que el genotipo V del virus de Newcastle ha sido aislado de varias especies de aves silvestres en los Estados Unidos. Estos resultados destacan la necesidad de una constante vigilancia epidemiológica para la enfermedad de Newcastle en las aves silvestres y de las medidas de bioseguridad constantes para prevenir la introducción del agente en las parvadas de aves comerciales.

Key words: Newcastle disease virus, NDV, Newcastle disease, cormorants, gulls, outbreak

Abbreviations: bp = base pairs; F = fusion protein; HI = hemagglutination-inhibition; ICPI = intracerebral pathogenicity index; L = large polymerase; M = matrix protein; ND = Newcastle disease; NDV = Newcastle disease virus; NP = nucleoprotein; OIE = World Organization for Animal Health; P = phosphoprotein; RRT-PCR = real-time reverse-transcription PCR; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen free; USDA = United States Department of Agriculture; V = V protein; vNDV = virulent Newcastle disease virus

Newcastle disease (ND) is a highly contagious disease that affects many species of birds and which frequently causes significant economic losses to the poultry industry worldwide (3). The causative agent of ND is Newcastle disease virus (NDV) or avian paramyxovirus type 1, a member of the genus *Avulavirus* of the family *Paramyxoviridae* (14). The NDV genome consists of a single-stranded, negative-sense RNA molecule of approximately 15.2 kb that contains six genes encoding

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seven proteins (nucleoprotein [N]; phosphoprotein [P]; V protein [V]; matrix protein [M]; fusion protein [F]; and large polymerase [L]) (14,20).

Newcastle disease is characterized by a wide range of clinical signs depending on the virulence of the virus strain and species of bird affected (15). Historically, NDV strains have been classified as velogenic (highly virulent), mesogenic (moderately virulent), and lentogenic (mildly virulent), based on the pathogenicity in chickens and chicken embryos (2,20). Velogenic strains are further divided into neurotropic and viscerotropic, with neurotropic strains causing respiratory and neurologic signs and viscerotropic strains causing hemorrhagic intestinal lesions (2,20). Virulent NDV (vNDV) outbreaks must be reported to the World Organisation for Animal Health (OIE). The OIE defines ND as an infection caused by an avian paramyxovirus type 1 that has an intracerebral pathogenicity index (ICPI) in 1-day-old chickens of 0.7 or greater, has multiple basic amino acid residues (at least three residues) at the C-terminus of the fusion (F) protein cleavage site (residues 113–116) and a phenylalanine residue at position 117 (17). All velogenic and some mesogenic strains of NDV are considered virulent for chickens.

NDV is known for its ability to infect many species of birds with more than 250 species in 27 orders being susceptible (3). NDV infections in wild birds represent a constant threat to the poultry industry and pose significant difficulties to disease control strategies (3). Although infections of wild birds are usually subclinical with most NDV isolates being lentogenic, outbreaks of velogenic NDV have been frequently reported in cormorants in several regions of the world including Canada and northern states of the United States (7,9,13,18). In this species, the disease is characterized mainly by neurologic signs including head tremors, ataxia, and paralysis of the legs and wings, circling, and in some cases blindness (12,13). High mortality rates (up to 92%) are observed in juvenile birds with adult birds being more resistant to the disease, although paresis and other neurologic signs have been reported (7,11).

NDV has been associated with cormorants and gulls since 1949, when hemagglutination-inhibition (HI) antibodies were detected in birds captured in Scotland (5). In North America, the first outbreak of NDV in cormorants and gulls was reported in 1975 in Canada (8) and then in 1990 in the provinces of Alberta, Saskatchewan, and Manitoba (26). In 1992, the disease was observed for the first time in cormorants in the United States, resulting in the death of approximately 20,000 double-crested cormorants (*Phalacrocorax auritus*) in seven northern states and in one province of Canada (7). Since then, vNDV has been repeatedly reported in cormorants in United States and Canada (7,9,12,13,18), indicating that it became established in this species (3,7). Notably, in the United States, pigeons and cormorants represent the only known natural reservoirs of vNDV in wild bird populations (3,16); therefore, it is important to continually evaluate the evolution and pathogenicity of NDV isolates obtained from these species.

Here, we present the characterization of 13 NDV isolates obtained from outbreaks of ND that occurred in 2010 in the states of Minnesota, Massachusetts, Maine, New Hampshire, and Maryland. NDV isolates were obtained from double-crested cormorants, great cormorants (*Phalacrocorax carbo*), herring gulls (*Larus argentatus*), and great black-backed gulls (*Larus marinus*). Detection of NDV in herring and great black-backed gulls in Maryland represents a significant geographic and host range expansion for the virus in wildlife (22). This is the first report of vNDV originating from cormorants isolated from wild birds in Maryland and, notably, the first time that genotype V vNDV has been isolated from multiple wild bird species in the United States. These observations highlight the need for constant epidemiologic surveillance for NDV in wild bird populations and for consistent biosecurity measures to prevent the introduction of the agent into domestic poultry flocks.

MATERIALS AND METHODS

Viruses and virus isolation. Thirteen NDV isolates were obtained from the United States Department of Agriculture (USDA) Southeast Poultry Research Laboratory (SEPRL) repository. The viruses were isolated from oral and/or cloacal swabs samples collected during outbreaks of ND affecting cormorants (double-crested and great cormorants) and gulls (herring and black-backed gulls) in the states of Minnesota, Massachusetts, Maine, New Hampshire, and Maryland in 2010. Viruses were isolated from swab samples by standard virus isolation in specific-pathogen-free (SPF) embryonated chicken eggs (2).

ICPI test. The pathogenicity of nine cormorant–gull NDV isolates was assessed by using the ICPI test (2). One-day-old SPF chickens were inoculated intracerebrally with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored during an 8-day observation period and scored as normal (score = 0), sick or paralyzed (score = 1), and dead (score = 2). Total scores were determined and the mean daily scores were calculated to obtain the ICPI (2).

RNA extraction and real-time RT-PCR (RRT-PCR). All NDV isolates (n = 13) were propagated in SPF embryonated chicken eggs. Total RNA was extracted from allantoic fluid using TRIzol® LS (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All isolates were tested using the USDA standard F-gene RRT-PCR assay (25) and a cormorant-specific F-gene RRT-PCR assay (18).

Sequencing of the fusion gene. The complete F-gene coding sequences or a 374-base pair (bp) region of the F-gene (1) were amplified from the genome of all NDV isolates by using the Superscript™ III One-step RT-PCR kit (Invitrogen) and F-gene-specific primers (1,25). PCR reactions were subjected to electrophoresis in 1% agarose gels and the DNA bands were excised from the gel and purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

All sequencing reactions were performed with fluorescent dideoxynucleotide terminators in the ABI 3700 automated sequencer (Applied Biosystems Inc., Foster City, CA). Sequence editing and assembly was performed with LaserGene sequence analysis software package (LaserGene, version 5.07; DNAStar, Inc., Madison, WI).

Phylogenetic analysis. A 373-bp region of the F-gene (1) was used to construct the phylogenetic trees and to localize the 2010 cormorant–gull NDV isolates (n = 13) among other class II genotype reference sequences (n = 67). Phylogenetic analysis was performed by using MEGA4 software (MEGA, version 4) (23). The evolutionary distances were inferred by using the neighbor-joining method (19) with 100 bootstrap replicates and included the first, second, and third coding and noncoding positions.

Accession numbers. The sequences of the F-gene of the NDV isolates characterized in the present study are available on GenBank under the accession numbers JN255784, JN255773, JN255774, JN255775, JN255776, JN255777, JN255778, JN255779, JN255780, JN255781, JN255782, JN255783, JN255784, JN255785.

RESULTS

During the 2010 cormorant–gull NDV outbreaks in Minnesota, Massachusetts, Maine, New Hampshire, and Maryland, the USDA SEPRL received 62 samples (oral and/or cloacal swabs) collected from various avian species including double-crested cormorants (*Phalacrocorax auritus*; n = 31), great cormorants (*Phalacrocorax carbo*; n = 2), great black-backed gulls (*Larus marinus*; n = 15), herring gulls (*Larus argentatus*; n = 2), and mallards (*Anas platyrhynchos*; n = 1). NDV was isolated or detected by RRT-PCR in 32 (62.7%) of these samples, including samples from double-crested and great cormorants and from great black-backed and herring gulls. Thirteen of these isolates were characterized in the present study. All isolates were subjected to

Table 1. Virulent Newcastle disease viruses isolated from cormorants and gulls in the United States in 2010.

SEPRL no.	GenBank Accession no.	Date collected	Species	Location	Fusion protein RRT-PCR: USDA F-test	Fusion protein RRT-PCR: Cormorant F-test	Sequencing	Fusion protein cleavage site aa sequence[A]	ICPI
648	JN255784	08/26/2010	DCCO[B]	Leech Lake, MN	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.43
649	JN255773	08/26/2010	DCCO	Leech Lake, MN	−	+	374 region	^{110}R-G-K-R-Q-K-R-F^{117}	ND[C]
650	JN255774	09/11/2010	DCCO	Barnstable County, MA	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.55
651	JN255775	06/13/2010	DCCO	Salisbury, MA	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.51
652	JN255785	09/17/2010	DCCO	Portsmouth, NH	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.39
653	JN255776	09/15/2010	DCCO	Barnstable County, MA	−	+	374 region	^{110}R-G-K-R-Q-K-R-F^{117}	ND
654	JN255777	09/17/2010	GRCO[D]	Seabrook, NH	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	ND
655	JN255778	09/2010	GBBG[E]	Poplar Island, MD	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.38
656	JN255779	09/2010	HERG[F]	Poplar Island, MD	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.39
657	JN255780	09/2010	GBBG	Poplar Island, MD	−	+	374 region	^{110}R-G-K-R-Q-K-R-F^{117}	ND
658	JN255781	10/02/2010	DCCO	Portland, ME	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.45
659	JN255782	10/11/2010	DCCO	Kennebunk, ME	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	1.53
660	JN255783	09/18/2010	DCCO	Lewiston, ME	−	+	Full fusion	^{110}R-G-K-R-Q-K-R-F^{117}	ND

[A]Fusion protein cleavage site amino acid residues 110 to 117.
[B]DCCO = double-crested cormorant (*Phalacrocorax auritus*).
[C]ND = not determined.
[D]GRCO = great cormorant (*Phalacrocorax carbo*).
[E]GBBG = great black-backed gull (*Larus argentatus*).
[F]HERG = herring gull (*Larus marinus*).

phylogenetic analysis to determine the evolutionary distances to other NDV strains and eight isolates were further characterized for their pathogenicity in 1-day-old chickens by standard ICPI tests.

Clinical signs observed in birds affected by ND during the 2010 outbreaks included torticollis, paralysis of the legs and wings, tremors, and lack of muscular coordination. Approximately 1100 birds died during these outbreaks and among the affected species were double-crested cormorants, great cormorants, black-backed gulls, and herring gulls. Other avian species such as ring-billed gulls, mallards, egrets, terns, and American white pelicans were found dead (22); however, no evidence of NDV infection was detected in these species (data not shown). A summary with the information about the NDV isolates characterized here is presented in Table 1.

To assess the pathogenicity of the 2010 cormorant NDV isolates, eight viruses were subjected to the ICPI test in 1-day-old chickens. All viruses presented ICPI values between 1.38 and 1.55 (Table 1), which is typical for viruses isolated from cormorants (18), and characterize them as vNDV based on the OIE standards. Additionally, all viruses presented a virulent fusion protein cleavage site with three basic amino acids at the C-terminus of the protein (positions 113–116) and a phenylalanine residue at position 117 (Table 1) (17).

All isolates were tested by the USDA-validated RRT-PCR F-gene assay (25) and by the cormorant RRT-PCR assay (18). Similar to the results obtained with the 2008 cormorant isolates (18), the USDA-validated F-gene assay failed to detect all 2010 isolates, whereas all isolates were detected by the cormorant F-gene RRT-PCR assay (Table 1).

The evolutionary distances between the 2010 cormorant isolates and other NDV strains were assessed by phylogenetic analysis. A 373-bp region of the F-gene (1) was used for the phylogenetic analysis of 13 isolates from 2010 and other reference strains (n = 67). All 2010 cormorant isolates clustered with the 2008 cormorant isolates within the class II genotype V group (Fig. 1). The nearest ancestors of the 2010 viruses are the viruses G/2008/US/MN/284019481, G/2008/US/MN/284019485, and G/2008/US/MN/284019495 obtained in the 2008 cormorant ND outbreak in Minnesota. Both 2008 and 2010 viruses are closely related to a virus isolate obtained during an outbreak in Nevada in 2005 (cormorant/2005/US/NV/19529-04), suggesting a common ancestor for these viruses (Fig. 1). Phylogenetic analysis of the complete coding sequences of the F-gene revealed a similar distribution of the viruses in the phylogenetic tree, with the 2010 isolates clustering with the 2008 viruses within the class II genotype V group (data not shown).

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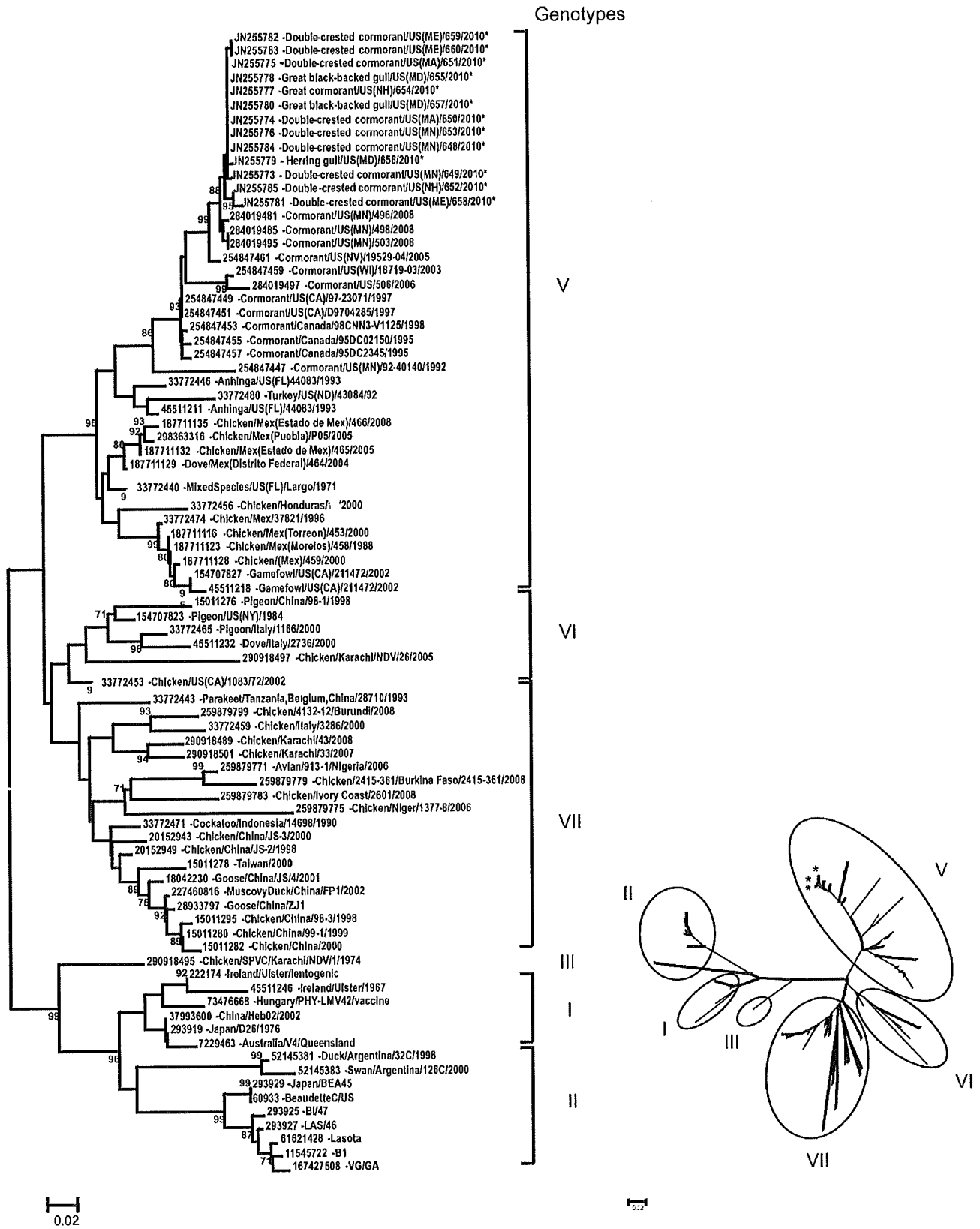
Fig. 1. Phylogenetic analysis of the 2010 cormorant NDV isolates and other reference strains on GenBank. A 373-bp region of the F-gene was used to construct the phylogenetic tree and to localize the 2010 isolates among other NDV strains. The evolutionary history was inferred using the neighbor-joining method (19). The optimal tree with the sum of branch length = 2.27030740 is shown. The percentage of replicate trees in which the associated taxa



clustered together in the bootstrap test (100 replicates) is shown next to the branches (6). 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 maximum composite likelihood method (24) and are in the units of the number of base substitutions per site. 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 14,903 positions in the final dataset. Phylogenetic analysis was conducted in MEGA4 (23). * = NDV isolates obtained during the 2010 outbreak.

DISCUSSION

In this study we present the characterization of 13 NDV isolates obtained during outbreaks of ND that affected cormorants and gulls in the states of Minnesota, Massachusetts, Maine, New Hampshire, and Maryland in 2010. Notably, NDV-positive gulls were captured on the eastern shore of Maryland, 60 mi away from commercial poultry houses that are part of the Delmarva Poultry Industry. This represents the first report of NDV isolation from wild birds in Maryland, indicating a significant eastward expansion of the virus since its emergence in North America (22).

NDV emerged in cormorants in North America in 1990 (26), and the first occurrence of the disease in cormorants in the United States was in 1992 (7). Since then, vNDV has been frequently associated with outbreaks of ND in cormorants, leading to variable mortality rates (<1 to 92%) in juvenile birds (7,9,12,13,18). The periodic occurrence of ND in cormorants indicates that vNDV became established in this species (7). Indeed, pigeons and cormorants are the only known natural reservoirs of vNDV in the wild in the United States (3,16), highlighting the importance of constant epidemiologic surveillance and proactive characterization of NDV isolates circulating in these species.

The pathogenicity of cormorant NDV isolates has not changed since its emergence in this species in 1949 (5), with all characterized isolates being classified as mesogenic or velogenic neurotropic viruses (7,9,12,13,18). The isolates characterized here presented ICPI values ranging from 1.38 to 1.55, which is consistent with vNDV strains (3,17). The clinical signs (torticollis, paralysis of the legs and wings, tremors, and lack of muscular coordination) observed in affected birds during the 2010 outbreaks (22) are consistent with the involvement of the central nervous system and further suggest that these viruses belong to the neurotropic NDV pathotype. However, the possibility that some of these isolates belong to the viscerotropic pathotype cannot be formally excluded.

Virulent NDV is exotic in poultry in the United States (3), but the frequent association of the virus with outbreaks in cormorants poses a significant threat to the poultry industry. Here, vNDV was isolated from wild birds (gulls) collected in close proximity (~60 miles) to the Delmarva poultry industry in Maryland, which is of great concern given the high concentration of poultry in this area. Isolation of vNDV from gulls represents an additional risk to poultry flocks, given that these birds are often found in poultry farms (10) and, therefore, could potentially introduce the virus into poultry flocks. Although of rare occurrence, there is strong evidence indicating that vNDV has been transmitted from cormorants to a turkey flock in North Dakota in 1992 (9,21), which highlights the importance of consistent biosecurity measures to prevent the introduction of vNDV, circulating in wild bird populations, into commercial poultry flocks.

Phylogenetic analysis revealed that the 2010 cormorant NDV isolates are closely related to the NDV strains that caused the outbreak in Minnesota in 2008 (18) with all 2010 isolates clustering with the 2008 viruses in the class II genotype V group (Fig. 1). Notably, since the emergence of ND in cormorants in North America, all viruses have been classified in the same genetic lineage of genotype V, suggesting co-evolution of the virus with this particular host species. Analysis of the nucleotide sequences of the F-gene cleavage site revealed that the evolutionary changes observed in the 2008 viruses, which led to failure of the USDA F-gene RRT-PCR assay in detecting the cormorant viruses (18), are also present in the 2010 viruses. Indeed, the USDA-validated F-gene RRT-PCR assay failed to detect the 2010 isolates, whereas all viruses were detected by the cormorant F-gene RRT-PCR assay (18). These findings suggest that the 2010 isolates may have originated from the 2008 viruses or that the two outbreaks may have been caused by the same epizootic virus.

Although vNDV has been frequently associated with outbreaks of ND in cormorants, the epidemiology of NDV infection in this species is still not completely understood. It has been hypothesized that adult birds may play a critical role in maintaining NDV in cormorant populations (4). Adult and older juvenile birds, which are resistant to clinical ND, may carry NDV subclinically, thus spreading the virus to birds from distinct geographic areas during wintering (4). Birds subclinically infected in wintering grounds reinitiate the disease cycle in the nesting sites after spring migration (4). This dynamic cycle of NDV in cormorants may partially explain the eastward expansion of the virus during the 2010 outbreaks when compared to the 2008 outbreaks. It is possible that adult birds subclinically infected during the 2008 outbreaks in Minnesota have spread the virus, during wintering, to birds from eastern nesting sites, thereby leading to the 2010 outbreaks in Massachusetts, Maine, New Hampshire, and Maryland. However, a complete epidemiologic–surveillance study is needed to draw definitive conclusions about the source of the 2010 outbreaks.

In summary, the 2010 cormorant NDV isolates are closely related to the 2008 viruses, following the new evolutionary trend observed in cormorant NDV isolates since 2005. Although such evolutionary changes had no apparent effect on virus virulence, they are responsible for the failure of the USDA-validated F-gene RRT-PCR assay to detect the cormorant and gull viruses, making necessary the use of a cormorant-specific F-gene RRT-PCR assay (18) for the diagnosis of NDV in these species. Isolation of virulent NDV from multiple wild bird species (cormorants and gulls) and in close proximity with the Delmarva poultry industry in Maryland poses a significant threat to poultry species and highlights the need for constant epidemiologic surveillance for NDV in wild bird populations and for consistent biosecurity measures to prevent the introduction of the agent into commercial poultry flocks.

REFERENCES

1. Aldous, E. W., J. K. Mynn, J. Banks, and D. J. Alexander. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32:239–256. 2003.

2. Alexander, D. J. Newcastle disease and other avian paramyxoviruses. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. International Book Distributing Co., Tallahassee, FL. pp. 156–163. 2006.

3. Alexander, D. J., and D. A. Senne. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Blackwell Publishing, Ames, IA. pp. 75–98. 2008.

4. Allison, A. B., N. L. Gottdenker, and D. E. Stallknecht. Wintering of neurotropic velogenic Newcastle disease virus and West Nile virus in double-crested cormorants (Phalacrocorax auritus) from the Florida Keys. Avian Dis. 49:292–297. 2005.

5. Blaxland, J. D. Newcastle disease in shags and cormorants and its significance as a factor in the spread of the disease among domestic poultry. Vet. Rec. 63:731–733. 1951.

6. Felsenstein, J. Confidence limits on phylogenesis: an approach using the bootstrap. Evolution 39:783–791. 1985.

7. Glaser, L. C., I. K. Barker, D. V. Weseloh, J. Ludwig, R. M. Windingstad, D. W. Key, and T. K. Bollinger. The 1992 epizootic of Newcastle disease in double-crested cormorants in North America. J. Wildl. Dis. 35:319–330. 1999.

8. Heckert, R. A. Ontario. Newcastle disease in cormorants. Can. Vet. J. 34:184. 1993.

9. Heckert, R. A., M. S. Collins, R. J. Manvell, I. Strong, J. E. Pearson, and D. J. Alexander. Comparison of Newcastle disease viruses isolated from

cormorants in Canada and the USA in 1975, 1990 and 1992. Can. J. Vet. Res. 60:50–54. 1996.

10. Kuiken, T. Review of Newcastle disease in cormorants. J. Waterbirds Soc.; Waterbirds 22:333–347. 1999.

11. Kuiken, T., D. Frandsen, and A. Clavijo. Newcastle disease in cormorants. Can. Vet. J. 39:299. 1998.

12. Kuiken, T., F. A. Leighton, G. Wobeser, K. L. Danesik, J. Riva, and R. A. Heckert. An epidemic of Newcastle disease in double-crested cormorants from Saskatchewan. J. Wildl. Dis. 34:457–471. 1998.

13. Kuiken, T., G. Wobeser, F. A. Leighton, D. M. Haines, B. Chelack, J. Bogdan, L. Hassard, R. A. Heckert, and J. Riva. Pathology of Newcastle disease in double-crested cormorants from Saskatchewan, with comparison of diagnostic methods. J. Wildl. Dis. 35:8–23. 1999.

14. Lamb, R. A., and G. D. Parks. Paramyxoviridae: the viruses and their replication. In: Field's virology, 5th ed. D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus, eds. Lippincott Williams & Wilkins, Philadelphia. pp. 1449–1496. 2007.

15. McLachland, N. J., and E. J. Dubovi. Paramyxoviridae. In: Fenner's veterinary virology, 4th ed. N. J. McLachland, and E. J. Dubovi, eds. Academic Press, London. pp. 299–325. 2011.

16. Miller, P. J., and C. L. Afonso. Newcastle disease virus. In: Encyclopedia of life sciences. John Wiley and Sons, Ltd., Chichester, U.K. pp. 1–9. 2011.

17. [OIE] World Organisation for Animal Health. Newcastle Disease. In: Manual of diagnostic tests for terrestrial animals: mammals, birds and bees, 7th ed. OIE, Paris, France. pp. 576–589. 2009.

18. Rue, C. A., L. Susta, C. C. Brown, J. M. Pasick, S. R. Swafford, P. C. Wolf, M. L. Killian, J. C. Pedersen, P. J. Miller, and C. L. Afonso. Evolutionary changes affecting rapid identification of 2008 Newcastle disease viruses isolated from double-crested cormorants. J. Clin. Microbiol. 48:2440–2448. 2010.

19. Saitou, N., and M. Nei. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425. 1987.

20. Samal, S. K. Paramyxoviruses of animals. In: Encyclopedia of virology, 3rd ed. Lippincott Williams and Wilkins, Philadelphia. pp. 40–47. 2008.

21. Seal, B. S. Analysis of matrix protein gene nucleotide sequence diversity among Newcastle disease virus isolates demonstrates that recent disease outbreaks are caused by viruses of psittacine origin. Virus Genes 11:217–224. 1995.

22. Sleeman, J. Summary of 2010 Newcastle disease virus outbreaks in wild birds in upper Midwest and the Northeast. Wildl. Health Bull. 2010:1–3. 2010.

23. Tamura, K., J. Dudley, M. Nei, and S. Kumar. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599. 2007.

24. Tamura, K., M. Nei, and S. Kumar. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101:11030–11035. 2004.

25. Wise, M. G., D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, D. R. Kapczynski, and E. Spackman. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Clin. Microbiol. 42:329–338. 2004.

26. Wobeser, G., F. A. Leighton, R. Norman, D. J. Myers, D. Onderka, M. J. Pybus, J. L. Neufeld, G. A. Fox, and D. J. Alexander. Newcastle disease in wild water birds in western Canada, 1990. Can. Vet. J. 34:353–359. 1993.

ACKNOWLEDGMENTS

We thank Dawn Williams-Coplin and Tim Olivier for technical assistance and the SEPRL sequencing facility personnel for nucleotide sequencing.

Infection, Genetics and Evolution xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes

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ARTICLE INFO

Article history:
Received 20 April 2012
Received in revised form 11 July 2012
Accepted 12 July 2012
Available online xxxx

Keywords:
APMV-1
NDV
Newcastle disease
Evolution
Epidemiology
Genotyping

ABSTRACT

The avian paramyxovirus type 1 (APMV-1), or Newcastle disease virus (NDV), comprise a diverse group of viruses with a single-stranded, negative-sense RNA genome. Historically, two systems have been simultaneously used to classify NDV isolates into lineages or genotypes, generating confusion in the nomenclature and discrepancies in the assignment of genetic groups. In the present study we assessed the genetic diversity of the avian paramyxovirus type-1 (APMV-1) and propose a unified nomenclature and a classification system based on objective criteria to separate NDV into genotypes. Complete F gene sequences of class I ($n = 102$) and class II ($n = 602$) viruses were used for the phylogenetic reconstruction and to identify distinct taxonomic groups. The mean interpopulational evolutionary distance was estimated (10%) and set as the cutoff value to assign new genotypes. Results of our study revealed that class I viruses comprise a single genotype, while class II contains 15 genetic groups including 10 previously established (I–IX, and XI) and five new genotypes (X, XII, XIII, XIV and XV). Sub-genotypes were identified among class I and class II genotypes. Adoption of a unified nomenclature and of objective criteria to classify NDV isolates will facilitate studies on NDV epidemiology, evolution, disease control and diagnostics.
Published by Elsevier B.V.

1. Introduction

Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1), is a member of the genus *Avulavirus* of the family *Paramyxoviridae* (Alexander and Senne, 2008). The APMV type 1 comprises a diverse group of viruses with a single-stranded, negative-sense RNA genome (Alexander and Senne, 2008). As for other RNA viruses, the evolutionary dynamics of NDV is determined mainly by positive and negative selection and by the inherent error rate of the viral RNA dependent RNA polymerase (Holland et al., 1982; Chare et al., 2003; Miller et al., 2009b). While selection and polymerase errors are the main forces driving the evolution of NDV, it has been suggested that recombination may also play a role in shaping the genetic structure of the APMV type 1 (Han et al., Q2 2008; Qin et al., 2008a,b; Miller et al., 2009b; Zhang et al., 2010).

Since the emergence of NDV in 1926, several genetic groups (lineages or genotypes) have been identified among the APMV-1(Aldous et al., 2003; Cattoli et al., 2010; Miller et al., 2010), and phylogenetic studies have shown that viruses of different genetic groups undergo simultaneous evolutionary changes in different geographic locations of the world (Miller et al., 2010, 2009b). This evolutionary dynamics impose significant difficulties for disease control and diagnosis (Cattoli et al., 2010; Kim et al., 2007; Miller et al., 2010; Rue et al., 2010). It has been shown, for example, that genotype-heterologous vaccines are less effective in preventing virus replication and shedding after challenge (Miller et al., 2007, 2009a). Therefore, genotype-homologous vaccines have been developed and are available in countries that experience significant economical burdens due to ND outbreaks (Hu et al., 2009, 2011; IASA – Investigación Aplicada S.A. de C.V., 2012). Evolutionary changes on the NDV genome have also been implicated in the failure of standard diagnostic tests to detect new genetic variants of the virus (Cattoli et al., 2010; Diel et al., 2011; Khan et al., 2010; Rue et al., 2010), requiring the development of new diagnostic tests to effectively detect variant viruses. These observations highlight the importance of constant epidemiologic and molecular surveillance for NDV.

Historically, NDV isolates have been classified into lineages or genotypes based on the phylogenetic analysis of the partial or complete nucleotide sequences of the fusion (F) gene (Aldous et al., 2003; Cattoli et al., 2010; Czeglédi et al., 2006; Kim et al., 2007, 2008; Liu et al., 2003; Perozo et al., 2008; Snoeck et al., 2009). The lineage classification, was defined by Aldous and collaborators, and initially grouped NDV isolates into six lineages (1–6) and 13 sub-lineages (Aldous et al., 2003), with one additional lineage (lineage 7) and seven sub-lineages being proposed later (Cattoli et al., 2010; Snoeck et al., 2009). The second system classifies

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1567-1348/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.meegid.2012.07.012

NDV in two major groups designated class I and class II (Czeglédi et al., 2006; Miller et al., 2010; Seal et al., 1995). Class I has been divided in nine genotypes (1–9) (Kim et al., 2007a,b; Miller et al., 2010), while class II comprises eleven genotypes (I–XI) (Czeglédi et al., 2006; Miller et al., 2010; Maminiaina et al., 2010; Tsai et al., 2004), with genotypes I, II, VI and VII being further divided in sub-genotypes Ia and Ib, II and IIa, VIa through VIf, and VIIa through VIIh (Kim et al., 2007a; Liu et al., 2003; Wehmann et al., 2003; Wu et al., 2010; Yu et al., 2001; Miller et al., 2009a, 2010). Both systems have been simultaneously used to classify NDV, generating confusion in the nomenclature and discrepancies in the assignment of multiple genetic groups. In the lineage system, for example, viruses of genotypes III, IV, V and VIII are classified into one large group named lineage 3 (Aldous et al., 2003). Other common problems of the current NDV classification systems include the large genetic diversity observed within sub-genotypes (sometimes larger than the genetic diversity between genotypes) (Miller et al., 2010), and the lack of objective criteria to define taxonomic groups. The genotype system is most widely used to classify NDV, as there is a stronger correlation among the phylogenetic relationship and the evolutionary distances between genetic groups. Rapid determination of the phylogenetic relationship between NDV isolates has, however, been hindered by the lack of uniformity in the nomenclature and by the lack of objective criteria to define major taxonomic groups. Therefore, the present study was conducted: (1). To obtain a clear representation of the genetic diversity of the APMV-1; (2). To develop a unified nomenclature for existing NDV taxonomic groups; (3). To define objective criteria to classify new NDV isolates; and 4. To provide a dataset with reference sequences for future classification of new NDV isolates.

Given the importance of the F gene for NDV virulence (de Leeuw et al., 2003, 2005), the large number of sequences available on GenBank, and the fact that current classification systems already use phylogenetic analysis of the F gene to classify NDV (Aldous et al., 2003; Kim et al., 2007b; Tsai et al., 2004), we have designed a classification system based on the phylogenetic analyses of all complete F gene sequences available on GenBank. Results of our analysis revealed that class I viruses contain a single genotype whereas class II NDV isolates can be effectively divided in fifteen genotypes.

2. Materials and methods

2.1. Phylogenetic analyses and inference of the evolutionary distances

A preliminary phylogenetic analysis was performed with 1995 sequences (available on GenBank as of 12/12/2011) corresponding to the partial coding sequence of the F gene (374 nt) (data not shown). However, here we present data based on the analysis of the complete F gene sequences (class I, $n = 102$; and class II, $n = 602$) (Supplementary Table S1). The phylogenetic analysis was performed with the software MEGA5 (MEGA, version 5) (Tamura et al., 2011) and the evolutionary history was inferred by using the Neighbor Joining (data not shown), and the Maximum likelihood methods (Tamura and Kumar, 2002), with standard errors being calculated based on 500 bootstrap replicates. To facilitate the display and to ensure that the genotype topology would be maintained if fewer isolates would be used, we have performed a similar analysis with a smaller dataset ($n = 118$) in which at least four sequences representing each genotype and sub-genotype were used for the phylogenetic reconstruction. Detailed information about the selected isolates is presented in Fig. 3. To assess if the results obtained with the analysis of the complete F gene sequences would be reflected at the genomic level, we performed a phylogenetic analysis with all complete genome sequences available on GenBank ($n = 103$). The topological congruence between the trees obtained with the complete genome and the corresponding complete F gene sequences was calculated according to the maximum agreement sub-tree (MAST) method using the on-line calculator of congruency index *Icong* (de Vienne et al., 2007).

Analysis of the best-fit substitution model was performed using MEGA5, and the goodness-of-fit of each model was measured by Bayesian Information Criterion (BIC) and corrected Akaine Information Criterion (AICc) (Tamura et al., 2011). The General Time Reversible (GTR) model with a discrete gamma distribution (+G) allowing for invariant sites (+I) was selected based on AICc and used in all data analyses (Tamura et al., 2011). Codon positions included in the analysis were the 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. A total of 1647 positions were included in the analysis of the complete F gene dataset, and 15,067 positions were included in the analysis of the complete genome dataset.

The smallest complete F gene sequence dataset ($n = 118$), was subjected to an alternative phylogenetic reconstruction algorithm using the software PhyML (version 2.4.4) (Guindon and Gascuel, 2003). The GTR + G + I model of nucleotide substitution was used for the analysis, with an estimated gamma shape parameter of 1.228. The robustness of the groups was assessed using the bootstrap approach with 100 replicates. The numbers presented in the phylogenetic trees represent the GenInfo Identifier (GI) sequence identification number in GenBank and the numbers in the tree nodes represent the bootstrap support.

The largest complete F gene dataset ($n = 602$), used for the phylogenetic reconstruction, was also used to infer the evolutionary distances within and between groups and to calculate the mean interpopulational evolutionary distance. The distances were inferred by pair-wise analysis using the MEGA5 software (Tamura and Kumar, 2002, 2011) and the number of base substitutions per site was calculated by two different methods. The most simplified method (p-distance) was performed by averaging all sequence pairs between groups, while the second method used the Maximum Composite Likelihood model (Nei and Kumar, 2000). The variation rate among sites was modeled with a gamma distribution (shape parameter = 1, set based on a preliminary estimation from our dataset) and the differences in the composition bias among sequences were considered in the evolutionary comparisons (Tamura et al., 2011). Codon positions included in the analysis were the 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated.

2.2. Recombination analysis

Recombination analysis was performed with all class II complete genome ($n = 103$) and complete F gene ($n = 602$) sequences using the program RDP3 (Martin et al., 2010). Four statistical methods (RDP, Genecomv, Maxchi and Chimera) were used to identify putative recombinant sequences. Sequences with recombination events identified by at least two detection methods ($p < 0.001$) were considered as true recombinants.

2.3. Genotype classification criteria

Genotypes and sub-genotypes were assigned based on the phylogenetic topology and on the evolutionary distances between different taxonomic groups. Initially, a large phylogenetic tree was obtained with 1995 sequences corresponding to the partial coding sequence of the F gene (374 nt). As low bootstrap support was observed for most taxonomic groups, large phylogenetic trees were constructed with the complete nucleotide sequences of the F gene from class I ($n = 102$) and class II ($n = 602$) viruses and genotypes/sub-genotypes were assigned according to the previous

Please cite this article in press as: Diel, D.G., et al. Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. (2012), http://dx.doi.org/10.1016/j.meegid.2012.07.012

classification. New genotypes were assigned in numerical order and identified by the presence of a distinct common node shared by at least four isolates (bootstrap >60% at the defining node). All complete F gene sequences of the genotypes and sub-genotypes identified in the phylogenetic analysis were used to infer the evolutionary distances between and within groups. To assign virus isolates to specific sub-genotypes, a separation into small clades was performed based on the Maximum Likelihood tree (complete F gene sequences; $n = 602$), and the distance between clades was inferred. The mean interpopulational evolutionary diversity (mean evolutionary distance between genotypes) was determined using the Maximum Composite Likelihood model (Tamura et al., 2004) (10%) and set as the cutoff value to confirm the classification based on the phylogenetic analysis. All taxonomic groups with less than four virus isolates were not classified into genotypes and were designated as "U" (unclassified). Similar classification criteria have been proposed by the World Health Organization, World Organization for Animal Health, Food and Agriculture Organization, and the H5N1 Evolution Working Group to classify highly pathogenic avian influenza virus isolates (WHO/OIE/FDA and Evolution Working Group, 2008).

2.4. New NDV isolates included in the study

The NDV isolates Malaysia/1041-632/2008, Malaysia/5091-633/2009, China/NDV04-23-647/2004, China/NDV03-45-641/2003, chicken/MX/NC02-634/2010, and chicken/MX/NC04-635/2010 were obtained from the repository of the USDA Southeast Poultry Research Laboratory. These viruses were isolated from poultry in Asia and Central America. The NDV isolates goose/China-GD/12/2011, goose/China-GD/17/2011, goose/China-GD/20/2011, goose/China-GD/1003/2010, and goose/China-GD/450/2011 were obtained from the repository of the Chinese National Reference Laboratory for Newcastle disease. These viruses were isolated from samples collected from geese in live bird markets in the province of Guangdong during a national surveillance program conducted between 2010 and 2011.

2.5. RNA isolation and nucleotide sequencing

The NDV isolates Malaysia/1041-632/2008, Malaysia/5091-633/2009, China/NDV04-23-647/2004, China/NDV03-45-641/2003, chicken/MX/NC02-634/2010, chicken/MX/NC04-635/2010, goose/China-GD/12/2011, goose/China-GD/17/2011, goose/China-GD/20/2011, goose/China-GD/1003/2010, and goose/China-GD/450/2011, were propagated in specific pathogen free (SPF) embryonated chicken eggs. Total RNA was extracted from allantoic fluids using Trizol LS (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The complete F gene nucleotide sequences were determined by using a RT-PCR/sequencing approach (Diel et al., 2011). Amplification reactions were performed with a one-step RT-PCR kit (Qiagen, Valencia, CA) and a set of F gene specific primers (fwd_upper-f1-ttgcttatagttagttcgcctgtc, and rev_down-f2-acccgtgtattgctctttgg). The PCR amplicons were subjected to electrophoresis in 1% agarose gels and the DNA bands were excised from the gels and purified by using the QuickClean DNA gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were cloned in the TOPO TA vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and subjected to DNA sequencing. All sequencing reactions were performed with fluorescent dideoxy-nucleotide terminators in an ABI 3700 automated sequencer (Applied Biosystems Inc., Foster City, CA). Sequence editing and assembly were performed with LaserGene sequence analysis software package (LaserGene, version 5.07; DNAStar, Inc., Madison, WI).

2.6. Accession numbers

The complete F gene sequences of the NDV isolates Malaysia/1041-632/2008, Malaysia/5091-633/2009, China/NDV04-23-647/2004, China/NDV03-45-641/2003, chicken/MX/NC02-634/2010, chicken/MX/NC04-635/2010, goose/China-GD/12/2011, goose/China-GD/17/2011, goose/China-GD/20/2011, goose/China-GD/1003/2010, goose/China-GD/450/2011 are available on GenBank under the accession numbers JQ697739, JQ697740, JQ697741, JQ697742, JQ697743, JQ697744, JN627508, JN627506, JN627505, JN627504, and JN627507.

3. Results and discussion

In the present study, we assessed the genetic diversity of APMV type-1 and developed a unified nomenclature and classification system to define NDV genotypes. Two previous classifications that grouped NDV isolates into lineages or genotypes were considered (Aldous et al., 2003; Miller et al., 2010; Pedersen et al., 2004; Seal et al., 1995). However, given that in the genotype classification there is a stronger correlation between the phylogenetic topology and the evolutionary distances within and between clades, we have used the genotype nomenclature and classification as the base to develop objective criteria to classify new NDV isolates. The classification criteria were determined based on the phylogenetic topology and on specific evolutionary distances that reflect the diversity of the APMV type 1. In the sections that follow, we will describe and discuss the results of our analysis and proposals for the future assignment of new NDV genotypes and sub-genotypes as they are discovered.

Table 1
Criteria used to define Newcastle disease virus genotypes.

Criteria	Description
1	Genotype and sub-genotype designations were maintained as previously described when possible (i.e. genotype I remains I and sub-genotype Ia remains Ia)
2	New genotypes and sub-genotypes were assigned based on the phylogenetic tree topology using the Maximum Likelihood method and the optimum nucleotide model as determined by MEGA5. Phylogenetic tree topology was supported by the evolutionary distances between groups
3	New genotypes or sub-genotypes were designated only when the complete F gene sequence of at least four independent isolates without a direct epidemiologic link (i.e. distinct outbreaks) were available
4	The mean nucleotide distance (evolutionary distances) between and within groups was inferred as base substitutions per site by averaging all sequence pairs with the MEGA5 software and the Maximum Composite Likelihood model
5	The mean interpopulational evolutionary distance (mean distance between genotypes) was estimated using the Maximum Composite Likelihood model (0.106; *standard error* = 0.0061) and set as the cutoff value to assign new genotypes
6	Different genotypes should have an average distance per site >10% (0.1).Different sub-genotypes should have an average distance per site between 3 (0.03) and 10% (0.1)
7	Bootstrap value at the genotype and sub-genotype defining node should be >60%

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3.1. Classification of NDV into genotypes

The rational for classifying viruses into genotypes is based on the need to determine the association of viruses within a genotype with their geographic and temporal distribution, their pathogenicity, and the host species from which they were isolated (Czeglédi et al., 2006). NDV isolates have been classified into multiple genotypes and sub-genotypes based on the phylogenetic relationship between virus isolates (Czeglédi et al., 2006; Khan et al., 2010; Kim et al., 2007a, 2008; Liu et al., 2003; Tsai et al., 2004; Wu et al., 2010; Yu et al., 2001). However, the lack of objective criteria to define major taxonomic groups makes the classification of new NDV isolates difficult. Here we designed a system to classify NDV based on the phylogenetic topology and on the evolutionary distances between different taxonomic groups (Table 1).

The evolutionary history of NDV was inferred by phylogenetic analysis using the partial (374 nt) or complete nucleotide sequences of the F gene, and compared to the evolutionary history inferred from the complete genome sequences. Phylogenetic analysis based on the partial (374 nt; $n = 1995$) or complete (1649 nt; $n = 602$) F gene sequences clearly separated class II NDV isolates into fifteen taxonomic groups (data not shown; Supplementary Fig. S1). However, when the partial F gene sequence (374 nt) was used for the analysis, inconsistencies in the phylogenetic tree topology and low bootstrap values (<60%) were observed for most taxonomic groups (data not shown). The overall phylogenetic topology based on the complete F gene sequences was consistent with the phylogenetic grouping obtained from the analysis of the complete genome sequences (congruence index [lcong] = 5.410, $p = 2.24e\text{-}45$). Additionally, when the alternative algorithm PhyML was used for the phylogenetic reconstruction ($n = 118$, complete F gene sequences), the grouping of all viruses was consistent with the grouping obtained in MEGA5, and all genotypes and sub-genotypes were supported by bootstrap values >60% (Supplementary Fig. S2, Fig. 3). Therefore, the complete F gene sequence was selected for all other analyses conducted here and is proposed for future classification of NDV isolates.

3.2. Classification criteria to define NDV genotypes

The specific classification criteria determined here by the analysis of all complete F gene sequences available on GenBank and used to define NDV genotypes are presented in Table 1. To avoid further inconsistencies in the classification of NDV isolates, previously assigned genotypes and sub-genotypes were maintained when possible. New genotypes were assigned when at least four isolates formed a distinct taxonomic group (bootstrap at the defining node >60%) with a mean evolutionary distance between other groups greater than 10%. The mean interpopulational evolutionary distance (10%) is proposed here as a cutoff to validate genotypes identified by phylogenetic analysis, because it represents the average diversity between distinct NDV taxonomic groups. The bootstrap value and the mean interpopulational evolutionary distance have also been proposed for the classification of highly pathogenic avian influenza viruses (WHO/OIE/FDA and Evolution Working Group, 2008). By using these specific criteria we identified one genotype in class I and fifteen genotypes in class II (described in detail below). These genotypes were identified from the consensus topology of large phylogenetic trees (class I, $n = 102$; class II, $n = 602$) and were confirmed by the analysis of the evolutionary distances between and within groups (Supplementary Fig. S1; Figs. 1–3; Table 2, Supplementary Tables S2, S3, S5). Genotypes and sub-genotypes of class II, assigned based on large phylogenetic trees, consistently grouped (Fig. 3) when a smaller number of isolates (at least four viruses) were used for the phylogenetic reconstruction. Although genotypes were consistently identified between the large and the small datasets, slight differences in the topologic organization and in the bootstrap values were observed (Supplementary Fig. S1; Fig. 3). These observations highlight the importance of using the largest dataset possible to classify NDV isolates into genotypes. The phylogenetic topology of each genotype was similar, and all viruses classified within the same taxonomic group when the Maximum Likelihood and the Neighbor-Joining phylogenetic reconstruction methods were used (data not shown).

3.3. Analysis of class I viruses revealed the existence of a single genotype

Class I comprise viruses that have been isolated mainly from waterfowl and shorebirds, usually from samples collected in live bird markets worldwide (Kim et al., 2007a,b; Miller et al., 2010, 2009a). These viruses have been previously classified by our group and by others into nine genotypes (1 to 9), based on phylogenetic analysis performed with partial nucleotide sequences (374 nt) of the F gene (Kim et al., 2007a; Miller et al., 2010; Wu et al., 2010). Results here, however, indicate that according to the new classification criteria these viruses comprise a single phylogenetic group, which was designated as genotype 1. The phylogenetic relationship between viruses of class I suggested the existence of at least three distinct sub-groups within genotype 1 (Fig. 1). Analysis of the evolutionary distances between these sub-groups (Table 2) confirmed the phylogenetic topology, indicating that genotype 1 is divided in three sub-genotypes (1a, 1b, and 1c; distances between groups varying from 0.076 [7.6%] to 0.079 [7.9%]) (Fig. 1; Table 2). The differences between the previous classification of class I viruses and the one proposed here are most likely due to the use of more stringent criteria (larger distances and requirement of four independent isolates) to clearly define the genotypes. However, the possibility that additional class I genotypes exist and were not identified in our analysis cannot be formally excluded (e.g. there are two clearly distinct groups of viruses in Fig. 1 that were assigned as 'U' because there is only three sequences in GenBank). An analysis involving a larger number of sequences should be performed in the future as more complete F gene sequences of class I viruses become available.

3.4. Analysis of class II viruses revealed the existence of fifteen genotypes

Class II contains viruses that have been isolated from multiple wild bird and poultry species (Supplementary Table S1; Fig. 3). Most viruses within this group are virulent (Miller et al., 2009a) and cause significant economic losses to the poultry industry worldwide. These viruses are also highly diverse and multiple phylogenetic studies have shown that they are continuously evolving (Czeglédi et al., 2006; Diel et al., 2011; Khan et al., 2010; Maminiaina et al., 2010; Miller et al., 2010; Pedersen et al., 2004; Perozo et al., 2008; Rue et al., 2010; Wu et al., 2010). Given the large genetic diversity observed within class II, these viruses have been classified in 11 genotypes (I–XI) (Tsai et al., 2004). In our study, we confirmed the existence of ten of these genotypes (I–IX, and XI), with genotype X, previously assigned by Tsai and collaborators (Tsai et al., 2004), not being included in our analysis due to the lack of complete F gene sequences on public databases. By using the genotyping criteria developed here (Table 1), we have also identified five new genotypes, which were designated as genotypes X, XII, XIII, XIV and XV (Figs. 2 and 3; Table 3). The classification of class II viruses into 15 genotypes (I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, and XV) is supported by the phylogenetic topology and by the evolutionary distances between (>10%) and within (<10%) groups (Figs. 2 and 3; Tables 3 and 4).

Fig. 1. Phylogenetic analysis based on the complete nucleotide sequence of the F gene of viruses representing Newcastle disease virus class I. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (−8604.4794) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.6182). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 27.9736% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 111 nucleotide sequences. Codon positions included were the 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

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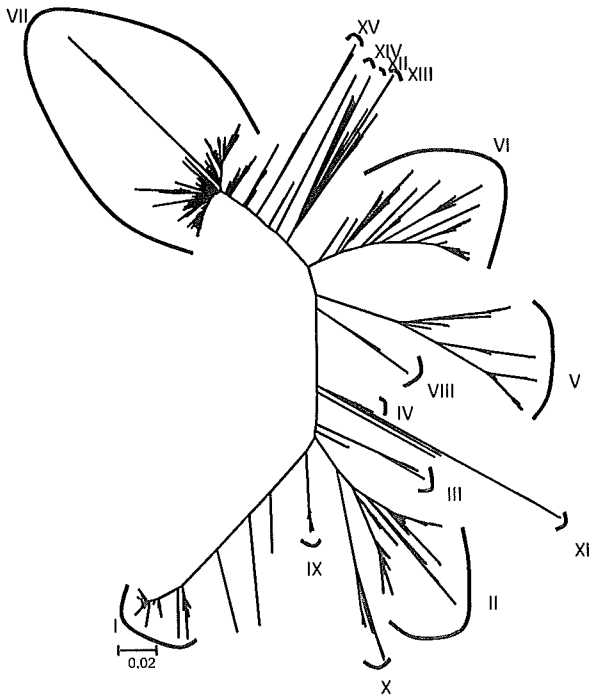

Fig. 2. Phylogenetic analysis based on the complete nucleotide sequence of the F gene of viruses representing Newcastle disease virus class II. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (−46323.5822) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.8546). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 8.7500% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 602 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1639 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

The evolutionary distances (inferred from the complete F gene sequences) between most class II genotypes was greater than 10% (Table 3), which is the mean interpopulational evolutionary distance set as the cutoff to define distinct genotypes. The exceptions here were genotypes III, IV and IX, which present slightly lower evolutionary distances between them (Table 3). Since the evolutionary distances between these three genotypes were close to 10% (the value set as cutoff for genotype assignment), and these groups represent mostly isolates obtained several years ago we decided to maintain their classification as distinct genotypes, to avoid inconsistencies with the existing literature. The phylogenetic tree topology (bootstrap >60%) (Supplementary Fig. S1), and the evolutionary distances inferred from the complete genome sequences (>10%) (Supplementary Table S2) further support their classification as distinct genotypes. The classification of class II viruses into fifteen genotypes was also supported by the phylogenetic and evolutionary distances inferred from the analysis of the nucleotide sequences of the complete genome (data not shown; Supplementary Table S2).

Genotypes I, II, V, VI and VII comprise highly diverse viruses, therefore these groups have been further divided in sub-genotypes (Kim et al., 2007a; Liu et al., 2003; Miller et al., 2010; Qin et al., 2008a,b; Tan et al., 2010; Wang et al., 2006; Wu et al., 2010). The criteria used here and proposed for future sub-genotype assignment are presented in Table 1. Genotype I has been previously classified in sub-genotypes Ia and Ib (Kim et al., 2007), which was confirmed here by the phylogenetic relationship and the evolutionary distances between viruses of these sub-genotypes (Supplementary Fig. S1; Fig. 3; Table 4A). Genotype II has been previously divided into two sub-genotypes named sub-genotype II and IIa (Kim et al., 2007; Miller et al., 2010). Here, however, analysis of the evolutionary distances between these two groups indicated that viruses of sub-genotype IIa form a distinct genotype, which was designated genotype X (Fig. 3; Supplementary Fig. S1; Supplementary Table S3). Although genotype V comprises a highly diverse group of viruses, this genotype has not been previously classified in sub-genotypes. Analysis of the evolutionary distances between different taxonomic groups identified within genotype V indicated the existence of at least two sub-genotypes among these viruses, which were designated here as sub-genotypes Va and Vb (Fig. 3, Supplementary Fig. S1; Table 4B). Genotype VI has been classified into eight sub-genotypes (Wang et al., 2006), and here we confirmed the existence of four of these sub-genotypes consisting of sub-genotypes VIa, VIb, VIc, and VIe (Fig. 3; Supplementary Fig. S1, Table 4B). Genotype VII comprises the largest number of NDV isolates and is the most diverse among all fifteen NDV genotypes, which led to its classification into in ten sub-genotypes (VIIa–h) (Liu et al., 2003; Tan et al., 2010; Wu et al., 2010; Yu et al., 2001). Here, however, we have identified only five sub-genotypes within genotype VII, which were named sub-genotypes VIIb, VIId, VIIe, VIIf, and VIIg (Fig. 3; Supplementary Fig. S1; Table 4A), according to the nomenclature previously used in the literature.

3.5. Recombination analysis

An increasing number of recombination events have been recently reported for NDV.

(Han et al., 2008; Qin et al., 2008a,b; Miller et al., 2009b; Zhang et al., 2010). However, the contribution of recombination for NDV evolution and its occurrence in nature are still largely debated (Afonso, 2008; Collins et al., 2008; Han and Worobey, 2011; Song et al., 2011). The dataset used in the present study was subjected to a recombination analysis, and using the program RDP3 we identified 14/103 (13.5%) recombinant sequences in the complete genome dataset and 15/602 (2.5%) recombination events in the complete F gene dataset (Supplementary Tables S3 and S4). To assess the effect of recombination on the evolutionary distances between genotypes (Table 3 and Supplementary Table S2), recombinant sequences were removed from the dataset and the distances between genotypes were re-inferred (Supplementary Tables S5 and S6). As shown on Supplementary Tables S5 and S6 the presence of recombinant sequences had only minimal effects on the evolutionary distances between NDV genotypes (Supplementary Tables S2 and S3). Therefore, recombinant sequences were kept in our dataset and labeled in the phylogenetic trees as recombinant forms (RF) (Fig. 3; Supplementary Fig. S1). Notably, all sequences identified as recombinants in our analysis, lack a common recombination breakpoint (Supplementary Table S5), suggesting that these recombination events may not be of evolutionary significance. In the future it might be valuable to improve the nomenclature system for recombinant forms of NDV, perhaps by recording the genotype composition and genomic breakpoint positions for each recombinant virus.

3.6. Epidemiologic characteristics of class II NDV genotypes

Genotypes I, II, III, IV, and IX emerged between 1930s and 1960s and are considered "early" genotypes (Czeglédi et al., 2006). Among these, genotype II viruses are of low virulence and strains of this genotype have been used as live vaccines against NDV for more than 40 years (Miller et al., 2010). Genotype IX includes the first virulent NDV strain obtained in China in 1948, and viruses of this genotype are still circulating in Asia with sporadic outbreaks

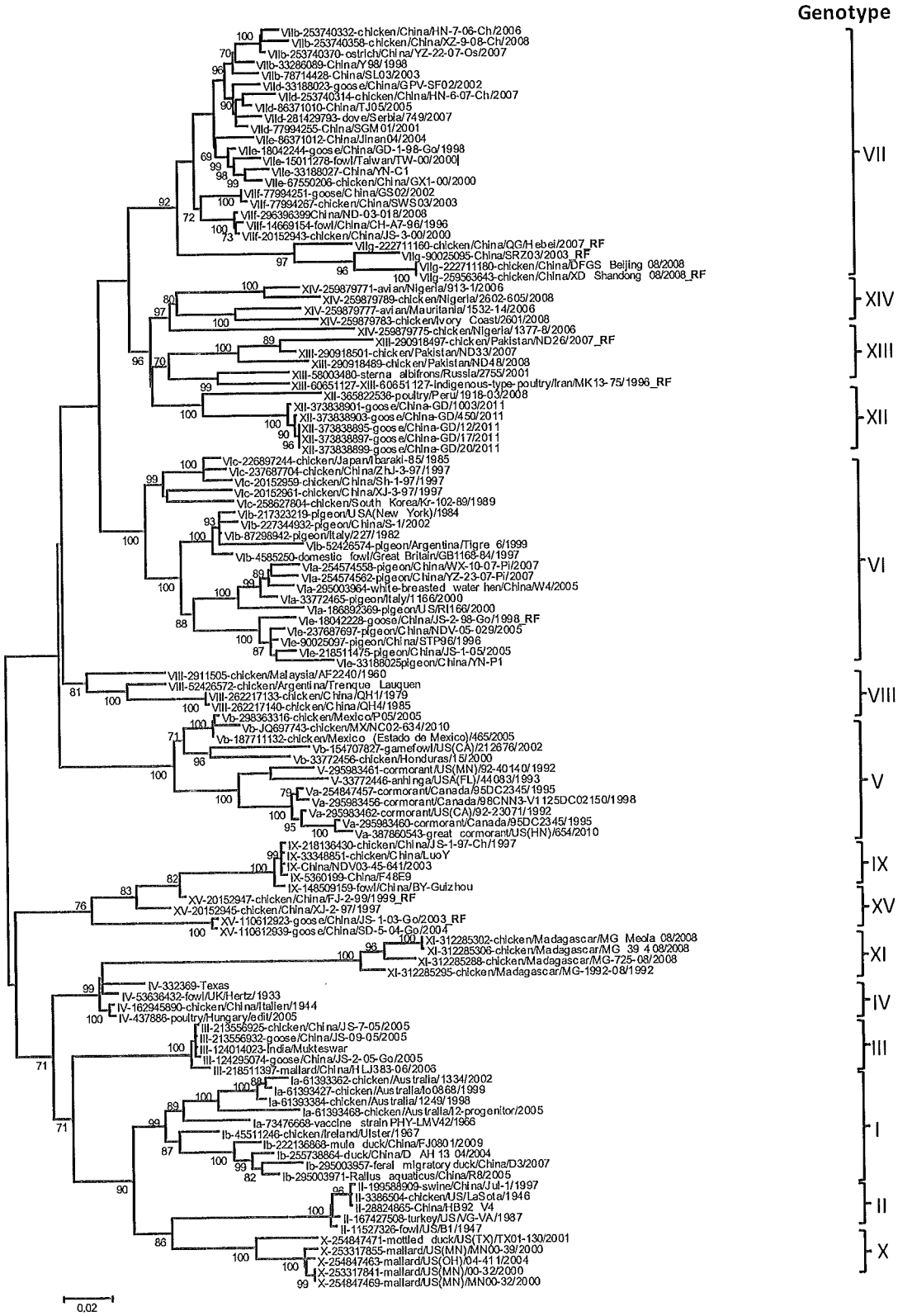

Fig. 3. Phylogenetic analysis based on the complete nucleotide sequence of the F gene of viruses representing Newcastle disease virus class II. At least four sequences representing each NDV genotype were used in the analysis. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (−23299.0541) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 1.2175). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30.6963% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 118 nucleotide sequences. Codon positions included were 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1647 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. RF = recombinant form identified with the program RDP3.

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Table 2
Estimates of evolutionary distances between class I sub-genotypes.[a]

Sub-genotype	No. of base substitutions/site in sub-genotypes[b]		
	1a	1b	1c
1a		(0.007)	(0.006)
1b	0.071		(0.007)
1c	0.079	0.076	

[a] Inferred from the complete nucleotide F gene sequence.
[b] The number of base substitutions per site from averaging all sequence pairs between class I sub-genotypes is shown. All results are based on the pair wise analysis of 102 sequences and the number of sequences analyzed per groups was: 1a, $n = 44$; 1b, $n = 22$; 1c, $n = 36$. Evolutionary distances were inferred by the analysis of 1662 nt positions of the F gene. The analysis was conducted using the Maximum Composite Likelihood method in MEGA5. Codon positions included were 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. Values in parentheses represent standard errors, obtained by a bootstrap procedure (500 replicates).

being reported in chickens and domestic ducks (Qiu et al., 2011; Wang et al., 2006; Zhang et al., 2011). Genotypes V, VI, VII, VIII and XI emerged after 1960s and are considered "late" genotypes (Czeglédi et al., 2006). Genotypes V, VI, VII and VIII contain only virulent viruses and are the predominant genotypes circulating worldwide (Miller et al., 2009a). Among these, genotype V viruses emerged in 1970s and are frequently isolated in South and Central America from poultry species and in North America from cormorants (Diel et al., 2011; Kinde et al., 2005; Pedersen et al., 2004; Perozo et al., 2008; Rue et al., 2010; Wehmann et al., 2003). Genotype VI, include viruses that have been isolated from multiple avian species (Alexander, 2011; Czeglédi et al., 2006; Kim et al., 2008; Liu et al., 2003; Wehmann et al., 2003). Viruses of this genotype are particularly important because of their frequent association with doves and pigeons and the consequent risk for introduction into poultry flocks (Alexander, 2011; Kim et al., 2008; Kommers et al., 2001, 2002). Currently, genotype VII is the genotype most frequently associated with outbreaks of ND in the Middle East and Asia (Miller et al., 2010), and outbreaks caused by these viruses are of particular concern given that some strains have shown increased virulence in poultry, while others have expanded their host range and are now able to cause disease in geese (Huang et al., 2004; Liu et al., 2003). Additionally, an outbreak of ND in South America has been recently attributed to a genotype VII virus, indicating that these viruses are spreading to other locations around the world (Perozo et al., 2012).

Genotypes X, XII, XIII, XIV and XV comprise viruses with different epidemiologic characteristics and geographic origins (Fig. 3; Supplementary Table S1). New genotype X, which contains viruses that have been previously classified into genotype IIa (Kim et al., 2007a), comprise viruses of low virulence that have been isolated from waterfowl and shorebirds in North America between late 1980s and early 2000s. Genotype XII is a novel genotype that contains virulent viruses recently isolated from poultry in South America (Diel et al., 2012) and from geese in China (isolates goose/China-GD/12/2011,goose/China-GD/17/2011,goose/China-GD/20/2011, goose/China-GD/1003/2010, and goose/China-GD/450/2011 characterized in the present study). The South American isolate of genotype XII (poultry/Peru/1918-03/2008) caused an outbreak of ND in Peru in 2008 (Diel et al., 2012), and phylogenetic related viruses have been recently isolated from healthy geese in live bird markets in China. The epidemiologic link between the Peruvian and the Chinese viruses is still unknown and further investigation is needed to define the origin and source of these viruses. Genotype XIII, previously classified as genotype VII (Khan et al., 2010), comprises virulent viruses that were isolated in Russia, Iran and Pakistan between 1995 and 2008 (Fig. 3). Genotype XIV contains virulent viruses obtained in West and Central Africa between 2006 and 2008 (Fig. 3). These viruses have been previously classified by using the lineage system into lineage 7 (Cattoli et al., 2010). Genotype XV, comprises viruses obtained from chickens and geese in China, which have been previously classified into sub-genotype VIId (isolates XJ-2/97 and FJ-2/99) or VIIe (isolate JX-2/99) (Liu et al., 2003). The re-classification proposed here, containing 15 genotypes, is supported by the phylogenetic relationship and the evolutionary distances between genotypes (Figs. 2 and 3; Tables 3 and 4). A summary with the epidemiologic information of all viruses used in the present study is presented in Supplementary Table S1.

Table 3
Estimates of evolutionary distances between class II genotypes.[a]

Genotype	No. of base substitutions/site in genotype[b]														
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
I		(0.010)	(0.008)	(0.007)	(0.012)	(0.012)	(0.012)	(0.011)	(0.008)	(0.007)	(0.013)	(0.014)	(0.012)	(0.012)	(0.009)
II	0.128		(0.012)	(0.012)	(0.015)	(0.015)	(0.017)	(0.014)	(0.011)	(0.009)	(0.017)	(0.018)	(0.016)	(0.016)	(0.010)
III	0.116	0.148		(0.007)	(0.011)	(0.012)	(0.012)	(0.011)	(0.009)	(0.009)	(0.013)	(0.013)	(0.012)	(0.013)	(0.010)
IV	0.101	0.133	0.086		(0.012)	(0.012)	(0.012)	(0.011)	(0.009)	(0.009)	(0.010)	(0.011)	(0.009)	(0.011)	(0.008)
V	0.194	0.213	0.184	0.151		(0.010)	(0.011)	(0.010)	(0.011)	(0.013)	(0.013)	(0.012)	(0.011)	(0.011)	(0.009)
VI	0.177	0.203	0.175	0.133	0.158		(0.008)	(0.007)	(0.012)	(0.013)	(0.013)	(0.012)	(0.007)	(0.008)	(0.010)
VII	0.179	0.215	0.169	0.140	0.160	0.128		(0.009)	(0.012)	(0.012)	(0.015)	(0.008)	(0.007)	(0.007)	(0.008)
VIII	0.147	0.172	0.140	0.104	0.137	0.119	0.128		(0.010)	(0.014)	(0.017)	(0.009)	(0.008)	(0.009)	(0.007)
IX	0.107	0.131	0.098	0.082	0.177	0.168	0.169	0.133		(0.011)	(0.013)	(0.009)	(0.012)	(0.012)	(0.008)
X	0.113	0.119	0.143	0.127	0.211	0.198	0.200	0.168	0.128		(0.014)	(0.015)	(0.013)	(0.014)	(0.010)
XI	0.200	0.228	0.190	0.130	0.232	0.231	0.242	0.201	0.176	0.222		(0.016)	(0.015)	(0.017)	(0.014)
XII	0.189	0.224	0.179	0.152	0.173	0.127	0.118	0.129	0.179	0.202	0.251		(0.007)	(0.008)	(0.010)
XIII	0.176	0.218	0.176	0.142	0.168	0.132	0.114	0.130	0.166	0.199	0.234	0.109		(0.007)	(0.008)
XIV	0.188	0.228	0.189	0.156	0.174	0.143	0.129	0.144	0.181	0.213	0.244	0.120	0.121		(0.009)
XV	0.146	0.141	0.140	0.112	0.167	0.142	0.103	0.133	0.107	0.163	0.215	0.148	0.141	0.153	

[a] Inferred from the complete nucleotide F gene sequence.
[b] The number of base substitutions per site from averaging all sequence pairs between class II genotypes is shown. All results are based on the pair wise analysis of 602 sequences and the number of sequences analyzed per groups was: I, $n = 70$; II, $n = 101$; III, $n = 9$; IV, $n = 6$; V, $n = 44$; VI, $n = 56$; VII, $n = 234$; VIII, $n = 4$; IX, $n = 18$; X, $n = 17$; XI, $n = 4$; XII, $n = 6$; XIII, $n = 8$; XIV, $n = 5$; XV, $n = 4$; Unclassified, $n = 12$. Evolutionary distances were inferred by the analysis of 1662 nt positions of the F gene. The analysis was conducted using the Maximum Composite Likelihood method in MEGA5. Codon positions included were 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. Values in parentheses are standard errors, obtained by a bootstrap procedure (500 replicates).

Please cite this article in press as: Diel, D.G., et al. Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. (2012), http://dx.doi.org/10.1016/j.meegid.2012.07.012

Table 4
Estimates of evolutionary distances between sub-genotypes of Class II.[a]

A

Genotype I sub-genotype	No. of base substitutions/site in sub-genotype		Genotype VII sub-genotype	No. of base substitutions/site in sub-genotype				
	a	b		b	d	e	f	g
a		(0.006)	b		(0.003)	(0.003)	(0.005)	(0.007)
b	0.07		d	0.035		(0.003)	(0.004)	(0.007)
–	–	–	e	0.039	0.036		(0.004)	(0.007)
–	–	–	f	0.052	0.048	0.041		(0.008)
–	–	–	g	0.088	0.082	0.089	0.096	

B

Genotype V sub-genotype	No. of base substitutions/site in sub-genotype		Genotype VI subgenotype	No. of base substitutions/site in sub-genotype			
	a	b		a	b	c	e
a		(0.008)	a		(0.005)	(0.008)	(0.006)
b	0.089	0.084	b	0.068		(0.007)	(0.007)
–	–	–	c	0.091	0.076		(0.008)
–	–		e	0.079	0.070	0.091	

[b] The percentage representing the number of base substitutions per site from averaging all sequence pairs between Class II sub-genotypes is shown. All results are based on the pair wise analysis of 602 sequences and the number of sequences analyzed per group was: Ia, $n = 37$; Ib, $n = 32$; Va, $n = 24$; Vb, $n = 12$; VIa, $n = 27$; VIb, $n = 16$; VIc, $n = 10$; VIe, $n = 7$; VIIb, $n = 86$; VIId, $n = 116$; VIIe, $n = 9$; VIIf, $n = 7$; VIIg, $n = 5$. Distances were inferred by the analysis of 1662 nt positions of the F gene. The analysis was conducted using the Maximum Composite Likelihood method in MEGA5. Codon positions included were 1st, 2nd, 3rd, and non-coding. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. There were a total of 1662 positions in the final dataset. Values in parentheses are standard errors, obtained by a bootstrap procedure (500 replicates).

[a] Inferred from the complete F gene sequence.

4. Conclusions

Given the rapid evolutionary dynamics of NDV, its broad host range (over 250 species of birds) and the use of mass vaccination strategies to control the disease worldwide, additional genetic variants will likely be discovered. To ensure their correct and consistent classification it is essential to use a unified nomenclature and classification system to define NDV taxonomic groups. Here, we propose to use only the genotype nomenclature and classification for the future assignment of NDV isolates into genetic groups. New NDV isolates should be classified based on phylogenetic topology, which need to be supported by the evolutionary distances between established genotypes or sub-genotypes. Additionally, the complete coding sequence of the F gene should be used for this analysis. The classification criteria used here and proposed for future NDV classification are presented on Table 1. It is important to note that the mean interpopulational evolutionary distance (0.1 or 10%) used here as the cutoff to validate NDV genotypes identified by phylogenetic analysis, is likely to change as NDV continues to evolve. Therefore, this value should be inferred on all future studies and set as the cutoff to assign new NDV genotypes. New genotype designations should be given according to the current numerical system (i.e. next genetic group should be named genotype XVI). New sub-genotypes should be designated with the next available sub-type letter on the genotype. Genotypes and sub-genotypes should be assigned only when complete F gene sequences of four or more NDV isolates with no epidemiologic link (i.e. obtained from different outbreaks) are available. We also provide a complete F gene dataset (Supplementary Table S1) to be used in future phylogenetic studies. Adoption of the proposed NDV nomenclature and classification system will facilitate studies on NDV epidemiology, evolution, disease control, and diagnostics.

Acknowledgments

We thank Dawn Williams-Coplin and Tim Olivier for technical assistance, and the SEPRL sequencing facility personnel for nucleotide sequencing.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.07.012.

References

Afonso, C.L., 2008. Not so fast on recombination analysis of Newcastle disease virus. J. Virol. 82 (18), 9303.
Aldous, E.W., Mynn, J.K., Banks, J., Alexander, D.J., 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32 (3), 239–256.
Alexander, D.J., Senne, D.A., 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: Saif, Y.M., Fadly, Aly M., Glisson, John R., McDougald, Larry R., Nolan, L.K., Swayne, D. (Eds.), Diseases of Poultry, 12th ed. Blackwell Publishing, Ames, IA, pp. 750–798.
Alexander, D.J., 2011. Newcastle disease in the European Union 2000 to 2009. Avian Pathol. 40 (6), 547–558.
Cattoli, G., Fusaro, A., Monne, I., Molia, S., Le Menach, A., Maregeya, B., Nchare, A., Bangana, I., Maina, A.G., Koffi, J.N., Thiam, H., Bezeid, O.E., Salviato, A., Nisi, R., Terregino, C., Capua, I., 2010. Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa implications for diagnosis and control. Vet. Microbiol. 142 (3–4), 168–176.
Chare, E.R., Gould, E.A., Holmes, E.C., 2003. Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. J. Gen. Virol. 84, 2691–2703.
Collins, P., Bukreyev, A., Murphy, B.R., 2008. What are the risks–hypothetical and observed of recombination involving live vaccines and vaccine vectors based on nonsegmented negative-strain RNA viruses? J. Virol. 82 (19), 9805–9806.
Czeglédi, A., Ujvári, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B., 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120 (1–2), 36–48.
de Leeuw, O.S., Hartog, L., Koch, G., Peeters, B.P.H., 2003. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. J. Gen. Virol. 84 (2), 475–484.
de Leeuw, O.S., Koch, G., Hartog, L., Ravenshorst, N., Peeters, B.P.H., 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J. Gen. Virol. 86 (6), 1759–1769.
de Vienne, D.M., Giraud, T., Martin, O.C., 2007. A congruence index for testing topological simirarity between trees. Bioinformat. 23 (23), 3119–3124.
Diel, D.G., Susta, L., Garcia, S. Cardenas, Killian, M.L., Brown, C.C., Miller, P.J., Afonso, C.L., 2012. Complete genome and clinicopathological characterization of a virulent newcastle disease virus isolate from south america. J. Clin. Microbiol. 50 (2), 378–387.

Diel, D.G., Miller, P.J., Wolf, P.C., Mickley, R.M., Musante, A.R., Emanuali, D.C., Shively, K.J., Pedersen, K., Afonso, C.L., 2011. Characterization of newcastle disease viruses isolated from cormorant and gull species in the united states in 2010. Avian Dis. 56, 128–133.

Guindon, S., Gascuel, O., 2003. A simplest, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.

Han, G.-Z., He, C.Q., Ding, N.Z., Ma, L.Y., 2008. Identification of a natural multi-recombinant of Newcastle disease virus. Virol. 371, 54–60.

Han, G.-Z., Worobey, M., 2011. Homologous recombination in negative sense RNA viruses. Viruses 3, 1358–1373.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., Vande, P.S., 1982. Rapid evolution of RNA genomes. Science 215, 1577–1585.

Hu, S., Ma, H., Wu, Y., Liu, W., Wang, X., Liu, Y., Liu, X., 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. Vaccine 27 (6), 904–910.

Hu, Z., Hu, S., Meng, C., Wang, X., Zhu, J., Liu, X., 2011. Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. Avian Dis. 55 (3), 391–397.

Huang, Y., Wan, H.Q., Liu, H.Q., Wu, Y.T., Liu, X.F., 2004. Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report. Arch. Virol. 149 (7), 1445–1457.

IASA – Investigación Aplicada S.A. de C.V., 2012. Genovax N5. Retrieved from <http://www.engormix.com/iasa-investigacion-aplicada/genovax-sh10043_pr26921.htm>.

Khan, T.A., Rue, C.A., Rehmani, S.F., Ahmed, A., Wasilenko, J.L., Miller, P.J., Afonso, C.L., 2010. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. J. Clin. Microbiol. 48 (5), 1892–1894.

Kim, L.M., King, D.J., Curry, P.E., Suarez, D.L., Swayne, D.E., Stallknecht, D.E., Slemons, R.D., Pedersen, J.C., Senne, D.A., Winker, K., Afonso, C.L., 2007a. Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. J. Virol. 81 (22), 12641–12653.

Kim, L.M., King, D.J., Guzman, H., Tesh, R.B., da Rosa, A.P.A. Travassos, Bueno, R., Dennett, J.A., Afonso, C.L., 2008. Biological and phylogenetic characterization of pigeon paramyxovirus serotype 1 circulating in wild North American pigeons and doves. J. Clin. Microbiol. 46 (10), 3303–3310.

Kim, L.M., King, D.J., Suarez, D.L., Wong, C.W., Afonso, C.L., 2007b. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription–PCR. J. Clin. Microbiol. 45 (4), 1310–1314.

Kinde, H., Hullinger, P.J., Charlton, B., McFarland, M., Hietala, S.K., Velez, V., Case, J.T., Garber, L., Wainwright, S.H., Mikolon, A.B., Breitmeyer, R.E., Ardans, A.A., 2005. The isolation of exotic Newcastle disease (END) virus from nonpoultry avian species associated with the epidemic of END in chickens in southern California: 2002–2003. Avian Dis. 49 (2), 195–198.

Kommers, G.D., King, D.J., Seal, B.S., Brown, C.C., 2001. Virulence of pigeon-origin Newcastle disease virus isolates for domestic chickens. Avian Dis. 45 (4), 906–921.

Kommers, G.D., King, D.J., Seal, B.S., Carmichael, K.P., Brown, C.C., 2002. Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. Vet. Pathol. 39 (3), 353–362.

Liu, X.F., Wan, H.Q., Ni, X.X., Wu, Y.T., Liu, W.B., 2003. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Arch. Virol. 148 (7), 1387–1403.

Maminiaina, O.F., Gil, P., Briand, F.-X., Albina, E., Keita, D., Andriamanivo, H.R., Chevalier, V., Lancelot, R., Martinez, D., Rakotondravao, R., Rajaonarison, J.J., Koko, M., Andriantsimahavandy, A.A., Jestin, V., de Almeida, R. Servan, 2010. Newcastle disease virus in Madagascar: identification of an original genotype possibly deriving from a died out ancestor of genotype IV. PloS One 5 (11), e13987.

Martin, D.P., Lemey, P., Lott, M., Moulton, V., Posada, D., Lefeuvre, P., 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinfomat 26, 2462–2463.

Miller, P.J., Decanini, E.L., Afonso, C.L., 2010. Newcastle disease: Evolution of genotypes and the related diagnostic challenges. Infect. Gen. Evol. 10 (1), 26–35.

Miller, P.J., Estevez, C., Yu, Q., Suarez, D.L., King, D.J., 2009a. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 53 (1), 39–49.

Miller, P.J., Kim, L.M., Ip, H.S., Afonso, C.L., 2009b. Evolutionary dynamics of Newcastle disease virus. Virol. 391 (1), 64–72.

Miller, P.J., King, D.J., Afonso, C.L., Suarez, D.L., 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine. 25 (41), 7238–7246.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. University Press, New York, NY, p. 333.

Pedersen, J.C., Senne, D.A., Woolcock, P.R., Kinde, H., King, D.J., Wise, M.G., Panigrahy, B., Seal, B.S., 2004. Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002–2003 outbreak in California and other recent outbreaks in North America. J. Clin. Microbiol. 42 (5), 2329–2334.

Perozo, F., Merino, R., Afonso, C.L., Villegas, P., Calderon, N., 2008. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. Avian Dis. 52 (3), 472–479.

Perozo, F., Marcano, R., Afonso, C.L., 2012. Biological and Phylogenetic Characterization of a Genotype VII Newcastle Disease Virus from Venezuela: Efficacy of field Vaccination. J. Clin. Microbiol.. http://dx.doi.org/10.1128/JCM.06,506-11.

Qin, Z., Sun, L., Ma, B., Cui, Z., Zhu, Y., Kitamura, Y., Liu, W., 2008a. F gene recombination between genotype II and VII Newcastle disease virus. Virus Res. 131, 299–303.

Qin, Z.-M., Tan, L.-T., Xu, H.-Y., Ma, B.-C., Wang, Y.-L., Yuan, X.-Y., Liu, W.-J., 2008b. Pathotypical characterization and molecular epidemiology of Newcastle disease virus isolates from different hosts in China from 1996 to 2005. J. Clin. Microbiol. 46 (2), 601–611.

Qiu, X., Sun, Q., Wu, S., Dong, L., Hu, S., Meng, C., Wu, Y., Liu, X., 2011. Entire genome sequence analysis of genotype IX Newcastle disease viruses reveals their early-genotype phylogenetic position and recent-genotype genome size. Virol. J. 8, 117.

Rue, C.A., Susta, L., Brown, C.C., Pasick, J.M., Swafford, S.R., Wolf, P.C., Killian, M.L., Pedersen, J.C., Miller, P.J., Afonso, C.L., 2010. Evolutionary changes affecting rapid identification of 2008 Newcastle disease viruses isolated from double-crested cormorants. J. Clin. Microbiol. 48 (7), 2440–2448.

Seal, B.S., King, D.J., Bennett, J.D., 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. J. Clin. Microbiol. 33 (10), 2624–2630.

Snoeck, C.J., Ducatez, M.F., Owoade, A.A., Faleke, O.O., Alkali, B.R., Tahita, M.C., Tarnagda, Z., Ouedraogo, J.B., Maikano, I., Mbah, P.O., Kremer, J.R., Muller, C.P., 2009. Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. Arch. Virol. 154 (1), 47–54.

Song, Q., Cao, Y., Li, Q., Gu, M., Zhong, L., Hu, S., Wan, H., Liu, X., 2011. Artificial recombination may influence the evolutionary analysis of Newcastle disease virus. J Virol. 85 (4), 10409–10414.

Tamura, K., Kumar, S., 2002. Evolutionary distance estimation under heterogeneous substitution pattern among lineages. Mol. Biol. Evol. 19 (10), 1727–1736.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Nat. Acad. Sci. USA 101, 11030–11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28 (10), 2731–2739.

Tan, S.W., Ideris, A., Omar, A.R., Yusoff, K., Hair-Bejo, M., 2010. Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. Arch. Virol. 155 (1), 63–70.

Tsai, H.-J., Chang, K.-H., Tseng, C.-H., Frost, K.M., Manvell, R.J., Alexander, D.J., 2004. Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. Vet. Microbiol. 104 (1–2), 19–30.

WHO/OIE/FDA and Evolution Working Group, 2008. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Em. Infect. Dis. 14 (7), e1.

Wang, Z., Liu, H., Xu, J., Bao, J., Zheng, D., Sun, C., Wei, R., Song, C., Chen, J., 2006. Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. An. N. Y. Acad. Sci. 1081, 228–239.

Wehmann, E., Czeglédi, A., Werner, O., Kaleta, E.F., Lomniczi, B., 2003. Occurrence of genotypes IV, V, VI and VIIa in Newcastle disease outbreaks in Germany between 1939 and 1995. Avian Pathol. 32 (2), 157–163.

Wu, S., Wang, W., Yao, C., Wang, X., Hu, S., Cao, J., Wu, Y., Liu, W., Liu, X., 2010. Genetic diversity of Newcastle disease viruses isolated from domestic poultry species in Eastern China during 2005–2008. Arch. Virol.. http://dx.doi.org/10.1007/s00705-010-0851-5, Epub ahead of print.

Yu, L., Wang, Z., Jiang, Y., Chang, L., Kwang, J., 2001. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. J. Clin. Microbiol. 39 (10), 3512–3519.

Zhang, R., Wag, X., Su, J., Zhao, J., Zhang, G., 2010. Isolation and analysis of two naturally occurring multi-recombination Newcastle disease viruses in China. Virus Res. 151, 45–53.

Zhang, S., Wang, X., Zhao, C., Liu, D., Hu, Y., Zhao, J., Zhang, G., 2011. Phylogenetic and Pathotypical Analysis of Two Virulent Newcastle Disease Viruses Isolated from Domestic Ducks in China. PloS One 6 (9), e25000.

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J. Clin. Microbiol. **2012, 50(2):378. DOI: 10.1128/JCM.06018-11.**
Published Ahead of Print 30 November 2011.

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Complete Genome and Clinicopathological Characterization of a Virulent Newcastle Disease Virus Isolate from South America

Diego G. Diel,[a] Leonardo Susta,[b] Stivalis Cardenas Garcia,[a,c] Mary L. Killian,[d] Corrie C. Brown,[b] Patti J. Miller,[a] and Claudio L. Afonso[a]

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Newcastle disease (ND) is one of the most important diseases of poultry, negatively affecting poultry production worldwide. The disease is caused by Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1), a negative-sense single-stranded RNA virus of the genus *Avulavirus*, family *Paramyxoviridae*. Although all NDV isolates characterized to date belong to a single serotype of APMV-1, significant genetic diversity has been described between different NDV isolates. Here we present the complete genome sequence and the clinicopathological characterization of a virulent Newcastle disease virus isolate (NDV-Peru/08) obtained from poultry during an outbreak of ND in Peru in 2008. Phylogenetic reconstruction and analysis of the evolutionary distances between NDV-Peru/08 and other isolates representing established NDV genotypes revealed the existence of large genomic and amino differences that clearly distinguish this isolate from viruses of typical NDV genotypes. Although NDV-Peru/08 is a genetically distinct virus, pathogenesis studies conducted with chickens revealed that NDV-Peru/08 infection results in clinical signs characteristic of velogenic viscerotropic NDV strains. Additionally, vaccination studies have shown that an inactivated NDV-LaSota/46 vaccine conferred full protection from NDV-Peru/08-induced clinical disease and mortality. This represents the first complete characterization of a virulent NDV isolate from South America.

Newcastle disease (ND), is one the most important diseases of poultry, negatively affecting poultry production worldwide (2). ND is caused by Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1), a negative-sense single-stranded RNA virus of the genus *Avulavirus*, family *Paramyxoviridae* (2). The NDV genome is ~15.2 kb in length and contains six genes encoding at least seven proteins named nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), RNA-dependent RNA polymerase (L), and V protein, which is produced through editing of the phosphoprotein mRNA (2). ND is characterized by a wide range of clinical manifestations, which vary in severity from subclinical respiratory or enteric infections to fatal neurological or enteric hemorrhagic disease (17).

Although all NDV isolates characterized to date belong to a single serotype, the avian paramyxovirus serotype 1 (APMV-1), significant genetic diversity has been recognized among different NDV isolates (16). Historically, NDV isolates have been classified into two major groups (class I and II), based on their genome lengths and the nucleotide sequences of their genomes (1, 4, 14, 15, 32). Class I viruses are distributed worldwide and have been isolated mainly from waterfowl and shorebirds (7, 8). Class II viruses have been divided into 11 genotypes (I to XI) (4, 14), with genotypes V, VI, VII, and VIII being the predominant genotypes circulating worldwide (14, 15). Among these, genotype VII viruses are particularly important given that they have been associated with many of the most recent outbreaks in Asia, Africa, and the Middle East (12, 14, 15, 32, 34). Additionally, a recent outbreak of ND in South America (Venezuela) has been attributed to a genotype VII virus, suggesting that viruses of this genotype are spreading worldwide (20). Notably, recent phylogenetic studies have shown that NDV is continuously evolving, with viruses of different genotypes undergoing simultaneous changes at different geographic locations, which eventually leads to diagnostic failures (14, 21, 23).

Virulent NDV (vNDV) occurs in at least six of the seven continents of the world and is enzootic in several countries, posing a constant threat to the poultry industry (14). In 2010, infection by vNDV was confirmed in 80 countries, including infections in wild birds in Israel, Kenya, Mongolia, Germany, Italy, Canada, and the United States and infections in domestic poultry in countries of North and South America, Europe, Africa, and Asia (18). In South America, vNDV infection is endemic in some of the northern countries, such as Venezuela, Colombia, and Suriname, while in southern countries, including Chile, Argentina, Uruguay, and Brazil, the disease is only occasionally reported (19). Although vNDV circulates and frequently causes outbreaks in South America, very little information is available on the epidemiology and evolutionary trends of the isolates circulating in that continent. Virulent NDV is exotic in poultry in the United States (31), and migration and/or illegal importation of birds from areas where vNDV is endemic represents a constant threat to the U.S. poultry industry (9, 10, 30). The last outbreak of ND in poultry in the U.S., for example, occurred in California during 2002 and 2003, likely as a result of illegal importation of birds and led to depopulation of more than 3 million birds and containment costs of more than U.S. $160 million (31). These observations highlight the importance of constant epidemiological surveillance for NDV and the need

Received 6 October 2011 Returned for modification 31 October 2011
Accepted 21 November 2011
Published ahead of print 30 November 2011
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doi:10.1128/JCM.06018-11

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0095-1137/12/$12.00 Journal of Clinical Microbiology p. 378–387

for a proactive characterization of the isolates circulating worldwide. Here we present the complete genome and the clinicopathological characterization of a genetically distinct NDV isolate (poultry/Peru/1918-03/2008 [NDV-Peru/08]) obtained during an outbreak of ND reported by the Official Veterinary Services (SENASA) in Peru in 2008.

MATERIALS AND METHODS

Viruses. The NDV isolate poultry/Peru/1918-03/2008 (NDV-Peru/08) was obtained from the USDA National Veterinary Services Laboratories (NVSL) repository (after authorization by the Official Veterinary Services of Peru [SENASA]). The virus was isolated from swab samples during an outbreak of ND affecting domestic poultry flocks in the region of Pachacutec, Arequipa, Peru, in 2008. Egg passage 2 (EP2) virus stocks were used for RNA extraction and in all pathogenesis experiments in the present study. The NDV strain LaSota/46 was obtained from the SEPRL repository and grown in 9- to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE). Allantoic fluid collected from NDV-LaSota/46-inoculated eggs was used for the production of an inactivated vaccine as described below.

Eggs and chickens. Embryonated chicken eggs and chickens were obtained from the SEPRL SPF white Leghorn flock. Birds were housed in negative pressure isolators under biosafety level 3 (BSL-3) enhanced containment and received food and water *ad libitum*.

ICPI test. Pathogenicity of NDV-Peru/08 was assessed by using the standard intracerebral pathogenicity index (ICPI) test (3). Day-old chicks were inoculated intracerebrally with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored during an 8-day observation period and scored as follows: normal, 0; sick or paralyzed, 1; or dead, 2. Total scores were determined, and the mean daily score was calculated to obtain the ICPI (3).

RNA isolation and sequencing. The NDV-Peru/08 isolate was propagated in SPF embryonated chicken eggs. Total RNA was extracted from allantoic fluids using TRIzol LS (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The F gene sequence was determined by PCR amplification of the complete gene (primers available upon request), followed by sequencing of the amplicon with fluorescent dideoxynucleotide terminators in an ABI 3700 automated sequencer (Applied Biosystems, Inc., Foster City, CA).

The complete genome sequence of NDV-Peru/08 was determined by using a shotgun reverse transcription (RT)-PCR/sequencing approach. Amplification reactions were performed with the one-step RT-PCR kit (Qiagen, Valencia, CA) and the following set of degenerated primers: beg2 (5′-CGCGTCGACTACTACGGGTAGA-3′) and end-r (5′-GTACCCGG GGATCCTTTTTTCTAA-3′) and FR26RV-N (5′-GCCGGAGCTCTGC AGATATC-3′) and FR20RV (5′-GCCGGAGCTCTGCAGATATC-3′). The PCR amplicons were subjected to electrophoresis in 1% agarose gels, and DNA bands with lengths of 500 to 1,000 bp and those longer than 1,001 bp were excised from the gels and purified by using the QuickClean DNA gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were cloned in the TOPO TA vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and subjected to DNA sequencing as described above. Sequence editing and assembly were performed with the LaserGene sequence analysis software package (LaserGene, version 5.07; DNAStar, Inc., Madison, WI).

Phylogenetic analysis. The complete F and the complete genome sequences were used to construct the phylogenetic trees and to localize NDV-Peru/08 among other class II reference strains. Phylogenetic analysis was performed by using the MEGA5 software (MEGA, version 5) (28). The evolutionary distances were inferred using MEGA 5 (27) and were expressed based on the number of nucleotide substitutions per site. The codon positions included in the analysis were the 1st, 2nd, 3rd, and noncoding. All positions containing gaps and missing data were eliminated from the data set (the "complete deletion" option). Analysis of recombination was performed using RDP3 (13). The numbers used in the phylogenetic trees represent the GenInfo Identifier (GI) sequence identification number in GenBank.

Sequence alignment. Alignment and comparison of the nucleotide and amino acid sequences between NDV-Peru/08 and selected strains representing established NDV genotypes were performed by using the software ClustalW (29).

Animal inoculations. The clinicopathological features of NDV-Peru/08 infection were assessed in chickens. Forty 4-week-old SPF white Leghorn chickens were randomly allocated to four experimental groups, consisting of two NDV-Peru/08-infected groups (group 1, $n = 10$; and group 2, $n = 10$) and two mock-infected (control) groups ($n = 10$). Birds were inoculated with a virus suspension (0.1 ml) containing 10^5 50% embryo infectious doses (EID_{50})/0.1 ml. Half of the inoculum (0.05 ml) was applied to the conjunctival sac, and half (0.05 ml) to the choanal cleft (26). Mock-infected control birds were inoculated as described above with 0.1 ml of phosphate-buffered saline (PBS). Birds were monitored daily for characteristic clinical signs of Newcastle disease. Two birds of groups 1 (NDV-Peru/08) and 2 (PBS control) were euthanized and necropsied at 2 and 5 days postinoculation (dpi), and all birds *in extremis* (presenting a severe clinical condition) were euthanized regardless of the sampling schedule. Tissue samples consisting of eyelid, spleen, bursa of Fabricius, thymus, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sacs, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of the femur, and nasal turbinate were collected and fixed in 10% neutral buffered formalin for 52 h. All sampled tissues were processed for histological examination and/or immunohistochemistry (IHC) using standard procedures (26). Oral and cloacal swabs were collected on days 2, 3 and 4 postinoculation (p.i.) and processed for virus isolation as described below. Birds of groups 2 (NDV-Peru/08) and 4 (PBS control) were monitored daily for characteristic clinical signs of Newcastle disease, and the time of death was recorded and used to plot the survival curves and to calculate the mean death time.

Virus isolation and quantitation. Virus shedding was assessed in oral and cloacal secretions from all inoculated birds. Swab samples were cleared by centrifugation at 1,000 × *g* for 20 min, and the supernatant was subjected to virus isolation in 9- to 10-day-old embryonated chicken eggs as previously described (26). Positive samples were tested by hemagglutination (HA) and hemagglutination inhibition (HI) to determine the presence of NDV. Additionally, positive samples were subjected to virus quantitation in embryonated chicken eggs. Serial 10-fold dilutions (10^{-5} to 10^{-10}) were inoculated in 9- to 10-day-old embryonated chicken eggs, and viral titers were determined as EID_{50}/0.1 ml according to the method of Reed and Muench.

IHC. Expression and distribution of the viral nucleoprotein (NP) were assessed by immunohistochemistry (IHC). Tissue sections (4 μm) were subjected to deparaffinization followed by antigen retrieval (Vector antigen unmasking solution; Vector Laboratories, Burlingame, CA) and blocking of nonspecific antigens (universal blocking reagent; Biogenex, San Ramon, CA) according to the manufacturer's instructions. Samples were incubated overnight with the anti-NP antibody at 4°C (26), washed, incubated with an alkaline phosphatase-labeled polymer anti-rabbit Fc (LabVision polymer; LabVision, Fremont, CA), and developed by using the chromogen Vector Red substrate (Vector Laboratories, Burlingame, CA) (26). Sections were counterstained with hematoxylin, mounted, and examined under a light microscope.

Vaccine production. Allantoic fluid collected from ECE inoculated with NDV-LaSota/46 or mock inoculated (sham) was clarified by centrifugation at 1,000 × *g* for 15 min. The virus titer was determined as EID_{50}/0.1 ml, as described above, and a water-in-oil emulsion inactivated vaccine was prepared as previously described (16).

Vaccination studies. The efficacy of a traditional inactivated vaccine in conferring protection against NDV-Peru/08 challenge was assessed in chickens. Four-week-old SPF white Leghorn chickens were randomly allocated into two experimental groups consisting of an NDV-LaSota/46-

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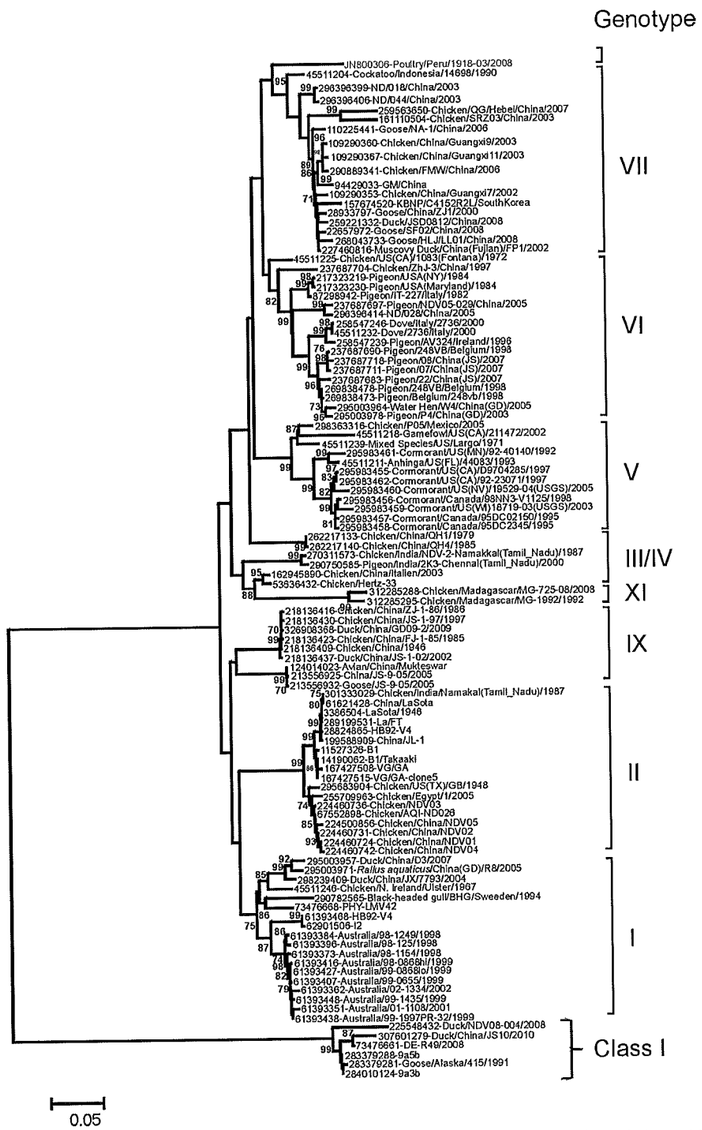

FIG 1 Phylogenetic analysis based on the complete fusion (F) gene sequence of 104 taxa available in GenBank. The evolutionary history was inferred using the neighbor-joining method (24). The optimal tree with the sum of branch lengths of 2.35304957 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (6). 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 maximum composite likelihood method (27) and are in units representing the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. All positions containing gaps and missing data were eliminated from the data set ("complete deletion" option). There were a total of 1,661 positions in the final data set. Phylogenetic analyses were conducted in MEGA5 (28).

vaccinated group ($n = 10$) and a control sham-vaccinated group ($n = 10$). Birds were vaccinated subcutaneously with 0.5 ml of the oil emulsion vaccines. Three weeks after vaccination, all birds were challenged with NDV-Peru/08 as described above and monitored for clinical signs of ND during a 14-day observation period. Serum samples were collected at days 0 and 21 postvaccination and day 14 postchallenge and tested for the presence of NDV antibodies by the hemagglutination inhibition assay.

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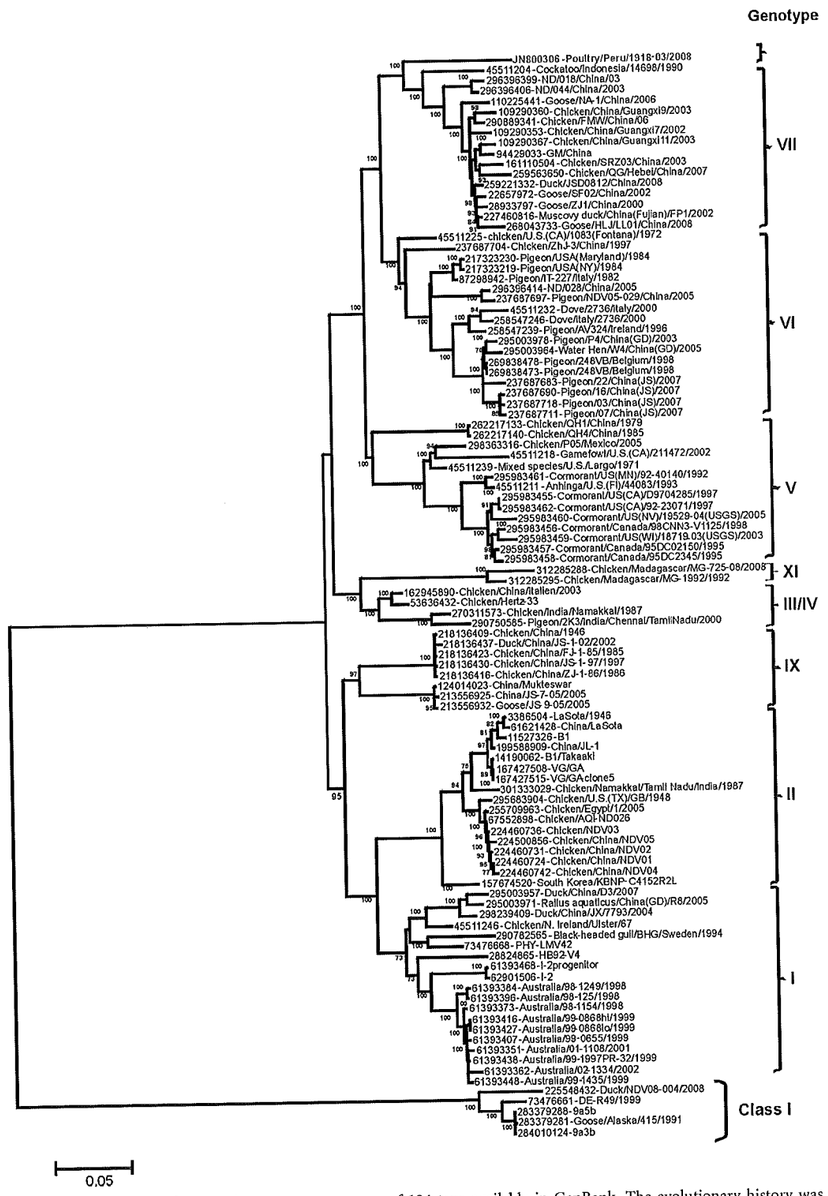

FIG 2 Phylogenetic analysis based on the complete genome sequence of 104 taxa available in GenBank. The evolutionary history was inferred using the neighbor-joining method (24). The optimal tree with the sum of branch lengths of 2.50688796 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (6). 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 maximum composite likelihood method (27) and are in units representing the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. All positions containing gaps and missing data were eliminated from the data set ("complete deletion" option). There were a total of 15,034 positions in the final data set. Phylogenetic analyses were conducted in MEGA5 (28).

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TABLE 1 Estimates of evolutionary distances between NDV-Peru/08 and viruses representing other established NDV genotypes

Genotype	No. of base substitutions/site in genotype[a]: I	II	III/IV	V	VI	VII	IX	NDV-Peru/08
I		(0.0037)	(0.0036)	(0.0043)	(0.0047)	(0.0048)	(0.0030)	(0.0056)
II	0.1423		(0.0047)	(0.0051)	(0.0051)	(0.0053)	(0.0041)	(0.0071)
III/IV	0.1464	0.1696		(0.0041)	(0.0036)	(0.0051)	(0.0030)	(0.0059)
V	0.2038	0.2289	0.1641		(0.0028)	(0.0035)	(0.0039)	(0.0041)
VI	0.1924	0.2226	0.1604	0.1576		(0.0033)	(0.0036)	(0.0041)
VII	0.2042	0.2262	0.1715	0.1655	0.1371		(0.0043)	(0.0036)
IX	0.1357	0.1576	0.1183	0.1772	0.1753	0.1864		(0.0053)
NDV-Peru/08	0.218	0.2432	0.1893	0.1804	0.1491	0.1255	0.2011	

[a] The number of base substitutions per site is shown for NDV-Peru/08 and genotypes of class II viruses. All results are based on the pairwise analysis of 98 sequences. The numbers of sequences analyzed per group were as follows: I, $n = 16$; II, $n = 18$; III/IV, $n = 6$; V, $n = 12$; VI, $n = 18$; VII, $n = 16$; IX, $n = 8$; and poultry/Peru/08 (NDV-Peru/08), $n = 1$. Analyses were conducted using the maximum composite likelihood method in MEGA5. The codon positions included were the 1st + 2nd + 3rd + noncoding. All positions containing gaps, and missing data were eliminated from the data set ("complete deletion" option). There were a total of 15,034 positions in the final data set. Values in parentheses are standard errors, obtained by a bootstrap procedure (500 replicates).

Nucleotide sequence accession number. The complete genome sequence of the NDV isolate poultry/Peru/1918-03/2008 is available in GenBank under accession no. JN800306.

RESULTS

NDV-Peru/08 belongs to the velogenic NDV pathotype. The pathogenicity of NDV-Peru/08 was initially assessed by sequencing of the fusion (F) protein cleavage site and by the standard ICPI test. Sequencing of the F protein cleavage site revealed the presence of three basic amino acid residues at positions 113, 115, and 116 and a phenylalanine at position 117 (^{112}R-R-Q-K-R-F^{117}). Intracerebral inoculation of NDV-Peru/08 in day-old chicks resulted in an ICPI of 1.78, which is typical of velogenic NDV strains (15, 31, 32).

Phylogenetic analysis of the complete F gene and the complete genome sequences demonstrate that NDV-Peru/08 is genetically distinct from other established NDV genotypes. Phylogenetic analysis based on the F gene revealed that NDV-Peru/08 is clearly distinct from viruses representing other known NDV genotypes (Fig. 1). To confirm the results obtained by the analysis of the F gene, we performed a phylogenetic analysis based on the complete genome sequence of NDV-Peru/08 and other reference NDV strains ($n = 104$). Similar to the results obtained in the analysis of the F gene, the compete genome phylogenetic reconstruction resulted in NDV-Peru/08 forming an isolated branch separate from the viruses of other known class II genotypes (Fig. 2). The viruses that appear most closely related to NDV-Peru/08 are the strains cockatoo/Indonesia/14698/90 (GI 45511204), ND/03/044 (GI 286396406), and ND/03/018 (GI 286396399), which were isolated in Asia and belong to genotype VII and subgenotypes VIIa (cockatoo/Indonesia/14698/90) and VIId (ND/03/044 and ND/03/018). Phylogenetic analysis performed with individual genes of the data set of 104 complete genomes (coding for F, NP, P, M, HN, and L) revealed a similar phylogenetic topology (data not shown). No recombination events were observed in the NDV-Peru/08 genome (data not shown).

The evolutionary distances between the complete genome of NDV-Peru/08 and those of strains of other class II genotypes (I, II, III/IV, V, VI, and VII) revealed that NDV-Peru/08 is significantly distant from viruses of all other NDV genotypes (Table 1). The distance between NDV-Peru/08 and the viruses of genotype VII, for example, is 0.1255, which is in the range of the distances observed between other genotypes (Table 1) and is markedly larger than the mean distance within genotype VII viruses (0.0406; standard error, 0.0009) (data not shown). Notably, the distances observed between NDV-Peru/08 and viruses of genotypes I and II (vaccine-like viruses) were 0.218 and 0.2432, respectively, and are the largest distances observed among all NDV genotypes (Table 1). Similar results were obtained when the F gene sequences were used to calculate the evolutionary distances (data not shown).

TABLE 2 Nucleotide and amino acid comparison between the Newcastle disease virus isolate NDV-Peru/08 and viruses representing other genotypes within class II[a]

Protein	% identity for virus by genotype[b]: I, Ulster/67 nt	I, Ulster/67 aa	II, LaSota nt	II, LaSota aa	III, JS/7/05/Ch nt	III, JS/7/05/Ch aa	IV, Italien nt	IV, Italien aa	V, Anhinga/93 nt	V, Anhinga/93 aa	VI, Fontana/72 nt	VI, Fontana/72 aa	VII, ZJ1/2000 nt	VII, ZJ1/2000 aa	IX, FJ/1/85/Ch nt	IX, FJ/1/85/Ch aa
NP	85	93	84	91	87	93	90	95	88	93	88	93	91	96	87	94
P	82	81	81	80	84	82	84	85	84	80	85	80	87	85	85	81
M	85	89	84	88	86	89	88	93	85	92	85	92	91	95	86	91
F	84	90	82	88	84	90	87	92	86	91	86	91	89	93	84	91
HN	85	89	83	86	84	87	86	89	85	89	86	89	90	93	84	88
L	87	94	85	92	87	94	88	95	88	95	88	95	91	96	87	94
Complete genome	84.8		82.5		85		86.8		85.9		89.9		89.6		84.8	

[a] Alignment was performed by using ClustalW.
[b] Accession numbers: I, AY562891; II, AF077761; III, FJ430159; IV, EU283914; V, AY562886; VI, AY562888; VII, AF431744; IX, FJ436304.

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TABLE 3 Genomic features of Newcastle disease virus isolate NDV-Peru/08

Protein	Gene start positions (nt)	3′ UTR (length nt)[a]	Coding sequence positions (nt)	5′ UTR (nt length)	Gene end positions (nt)	Length of: Intergenic region (nt)	Gene (nt)	Protein (aa)
NP	56–65	66	122–1591	217	1798–1808	1	1,469	489
P	1810–1819	73	1883–3080	180	3250–3260	1	1,197	395
M	3262–3271	34	3286–4390	112	4493–4502	1	1,073	364
F	4504–4513	46	4550–6211	84	6285–6285	31	1,661	553
HN	6327–6336	91	6418–8133	195	8318–8328	47	1,715	571
L	8376–8387	11	8387–15001	77	15069–15078		6,614	2,204

[a] UTR, untranscribed region.

A comparison of the nucleotide and amino acid sequences between NDV-Peru/08 and selected class II reference strains representing genotypes I, II, III, IV, V, VI, VII, and IX is presented in Table 2. Alignment of the complete genome sequences revealed that NDV-Peru/08 shares 89.9% and 89.6% nucleotide identity with NDV strains Fontana/72 (genotype VI) and ZJ1/2000 (genotype VII), respectively. Notably, the lowest nucleotide identity was observed between NDV-Peru/08 and the vaccine strains Ulster/67 (84.4%; genotype I) and LaSota/46 (82.5%; genotype II). Similar to the complete genome nucleotide sequence analysis, comparison of the nucleotide and amino acid sequences of each gene confirmed that NDV-Peru/08 shares the highest nucleotide and amino acid identity with the strains of genotypes VI and VII, while the lowest degree of identity was observed between NDV-Peru/08 and the vaccine-like strains of genotypes I and II (Table 2).

Genomic features of NDV-Peru/08. The genome of NDV-Peru/08 is 15,192 nucleotides (nt) in length, it contains a 6-nt insertion in the 5′ noncoding region of the NP gene between positions 1738 and 1743 and a G+C content of 46.2%. A summary of the genomic features of NDV-Peru/08, including the gene start, gene end, and intergenic and coding regions is presented in Table 3.

Infection of chickens with NDV-Peru/08 results in characteristic velogenic viscerotropic Newcastle disease. The clinicopathological characteristics and the virulence of NDV-Peru/08 were assessed in chickens. All NDV-Peru/08-inoculated birds presented severe prostration, had mucous diarrhea, and died or were euthanized by day 5 postinoculation (mean death time, 4.7 days) (Fig. 3). Gross lesions were initially observed by day 2 p.i. and consisted of conjunctivitis, multifocal necrosis, and hemorrhages in the intestine and cecal tonsils, as well as mottling and multifocal necrosis in the spleen. Additionally, atrophy of the thymus was observed by day 3 p.i. and was characterized by a marked reduction in size and by the presence of gelatinous edema and perithymic hemorrhages. Multifocal hemorrhages were also observed in the pharynx by day 4 p.i. Control group birds did not exhibit any clinical signs or pathological changes.

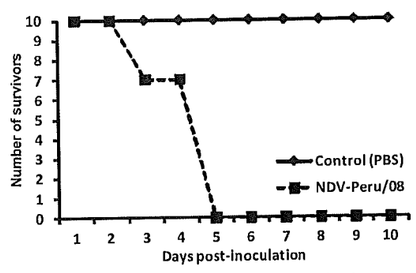

FIG 3 Survival curve. Three birds of the NDV-Peru/08-inoculated group (group 2) died on day 3 postinoculation, and the remaining birds died on day 5 p.i. (mean death time, 4.7 days).

Typical histological changes associated with velogenic viscerotropic NDV infection, including lymphoid depletion and necrosis, accumulation of macrophages, necrotic debris, and scattered heterophils, were observed in multiple organs of NDV-Peru/08-inoculated birds (Fig. 4A, panels b and d; Table 4). Lesions were more severe as the disease progressed and peaked at day 4 p.i. A summary of the severity and distribution of the lesions is presented in Table 4. Control group birds did not present histological changes.

Immunohistochemical staining for NDV nucleoprotein (NP) revealed a widespread distribution of the virus (Table 4), with antigen load and distribution peaking at 4 dpi. The strongest staining intensity was observed in the eyelids, lymphoid organs, and mucosa-associated-lymphoid tissue (MALT) aggregates of various organs (Table 4; Fig. 4B, panels a and b), with positive cells consisting mainly of lymphocytes and macrophages.

All birds inoculated with NDV-Peru/08 shed the virus in oral and cloacal swabs. The virus was isolated from samples collected on days 2, 3, and 5 p.i., with virus titers ranging from $10^{3.7}$ to $10^{5.9}$ EID_{50}/0.1 ml and from $10^{2.9}$ to $10^{5.4}$ EID_{50}/0.1 ml in oral and cloacal secretions, respectively.

Inactivated NDV-LaSota/46 vaccine confers protection against NDV-Peru/08-induced disease and mortality. The efficacy of an inactivated NDV-LaSota/46 vaccine in conferring protection against NDV-Peru/08 challenge was assessed in chickens. All NDV-LaSota/46-vaccinated birds seroconverted (HI titers between 64 and 128, geometric mean titer [GMT] of 105.95) and were fully protected from clinical disease and mortality induced by NDV-Peru/08 infection, whereas all birds from the sham-vaccine group presented typical clinical signs of velogenic viscerotropic NDV strains, including severe prostration and diarrhea, and died by day 5 p.i.

DISCUSSION

Virulent Newcastle disease virus (vNDV) is endemic in many countries of North, Central, and South America, and outbreaks of ND are frequently reported to the World Organization for Animal Health (OIE). In June and July of 2008, simultaneous outbreaks of ND were confirmed in domestic poultry in Peru (provinces of Arequipa and Cusco). The high morbidity and

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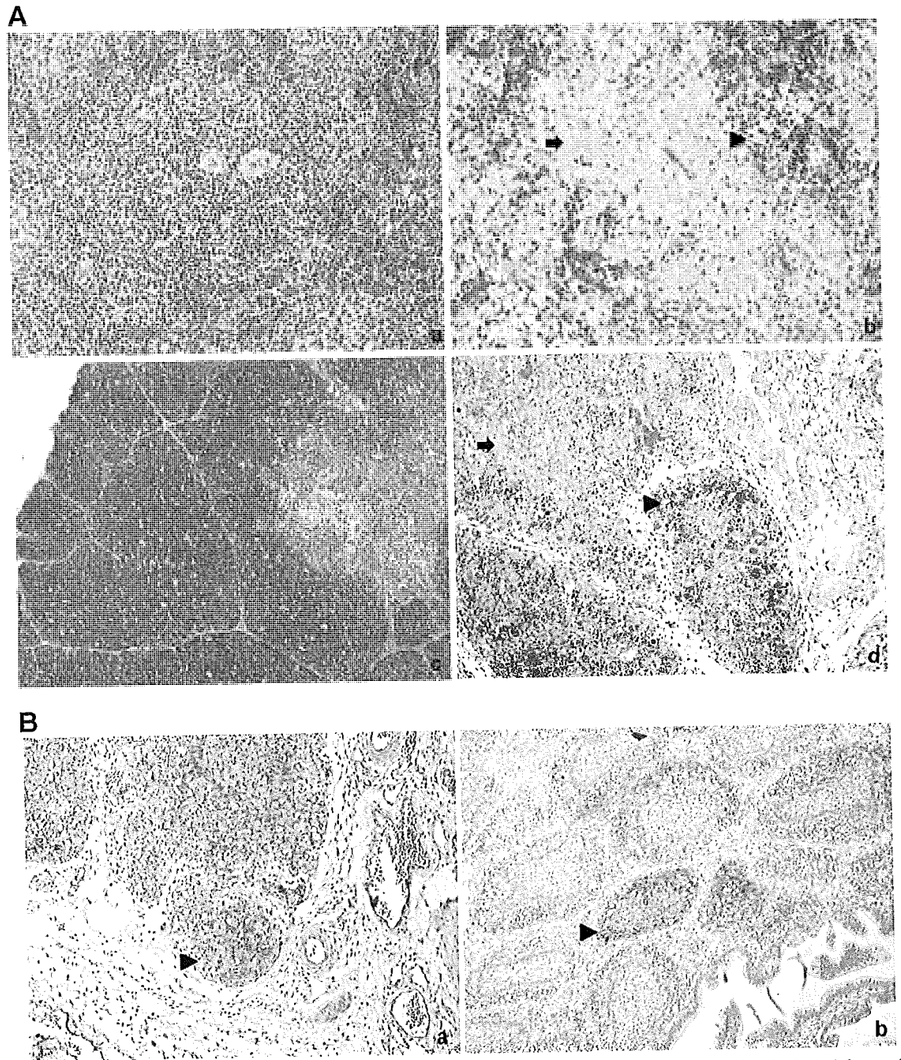

FIG 4 Histological changes and distribution of Newcastle disease virus nucleoprotein in NDV-Peru/08-infected tissues. (A) Histological changes (panels b and d) consisted of lymphoid depletion and necrosis (arrows), accumulation of macrophages (arrowheads), and scattered heterophils. Panel a, spleen, PBS control, 40×; panel b, spleen, NDV-Peru/08, 40×; panel c, thymus, PBS control, 20×; panel d, thymus, NDV-Peru/08, hematoxylin and eosin staining. (B) Immunohistochemical staining for Newcastle disease virus nucleoprotein. Shown is positive staining in the thymus (panel a, 20×) and bursa of Fabricius (panel b, 20×). Positive cells consisted mainly of lymphocytes and macrophages (arrowheads).

mortality rates (75.6%) of the 2008 outbreak and the clinicopathological characteristics of NDV-Peru/08 resemble the clinical presentation described for the highly virulent viruses of genotypes V and VII that circulate in Central America, Africa, Asia, and the Middle East (26). Although the source of the 2008 outbreak in Peru is still unknown, field epidemiological investigations reported to the OIE suggest that the virus was likely introduced through live birds that were added to the affected flocks (19). However, a complete epidemiological investigation, including sampling of domestic and wild bird species, is needed to define the actual source of the outbreak and origin of the virus.

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TABLE 4 Distribution and severity of lesions and distribution and intensity of viral antigen staining in tissues of NDV-Peru/08-inoculated birds

Tissue[a]	Result on postinoculation day[b]: 2	3	4
Eyelid			
HE	++	++	++
IHC	++	+++	+++
Spleen			
HE	++	+++	+++
IHC	++	++	+++
Thymus			
HE	+	++	+++
IHC	–	++	++++
Bursa			
HE	+	+	+++
IHC	+	++	++
Harderian gland			
HE	–	+	–
IHC	–	+	+++
Proventriculus			
HE	+	++	–
IHC	–	++++	+++
Pancreas			
HE	+	–	++
IHC	–	–	++
Small intestine			
HE	–	–	+
IHC	+	–	++
Mekel's diverticulum			
HE	+	–	++
IHC	–	–	++
Cecal tonsils			
HE	+	++	+++
IHC	+++	+++	++++
Large intestine			
HE	–	+	+
IHC	–	++	++
Air sacs			
HE	–	–	–
IHC	–	–	+
Trachea			
HE	–	–	–
IHC	–	+	–
Lung			
HE	–	–	+ (BALT)
IHC	–	+ (BALT)	++ (BALT)
Pharynx			
HE	+	++	++
IHC	–		+

TABLE 4 (Continued)

Tissue[a]	Result on postinoculation day[b]: 2	3	4
Brain			
HE	–	–	–
IHC	–	–	+ (cerebellum)
Liver			
HE	–	–	+ (MALT)
IHC	–	–	–
Kidney			
HE	–	–	–
IHC	–	–	+
Comb			
HE	–	–	–
IHC	–	–	++
Femur			
HE	–	–	+
IHC	–	–	+

[a] HE, hematoxylin and eosin staining; IHC, immunohistochemistry staining for NDV nucleoprotein.

[b] BALT, bronchus-associated lymphoid tissue; MALT, mucosa-associated lymphoid tissue. For HE, the results are scored by tissue as follows: (i) spleen, +, moderate hyperplasia, ++, lymphocytic depletion, +++, moderate (<50%) lymphocyte depletion, histiocytic accumulation, and multifocal necrosis, and ++++, severe (>50%) lymphocytic depletion, histiocytosis, and necrosis; (ii) thymus, cecal tonsil, gut-associated lymphoid tissue (GALT), and bursa, +, mild lymphocytic depletion, ++, moderate (<50%) lymphocytic depletion with necrosis and histiocytosis, and +++, severe (>50%) lymphocytic depletion, necrosis, and histiocytosis; (iii) bone marrow, +, mild (<20%) bone marrow necrosis, ++, moderate (20 to 50%) bone marrow necrosis, and +++, severe (>50%) bone marrow necrosis; (iv) pancreas, +, mild (<3 areas) vacuolation and degeneration, and ++, moderate (>3 areas) vacuolation and degeneration; and (v) brain, +, vascular reactivity, ++, vascular reactivity and perivascular cuffing, and +++, vascular reactivity, perivascular cuffing, and gliosis. For IHC staining, the results are scored as follows: –, no IHC signal detected; +, rare cells in the section are positive on IHC; ++, positive cells seen in <50% of all high-magnification fields (HMF); +++, positive signal seen in 50 to 75% of HMF; and ++++, abundant positive signal in more than 75% of HMF.

Recent phylogenetic studies have shown that NDV is continuously evolving, with viruses of different genotypes undergoing simultaneous changes in distinct geographic locations (4, 5, 11, 22, 25, 33, 34). The results presented here indicate that NDV-Peru/08 has evolved and significantly diverged from viruses representing all known NDV genotypes. Classification of NDV-Peru/08 into one of the existing NDV genotypes or into a new genotype, however, is still uncertain, and isolation of additional viruses with similar genetic and phylogenetic properties is needed in order to classify this isolate.

Historically, NDV isolates have been classified as lentogenic, mesogenic, or velogenic based on to the clinicopathological outcome of the infection in chickens (3). Clinicopathological characterization of NDV-Peru/08 revealed that this virus behaves as a typical velogenic viscerotropic strain. The mortality rate (100%) and mean death time (4.7 days) observed in NDV-Peru/08-inoculated birds are characteristic of highly virulent strains of genotypes V, VI, and VII (21, 26, 31). Histological examination and immunohistochemical staining of tissue samples collected from multiple organs confirmed the tropism of NDV-Peru/08 for lymphoid tissues associated with the gastrointestinal tract and, to a

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lesser extent, with the respiratory system. A similar tropism is seen in infections with the highly virulent genotype VII viruses (26).

The data presented here provide evidence that a genetically distinct virus, most closely related to but different from isolates of genotypes VI and VII caused the outbreak of ND in Peru. Notably, the evolutionary distances between NDV-Peru/08 and vaccine-like viruses of genotypes I and II are the largest distances observed between all genotypes. Given the genetic differences observed between NDV-Peru/08 and vaccine-like viruses (Table 1 and 2), vaccination studies were performed with chickens. Results of these experiments demonstrated that, under biosafety level 3 conditions and using SPF birds, a traditional inactivated NDV vaccine strain from genotype II (NDV-LaSota/46) fully protected chickens from NDV-Peru/08-induced clinical disease and mortality. These observations suggest that the genetic diversity of NDV-Peru/08 is not reflected in antigenic changes that significantly affect the immune response elicited by the inactivated NDV-LaSota/46 vaccine. However, whether these findings will be observed under field conditions and with vaccine strains from other NDV genotypes remains to be determined.

In summary, complete genome- and protein-level analysis of the evolutionary distances between NDV-Peru/08 and strains of established NDV genotypes demonstrated that this isolate is distinct from viruses of typical NDV genotypes. Given the lack of epidemiological data from the 2008 outbreak and from other NDV isolates circulating in South America, it is unclear whether similar viruses are still circulating in that continent, or whether that outbreak was a unique episode caused by this isolate. Continuous characterization of novel NDV isolates that occasionally emerge and cause outbreaks or of those that frequently circulate worldwide are important to improve the current understanding of NDV epidemiology and evolution and for the development of improved control and diagnostic strategies.

ACKNOWLEDGMENTS

We thank Dawn Williams-Coplin and Tim Olivier for technical assistance, Roger Brock for help with animal experiments, and the SEPRL sequencing facility personnel for nucleotide sequencing. We also acknowledge the generous support of CEVA Biomune for the vaccination studies and the National Direction of Animal Health of Peru (SENASA) for invaluable collaboration and for sharing information on the outbreak.

This work was supported by USDA funding CRIS 6612-32000-049.

REFERENCES

1. Aldous EW, Mynn JK, Banks J, Alexander DJ. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32:239–256.
2. Alexander DJ, Senne DA. 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections, p 75–98. *In* Saif M, et al (ed), Diseases of poultry, 12th ed. Blackwell Publishing, Ames, IA.
3. Alexander DJ, Swayne DE. 1998. Newcastle disease virus and other avian paramyxoviruses, p 156–163. *In* Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM (ed), A laboratory manual for the isolation and identification of avian pathogens, vol 4. American Association of Avian Pathologists, Kennett Square, PA.
4. Czegledi A, et al. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120:36–48.
5. Ding Z, et al. 2010. Genetic analysis of avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from swine populations in China related to commonly utilized commercial vaccine strains. Virus Genes 41:369–376.
6. Felsenstein J. 1992. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. Genet. Res. 60: 209–220.
7. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. 2007. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. J. Clin. Microbiol. 45:1310–1314.
8. Kim LM, Suarez DL, Afonso CL. 2008. Detection of a broad range of class I and II Newcastle disease viruses using multiplex real-time reverse transcription polymerase chain reaction assay. J. Vet. Diagn. Invest. 20:414–425.
9. Kinde H, et al. 2005. The isolation of exotic Newcastle disease (END) virus from nonpoultry avian species associated with the epidemic of END in chickens in southern California: 2002–2003. Avian Dis. 49:195–198.
10. Lancaster JE, Alexander DJ. 1975. Newcastle Disease virus and spread, p 1–79.Canada Department of Agriculture, monograph no. 11. Canada Department of Agriculture, Ottawa, Canada.
11. Linde AM, et al. 2010. Complete genome characterisation of a Newcastle disease virus isolated during an outbreak in Sweden in 1997. Virus Genes 41:165–173.
12. Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB. 2003. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Arch. Virol. 148:1387–1403.
13. Martin DP. 2009. Recombination detection and analysis using RDP3. Methods Mol. Biol. 537:185–205.
14. Miller PJ, Decanini EL, Afonso CL. 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. 10: 26–35.
15. Miller PJ, Kim LM, Ip HS, Afonso CL. 2009. Evolutionary dynamics of Newcastle disease virus. Virology 391:64–72.
16. Miller PJ, King DJ, Afonso CL, Suarez DL. 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 25:7238–7246.
17. OIE. 2009. Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees, 5th ed, vol 1, part 2, chapter 2.3.14, p 576–589. Biological Standards Commission, World Organization for Animal Health, Paris, France.
18. OIE. 2011. World Animal Health Information Database (WAHID) Interface. http://web.oie.int/wahis/public.php?page=home&WAHIDPHPSESSID=4d95e8777ff8b8a615b31617ac4c7835.
19. OIE. 2008. World Animal Health Information Database (WAHID) Interface. Event summary: Newcastle disease, Peru. World Organization for Animal Health, Paris, France. http://web.oie.int/wahis/public.php?page=event_summary&reportid=7326.
20. Perozo F, Afonso RMCL, Fernandez R, Rojo F. 2011. Genotype VII velogenic viscerotropic Venezuelan Newcastle disease virus isolate: live (AvinewR) and killed (Gallimune NDR) vaccination, abstr 169P, p 49. Abstr. 2011 Int. Poultry Scientific Forum, Atlanta, GA, 24 to 25 January 2011.
21. Perozo F, Merino R, Afonso CL, Villegas P, Calderon N. 2008. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. Avian Dis. 52:472–479.
22. Qiu X, et al. 2011. Entire genome sequence analysis of genotype IX Newcastle disease viruses reveals their early-genotype phylogenetic position and recent-genotype genome size. Virol. J. 8:117.
23. Rue CA, et al. 2010. Evolutionary changes affecting rapid identification of 2008 Newcastle disease viruses isolated from double-crested cormorants. J. Clin. Microbiol. 48:2440–2448.
24. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
25. Seal BS. 2004. Nucleotide and predicted amino acid sequence analysis of the fusion protein and hemagglutinin-neuraminidase protein genes among Newcastle disease virus isolates. Phylogenetic relationships among the Paramyxovirinae based on attachment glycoprotein sequences. Funct. Integr. Genomics 4:246–257.
26. Susta L, Miller PJ, Afonso CL, Brown CC. 2010. Clinicopathological characterization in poultry of three strains of Newcastle disease virus isolated from recent outbreaks. Vet. Pathol. 48:349–360.
27. Tamura K, Nei M, Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101:11030–11035.
28. Tamura, K., et al. MEGA5: molecular evolutionary genetics analysis using

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maximum likelihood, evolutionary distance, and maximum parsimony methods Mol. Biol. Evol. 28:2731–2739.
29. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.
30. Utterback WW, Schwartz JH. 1973. Epizootiology of velogenic viscerotropic Newcastle disease in southern California, 1971–1973. J. Am. Vet. Med. Assoc. 163:1080–1088.
31. Wakamatsu N, King DJ, Kapczynski DR, Seal BS, Brown CC. 2006. Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002–2003. Vet. Pathol. 43:925–933.
32. Wang Z, et al. 2006. Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. Ann. N. Y. Acad. Sci. 1081:228–239.
33. Wu S, et al. 9 November 2010, posting date. Genetic diversity of Newcastle disease viruses isolated from domestic poultry species in Eastern China during 2005–2008. Arch. Virol. [Epub ahead of print.] doi:10.1007/s00705-010-0851-5.
34. Yu L, Wang Z, Jiang Y, Chang L, Kwang J. 2001. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. J. Clin. Microbiol. 39:3512–3519.

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Viruses **2012**, *4*, 28-61; doi:10.3390/v4010028

OPEN ACCESS

viruses

ISSN 1999-4915

www.mdpi.com/journal/viruses

Article

Analysis of the Genome of the Sexually Transmitted Insect Virus *Helicoverpa zea* Nudivirus 2

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Received: 29 November 2011; in revised form: 30 December 2011 / Accepted: 31 December 2011 / Published: 6 January 2012

Abstract: The sexually transmitted insect virus *Helicoverpa zea* nudivirus 2 (HzNV-2) was determined to have a circular double-stranded DNA genome of 231,621 bp coding for an estimated 113 open reading frames (ORFs). HzNV-2 is most closely related to the nudiviruses, a sister group of the insect baculoviruses. Several putative ORFs that share homology with the baculovirus core genes were identified in the viral genome. However, HzNV-2 lacks several key genetic features of baculoviruses including the late transcriptional regulation factor, LEF-1 and the palindromic *hrs*, which serve as origins of replication. The HzNV-2 genome was found to code for three ORFs that had significant sequence homology to cellular genes which are not generally found in viral genomes. These included a presumed juvenile hormone esterase gene, a gene coding for a putative zinc-dependent matrix metalloprotease, and a major facilitator superfamily protein gene; all of which are believed to play a role in the cellular proliferation and the tissue hypertrophy observed in the malformation of reproductive organs observed in HzNV-2 infected corn earworm moths, *Helicoverpa zea*.

Keywords: nudivirus; sterile insects; baculovirus; sexually transmitted virus; virus genome; juvenile hormone esterase; HzNV-2; HzNV-1; corn earworm; *Helicoverpa zea*

1. Introduction

Helicoverpa zea nudivirus 2 (HzNV-2) was originally identified as being responsible for the sterility of infected corn earworm moths (*H. zea*) in a colony of this insect established and maintained at Stoneville MS (USA). HzNV-2 [a.k.a. gonad specific virus (GSV), Hz-2V] is an enveloped, rod-shaped, double stranded DNA (dsDNA) virus which is a member of the newly described *Nudivirus* genus [1]. Other members of this group include: the *Oryctes rhinoceros* nudivirus (OrNV), *Gryllus bimaculatus* nudivirus (GbNV) and the nudivirus (HzNV-1), initially identified in a cell line established from ovarian tissues of *H. zea* moths. Although the nudiviruses (NV) are considered a loosely associated group of non-occluded insect pathogenic dsDNA viruses, the recent analysis of OrNV genome has provided a foundation for common elements of some of the disparate members of this group [2,3]. In addition, this work describes possible evolutionary linkages between these viruses and other large dsDNA viruses of arthropods including the baculoviruses, the hytrosaviruses and the nimaviruses. Significant homology has also been found between several nudivirus genes and genes coding for both nucleocapsid proteins of bracoviruses and genes involved in the replication of these polydnaviruses. These findings have led to the suggestion that polydnavirus originated from the integration of a nudivirus ancestor into the genome of a braconid wasp [4].

Our initial characterization of the HzNV-2 genome revealed that this virus shared a 93% sequence identity with HzNV-1 [1]. This was an interesting finding in view of the fact that, while both viruses can replicate in tissue culture cells, only HzNV-2 has the ability to replicate in an insect host. In as much as the replication of either of these two viruses in cell culture can lead to establishment of persistently infected cell lines, persistent replication of HzNV-2 in the insect host, results in fertile *H. zea* moths, which are asymptomatic carriers of the virus. Productive replication of HzNV-2 on the other hand appears to be limited to the reproductive tissues of the infected moth resulting in the malformation of these tissues and sterility of the infected host. These infected moths then serve to transmit the virus to other moths during mating attempts making HzNV-2 the causative agent of the only known sexually transmitted viral diseases of insects.

HzNV-2 infected female moths lack some reproductive structures including, ovaries, bursa copulatrix, accessory glands and spermatheca, and have grossly deformed, enlarged common and lateral oviducts, which appear as a large "Y-shaped" structure. This is a condition which has been referred as being agonadal [5,6]. In both pupae and adult females, the branches of the "Y-shaped" structure are much larger than the corresponding normal, lateral oviducts. EM observations of the branch structure in 7-day-old, infected pupae shows a high level of virus replication in cells within this structure. Viral replication in these structures results in hypertrophy of the oviducts and proliferation of cells that make up these tissues [6]. In female reproductive tissues, virus replication culminates in the formation of a "viral plug" which serves to protract the mating behavior of these females, as well as serve as a source of contaminating virus for males attempting to mate [7]. In addition, infected females

produce five to seven times more sex pheromone than uninfected females and attract twice as many male mates [8].

The ability of HzNV-2 to manipulate molecular processes of the host, particularly the massive cellular and tissue transformations observed in oviducts of infected females, suggests this virus is able to direct the alteration of the physiology and gene expression in infected host tissues. In order to better understand the evolutionary relationship between HzNV-1 and HzNV-2, and to begin to identify viral genes responsible for the pathology and unique biology of this insect pathogenic virus, we undertook the sequencing of the genome of HzNV-2.

2. Results and Discussion

2.1. Genome Features

The genome of HzNV-2 was found to be a circular dsDNA molecule of 231,621 bp. This is in good agreement with the previous restriction endonuclease size estimate of 225 to 235 kb [9], making it the largest of the insect viruses with dsDNA genomes.

The HzNV-2 genome has a G+C content of 41.9% and contains 376 ORFs coding more than 60 aa, of which 113 ORFs are likely to encode proteins. Of these putative genes, 66 ORFs are found on the forward strand and 47 on the reverse, all arranged evenly, producing 29 clusters of 1 to 6 ORFs in the viral genome. With sizes ranging from 5.7 kb to 189 bp, the average identified ORF was 1.4 kb in length. The gene density was one gene per 2.05 kb, and the coding density was 68%. The location, size, and direction of these ORFs is shown in Table 1 and Figure 1. For descriptive purposes, the HzNV-2 genome map is presented in linear form, and gene homologues of HzNV-1 are excluded (from Table 1, with the exception of HzNV-1 p34 and p51 late proteins which were the best blast matches) because of their high sequence homologies, which were over 90% in most cases. HzNV-1 and other nudivirus homologues are also listed in Table 1.

Unlike the baculoviruses, which have clusters of repeated, imperfect palindromic sequences, the so-called homologous regions (*hrs*) [10], no *hrs* were found in HzNV-2 genome. No *hrs* have been found thus far in any of the other NV, including HzNV-1 [11], and GbNV [12]. The *hrs* found in baculoviruses serve as origins of viral DNA replication [13], and all of the members of *Baculoviridae* family sequenced to date have *hrs* as a common feature of their genome. Although no *hrs* were identified in the HzNV-2 genome, abundant direct tandem repeated sequences were present. Six of the most significant direct repeats (*drs*) regions of 24 to 81 bp, which repeat 7 to 25 times (Table 2), were found by EMBOSS Etandem software. Five of the six *drs* in the HzNV-2 genome form a cluster at the 174.2–180 kb region.

In a search of GenBank database release 166, only 38 of the 113 putative HzNV-2 genes demonstrated clear homologies to genes in the current database other than putative HzNV-1 genes. These include six putative genes involved in DNA replication, four in transcription, five in nucleic acid metabolism, three structural proteins, and 14 with other functions. Hz2V058 and Hz2V073 had high homologies to the two HzNV-1 late genes, p34 and p51, respectively. The other 75 of 113 ORFs had poor or no homology to any known genes.

Figure 1. Genome map of HzNV-2. For descriptive purpose, circular genomic DNA is presented in linear form. The position of the first nucleotide was arbitrarily chosen. ORFs are numbered from left to right based on the position of the methionine initiation codon. ORFs transcribed to the right are located above the horizontal line; ORFs transcribed to the left are below.

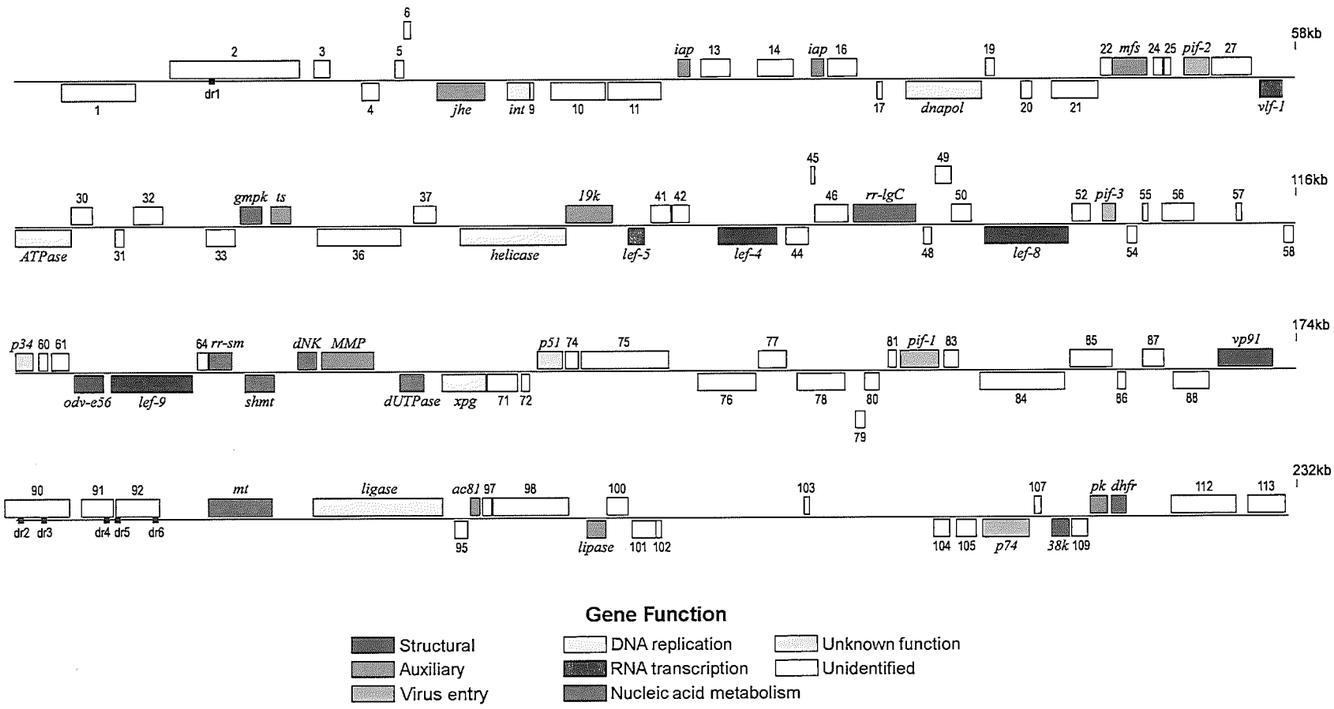


Table 1. HzNV-2 putative ORFs.

ORF	Strand [a]	Position		Length		Best Blast match			Signature [b]	HzNV-1 [c]
		start	end	(nt)	(aa)	Predicted function	Species/Viruses	E-value		
Hz2V001	R	5630	2298	3333	1111				TAF, H3	1
Hz2V002	F	7183	13047	5865	1955					154
Hz2V003	F	13677	14378	702	234					151
Hz2V004	R	16594	15830	765	255					150
Hz2V005	F	17366	17803	438	146					148
Hz2V006	F	17779	18063	285	95					
Hz2V007	R	21403	19241	2163	721	Carboxylesterase	*Drosophila pseudoobscura*	3×10^{-21}	Coesterase	145
Hz2V008	R	23425	22421	1005	335	Integrase	Monodon baculovirus	1×10^{-29}	INT_REC_C	144
Hz2V009	R	23621	23430	192	64					143
Hz2V010	R	26839	24425	2415	805					141
Hz2V011	R	29383	26969	2415	805					140
Hz2V012	F	30140	30700	561	187	Inhibitor of apoptosis protein	*Trichoplusia ni SNPV*	7×10^{-25}	BIR	138
Hz2V013	F	31151	32494	1344	448					137
Hz2V014	F	33735	35312	1578	526					
Hz2V015	F	36168	36713	546	182	Inhibitor of apoptosis protein	*Adoxophyes orana* GV	120	BIR	135
Hz2V016	F	36879	38201	1323	441					134
Hz2V017	R	39326	39138	189	63					133
Hz2V018	R	43848	40441	3408	1136	DNA polymerase	*Oryctes rhinoceros NV*	1×10^{-25}	POLBc	131
Hz2V019	F	44032	44442	411	137					130
Hz2V020	R	46083	45655	429	143					129
Hz2V021	R	49108	47033	2076	692					128
Hz2V022	F	49210	49731	522	174					
Hz2V023	F	49772	51307	1536	512	Membrane transporter	*Ades aegyptii*	1×10^{-135}	MFS_1	126
Hz2V024	F	51575	52000	426	142					125

Table 1. *Cont.*

ORF	Strand [a]	Position		Length		Best Blast match			Signature [b]	HzNV-1 [c]
		start	end	(nt)	(aa)	Predicted function	Species/Viruses	E-value		
Hz2V025	F	52066	52392	327	109					124
Hz2V026	F	52998	54137	1140	380	Per-os infectivity factor 2	*Gryllus bimaculatus* NV	5×10^{-37}	Baculo_44	123
Hz2V027	F	54224	56023	1800	600					122
Hz2V028	R	57374	56376	999	333	Very late expression factor 1	Monodon baculovirus	1×10^{-8}		121
Hz2V029	R	60667	58199	2469	823	DNA repair related ATPase	*Gryllus bimaculatus* NV	3×10^{-4}	SbcC	119
Hz2V030	F	60709	61674	966	322					118
Hz2V031	R	63063	62671	393	131					117
Hz2V032	F	63532	64803	1272	424					115
Hz2V033	R	68086	66803	1284	428					112
Hz2V034	F	68319	69323	1005	335	Guanosine monophosphate kinase	Monodon baculovirus	4×10^{-5}	GMPK	111
Hz2V035	F	69749	70621	873	291	Thymidylate synthase	*Bombyx mori*	4×10^{-131}	Thymidylat_synt	109
Hz2V036	R	75567	71800	3768	1256					107
Hz2V037	F	76171	77190	1020	340					106
Hz2V038	R	83046	78304	4743	1581	Helicase	*Culex nigripalpus* NPV	2.8	Pox_D5	104
Hz2V039	F	83048	85156	2109	703	19kDa protein	*Gryllus bimaculatus* NV	9×10^{-9}	Baculo_19	103
Hz2V040	R	86584	85862	723	241	Late expression factor 5	*Spodoptera exigua* NPV	6×10^{-3}	Baculo_LEF5	101
Hz2V041	F	86863	87756	894	298					100
Hz2V042	F	87845	88594	750	250					99
Hz2V043	R	92546	89928	2619	873	Late expression factor 4	*Gryllus bimaculatus* NV	3×10^{-18}		98
Hz2V044	R	94003	92963	1041	347					97
Hz2V045	F	94110	94313	204	68					
Hz2V046	F	94306	95775	1470	490					96
Hz2V047	F	96009	98822	2814	938	Ribonuclease reductase	*Spodoptera litura* NPV	0	Ribonuc_red_lgC	95
Hz2V048	R	99573	99214	360	120					94

Table 1. *Cont.*

ORF	Strand [a]	Position		Length		Best Blast match			Signature [b]	HzNV-1 [c]
		start	end	(nt)	(aa)	Predicted function	Species/Viruses	E-value		
Hz2V049	F	99730	100473	744	248					93
Hz2V050	F	100458	101336	879	293					
Hz2V051	R	105733	101939	3795	1265	Late expression factor 8	*Gryllus bimaculatus* NV	1×10^{-12}	RNA_pol_Rpb2_6	90
Hz2V052	F	105870	106712	843	281					89
Hz2V053	F	107253	107894	642	214	Per-os infectivity factor 3	*Gryllus bimaculatus* NV	2×10^{-10}		88
Hz2V054	R	108853	108413	441	147					87
Hz2V055	F	109110	109391	282	94					85
Hz2V056	F	109953	111365	1413	471					83
Hz2V057	F	113318	113530	213	71					
Hz2V058	R	115909	115496	414	138					81
Hz2V059	F	116202	116963	762	254	p34 late protein	*Helicoverpa zea* NV-1 [d]	8×10^{-147}		79
Hz2V060	F	117224	117595	372	124					78
Hz2V061	F	117803	118570	768	256					77
Hz2V062	R	120105	118786	1320	440	Odv-e56 structural protein	*Gryllus bimaculatus* NV	2×10^{-9}		76
Hz2V063	R	124121	120513	3609	1203	Late expression factor 9	Monodon baculovirus	1×10^{-60}		75
Hz2V064	F	124357	124818	462	154					74
Hz2V065	F	124932	125930	999	333	Ribonuclease reductase	*Xenopus tropicalis*	5×10^{-128}	Ribonuc_red_sm	73
Hz2V066	R	127832	126510	1323	441	Serine hydroxymethyltransferase	*Bombyx mori*	0	SHMT	72
Hz2V067	F	128939	129760	822	274	Deoxynucleotide kinase	*Drosophila melanogaster*	1×10^{-38}	dNK	71
Hz2V068	F	129972	132338	2367	789	Matrix metalloprotease	*Acyrthosiphon pisum*	3×10^{-69}	ZnMc_MMP, HX	70
Hz2V069	R	134585	133536	1050	350	dUTPase	*Culex quinquefasciatus*	2×10^{-38}	dUTPase	69
Hz2V070	R	137400	135430	1971	657	DNA excision repair enzyme	*Musca domestica* SGHV	2×10^{-7}	XPG	68
Hz2V071	R	138863	137478	1386	462					67
Hz2V072	R	139354	139019	336	112					66

Table 1. *Cont.*

ORF	Strand [a]	Position		Length		Best Blast match			Signature [b]	HzNV-1 [c]
		start	end	(nt)	(aa)	Predicted function	Species/Viruses	E-value		
Hz2V073	F	139721	140869	1149	383	p51 late protein	*Helicoverpa zea* NV-1 [d]	0		64
Hz2V074	F	141013	141564	552	184					63
Hz2V075	F	141693	145637	3945	1315					62
Hz2V076	R	149608	146981	2628	876					60
Hz2V077	F	149749	150978	1230	410					59
Hz2V078	R	153629	151443	2187	729					58
Hz2V079	R	154524	154090	435	145					
Hz2V080	R	155191	154481	711	237					
Hz2V081	F	155556	155936	381	127					56
Hz2V082	F	156150	157853	1704	568	Per-os infectivity factor 1	*Gryllus bimaculatus* NV	6×10^{-23}	DUF686	55
Hz2V083	F	158080	158742	663	221					54
Hz2V084	R	163557	159694	3864	1288					52
Hz2V085	F	163804	165702	1899	633					51
Hz2V086	R	166298	165954	345	115					
Hz2V087	F	167038	168003	966	322					49
Hz2V088	R	170084	168429	1656	552					47
Hz2V089	F	170456	172948	2493	831	vp91 capsid protein	*Gryllus bimaculatus* NV	4×10^{-14}		46
Hz2V090	F	173649	176570	2922	974					
Hz2V091	F	177136	178524	1389	463					
Hz2V092	F	178677	180614	1938	646					
Hz2V093	F	182846	185689	2844	948	Methyltransferase	*Helicoverpa zea* NPV	8×10^{-28}	FtsJ	37
Hz2V094	F	187572	193430	5859	1953	DNA ligase	*Apis mellifera*	4×10^{-86}	DNA_ligase_A_M	36
Hz2V095	R	194548	193964	585	195					34
Hz2V096	F	194663	195130	468	156	Ac81	*Gryllus bimaculatus* NV	6×10^{-12}	DUF845	33
Hz2V097	F	195232	195630	399	133					32

Table 1. *Cont.*

ORF	Strand [a]	Position		Length		Best Blast match			Signature [b]	HzNV-1 [c]
		start	end	(nt)	(aa)	Predicted function	Species/Viruses	E-value		
Hz2V098	F	195699	199133	3435	1145					31
Hz2V099	R	200794	199931	864	288	Esterase/lipase	*Psychromonas ingrahamii*	0.16	Aes	30
Hz2V100	F	200858	201796	939	313					29
Hz2V101	R	203059	201992	1068	356					28
Hz2V102	R	203298	203092	207	69					
Hz2V103	F	209783	209980	198	66					
Hz2V104	R	216330	215599	732	244					13
Hz2V105	R	217543	216668	876	292					12
Hz2V106	R	219936	217846	2091	697	p74 envelope protein	*Gryllius bimaculus* NV	5×10^{-57}	Baculo_p74	11
Hz2V107	F	220177	220464	288	96					
Hz2V108	R	221727	220951	777	259	38kDa protein	Monodon baculovirus	3×10^{-15}	DUF705	10
Hz2V109	R	222547	221819	729	243					9
Hz2V110	F	222677	223438	762	254	Protein kinase	*Trichomonas vaginalis*	5×10^{-18}	S_TKc	8
Hz2V111	F	223656	224297	642	214	Dihydrofolate reductase	*Heliothis virescens*	1×10^{-45}	DHFR	7
Hz2V112	F	226341	229232	2892	964					4
Hz2V113	F	229783	231468	1686	562					3

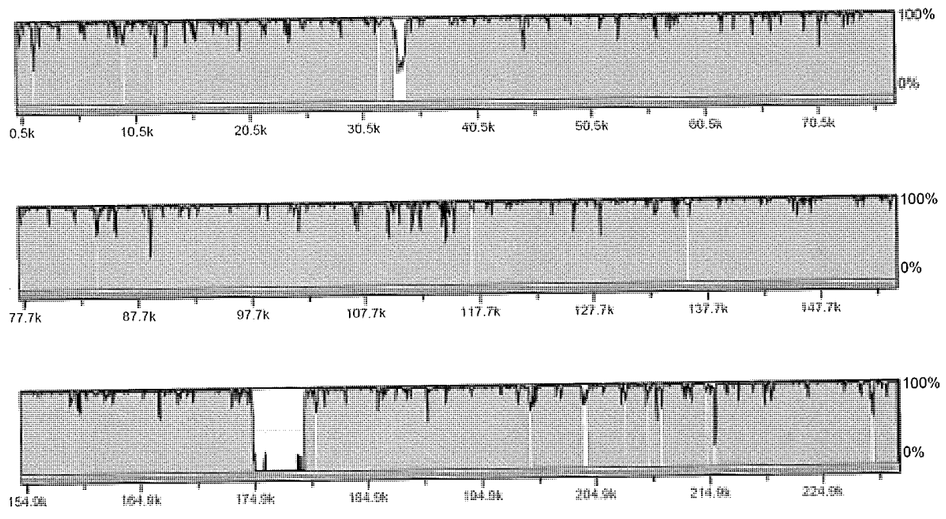
[a] The direction of the transcripts are indicated by F (forward) and R (reverse); [b] Functional motif found in Pfam and CDD database; [c] HzNV-1 ORFs corresponding to that of HzNV-2; [d] HzNV-1 ORFs which are the best match to that of HzNV-2.

Table 2. HzNV-2 direct repeat (*drs*) sequences.

	start	end	score	size	Count	%id	consensus
dr1	11711	12310	134	24	25	63.2	atgaagctgaggatgaatctgaac
dr2	174285	174572	132	36	8	79.2	gaaactcctaaatcaaaggatgaa cctaaagcaaag
dr3	175417	175656	124	60	4	88.3	atgaaaaagcaaaggctgaggcga aggctaaagccgatgctgctgcaa aagccaaagctg
dr4	178103	178390	182	24	12	85.8	ttataccagagagcaagccagaaa
dr5	178517	178921	104	81	5	72.8	acctaaagttgaatctaaagtagt ggaaccacctaaagcggaatctaa aacagtggaagctcctactaaaac agttgaagt
dr6	180236	180445	156	30	7	94.3	agctgccgctaaacgcaaagccg aggctga

Direct repeat sequences were found by using EMBOSS Etandem software with a cutoff score of 100.

Figure 2. Comparison of HzNV-1 and HzNV-2 genome sequence. The 228089 bp of the HzNV-1 genome sequence was subjected to rVISTA software to compare with 231621 bp of the HzNV-2 genome sequence. HzNV-2 is the base sequence, the sequence identity is plotted in 0~100% scale, and a region with 95% or higher identity is presented in red. The sequence window size is 100 bp.



2.2. Evaluation of ORFs

Within the HzNV-2 genome sequence, a total of 376 virtual ORFs were identified that were longer than 60 aa and started with a methionine initiation codon. Minor ORFs, which were completely included within larger ORFs, were excluded. A total of 163 ORFs with a Glimmer score greater than 90 points were found among the 376 putative ORFs and were subjected to further evaluation. Hexamer scores were used to exclude ORFs with a low coding potential, and to eliminate ORFs which had a significant overlap with a portion of adjacent ORFs on either the same or complimentary strand. A

total of 113 ORFs among the 163 ORFs that passed the GLIMMER cutoff were selected for database search and physical characterization to identify possible functions of the putative genes.

In the initial analysis of the viral genome, it became clear that HzNV-2 was very closely related to the insect virus HzNV-1. Gene parity plot results demonstrated that 97 out of 113 putative HzNV-2 ORFs were found in the HzNV-1 genome without any overlap or inversion (Table 1). The global DNA sequence alignment between HzNV-1 and HzNV-2 by EMBOSS Matcher and rVISTA software showed 93.5% sequence identity with a 5.1 kb area at 174.7–179.8 kb region of HzNV-2 that showed significant gaps in the corresponding region of HzNV-1 genome (Figure 2). There were 3 ORF found in region: Hz2V090, Hz2V091, and Hz2V092. There were a total of 14 ORFs identified in the HzNV-2 genome that were not found in the HzNV-1 genome. None of these ORFs were determined to share sequence homology with any genes of know function.

The Genbank accession number of the Hz-2V genome sequence is JN418988.

2.3. Enzymes Related to DNA Replication and Repair

Five putative genes, Hz2V008 (integrase), Hz2V018 (dnapol), Hz2V029 (ATPase), Hz2V038 (helicase), Hz2V070 (excision\repair), and Hz2V094 (ligase) were orthologues of genes likely involved in DNA replication and repair. The C-terminal region of Hz2V008 had homology to the catalytic domain for integrase/recombinase, which has DNA breaking-rejoining activity. This region of Hz2V008 had relatively good similarity ($E = 2 \times 10^{-9}$) to the INT_REC_C conserved domain, which is related to phage integrase and bacterial and yeast recombinase. It contained well-conserved functional motif cores for DNA binding sites, catalytic residues, the active sites, and topoisomerase. The integrases from monodon baculovirus (MBV) and GbNV showed the best similarity ($E = 1 \times 10^{-29}$ and 2×10^{-11}, respectively) to Hz2V008 suggesting a possible common ancestral origin for these viral genes. Hz2V008 did not show homology with any baculovirus genes.

Hz2V018 had homology to DNA polymerase. The C-terminal region of Hz2V018 had ($E = 7 \times 10^{-10}$) similarity to the DNA dependent DNA polymerase type-B family (POLBc) conserved domain, which has DNA binding, polymerase, and 3'-5' exonuclease activity [14]. In this region, the well-conserved nucleotide binding motif, K(3x)NS(x)YG(2x)G, and the polymerase catalytic motif, YxDTD, were found at position 842–853 aa, and 892–897 aa, respectively. Hz2V018 also showed homology to the OrNV DNA polymerase.

Hz2V029 is an 823 aa long protein, with 256 aa at the N-terminal region which showed similarity ($E = 2 \times 10^{-3}$) to the C-terminal region of the SbcC conserved domain. The SbcC gene is a prokaryotic ortholog of the rad50 gene of humans that has ATPase activity and plays an important role in DNA double-strand break repair [15,16]. The SbcC gene is known to play a similar role during DNA replication in prokaryotes [17]. The highest similarities were detected for GbNV putitive desmoplakin ($E = 3 \times 10^{-4}$) and *Spodoptera frugiperda* ascovirus 1a SbcC/ATPase domain ($E = 4 \times 10^{-4}$).

Hz2V038 had homology to DNA helicase and was identified by the presence of a poxvirus D5 protein-like domain (Pox_D5) at its C-terminal region), [18]. Helicase is an essential gene for virus replication, and all baculoviruses contain a conserved helicase domain. 57.9% of residues of the conserved helicase domain aligned to over 186 aa of the Hz2V038 C-terminal region at position 1138–1323 aa, however, this similarity was relatively low ($E = 4 \times 10^{-3}$). *Culex nigripalpus*

helicase-1 was detected by a BLASTP search with Hz2V038, but with a poor score (E = 2.3). Hence PSI-BLAST was used to detect homologous genes. In the second iteration of PSI-BLAST, homologies to helicase genes of various baculoviruses were detected near the region of Pox_D5 domain. The *Ectoropis oblique* nucleopolyhedrovirus (NPV) helicase showed highest homology.

Hz2V070 had homology to xeroderma pigmentosum G (XPG) enzyme, which is related to other enzymes involved in nucleotide-excision repair and transcription-coupled repair of oxidative DNA damage. XPG has been shown to function by making a nick at the 3' side of the DNA strand damaged by UV radiation, which is then mended via the DNA excision repair system [19,20]. XPG is functionally and structurally related to flap endonuclease (FEN-1) enzyme [21], which functions in a manner analogous to the base excision DNA repair mechanism [22]. The homology between Hz2V070 and XPG was detected in the N-terminal region at position 28–256 aa, and CD-Search detected that the XPG domain had 245 of 319 residues aligned to the N-terminal region of Hz2V070 with relatively low similarity ($E = 6 \times 10^{-3}$). Because of the subtle homology of Hz2V070 to the XPG domain, BLASTP was able to detect only three homologues, found in genomes of the insect pathogenic, DNA viruses GbNV, OrNV and the salivary gland hypertrophy of *Musca domestica*, (MdSHGV), when the search was limited to the database subset of virus sequences.

Hz2V094 codes for a large putative protein composed of 1953 aa. This putative gene product had a highly conserved ($E = 3 \times 10^{-46}$) ATP-depentent DNA ligase domain of 201 aa in length that had high similarity to the DNA_Ligase_A_M conserved domain. The best match was to *Apis melifera* DNA ligase III ($E = 5 \times 10^{-84}$). The DNA ligase catalytic domain was located at the end of C-terminal region of Hz2V094. It also showed homology to other ligases at positions 480–714 aa and 1541–1929 aa regions. There was a more than 800 aa insertion between these two regions that did not show homology to any other genes.

2.4. Proteins Involved in RNA Transcription

There are five putative genes, Hz2V028 (*vlf-1*), Hz2V040 (*lef-5*), Hz2V043 (*lef-4*), Hz2V051 (*lef-8*), and Hz2V063 (*lef-9*), identified in the HzNV-2 genome that shared homology with baculovirus genes known to be involved in transcription. This is a very interesting result since these genes are all conserved baculovirus core genes that function in transcription and expression of late and very late genes. Baculoviruses share six common genes related to transcription, *p47*, *lef-4*, *lef-5*, *lef-8*, *lef-9*, and *vlf-1* [23]. The recent analysis of the OrNV genome sequence identified all six of these core genes in the genome of this virus, as well as in the genome of HzNV-1, and GbNV [2]. Our analysis identified all of these genes as well, except for *p47*.

These five common genes can be categorized into two groups. Both *lef-5* and *vlf-1* are transcription initiation factors, while *lef-4*, *lef-8* and *lef-9* along with *p47* encode subunits of RNA polymerase [24]. Hz2V028 showed homology to baculovirus very late expression factor 1 (vlf-1), which is essential for burst expression of the very late baculovirus genes, *polyhedrin* and *p10* [25]. *Vlf-1* is a transcription initiation factor that recognizes and binds to the promoter motif, DTAAG, of baculovirus very late genes [26] and is also involved in capsid assembly and DNA packaging [27]. Transient expression studies with the baculovirus very late promoter motif and HzNV-1 RNA polymerase demonstrated however, that HzNV-1 does not recognize the DTAAG motif [28]. This result indicates that HzNV-2

vlf-1, which has a 93% aa sequence similarity with the HzNV-1 *vlf-1* homologue, possibly recognizes a different late or very late promoter motif than does the baculovirus *vlf-1*. This is not surprising since the similarity of Hz2V028 to baculovirus *vlf-1* was relatively low ($E = 2 \times 10^{-2}$).

Hz2V040 had homology to late expression factor 5 (*lef-5*). LEF-5 has a characteristic zinc ribbon motif for DNA binding, which is often found in eukaryotic transcription elongation factors. However, LEF-5 along with baculovirus RNA polymerase, has transcriptional initiation activity at the late gene promoter [29]. Hz2V040 showed relatively low similarity to the C-terminal region of the Baculo_LEF-5 conserved domain ($E = 6 \times 10^{-3}$) at position 165–193 aa. This region also showed homology to the zinc finger motif (ZnF_C2C2) which is well conserved, especially at the positions of cysteine residues. Hence, Hz2V040 probably has a secondary structure similar to the DNA binding motif of baculovirus LEF-5 and therefore, DNA binding activity. Hz2V040 showed the best homology to *Spodoptera furugiperda* NPV *lef*-5 ($E = 6 \times 10^{-3}$). Interestingly, *lef*-5 homologues were also found in the other NV, GbNV and OrNV, however, these homologues were not detected by BLASTP because of their subtle similarities. None the less, these nudivirus homologues were detected by PSI-BLAST at their zinc ribbon domain.

The best match of Hz2V043 was to late expression factor 4 (*lef*-4) of GbNV ($E = 3 \times 10^{-18}$). LEF-4 has guanyltransferase and RNA triphosphatase activity and is thought to play a role in mRNA capping, which is essential for baculovirus replication [24,30,31]. Hz2V043 (873 aa) was almost twice as long as the homologue of *Autographa californica* NPV (AcMNPV) (464 aa), and the C-terminal region of this ORF showed homology to the *lef*-4 gene of GbNV and OrNV. Two KxDG motifs, characteristic of RNA capping enzymes capable of forming a stable enzyme-nucleotide monophosphate complex for guanylation [32], were found at positions 641 aa and 764 aa. This result supports the possibility of Hz2V043 having mRNA 5'-capping activity, despite its low similarity to baculovirus *lef-4* genes.

Hz2V051 showed homology to late expression factor 8 (*lef-8*). *Lef-8*, in conjunction with *lef-9*, encodes one of the main catalytic subunits of the baculovirus RNA polymerase. Hz2V051 showed the best similarity to GbNV *lef-8* ($E = 1 \times 10^{-12}$); however, the similarity to other baculovirus *lef-8* genes was relatively low. Hz2V051 was 1265 aa long, and the region at position 650–1112 aa was detected as an RNA_pol_Rpb2_6 conserved domain. This C-terminal region of Hz2V051 contains a conserved sequence motif GxKx4HGQ/NKG at 858–869 aa, which is found in both prokaryotic and eukaryotic RNA polymerases as well as those of the insect baculoviruses [33].

Hz2V063 had homology to the N-terminal region of the NV and baculovirus late expression factor 9 (LEF-9). OrNV LEF-9 showed good similarity ($E = 2 \times 10^{-8}$) at this region, however, the similarity of baculovirus LEF-9 detected by PSI-BLAST at this region was low. Hz2V063 contained an HADQDGD motif at position 286–292 aa, which is similar to the structure of the NTDCDGD motif of AcMNPV LEF-9 [34] and the NADFDGD motif of the vaccinia virus RNA polymerase large subunit [35]. LEF-9, in association with LEF 8, comprises the baculovirus RNA polymerase [36]. The exact function of LEF-9 in the polymerase, however, is not clear.

2.5. Genes Involved in Virus Entry

Baculoviruses also have common core genes encoding structural proteins related to their *per os* infectivity. HzNV-2 was found to have four genes, Hz2V026, Hz2V053, Hz2V082, and Hz2V106, which are homologues to these baculovirus *per os* infectivity factors (PIF). These *pif* genes are also well conserved in other NVs [1,2]. The identification of putative HzNV-2 *pif* genes is a very interesting result since, in contrast to the baculoviruses and other NVs, HzNV-2 is not highly infectious *per os* [5]. The presence of *pif* gene homologues in the HzNV-2 genome suggests a common mode of entry into cells which has been evolutionarily conserved among these different viruses.

Hz2V026 showed best similarity to GbNV *pif-2* gene ($E = 5 \times 10^{-37}$) and showed good similarity to AcMNPV *pif-2* ($E = 7 \times 10^{-11}$). The *pif-2* gene product which in addition to playing a role in oral infection by baculoviruses, is also a structural component of the occlusion-derived virus envelope [37]. Interestingly, the positions of cysteine residues were well conserved between Hz2V026 and the other *pif-2* genes. This result indicates that the HzNV-2 PIF-2 homologue might form disulfide bonds in the same manner and therefore have a very similar secondary structure to the *pif-2* gene products of these other viruses.

Hz2V053 was found to be a homologue of the baculovirus *pif-3* gene and showed good similarity to GbNV *pif-3* gene ($E = 2 \times 10^{-10}$). Similarity to other baculovirus *pif-3* genes was low, but as with PIF-2, the positions of cysteine residues were well conserved between Hz2V053, the PIF-3 of GbNV and those of other baculoviruses [38]. Despite the low similarity to baculovirus *pif-3*, Hz2V053 does contain an N-terminal transmembrane domain at position 3-19 aa, characteristic of other PIF-3s.

Hz2V082 is a homologue of the baculovirus *pif-1* gene. This gene showed the best similarity to the GbNV *pif-1* gene ($E = 6 \times 10^{-23}$) and homology with other baculovirus *pif-1* genes at the region of 70–350 aa. PIF-1 is a viral structural protein which, along with PIF-2, is responsible for oral infectivity by mediating the direct binding of the virus particle to host cells [39]. As with the other PIFs, the position of cysteine residues in PIF-1 and Hz2V08 are well conserved. When Hz2V082 was subjected to ANTHEPROT, three transmembrane domains and a putative N-terminal signal peptide cleavage site were found. This result suggests that the localization of Hz2V082 gene product in the virus membrane is similar to that of the *Spodoptera Littoralis* NPV (SlNPV) PIF-1. However, the topology of the HzNV-2 protein in the membrane might be slightly different, since the SlNPV PIF-1, and the PIF-1 other baculovirus have an additional fourth putative transmembrane domain [40].

Hz2V106 showed homology with the bacuolvirus *p74* gene, which codes for a critical oral infection factor of baculoviruses. P74 is a highly conserved envelope protein of baculoviruses that mediates specific binding of the virus particle to host cells [41]. The best similarity found for Hz2V106 was with the GbNV *p74* gene ($E = 5 \times 10^{-57}$). The proposed secondary structure of the *p74* gene product of *Choristoneura fumiferana* granulovirus (GV) illustrates that it has a characteristic positioning of cysteine residues that allows for the formation of disulfide bonds within its C-terminal transmembrane membrane anchoring domain [42]. Hz2V106 has six cysteine residues at positions 99, 104, 138, 169, 217, and 231 with three C-terminal transmembrane domains at 494–516 aa, 639–661 aa, and 665–687 aa, which are in a very similar context to those in the highly conserved baculovirus P74 protein.

2.6. Enzymes Involved in Nucleic Acid Metabolism

Seven putative HzNV-2 ORFs, Hz2V035, Hz2V047, Hz2V065, Hz2V066, Hz2V067, Hz2V069, and Hz2V093, showed homology to previously identified genes from eukaryotes, prokaryotes, and viruses that coded for proteins having activity related to nucleic acid metabolism.

Hz2V035 was a homologue of thymidylate synthase (TS). It contained the highly conserved TS functional domain and showed over 80% sequence identity to other TS genes. The HzNV-2 TS gene showed the best similarity to that of *Bombyx mori* TS ($E = 4 \times 10^{-131}$) and also showed high similarity to the TS genes of herpesvirus, NV, iridovirus, and other viruses. So far, no baculovirus TS genes have been found. TS is well conserved across species and is involved in the synthesis of dTMP precursors from dUTP [43,44]. Interestingly, all identified viral TS genes are thought to be acquired from their hosts in independent incidents rather than originating from a common ancestor [45,46].

Hz2V047 and Hz2V065 were homologues to ribonucleotide reductase large (RR1) and small subunit (RR2), respectively. These genes also showed homology to a variety of RR genes with over 70% amino acid similarities. The best match was to SINPV RR1 ($E = 0$). RR1 and RR2 are involved in reducing ribonucleotides into deoxyribonucleotides to produce precursors of DNA [47] and are commonly found in viruses as well as prokaryotes and eukaryotes.

Hz2V066 showed homology to the serine hydroxymethyltransferase (SHMT) conserved domain ($E = 7 \times 10^{-171}$). SHMT catalyzes the reversible interconversion of serine and glycine with tetrahydrofolate [48]. This is the major source of one-carbon groups used in the biosynthesis of compounds containing methyl groups and purine rings, as well as in thymidylate biosynthesis [49]. Hz2V066 showed the greatest similarity to the *Bombyx mori* SHMT gene ($E = 0$). SHMT is a cellular gene, and to date has not been found in any other eukaryotic viruses.

Hz2V067 was homologous to deoxynucleotide kinase (dNK), with the best match to dNK of *Drosophila melanogaster* ($E = 1 \times 10^{-38}$) having 57% similarity. dNK catalyzes the phosphorylation of deoxyribonucleosides to yield corresponding monophosphates and is a key enzyme involved in the salvage of deoxyribonucleosides [50].

Hz2V069 is a 350 aa long ORF, of which over 150 aa of the N-terminal region showed homology with the dUTP phosphatase (dUTPase) domain ($E = 1 \times 10^{-31}$). The best match was *Culex quinquefasciatus* dUTPase ($E = 2 \times 10^{-38}$), however, this gene product is also often found as a protein component of animal viruses [51]. dUTPase provides a substrate for TS and maintains low dUTP:dTTP ratio to minimize the misincorporation of uracil into virus DNA during replication [52]. dUTPase also plays an important role during virus replication in terminally-differentiated or non-dividing cells that have a low level of cellular dUTPase expression [53]. Hence, dUTPase might be a key enzyme related to HzNV-2 replication and latency in asymptomatic carriers.

Hz2V093 encoded a 948 aa long protein that showed homology to methyltransferase (MT) at the N-terminal region of this protein. The best match was *H. zea* NPV ORF65 ($E = 8 \times 10^{-28}$). MT is often found in NPVs, but other than HzNV-1, it has not been found in NVs. Baculovirus methyltransferase along with LEF-4 is believed to be involved in RNA capping of late and very late gene transcripts, however, it is not essential for virus replication [54].

Hz2V111 was a homologue of dihydrofolate reductase (DHFR). The best match was *Heliothis virescens* DHFR (1×10^{-45}), with good homology to herpesvirus DHFR. DHFR reduces dihydrofolate

to tetrahydrofolate, which is required to regenerate methylenetetrahydrofolate by thymidylate synthase, an essential step in the biosynthesis of deoxythymine phosphate for DNA synthesis [55]. DHFR was also found in MdSGHV, however, it has not yet been found in any baculoviruses.

2.7. Structural Proteins

Hz2V062 was a homologue of the baculovirus *odv-e56* gene, which encodes an occlusion-derived virus envelope protein. It showed the best match to GbNV *odv-e56*, while the similarity to other baculovirus *odv-e56* was very low. Odv-e56 is one of the baculovirus late genes which utilize a TAAG promoter motif for its expression [56]. A TAAG motif, however, was not found within the 500 bp region upstream of Hz2V062. This observation is also indicative of the possibility that the late or very late gene regulation mechanism of HzNV-1 and HzNV-2 has evolved differently from that of baculoviruses.

Hz2V089 was a homologue of baculovirus *vp91*. The VP91 in baculoviruses is localized near the nuclear membrane of the infected cell and is associated with the virion capsid [57]. The C-terminal region of Hz2V089 showed homology to the GbNV *vp91* gene ($E = 4 \times 10^{-14}$) and also other baculovirus *vp91* genes as well.

Hz2V108 was a homologue of baculovirus 38K protein gene which codes a structural protein associated with the virus nucleocapsid [58]. The 38K protein plays an important role in nucleocapsid assembly, interacting with the other nucleocapsid proteins, VP1054, VP39, VP80, and 38K itself [58]. The best match was MBV baculovirus (3×10^{-15}).

2.8. Auxiliary and Undefined Genes

Hz2V007 codes for a putative protein composed of 721 aa. This putative gene product showed highest similarity to the *Bombyx mori* carboxylesterase (COE) ($E = 4 \times 10^{-21}$) and also had a high similarity to *Anopheles gambiae* juvenile hormone esterase (JHE) ($E = 2 \times 10^{-19}$). Hz2V007 had a COE domain at position 25–202 aa that had significant similarity to other highly conserved COE domains ($E = 6 \times 10^{-31}$). Almost the entire peptide sequence of Hz2V007 at position 1–632 aa lined up with the COE domain with relatively high similarity ($E = 5 \times 10^{-11}$).

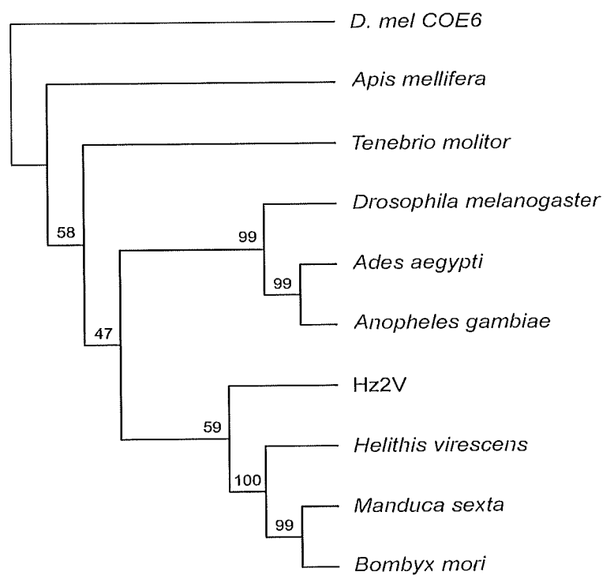
Juvenile hormone (JH) is produced in the corpora allata, of insects, and acts as a transcriptional regulator, controlling the expression of a variety of genes involved in physiological, developmental, and reproductive processes in insects. One pathway for the control of JH titer in insects is by degradation via JHE [59]. The high similarity between Hz2V007 and insect JHEs is a very interesting result since it suggests that HzNV-2 can control the physiology of the infected host insects by regulating JH levels and ultimately the level of gene expression in various tissues during their development, at specific times during the replication cycle of the virus.

To compare Hz2V007 with other insect JHEs, nine JHEs peptide sequences from four different insect orders were selected from Genbank including *D.mel* COE-6, one hymenopteran (*Apis melifera*), one coleopteran (*Tenebrio molitor*), three dipterans (*Drosophila melanogaster*, *Aedes aegypti*, and *Anopheles gambiae*), and three lepidopterans (*Heliothis virecens*, *Manduca sexta*, and *Bombyx mori*). Sequence alignments of the entire coding region of each JHE with Hz2V007 were performed by EMBOSS Water software. Hz2V007 showed highest peptide sequence identity and similarity within

the catalytic domain of *A. gambiae* JHE (30.1% and 43.9% respectively), and the lowest with *H. virescens* JHE (20.4% and 34.6%). This level of similarity was relatively low compared with the range of sequence identities and similarities found among the nine insects JHEs, which had identities ranged between ~28.6 and 61.2% and similarities between ~61.2 and 77.1%.

To investigate the phylogenetic positioning of Hz2V007 among these insect JHEs, molecular phylogenetic analysis using neighbor-joining in MUSCLE and ClustalX was performed. The results demonstrated that Hz2V007 grouped with the JHEs from lepidopteron insects, rather than to dipteran JHEs to which it had the highest sequence similarity (Figure 3). This result may reflect the closer relationship between HzNV-2 and lepidopteran insects, from which the virus was most likely to have obtained the ancestral form of Hz2V007.

Figure 3. Phylogenetic analysis of JHEs and Hz2V007. Phylogenetic analysis of selected JHEs and Hz2V007. Multiple sequences were aligned using MUSCLE, and the tree was inferred using neighbor-joining in ClustalX. *D. melanogaster* corboxylesterase-6 gene was used as the outgroup. The robustness of the tree was tested using bootstrap analysis (1000 replicates). The percent values are presented at the nodes.



Another characteristic feature that Hz2V007 shares with other JHEs was a signal peptide. A possible cleavable signal peptide was found at position 1–20 aa, flanked by a putative cleavage site between 20 and 21 aa. The signal peptide sequences, which are responsible for secretion of insect JHE, were found in all nine JHEs.

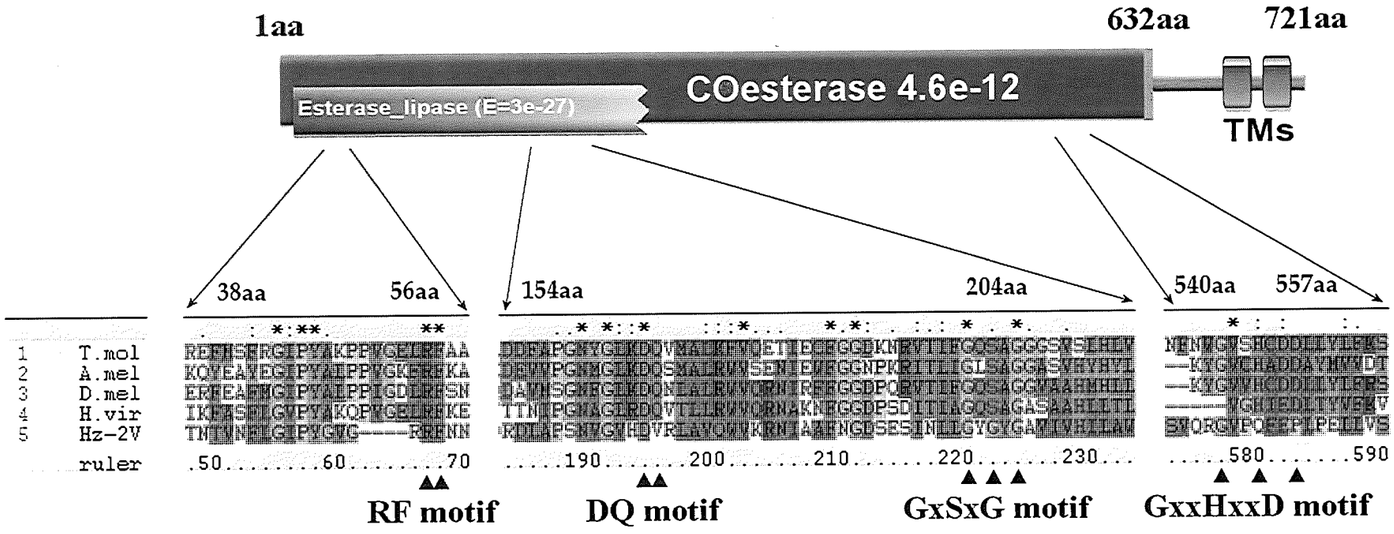
The signal peptide sequence prediction and phylogenetic analysis results alone are not sufficient to predict the JHE activity of Hz2V007. To further assess its possible JHE activity, Hz2V007 and four JHEs from each order were chosen, and multiple sequence alignment was performed to compare

functional motifs within the catalytic domain of the JHEs with corresponding regions of Hz2V007. Most of the JHEs have five conserved catalytic motifs, RF, DQ, GxSxG, E, and GxxHxxD/E [60]. In Hz2V007, an intact RF motif was found at position 53 aa and partially conserved DQ, GxSxG and GxxHxxD motifs were found at 165 aa, 191 aa, and 544 aa, respectively. An E motif was not identified (Figure 4).

The average polypeptide length of the nine JHEs is 573 aa, and the entire polypeptide sequence of JHE corresponded to the region at 1–632 aa of Hz2V007. This means that Hz2V007 has 89 aa of C-terminal flanking sequence beyond the JHE sequence. This region showed no significant homology to any known sequences in the database. Interestingly, the SMART program predicted a transmembrane domain in this C-terminal region of Hz2V007 at position 686–719 aa, while it predicted an N-terminal signal peptide sequence for secretion. This result suggests a unique localization of the Hz2V007 gene product. TMHMM analysis results predicted that the N-terminal JHE-like region would reside outside of the cell, anchored on the cell membrane by the C-terminal transmembrane domain. From these results, it can be inferred that HzNV-2 captured the JHE gene from its lepidopteran host and evolved to alter its catalytic characteristics and specifically localize the Hz2V007 product at the surface of cells in infected tissue. When one considers the role of JH and JHE in controlling development and reproduction of insects, Hz2V007 might be a key gene responsible for the malformation of reproductive tissue functioning to regulate JH titers and gene expression in these tissues of the infected hosts.

Hz2V012 and Hz2V015 encoded inhibitor of apoptosis (IAP) homologues. The IAP gene was first found in baculoviruses suppressing apoptosis of virus infected host cells [61]. The baculovirus IAPs have 1~3 baculovirus IAP repeat (BIR) conserved domains, and inhibit apoptosis by acting as inhibitors of the caspase family of protease enzymes. Baculovirus IAPs also contain a RING-finger motif and a zinc-binding site in their C-terminal region. The RING-finger motif is not required for anti-apoptotic activity [62,63]; however, it is important for regulation of IAPs by ubiquitin ligase [64,65]. Hz2V012 and Hz2V015 (termed IAP 1 and IAP 2) were 187aa and 182aa long peptides, respectively. These were both much shorter than other cellular or baculovirus IAPs. The best match of IAP 1 was the *Trichplusia ni* (cabbage looper) NPV IAP gene ($E = 7 \times 10^{-25}$), and the best match of IAP 2 was *Adoxophyes orana* GV IAP 3 ($E = 1 \times 10^{-26}$). IAP-1 had two BIR domains without a RING-finger motif, while IAP 2 had one BIR domain with a C-terminal RING-finger motif. These are interesting results because most of the baculovirus IAPs have two BIR domains with one RING-finger motif at the C-terminal region. The exact role of the IAP genes of HzNV-2 still needs to be confirmed experimentally.

Figure 4. A schematic presentation of functional domains and motifs in Hz2V007. The functional domains identified by CDD and pfam database searches are presented in orange and blue, respectively. The transmembrane domains are presented in red. Multiple JHE sequence alignments are presented using ClustalX, and the locations of conserved residues in the active sites of JHE are indicated with triangles. T. mol (*T. molitor*), A.mol (*A. melifera*), D.mel (*D. melanogaster*), H.vir (*H. virecens*).



Hz2V023 had total of 12 characteristic transmembrane domains and showed homology to the major facilitator superfamily (MFS) conserved domain ($E = 5 \times 10^{-10}$). MFS is a family of transporter genes found ubiquitously in living organisms, which code for single-polypeptide carrier proteins involved in the uptake or efflux of small molecules. The primary functions of the MFS is uptake of sugar, and drug efflux [66]. Hz2V023 showed the best match to *A. aegypti* adenylate cyclase ($E = 1 \times 10^{-135}$).

Hz2V034 had homology to the guanosine monophosphate kinase (GMPK) motif at position 57–96 aa. The similarity was relatively low ($E = 1 \times 10^{-3}$), but the conserved catalytic motif of GMPK, G-X2-G-X-G-K was found at position 61–67 aa as G-M-S-C-S-G-K with one aa mismatch at position 64 aa. GMPK catalyzes the reversible transfer of the terminal phosphoryl group of ATP and GMP to yield ADP and GDP [67]. This is an important step in the biosynthesis of GTP. GMPK also plays an important role in the recycling of the secondary messenger cGMP [68]. Hz2V034 showed best similarity to the hypothetical protein of MBV ($E = 4 \times 10^{-5}$).

Hz2V039 had homology to the baculovirus 19K protein gene (AcMNPV ORF96) which codes for a protein of unknown function. The 19K gene homologues are commonly found in baculoviruses, and Hz2V039 showed best similarity to GbNV ORF87 ($E = 9 \times 10^{-9}$). Hz2V059 and Hz2V073 encoded HzNV-1 *p34* and *p51* late gene homologues, respectively. Originally, *p34* and *p51* genes, which were detected from 8 to 24 hr post infection, were found in a cDNA library of HzNV-1 infected Tn-368 culture cells [28,69].

Hz2V068 encoded a zinc-dependent matrix metalloprotease (ZnMc_MMP) conserved domain ($E = 2 \times 10^{-38}$) and hemopexin-like repeat (HX) domains ($E = 6 \times 10^{-26}$) at the C-terminal flanking region. MMPs are zinc and calcium dependent enzymes, which are synthesized as proenzymes in connective tissues [70]. MMPs play an important role in cellular differentiation and morphogenesis being responsible for pericellular proteolysis of the extracellular matrix and other cell surface molecules. The activities of MMPs are regulated by a tissue inhibitor of metalloprotease (TIMP). [71]. The HX domain is often found in MMPs and their TIMPs. The signature of the MMP active site, HEXXHXUGUXH (U = bulky hydrophobic aa), was found at position 412–122 aa of Hz2V068, and putative TIMP-binding sites were found at the position 346–434 aa region. A possible cleavable signal peptide for secretion was also found at position 1–18 aa. Hz2V068 showed best similarity to *Acyrthosiphon pisum* (aphid) MMP ($E = 3 \times 10^{-69}$). Baculoviruses also have a metalloprotease, known as enhancin. Enhancin is incorporated in the occlusion body derived virus (ODV) and is thought to aid in the infection pathway via the degradation of intestinal mucin, a major protein component of the insect peritrophic membrane [72]. Hz2V068, however, shares a greater homology with cellular MMPs than with the baculoviral enhancins, which lack the hemopexin domain and a signal peptide.

Hz2V096 showed homology to AcMNPV ORF81 (*ac81*). The *ac81* homologue is found in all baculoviruses, and is hence considered a baculovirus core gene [73]. A recent study of BmNPV ORF67, another homologue of Ac81, revealed that although its exact function is not known, it is a non-structural, late protein, detected in the cytoplasm of infected cells interacting with the cellular protein actin A3 [74]. Hz2V096 showed the best match to GbNV ORF14 ($E = 6 \times 10^{-14}$) and relatively low similarity to other baculovirus *ac81* homologues; however, the C-terminal transmembrane domain and the positions of cysteine residues common to Ac81s were conserved. Hence Ac81 homologues and Hz2V096 probably share similar secondary structure and perform similar functions during virus replication.

Hz2V099 showed homology to a prokaryotic acetylesterase (Aes) homologue. Aes is a member of esterase/lipase superfamily, and the Aes conserved domain was detected in Hz2V099 with relatively low similarity ($E = 9 \times 10^{-4}$). The Aes gene was first found in *Escherichia coli* and showed homology to lipase and esterase. Functional studies of the Aes protein demonstrated it had acetylesterase activity [75] and functioned in the control of a transcriptional activator [76].

Hz2V110 showed homology to the serine/threonine protein kinase (S_TKc) conserved domain ($E = 1 \times 10^{-28}$). Serine/threonine protein kinase catalyzes phosphorylation of serine and threonine residues, a major process in regulating cellular functions, especially protein phosphorylation in signal transduction [77].

2.9. Comparison of HzNV-2 and Baculovirus Genome Contents

A total of 13 complete baculovirus genome sequences have been compared, and 30 conserved core genes were found to be present in all baculoviruses [73]. These genes were organized into six categories based on their functions; replication, transcription, virus entry, structural proteins, auxiliary, and unknown. A total of 16 HzNV-2 genes found to share homology with the some of these baculovirus conserved core genes (Table 3). A total of 20 HzNV-1 genes have been reported to share homologies with baculovirus core genes (2). The HzNV-2 was found to contain homologues of all of these HzNV-1 genes however, no ORFs having homology with the 4 baculovirus core genes p47, ac68, vp39 and p33 were found in the HzNV-2 genome.

Among the DNA replication related baculovirus core genes, the most essential ones, DNA polymerase and helicase, were found in HzNV-2. However, LEF 1, DNA primase [78], and LEF 2, the LEF 1 associated protein [79], were not found in HzNV-2. Also *hrs*, the characteristic palindromic structures in baculovirus genomic DNA that function as transcriptional enhancers [80] and/or as origins of replication, were not found in HzNV-2. The absence of *lef-1*, *lef-2*, and *hrs* demonstrate that HzNV-2 possibly has a different viral DNA replication initiation mechanism than that of baculoviruses.

Five of the six conserved baculovirus core genes involved in transcription were found in HzNV-2. These five genes were *lef-4*, *lef-8*, and *lef-9*, which are the subunits of the baculovirus RNA polymerase, and LEF-5 and VLF-1, which are the late gene transcriptional factors of the baculovirus. The only conserved baculovirus gene involved in transcription that was not found in HzNV-2 was *p47*, one of the RNA polymerase subunits with unknown function [30]. It is interesting that HzNV-2 has baculovirus RNA polymerase and transcription factor homologues because having an alpha-amanitin resistant RNA polymerase is the one of the most distinctive features of the baculovirus compared with other DNA viruses [81]. Therefore, the presence of conserved homologues in HzNV-2 related to the transcription mechanism of baculoviruses supports the hypothesis that HzNV-2 and baculoviruses possibly evolved from a common ancestor.

Another group of conserved baculovirus core genes are the genes related to virus entry. The *p74*, *pif-1*, *pif-2*, and *pif-3* genes which are the *per os* infectivity factors, belong to this group. In the HzNV-2 genome, all four of the *per os* infectivity related genes were found, while the fusion protein gene *p47* was not. This is a very interesting result since the primary route of transmission of HzNV-2 is not *per os* but rather by mating or through transovarial transmission. The similarity of *p74* and the

pif genes to the HzNV-2 homologues were very low, so it is possible that these genes evolved in HzNV-2 to target reproductive tissue cells instead of the midgut cells.

The baculovirus fusion protein, P47 is thought to play a role in systemic viral infections [82], The hymenopteran baculoviruses, *Neodiprion sertifer* NPV [83], and *Neodiprion lecontei* NPV [84], which also lack a fusion protein, replicate only in the midgut cells and do not exhibit systemic infection. This localized pathology compares with that of HzNV-2 in terms of non-systematic and tissue-specific infection. Hence, it is possible that the progeny virus of HzNV-2 in the target tissue could spread by cell surface specific binding of virus utilizing P74 and PIFs and not systemically via the fusion protein as do baculoviruses.

HzNV-2 shares three of the eight baculovirus conserved genes coding for structural proteins, *odv-e56*, *vp91*, and *38K*, a capsid protein gene. ODV-e56 and VP91 are the virus structural proteins, while 38K is related to nucleocapsid assembly. The other five baculovirus conserved genes, *odv-e27*, *gp41*, *p6.9*, *vp39*, and *vp1054*, were not found in HzNV-2. GP41, P6.9, and VP39 are structural proteins which compose the nucleocapsid. VP1054 is responsible for nucleocapsid assembly and directly interacts with VP39 and 38K [84]. Only the 38K homologue was found in HzNV-2. The ODV-E27 is a structural protein and is considered a viral cyclin. It arrests host cells at the G_2/M phase of the cell cycle with continuing viral DNA replication [85]. The absence of an *odv-e27* homologue in HzNV-2 might be expected since the pathogenesis of this virus in infected reproductive tissue is thought to occur with the development of reproductive tissue during the late larval and early adult stages of host insects.

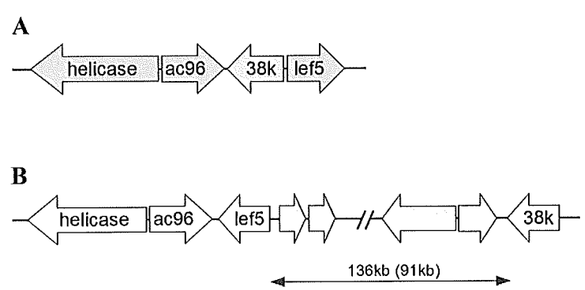
Baculovirus alkaline exonuclease (alk-exo) while categorized as an auxiliary gene, has both 5'-3' exonuclease and endonuclease activity, as well as the ability to form complexes with the single strand binding protein and LEF 3. Alk-exo enzyme participates in dsDNA repair and is possibly responsible for the maturation of virus DNA in addition to maintenance of virus genome integrity [86,87]. An alk-exo homologue was not found in HzNV-2, perhaps due to HzNV-2's use of different DNA replication and repair mechanisms. Of the six baculovirus conserved genes, *ac68*, *ac81*, *ac92*, *ac96*, *ac109* and *ac142*, with unknown function, only *ac81* and *ac96* were found in HzNV-2.

Table 3. Comparison of conserved baculovirus core genes and HzNV-2.

Gene Function	Genes present in HzNV-2	Genes absent in HzNV-2
Replication	*dnapol* (*ac65*), *helicase* (*ac95*)	*lef-1* (*ac14*), *lef-2* (*ac6*)
Transcription	*lef-4* (*ac90*), *lef-5* (*ac99*), *lef-8* (*ac50*), *lef-9* (*ac62*), *vlf-1* (*ac77*)	*p47* (*ac40*)
Virus entry	*p74* (*ac138*), *pif-1* (*ac119*), *pif-2* (*ac22*), *pif-3* (*ac115*)	*ld130* (*ac23*)
Structural	*p91* (*ac83*), *38K* (*ac98*), *odv-e56* (*ac148*)	*odv-e27* (*ac144*), *gp41* (*ac80*), *p6.9* (*ac100*), *vp39* (*ac89*), *vp1054* (*ac54*)
Auxiliary		*alk-exo* (*ac133*)
Unknown	*ac81*, *19K* (*ac96*)	*ac68*, *ac92*, *ac109*, *ac142*

Another characteristic of baculovirus conserved genes is a core gene cluster, which is a set of four genes (*helicase*, *ac96*, *38K*, and *lef-5*) found in the same relative position and direction in all known baculovirus genomes. This conserved gene cluster is very distinctive since extensive genome rearrangements are believed to have occurred in the evolution of baculoviruses [73]. HzNV-2 has a baculovirus core gene cluster-like region; however, it was observed to be different from that found in baculoviruses. The location and orientation of *helicase* and *ac96* were the same as in the baculovirus gene cluster, however *lef-5* was on the opposite strand, and orientation compare to its position in the baculovirus core cluster. The most distinctive difference was the location of *38k* gene. In baculoviruses, *38k* is located between the *ac96* and *lef-5* gene on the opposite DNA strand. The HzNV-2 *38k* homologue, however, is located 91 kb downstream of the *helicase* gene (Figure 5). This result may again reflect the divergence of HzNV-2 and baculoviruses during evolution from a common ancestor.

Figure 5. Comparison of the conserved core cluster of a baculovirus and corresponding region in HzNV-2. (**A**) Gene arrangement in the conserved core cluster of baculovirus; (**B**) Gene arrangement around HzNV-2 gene. The relative positions and orientations of *helicase* and *ac96* are the same in both baculovirus and HzNV-2, however, HzNV-2 has *lef-5* on the opposite DNA strand and *38 K* at 136 kb downstream (also 91 kb upstream) of the *helicase* gene.



2.11. Phylogenetic Analysis

DNA polymerase is one of key phylogenetic characteristics of virus taxonomy, therefore, a phylogenetic analysis was performed using this gene to assess the relationship of HzNV-2 to other insect DNA viruses. For this analysis, eleven DNA polymerase sequences were chosen from NPVs, GVs, entomopoxviruses, and NVs. A multiple sequence alignment was performed by MUSCLE, and a phylogenetic tree was built by neighbor-joining using MEGA4. The result demonstrated that Hz2V018 (the HzNV-2 DNA polymerase) grouped with GbNV, OrNV, and HzNV-1 DNA polymerase, while the baculovirus and entomopoxvirus DNA polymerases have monophyletic lineages (Figure 6A). This result showed that as expected, HzNV-2 DNA polymerase is more closely related to other NV DNA polymerases than to those of other insect viruses.

A similar approach was applied to the virus entry related protein genes. Although the similarities between these viruses are relatively low, *p74* and the *pif* genes are well-conserved genes in

baculoviruses and NV. A possible explanation might be a diverging evolution of the *p74* and *pif* genes from an ancestral virus during host range expansion. The attachment and entry of viruses into cells is a key process in replication and is obligatory for horizontal transmission between susceptible hosts. The conservation of genes involved in this process across these different dsDNA viruses of insects (2), suggests that they share features of this process which have been maintained during their evolution. A total of 14 sets of *p74*, *pif-1*, *pif-2*, and *pif-3* genes chosen from baculoviruses and NV were combined for phylogenetic analysis. As with the DNA polymerase tree result, HzNV-2 grouped with other NV while the baculoviruses had a monophyletic lineage. This result demonstrated that the *p74* and *pif* genes of HzNV-2 are more closely related to those of NV and more distantly related to those of baculoviruses (Figure 6B). This result suggest that while there are some common elements in the process used by these viruses to enter host cells, and that NVs may share an aspect of this pathway which is different from that used by baculoviruses. Similar phylogenic analysis was performed in the genome analysis of the MdSGHV with results similar to those presented here (Figures 6A,B) [46].

Figure 6. Phylogenetic analysis of selected insect DNA viruses and HzNV-2. (**A**) DNA polymerase; (**B**) combined sequence analysis *of p74*, *pif-1*, *pif-2* and *pif-3*; Multiple sequence alignments were performed by MUSCLE, and then the tree was inferred using neighbor-joining in MEGA4. The robustness of the tree was tested using bootstrap analysis (1000 replicates). The percent values are presented at the nodes. Organisms included in this analysis, with abbreviated names: *Autographa californica* NPV (AcNPV), *adoxophyes honmai* NPV (AdhoNPV), *Amsacta moorei* entomopoxvirus (AmEntPox), *Bombyx mori* NPV (BmNPV), *Culex nigripalpus* NPV (CuniNPV), *Cydia pomonella* granulovirus (CpGV), *Euproctis pseudoconspersa* NPV (EpNPV), *Gryllus bimaculatus* nudivirus (GbNV), *Lymantria dispar* MNPV (LdMNPV), *Melanoplus sanguinipes* entomopoxvirus (MsEPV), *Neodiprion lecontei* NPV (NeleNPV), *Neodiprion sertifer* NPV (NeseNPV), *Orgyia pseudotsugata* NPV (OpNPV), *Oryctes rhinoceros nudivirus* (OrNV), *Plutella xylostella* GV (PxGV).

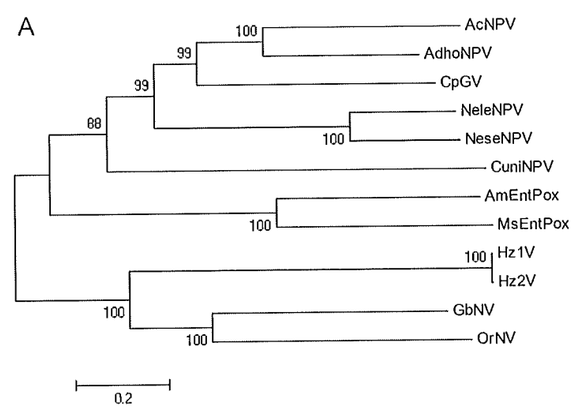
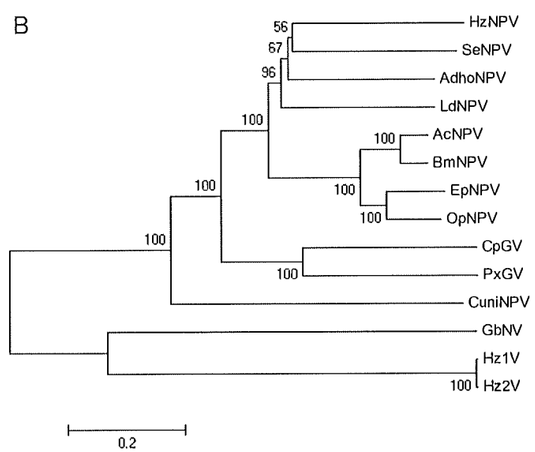


Figure 6. *Cont.*



3. Experimental Section

3.1. Source of Viruses

HzNV-2 viral particles were injected into newly emerged female moths from a laboratory colony of healthy *H. zea*, as outlined by Rallis and Burand [6]. After mating with healthy male moths, eggs laid by the injected female moths were collected from the mating chambers, then transferred into hatching chambers. Newly hatched larvae were reared on an artificial diet in environmental chambers under a light:dark cycle of 16:8 hours at 26 °C and upon emergence, progeny individuals were examined for signs of HzNV-2 infection, as described by Raina and Adams [88] and Hamm *et al.* [5]. Infected female moths with a visible, viral plug were collected as the source of viruses. The virus in the plugs were suspended in phosphate buffered saline (PBS) (136 mM NaCl, 1.4 mM KH2PO4, 8 mM Na2HPO4, 2.6 mM KCl, pH7.3), and used for virus particle preparation.

3.2. Virus Purification and DNA Extraction

The crude virus preparation in PBS, collected from the plugs, was layered on a sucrose gradient (25–55%), and centrifuged at 22,000 rpm at 4 °C for 1 h using a Beckman SW28 rotor. The purified virus band in the sucrose gradient was collected, diluted in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) or PBS, and centrifuged to pellet the purified virus particles. The purified virus particles were then suspended in TE or PBS and stored at −20 °C for DNA extraction.

The purified virus particles were incubated at 37 °C for 1 h with protease K at 1 mg/mL and sodium dodecyl sulfate (SDS) at 1% to solublize the virus envelope and structural proteins. The viral DNA was extracted twice with phenol, chloroform, and isoamylalcohol (25:24:1), followed by DNA precipitation with 0.3 M sodium acetate and 100% ethanol at −80°C for 1 hour. The extracted viral DNA was centrifuged at 12,000 rpm for 15 min at 4 °C, washed three times with 70% ethanol, air-dried, resuspended in TE and stored at −20°C.

3.3. DNA Sequencing

Random DNA fragments of 1.5 to 6.5 kbp were obtained by partial enzymatic digestion with *Tsp*509I (New England Biolabs). The DNA fragments were cloned into the *Eco*RI site of pUC19 plasmid, and grown in *Escherichia coli* DH10B cells. Plasmids containing virus DNA fragments were purified by an alkaline lysis method. The fragments were sequenced from both ends of the cloning site with M13 forward and reverse primers using the dideoxy chain termination method [89] on a PRISM 3700 automated DNA sequencer (PE Biosystems). Chromatogram traces were base-called with PHRED and assembled with PHRAP [90,91]. Confirmatory assemblies were performed with the TIGR [92] and CAP3 [93] sequence assembly computer programs. The HzNV-2 virus DNA was sequenced to an average of 13-fold coverage at each base position.

3.4. Sequence Analysis

Open reading frames (ORFs) longer than 60 amino acids (aa) with a methionine start codon were evaluated for coding potential using the Hexamer [94] and Glimmer [95,96] computer programs. Protein homology searches were performed using the BLAST, PSI-BLAST [97], FASTA [98], and HMMER [99] computer programs with the databases: PROSITE, Pfam, Prodom, and GenBank [100]. Global sequence alignment was performed using rVISTA [101]. Conserved domain searches were performed using the SMART [102] and CD-Search [103] computer programs with Swiss-Prot and CDD [104] databases. CGC [105], EMBOSS [106], Psort [107], TMHMM [108,109], SignalP 3.0 [110], and ANTHEPROT [111] computer programs were used for general analysis, signal peptide prediction, transmembrane domain prediction, and physical characterization of proteins. Phylogenetic analysis was performed by using ClustalX [112], MUSCLE [113], and MEGA4 [114].

4. Summary and Conclusions

We report here the complete nucleotide sequence and the annotation of the genome of the NV HzNV-2. This analysis showed HzNV-2 and HzNV-1 to have 93% sequence identity. Based on the high degree of sequence identity between these two NVs, it now appears that the IMC-Hz-1 ovarian cell line, from which HzNV-1 was originally isolated [115,116], was established from the ovaries of *H. zea* moths persistently infected with a NV which resembled HzNV-2. If this were the case, it is highly unlikely that females with grossly malformed reproductive tissues, characteristic of HzNV-2 infected insects, would have been selected for use in establishing an ovarian cell line. This possibility along with the absence of any observed pathology in HzNV-1 infected insects, suggest that genes responsible for the pathology caused by HzNV-2 may be missing in the HzNV-1 genome [11]. In this study, a total of 16 ORFs were identified in the HzNV-2 genome, that were not previously found in HzNV-1. Unfortunately, none of these 16 ORFs were determined to share sequence homology with any genes of know function, and therefore could not be directly associated with the pathology observed in HzNV-2 infected insects. Nonetheless, the genetic similarity between HzNV-1 and HzNV-2 indicates that these two viruses are very closely related, having a very recent, common ancestor.

In a recent study, 20 HzNV-1 genes were found to share homologies with baculovirus core genes (2). Homologues of all of these HzNV-1 genes were found in the HzNV-2 genome however, there

were no ORFs in HzNV-2 found to have homology with the 4 baculovirus core genes *p47*, *ac68*, *vp39* and *p33*. Interestingly, Hz1V075, which was found to be homologues to the OrNV *p47* was also reported to be homologous to OrNV *lef-9* (2). In our analysis, Hz2V063 was found to be homologous to Hz1V075 and MBV *lef-9,* but not to any *p47* ORFs. If Hz2V063 and Hz1V075 both have activity found associated with the two separate ORFs encoded by baculoviruses and other NVs, then this would suggest an important point of divergence of HzNVs from other N*Vs.*

An interesting feature of the HzNV-2 genome is the presence of a large number of cellular gene homologues. The HzNV-2 genome contains 12 genes which have homology to cellular genes. The majority of the cellular gene homologues in HzNV-2 are related to nucleic acid metabolism and DNA replication; 9 of the 12 cellular genes found in HzNV-2 are TS, dUTPase, DHFR, RR1, RR2, dNK, SHMT and ligase. The three other HzNV-2 genes found to more closely resemble host genes than viral genes (Hz2V023, Hz2068 and Hz2V007) are thought to code for proteins which could be involved in the regulation of molecular processes in the infected host leading to cell proliferation and tissue malformation observed in the reproductive structures of infected insects.

It was interesting to find that HzNV-2 coded for a major facilitator superfamily (MFS) protein gene (Hz2V023). MFS genes are found ubiquitously in living cells [66] and have never before been identified in a viral genome. These transmembrane proteins are involved in the movement of small molecules, in and out of cells and could act to facilitate the enhanced metabolism required for the cell proliferation observed in HzNV-2 infected reproductive tissues.

Hz2V068 which encodes both an MMP domain and an HX domain could also play an important role in altering the pattern of cell differentiation and morphogenesis. It is possible that this viral gene product could function in the proteolysis of the extracellular matrix and cell surface molecules, altering cell differentiation during HzNV-2 replication, resulting in the malformation of reproductive tissues during their development in infected insects.

It was very surprising to find that HzNV-2 contains, Carboxylesterase (Hz2V007) which shares significant homology with *A. gambiae* JHE, suggesting that Hz2V007 codes for a protein that may have JHE activity. In holometabolous insects such as *H. zea* and *Drosophila*, the developmental process of molting is controlled by two hormones, the steroid, 20-hydroxyecdysone (ecdysone) and the sesquiterpenoid, juvenile hormone (JH). These two hormones work in concert to regulate gene expression during molting, directing insect growth and tissue development [117]. The predicted JHE activity of Hz2V007, coupled with increased cellular metabolic functions resulting from Hz2V023, MFS activity and tissue remodeling by Hz2V068, coded MMP could all contribute to malformation of virus infected tissues and the unique pathology observed in HzNV-2 infected moths. It has not yet been determined how each of these novel virus genes function, but we believe that it is the expression of these genes, along with several of the other cellular gene homologues identified in the HzNV-2 genome, which are responsible for the pathology and unique biology of this insect pathogenic virus.

Acknowledgments

This material is based upon work supported by USDA NRICGP Grant #2001-35302-10885, the Cooperative State Research Extension, Education Service, U.S. Department of Agriculture, the

Massachusetts Agricultural Experiment Station and the Department of Plant Soil and Insect Science, under Projects No. MAS00909 and MAS00972.

Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Wang, Y.-J.; Burand, J.P.; Jehle, J.A. Nudivirus genomics: Diversity and classification. *Virol. Sin.* **2007**, *22*, 128–136.
2. Wang, Y.; Bininda-Emonds, O.R.P.; van Oers, M.M.; Vlak, J.M.; Jehle, J.A. The genome of oryctes rhinoceros nudivirus provides novel insight into the evolution of nuclear arthropod-specific large circular double-stranded DNA viruses. *Virus Genes* **2011**, *42*, 444–456.
3. Wang, Y.; Jehle, J.A. Nudiviruses and other large, double-stranded circular DNA viruses of invertebrates: New insights on an old topic RID C-2243-2009. *J. Invertebr. Pathol.* **2009**, *101*, 187–193.
4. Bezier, A.; Annaheim, M.; Herbiniere, J.; Wetterwald, C.; Gyapay, G.; Bernard-Samain, S.; Wincker, P.; Roditi, I.; Heller, M.; Belghazi, M.; *et al.* Polydnaviruses of braconid wasps derive from an ancestral nudivirus. *Science* **2009**, *323*, 926–930.
5. Hamm, J.J.; Carpenter, J.E.; Styer, E.L. Oviposition day effect on incidence of agonadal progeny of Helicoverpa zea (Lepidoptera: Noctuidae) infected with a virus. *Ann. Entomol. Soc. Am.* **1996**, *89*, 266–275.
6. Rallis, C.P.; Burand, J.P. Pathology and ultrastructure of Hz-2V infection in the agonadal female corn earworm, Helicoverpa zea. *J. Invertebr. Pathol.* **2002**, *81*, 33–44.
7. Burand, J.P.; Rallis, C.P. *In vivo* dose-response of insects to Hz-2V infection. *Virol. J.* **2004**, *1*, 15.
8. Burand, J.P.; Tan, W.; Kim, W.; Nojima, S.; Roelofs, W. Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female Helicoverpa zea moths. *J. Insect Sci.* **2005**, *5*, 6.
9. Lu, H. Characterization of a novel baculovirus, gonad-specific virus, GSV. Ph.D. dissertation, University of Massachusetts, Amherst, MA, USA, 1997.
10. Cochran, M.A.; Faulkner, P. Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. *J. Virol.* **1983**, *45*, 961–970.
11. Cheng, C.H.; Liu, S.M.; Chow, T.Y.; Hsiao, Y.Y.; Wang, D.P.; Huang, J.J.; Chen, H.H. Analysis of the complete genome sequence of the Hz-1 virus suggests that it is related to members of the Baculoviridae. *J. Virol.* **2002**, *76*, 9024–9034.
12. Wang, Y.; Kleespies, R.G.; Huger, A.M.; Jehle, J.A. The genome of Gryllus bimaculatus nudivirus indicates an ancient diversification of baculovirus-related nonoccluded nudiviruses of insects. *J. Virol.* **2007**, *81*, 5395–5406.
13. Pearson, M.N.; Rohrmann, G.F. Lymantria dispar nuclear polyhedrosis virus homologous regions: characterization of their ability to function as replication origins. *J. Virol.* **1995**, *69*, 213–221.

14. Shamoo, Y.; Steitz, T.A. Building a replisome from interacting pieces: Sliding clamp complexed to a peptide from DNA polymerase and a polymerase editing complex. *Cell* **1999**, *99*, 155–166.
15. Bressan, D.A.; Baxter, B.K.; Petrini, J.H. The Mre11-Rad50-Xrs2 protein complex facilitates homologous recombination-based double-strand break repair in Saccharomyces cerevisiae. *Mol. Cell Biol.* **1999**, *19*, 7681–7687.
16. Hopfner, K.P.; Karcher, A.; Shin, D.S.; Craig, L.; Arthur, L.M.; Carney, J.P.; Tainer, J.A. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **2000**, *101*, 789–800.
17. Mascarenhas, J.; Sanchez, H.; Tadesse, S.; Kidane, D.; Krisnamurthy, M.; Alonso, J.C.; Graumann, P.L. Bacillus subtilis SbcC protein plays an important role in DNA inter-strand cross-link repair. *BMC Mol. Biol.* **2006**, *7*, 20.
18. Evans, E.; Klemperer, N.; Ghosh, R.; Traktman, P. The vaccinia virus D5 protein, which is required for DNA replication, is a nucleic acid-independent nucleoside triphosphatase. *J. Virol.* **1995**, *69*, 5353–5361.
19. O'Donovan, A.; Davies, A.A.; Moggs, J.G.; West, S.C.; Wood, R.D. XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature* **1994**, *371*, 432–435.
20. Cooper, P.K.; Nouspikel, T.; Clarkson, S.G.; Leadon, S.A. Defective transcription-coupled repair of oxidative base damage in Cockayne syndrome patients from XP group G. *Science* **1997**, *275*, 990–993.
21. Hosfield, D.J.; Mol, C.D.; Shen, B.; Tainer, J.A. Structure of the DNA repair and replication endonuclease and exonuclease FEN-1: Coupling DNA and PCNA binding to FEN-1 activity. *Cell* **1998**, *95*, 135–146.
22. Kim, K.; Biade, S.; Matsumoto, Y. Involvement of flap endonuclease 1 in base excision DNA repair. *J. Biol. Chem.* **1998**, *273*, 8842–8848.
23. Jehle, J.A.; Blissard, G.W.; Bonning, B.C.; Cory, J.S.; Herniou, E.A.; Rohrmann, G.F.; Theilmann, D.A.; Thiem, S.M.; Vlak, J.M. On the classification and nomenclature of baculoviruses: A proposal for revision. *Arch. Virol.* **2006**, *151*, 1257–1266.
24. Guarino, L.A.; Xu, B.; Jin, J.; Dong, W. A virus-encoded RNA polymerase purified from baculovirus-infected cells. *J. Virol.* **1998**, *72*, 7985–7991.
25. Mclachlin, J.R.; Miller, L.K. Identification and characterization of Vlf-1, a baculovirus gene involved in very late gene-expression. *J. Virol.* **1994**, *68*, 7746–7756.
26. Yang, S.; Miller, L.K. Activation of baculovirus very late promoters by interaction with very late factor 1. *J. Virol.* **1999**, *73*, 3404–3409.
27. Vanarsdall, A.L.; Okano, K.; Rohrmann, G.F. Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing. *J. Virol.* **2006**, *80*, 1724–1733.
28. Guttieri, M.C.; Burand, J.P. Nucleotide sequence, temporal expression, and transcriptional mapping of the p34 late gene of the Hz-1 insect virus. *Virology* **1996**, *223*, 370–375.
29. Guarino, L.A.; Dong, W.; Jin, J. *In vitro* activity of the baculovirus late expression factor LEF-5. *J. Virol.* **2002**, *76*, 12663–12675.
30. Jin, J.; Dong, W.; Guarino, L.A. The LEF-4 subunit of baculovirus RNA polymerase has RNA 5'-triphosphatase and ATPase activities. *J. Virol.* **1998**, *72*, 10011–10019.

31. Knebel-Morsdorf, D.; Quadt, I.; Li, Y.; Montier, L.; Guarino, L.A. Expression of baculovirus late and very late genes depends on LEF-4, a component of the viral RNA polymerase whose guanyltransferase function is essential. *J. Virol.* **2006**, *80*, 4168–4173.
32. Shuman, S.; Liu, Y.; Schwer, B. Covalent catalysis in nucleotidyl transfer reactions: essential motifs in Saccharomyces cerevisiae RNA capping enzyme are conserved in Schizosaccharomyces pombe and viral capping enzymes and among polynucleotide ligases. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 12046–12050.
33. Passarelli, A.L.; Todd, J.W.; Miller, L.K. A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. *J. Virol.* **1994**, *68*, 4673–4678.
34. Lu, A.; Miller, L.K. Identification of three late expression factor genes within the 33.8- to 43.4-map-unit region of Autographa californica nuclear polyhedrosis virus. *J. Virol.* **1994**, *68*, 6710–6718.
35. Broyles, S.S.; Moss, B. Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: Nucleotide sequence and transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 3141–3145.
36. Acharya, A.; Gopinathan, K.P. Characterization of late gene expression factors lef-9 and lef-8 from Bombyx mori nucleopolyhedrovirus. *J. Gen. Virol.* **2002**, *83*, 2015–2023.
37. Pijlman, G.P.; Pruijssers, A.J.; Vlak, J.M. Identification of pif-2, a third conserved baculovirus gene required for per os infection of insects. *J. Gen. Virol.* **2003**, *84*, 2041–2049.
38. Li, X.; Song, J.; Jiang, T.; Liang, C.; Chen, X. The N-terminal hydrophobic sequence of Autographa californica nucleopolyhedrovirus PIF-3 is essential for oral infection. *Arch. Virol.* **2007**, *152*, 1851–1858.
39. Ohkawa, T.; Washburn, J.O.; Sitapara, R.; Sid, E.; Volkman, L.E. Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of Heliothis virescens larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. *J. Virol.* **2005**, *79*, 15258–15264.
40. Kikhno, I.; Gutierrez, S.; Croizier, L.; Croizier, G.; Ferber, M.L. Characterization of pif, a gene required for the per os infectivity of Spodoptera littoralis nucleopolyhedrovirus. *J. Gen. Virol.* **2002**, *83*, 3013–3022.
41. Haas-Stapleton, E.J.; Washburn, J.O.; Volkman, L.E. P74 mediates specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to primary cellular targets in the midgut epithelia of Heliothis virescens larvae. *J. Virol.* **2004**, *78*, 6786–6791.
42. Rashidan, K.K.; Nassoury, N.; Tazi, S.; Giannopoulos, P.N.; Guertin, C. Choristoneura fumiferana Granulovirus p74 protein, a highly conserved baculoviral envelope protein. *J. Biochem. Mol. Biol.* **2003**, *36*, 475–487.
43. Carreras, C.W.; Santi, D.V. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **1995**, *64*, 721–762.
44. Perryman, S.M.; Rossana, C.; Deng, T.L.; Vanin, E.F.; Johnson, L.F. Sequence of a cDNA for mouse thymidylate synthase reveals striking similarity with the prokaryotic enzyme. *Mol. Biol. Evol.* **1986**, *3*, 313–321.

45. Chen, H.H.; Tso, D.J.; Yeh, W.B.; Cheng, H.J.; Wu, T.F. The thymidylate synthase gene of Hz-1 virus: A gene captured from its lepidopteran host. *Insect Mol. Biol.* **2001**, *10*, 495–503.
46. Garcia-Maruniak, A.; Maruniak, J.E.; Farmerie, W.; Boucias, D.G. Sequence analysis of a non-classified, non-occluded DNA virus that causes salivary gland hypertrophy of Musca domestica, MdSGHV. *Virology* **2008**, *377*, 184–196.
47. Jordan, A.; Reichard, P. Ribonucleotide reductases. *Annu. Rev. Biochem.* **1998**, *67*, 71–98.
48. Blakley, R.L. The interconversion of serine and glycine: Participation of pyridoxal phosphate. *Biochem. J.* **1955**, *61*, 315–323.
49. Blakley, R.L. *The Biochemistry of Folic Acid and Related Pteridines*; North-Holland Pub. Co.: Amsterdam, The Netherlands, 1969; pp. xxi, 570.
50. Ives, D.H.; Ikeda, S. Life on the salvage path: The deoxynucleoside kinase of Lactobacillus acidophilus R-26. *Progr. Nucleic Acid Res. Mol. Biol.* **1998**, *59*, 205–255.
51. Elder, J.H.; Lerner, D.L.; Hasselkus-Light, C.S.; Fontenot, D.J.; Hunter, E.; Luciw, P.A.; Montelaro, R.C.; Phillips, T.R. Distinct subsets of retroviruses encode dUTPase. *J. Virol.* **1992**, *66*, 1791–1794.
52. Chen, R.; Wang, H.; Mansky, L.M. Roles of uracil-DNA glycosylase and dUTPase in virus replication. *J. Gen. Virol.* **2002**, *83*, 2339–2345.
53. Miller, R.J.; Cairns, J.S.; Bridges, S.; Sarver, N. Human immunodeficiency virus and AIDS: Insights from animal lentiviruses. *J. Virol.* **2000**, *74*, 7187–7195.
54. Wu, X.; Guarino, L.A. Autographa californica nucleopolyhedrovirus orf69 encodes an RNA cap (nucleoside-2'-O)-methyltransferase. *J. Virol.* **2003**, *77*, 3430–3440.
55. Schnell, J.R.; Dyson, H.J.; Wright, P.E. Structure, dynamics, and catalytic function of dihydrofolate reductase. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 119–140.
56. Braunagel, S.C.; Elton, D.M.; Ma, H.; Summers, M.D. Identification and analysis of an Autographa californica nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. *Virology* **1996**, *217*, 97–110.
57. Russell, R.L.; Rohrmann, G.F. Characterization of P91, a protein associated with virions of an Orgyia pseudotsugata baculovirus. *Virology* **1997**, *233*, 210–223.
58. Wu, W.; Liang, H.; Kan, J.; Liu, C.; Yuan, M.; Liang, C.; Yang, K.; Pang, Y. Autographa californica multiple nucleopolyhedrovirus 38K is a novel nucleocapsid protein that interacts with VP1054, VP39, VP80, and itself. *J. Virol.* **2008**, *82*, 12356–12364.
59. Gilbert, L.I.; Granger, N.A.; Roe, R.M. The juvenile hormones: Historical facts and speculations on future research directions. *Insect Biochem. Mol. Biol.* **2000**, *30*, 617–644.
60. Ward, V.K.; Bonning, B.C.; Huang, T.; Shiotsuki, T.; Griffeth, V.N.; Hammock, B.D. Analysis of the catalytic mechanism of juvenile-hormone esterase by site-directed mutagenesis. *Int. J. Biochem.* **1992**, *24*, 1933–1941.
61. Crook, N.E.; Clem, R.J.; Miller, L.K. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* **1993**, *67*, 2168–2174.
62. Uren, A.G.; Coulson, E.J.; Vaux, D.L. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. *Trends Biochem. Sci.* **1998**, *23*, 159–162.

63. Miller, L.K. An exegesis of IAPs: Salvation and surprises from BIR motifs. *Trends Cell Biol.* **1999**, *9*, 323–328.
64. Joazeiro, C.A.; Weissman, A.M. RING finger proteins: Mediators of ubiquitin ligase activity. *Cell* **2000**, *102*, 549–552.
65. Wilson, R.; Goyal, L.; Ditzel, M.; Zachariou, A.; Baker, D.A.; Agapite, J.; Steller, H.; Meier, P. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat. Cell Biol.* **2002**, *4*, 445–450.
66. Pao, S.S.; Paulsen, I.T.; Saier, M.H., Jr. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1–34.
67. Konrad, M. Cloning and expression of the essential gene for guanylate kinase from yeast. *J. Biol. Chem.* **1992**, *267*, 25652–25655.
68. Hall, S.W.; Kuhn, H. Purification and properties of guanylate kinase from bovine retinas and rod outer segments. *Eur. J. Biochem.* **1986**, *161*, 551–556.
69. Guttieri, M.C.; Burand, J.P. Location, nucleotide sequence, and regulation of the p51 late gene of the Hz-1 insect virus: Identification of a putative late regulatory element. *Virus Genes* **2001**, *23*, 17–25.
70. Murphy, G.J.; Murphy, G.; Reynolds, J.J. The origin of matrix metalloproteinases and their familial relationships. *FEBS Lett.* **1991**, *289*, 4–7.
71. Nagase, H.; Woessner, J.F., Jr. Matrix metalloproteinases. *J. Biol. Chem.* **1999**, *274*, 21491–21494.
72. Wang, P.; Granados, R.R. An intestinal mucin is the target substrate for a baculovirus enhancin. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 6977–6982.
73. Herniou, E.A.; Olszewski, J.A.; Cory, J.S.; O'Reilly, D.R. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol.* **2003**, *48*, 211–234.
74. Chen, H.Q.; Chen, K.P.; Yao, Q.; Guo, Z.J.; Wang, L.L. Characterization of a late gene, ORF67 from Bombyx mori nucleopolyhedrovirus. *FEBS Lett.* **2007**, *581*, 5836–5842.
75. Peist, R.; Koch, A.; Bolek, P.; Sewitz, S.; Kolbus, T.; Boos, W. Characterization of the aes gene of Escherichia coli encoding an enzyme with esterase activity. *J. Bacteriol.* **1997**, *179*, 7679–7686.
76. Joly, N.; Danot, O.; Schlegel, A.; Boos, W.; Richet, E. The Aes protein directly controls the activity of MalT, the central transcriptional activator of the Escherichia coli maltose regulon. *J. Biol. Chem.* **2002**, *277*, 16606–16613.
77. Edelman, A.M.; Blumenthal, D.K.; Krebs, E.G. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **1987**, *56*, 567–613.
78. Mikhailov, V.S.; Rohrmann, G.F. Baculovirus replication factor LEF-1 is a DNA primase. *J. Virol.* **2002**, *76*, 2287–2297.
79. Evans, J.T.; Leisy, D.J.; Rohrmann, G.F. Characterization of the interaction between the baculovirus replication factors LEF-1 and LEF-2. *J. Virol.* **1997**, *71*, 3114–3119.
80. Guarino, L.A.; Summers, M.D. Interspersed homologous DNA of autographa californica nuclear polyhedrosis virus enhances delayed-early gene expression. *J. Virol.* **1986**, *60*, 215–223.
81. Grula, M.A.; Buller, P.L.; Weaver, R.F. alpha-Amanitin-Resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected Heliothis zea Larvae and Spodoptera frugiperda cells. *J. Virol.* **1981**, *38*, 916–921.

82. Pearson, M.N.; Groten, C.; Rohrmann, G.F. Identification of the lymantria dispar nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. *J. Virol.* **2000**, *74*, 6126–6131.
83. Garcia-Maruniak, A.; Maruniak, J.E.; Zanotto, P.M.; Doumbouya, A.E.; Liu, J.C.; Merritt, T.M.; Lanoie, J.S. Sequence analysis of the genome of the Neodiprion sertifer nucleopolyhedrovirus. *J. Virol.* **2004**, *78*, 7036–7051.
84. Lauzon, H.A.; Lucarotti, C.J.; Krell, P.J.; Feng, Q.; Retnakaran, A.; Arif, B.M. Sequence and organization of the Neodiprion lecontei nucleopolyhedrovirus genome. *J. Virol.* **2004**, *78*, 7023–7035.
85. Belyavskyi, M.; Braunagel, S.C.; Summers, M.D. The structural protein ODV-EC27 of Autographa californica nucleopolyhedrovirus is a multifunctional viral cyclin. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 11205–11210.
86. Mikhailov, V.S.; Okano, K.; Rohrmann, G.F. Baculovirus alkaline nuclease possesses a 5'-->3' exonuclease activity and associates with the DNA-binding protein LEF-3. *J. Virol.* **2003**, *77*, 2436–2444.
87. Li, L.; Rohrmann, G.F. Characterization of a baculovirus alkaline nuclease. *J. Virol.* **2000**, *74*, 6401–6407.
88. Raina, A.K.; Adams, J.R. Gonad-specific virus of corn-earworm. *Nature* **1995**, *374*, 770–770.
89. Sanger, F.; Air, G.M.; Barrell, B.G.; Brown, N.L.; Coulson, A.R.; Fiddes, J.C.; Hutchison, C.A.; Slocombe, P.M.; Smith, M. Nucleotide-sequence of bacteriophage Phichi174 DNA. *Nature* **1977**, *265*, 687–695.
90. Ewing, B.; Hillier, L.; Wendl, M.C.; Green, P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **1998**, *8*, 175–185.
91. Ewing, W.R.; Choi, Y.M.; Becker, M.; Manetta, V.; Green, D.; Davis, R.; Mason, H.; Ly, C.; Cha, D.; McGarry, D.; *et al.* Sulfonamidopyrrolidinones: Design, SAR and parenteral activity of a novel class of factor Xa inhibitors. *Abstr. Paper. Am. Chem. Soc.* **1998**, *215*, U920–U920.
92. Sutton, G.G.; White, O.; Adams, M.D.; Kerlavage, A.R. TIGR assembler: A new tool for assembling large shotgun sequencing projects. *Genome Sci. Technol.* **1995**, *1*, 9–19.
93. Huang, X.; Madan, A. CAP3: A DNA sequence assembly program. *Genome Res.* **1999**, *9*, 868–877.
94. *Hexamer*. Available online: ftp://ftp.sanger.ac.uk/pub/rd/ (accessed on 1 January 2012).
95. Salzberg, S.L.; Delcher, A.L.; Kasif, S.; White, O. Microbial gene identification using interpolated Markov models. *Nucleic Acids Res.* **1998**, *26*, 544–548.
96. Delcher, A.L.; Harmon, D.; Kasif, S.; White, O.; Salzberg, S.L. Improved microbial gene identification with GLIMMER. *Nucleic Acids Res.* **1999**, *27*, 4636–4641.
97. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* **1997**, *25*, 3389–3402.
98. Pearson, W.R. Rapid and sensitive sequence comparison with FASTP and FASTA. *Meth. Enzymol.* **1990**, *183*, 63–98.
99. Eddy, S.R.; Mitchison, G.; Durbin, R. Maximum discrimination hidden Markov models of sequence consensus. *J. Comput. Biol.* **1995**, *2*, 9–23.
100. Burks, C. Molecular biology database list. *Nucleic Acids Res.* **1999**, *27*, 1–9.

101. Loots, G.; Ovcharenko, I. VISTA 2.0: Evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* **2004**, *32*, W217–W221.
102. Schultz, J.; Milpetz, F.; Bork, P.; Ponting, C.P. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 5857–5864.
103. Marchler-Bauer, A.; Bryant, S.H. CD-Search: Protein domain annotations on the fly. *Nucleic Acids Res.* **2004**, *32*, W327–331.
104. Marchler-Bauer, A.; Anderson, J.B.; DeWeese-Scott, C.; Fedorova, N.D.; Geer, L.Y.; He, S.; Hurwitz, D.I.; Jackson, J.D.; Jacobs, A.R.; Lanczycki, C.J.; *et al.* CDD: A curated Entrez database of conserved domain alignments. *Nucleic Acids Res.* **2003**, *31*, 383–387.
105. Devereux, J.; Haeberli, P.; Smithies, O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **1984**, *12*, 387–395.
106. Rice, P.; Longden, I.; Bleasby, A. EMBOSS: The european molecular biology open software suite. *Trends Genet.* **2000**, *16*, 276–277.
107. Nakai, K.; Horton, P. PSORT: A program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **1999**, *24*, 34–36.
108. Sonnhammer, E.L.; von Heijne, G.; Krogh, A. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **1998**, *6*, 175–182.
109. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* **2001**, *305*, 567–580.
110. Bendtsen, J.D.; Nielsen, H.; von Heijne, G.; Brunak, S. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **2004**, *340*, 783–795.
111. Deleage, G.; Combet, C.; Blanchet, C.; Geourjon, C. ANTHEPROT: An integrated protein sequence analysis software with client/server capabilities. *Comput. Biol. Med.* **2001**, *31*, 259–267.
112. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25*, 4876–4882.
113. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797.
114. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.
115. Granados, R.R.; Nguyen, T.; Cato, B. An insect cell line persistently infected with a baculovirus-like particle. *Intervirology* **1978**, *10*, 309–317.
116. Hink, W.F.; Ignoffo, C.M. Establishment of a new cell line (IMC-HZ-1) from ovaries of cotton bollworm moths, Heliothis zea (boddie). *Exp. Cell Res.* **1970**, *60*, 307–309.
117. Dubrovsky, E.B. Hormonal cross talk in insect development. *Trends Endocrinol. Metab.* **2005**, *16*, 6–11.

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***J. Clin. Microbiol.* 2012, 50(4):1204. DOI: 10.1128/JCM.06506-11.**
Published Ahead of Print 11 January 2012.

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Here we report the biological and molecular characterization of a virulent genotype VII Newcastle disease virus (NDV) circulating in Venezuela and the assessment of the vaccination efficacy under field conditions compared to controlled rearing conditions. Biological pathotyping showed a mean embryo dead time of 50 h and an intracerebral pathogenicity index of 1.86. Sequence-based phylogenetic analysis demonstrated that the virus belongs to genotype VII in class II (a genotype often found in Asia and Africa), representing the first report of the presence of this genotype in the continent of South America. A vaccine-challenge trial in commercial broilers reared in fields or in a experimental setting included dual (live/killed) priming of 1-day-old chicks plus two live NDV and infectious bursal disease virus (IBDV) field vaccinations at days 7 and 17, followed by a very stringent genotype VII NDV challenge at day 28. Serology for NDV and IBDV, bursal integrity, and protection against NDV lethal challenge were assessed. At 28 days, field vaccinates showed significantly lower NDV (1,356 versus 2,384) and higher IBD (7,295 versus 1,489) enzyme-linked immunosorbent assay (ELISA) antibody titers than the experimentally reared birds. A lower bursal size and bursa-body weight ratio ($P < 0.05$) and higher bursa lesion score were also detected in the field set. Only 57.1% of field vaccinates survived the lethal challenge, differing ($P < 0.05$) from 90.5% survival in the experimental farm. Overall, results confirmed the presence of the genotype VII viruses in South America and suggest that field-associated factors such as immunosuppression compromise the efficacy of the vaccination protocols implemented.

Newcastle disease virus (NDV) is one of the most important infectious agents in veterinary medicine and the causative agent of Newcastle disease (ND), which affects commercial poultry and causes important economical losses (2). The virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, in the genus *Avulavirus* (15), which encompasses a diverse group of single-stranded, negative-sense, nonsegmented RNA viruses. Due to variations in virulence and host susceptibility, the symptoms of NDV infection in domestic species (chicken, turkey, goose, duck, and pigeon) range from unapparent to severe; infection causes respiratory, enteric, and nervous system disease, leading to high mortality rates (2).

Antigenic and genetic diversity within the NDV isolates is recognized (1). Different genotypes of NDV circulate throughout the world, albeit they are all members of a unique avian paramyxovirus group 1 (APMV-1) serotype. Since 1926 and based on nucleotide sequence, 9 class I NDV and 10 distinct lineages of class II NDV (I to IX and XI) strains have been identified (6, 11, 17). Molecular characterization is of paramount importance for the epidemiology studies required in the development and adaptation of control strategies (10). Genotypes V, VI, and VII of virulent viruses are the predominant genotypes circulating worldwide (16, 17); of these, genotype VII is particularly important given that it has been associated with many of the most recent outbreaks in Asia, Africa, and the Middle East (10, 11, 12, 28, 29).

Vaccination of commercially reared birds is the best way to reduce losses resulting from NDV infection (2, 21). NDV vaccine strains of genotypes I and II are used to control detrimental effects of subclinical forms of the disease and severe clinical disease during outbreaks (2, 23, 27). Additionally, control of risk factors, including immunosuppressive agents, biosecurity breaks, inadequate management practices, and harsh environments, together with common sense, is required to diminish the economic impact of this and other poultry diseases (5). The aim of this work was to fully characterize a novel genotype VII virulent NDV from Venezuela and to evaluate the efficacy of a currently used commercial vaccination scheme under field and controlled rearing conditions.

MATERIALS AND METHODS

Field virus. A NDV isolate that was negative in hemagglutination inhibition tests for avian influenza virus and avian adenoviruses (VEN-611) was obtained from a field outbreak (May 2008) in commercial pullet flocks experiencing high mortality rates and showing ND clinical signs. Virus isolation was performed using standard virus isolation procedures and specific-pathogen-free (SPF) embryonated eggs (3).

Biological pathogenicity assessment. The pathogenic evaluation of the isolate was carried out using standard assay methods to determine the intracerebral pathogenicity index (ICPI) in 1-day-old chicks (3). Briefly, 1-day-old chicks were inoculated intracerebrally with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored during an 8-day observation period and scored as normal (0), sick or paralyzed (1), and dead (2). Total scores were determined, and the mean daily score was calculated to obtain the ICPI. Mean death time (MDT) determinations were performed as previously reported using SPF embryonated chicken eggs (3).

Vaccine viruses. The NDV vaccine strains used included a Villegas-Glisson/University of Georgia (VG/GA) strain (AVINEW) for initial live vaccination, an Ulster strain (GALLIMUNE ND) inactivated in oil adjuvant for day-old subcutaneous (SC) application, and strain LaSota

Received 28 November 2011 Returned for modification 20 December 2011
Accepted 29 December 2011
Published ahead of print 11 January 2012
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doi:10.1128/JCM.06506-11

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0095-1137/12/$12.00 Journal of Clinical Microbiology p. 1204–1208

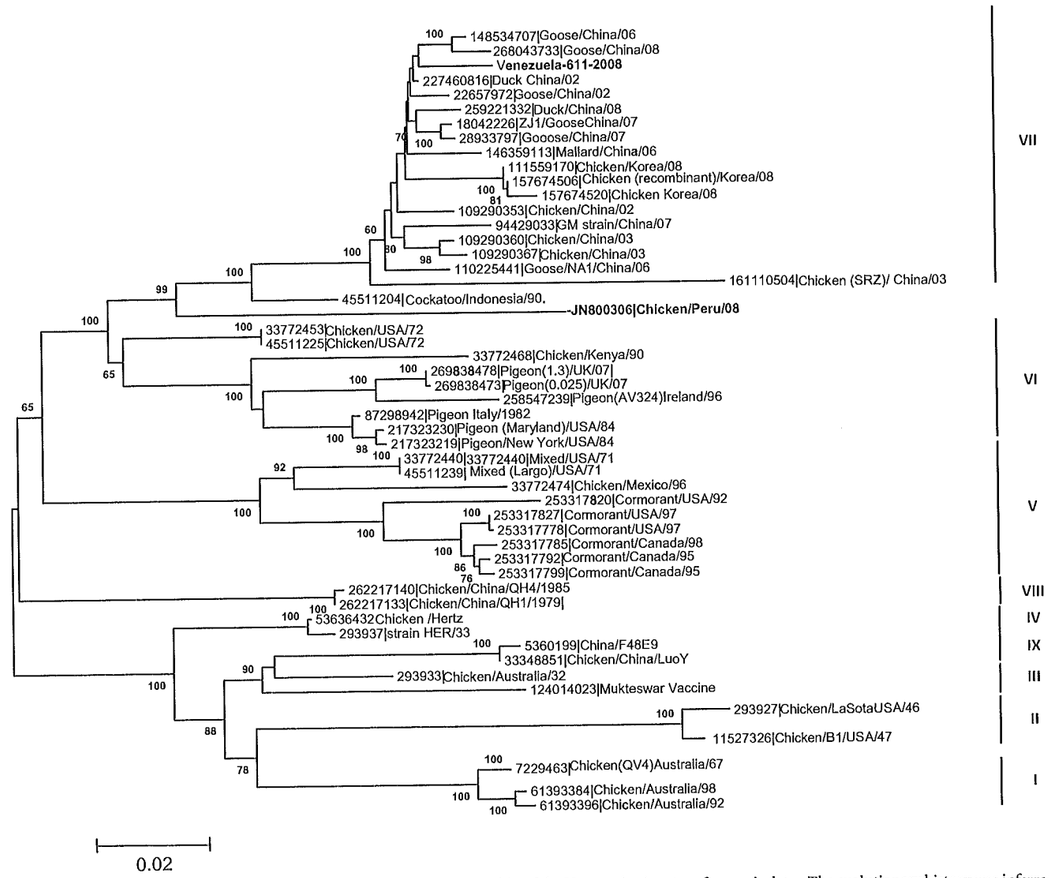

FIG 1 Complete F gene nucleotide comparison and evolutionary relationship of the Venezuela virus to reference isolates. The evolutionary history was inferred using the neighbor-joining method as described in Materials and Methods. The numbers used in the phylogenetic trees represent the GenInfo (GI) sequence identification numbers in GenBank and are followed by a brief description of the viruses. The tree is drawn to scale, with branch lengths presented in the same units as those used for the evolutionary distances used to infer the phylogenetic tree.

and an intermediate live infectious bursal disease virus (IBDV) strain (BURSABLEN) for field vaccination. All commercial vaccines were from Merial Select, Inc., Gainesville, GA, and were applied following the manufacturer's instructions.

Viral RNA extraction and amplification. NDV-positive allantoic fluid was inactivated and transported to the Southeast Poultry Research Laboratory (Athens, GA) for molecular studies, using Flinders technology filter paper (FTA cards; Whatman International Ltd., Springfield Mill, United Kingdom). Fifty microliters of each NDV-positive allantoic fluid sample was added to the matrix in the card, and RNA extraction was performed as previously explained (22). Reverse transcriptase PCR (RT-PCR) amplification of the complete coding region for the F gene was performed using a Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA) as previously described (24).

Sequence analysis. Amplified products were separated on a 1% agarose gel; the bands were excised and eluted using a QIAquick gel extraction kit (Qiagen Valencia, CA) and then sequenced at the DNA sequencing facility at Southeast Poultry Research Laboratory in Athens, GA. An ABI BigDye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) was used, and products were run on an ABI 3730XL DNA analyzer (Applied Biosystems). Sequences were edited and aligned with the DNAStar Lasergene 8.0 program (Madison, WI) using the Clustal W algorithm. Determinations of percentages of amino acid identity for the Fusion protein were performed by obtaining a comparison of the full fusion protein of the Venezuelan isolate to those of vaccine and reference strains. The optimal tree with the sum of branch length = 1.10958014 is shown (Fig. 1). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (150 replicates) are shown next to the branches (8). 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 maximum composite likelihood method and the Kimura 2-parameter method (data not shown), and the units represent the number of base substitutions per site. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were the 1st, 2nd, 3rd, and noncoding positions. All positions containing

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gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 1,653 positions in the final data set. Phylogenetic analyses were conducted using MEGA5 software (26).

Vaccine-challenge trial. To evaluate the level of protection conferred by the NDV vaccination protocol recommended to producers in Venezuela, a vaccine-challenge trial using three groups of commercial broilers was designed and implemented as follows. At day 1, two (2) groups were vaccinated by spray with live strain VG/GA followed by subcutaneous (SC) application of 0.1 ml of the Ulster strain inactivated in oil adjuvant (G01 and G02). The third group was the unvaccinated control (G03). The G01 and G03 groups were transported to a controlled environment (experimental farm) with water and feed provided *at libitum*. The group G02 birds were wing banded and transferred to a 20,000-bird commercial chicken house. Birds in groups G01 and G02 were vaccinated against NDV and IBDV at days 7 and 17 by the use of drinking water following the manufacturer's instructions. At 1, 14, 21, and 28 days of age, 10 birds per group were bled and assessed for ND and IBDV response to vaccination by the use of an enzyme-linked immunosorbent assay (ELISA), with FlockCheck Newcastle disease and IBDV antibody tests performed following the instructions of the manufacturer (IDEXX, Westbrook, ME). A total of 63 commercial boilers (three groups of seven birds per treatment with three replicates) were used in the challenge. Birds were challenged at 28 days by the intraocular route with 0.1 ml of the local genotype VII virus-infected allantoic fluid diluted 10^4 in phosphate-buffered saline (PBS) to a 50% tissue culture infective dose (CID_{50})/ml in 0.1 ml. Birds were observed up to 42 days of age for survival and clinical manifestations.

Bursal integrity assessment. At 14, 21, and 28 days of age, three birds per group were weighed, euthanatized by cervical dislocation, and subjected to necropsy. The bursae were collected, weighed, and evaluated for the presence of macroscopic lesions, and a portion of each was fixed immediately by immersion in 10% neutral buffered formalin for 24 h. Tissues were then processed and embedded in paraffin, using routine histological techniques. The relative bursa/body weight ratio was obtained using the following formula: relative bursa weight = (bursa weight/body weight) × 1,000. The extent of bursal histological damage was graded on a scale from 1 to 4 as previously described (24): briefly, 1 = normal to 10% follicular atrophy; 2 = focal, mild scattered cell depletion up to 10% to 30% follicular atrophy; 3 = multifocal follicular atrophy at 30% to 70%; 4 = diffuse atrophy of >70% of the follicles or any evidence of acute necrosis.

Statistical analysis. All statistical analysis was performed using Sigma Stat 3.0 software. Serology data are presented as mean titers. Group means were analyzed by analysis of variance (ANOVA) with Tukey's multiple-comparison test. Significance is reported at the level of $P \leq 0.05$.

Nucleotide sequence accession number. The full F protein gene sequence has been reported to GenBank (accession number JQ319052).

RESULTS AND DISCUSSION

In this trial, biological pathotyping confirmed that the Venezuelan virus is velogenic; 50-h MDT and 1.86 ICPI results indicate high virulence and are similar to previously reported values for Venezuelan NDV from domestic and waterfowl origins (19). Additionally, we previously reported comparable results for virulent NDV isolates obtained between 1996 and 2006 in Mexico, where the MDT test results ranged between 39.7 and 61.5 h and the lowest and highest intracerebral pathogenicity index (ICPI) values were 1.59 and 1.94, respectively (24).

The molecular approach for NDV identification and pathotyping using reverse transcriptase PCR (RT-PCR) followed by direct sequencing and analysis of the fusion protein gene cleavage site is currently used for NDV research and surveillance (1, 7, 10, 16, 17, 18, 25). In this trial, the full F protein gene was sequenced and reported to GenBank (see above). The full gene sequence was compared to those of reference strains belonging to genotypes I to VII of class II. Most of the North and Central American NDV virulent isolates reported during the last decade belonged to class II in genotype V (United States, Mexico [cormorants]) or VI ([pigeons]), supporting the idea of a local evolutionary trend with regional viral dissemination (16, 17, 25). The phylogenetic analysis for the Venezuelan strain shown in Fig. 1 demonstrated that the isolate belongs to genotype VII in class II, representing the first report of the presence of this genotype in the continent of South America. Additional work, including a higher number of archived and current Venezuelan NDV isolates, is required to assess whether VII is the sole genotype present in the country or whether there are other genotypes circulating.

A comparison of the percentages of amino acid identity between the Venezuelan full F protein and those of reference strains resulted in similarities of up to 98.6% (1.4% amino acid differences) with genotype VII viruses, mainly several goose isolates from China, suggesting a close relationship between the Venezuelan isolate and Asian genotype VII isolates. Amino acid differences of up to 5.8% from a Peru 2008 isolate suggest that those isolates are more distantly related and not likely part of the same introduction. As shown in Table 1, the Venezuelan strain F pro-

TABLE 1 Estimates of evolutionary divergence between amino acid sequences of the full fusion protein of the Venezuelan isolate and those of commercial vaccines and reference strains of different genotypes[a]

Strain	% amino acid substitutions0 compared to:	
	293927 LaSota vaccine	Venezuela 2008
JN800306 Venezuela 2008	12.4	0.0
148534707 Goose/China/2006	12.7	1.4
146359113 Mallard/China/2006	11.8	1.8
268043733 Goose/China/2008	12.8	1.8
28933797 Goose/China/2007	13.3	1.8
110225441 Goose/NA1/China/2006	12.0	1.9
18042226 ZJ1/Goose/China/2007	13.5	1.9
109290367 Chicken/China/2003	12.1	2.3
109290353 Chicken/China/2002	12.8	2.3
45511225 Chicken/USA/1972	10.1	4.3
217323230 Pigeon (MD)/USA/1984	10.9	5.2
269838473 Pigeon/UK/2007	10.9	5.2
Chicken/Peru/2008	12.6	5.8
258547239 Pigeon (AV324)/Ireland/1996	12.1	6.6
262217133 Chicken/China/QH1/1979	10.4	6.6
53636432 Chicken/Hertz	8.6	6.6
161110504 Chicken (SRZ)/China/2003	7.3	7.0
45511239 Mixed (Largo, MD)/USA/1971	12.4	8.0
253317778 Cormorant/USA/1997	12.9	8.4
61393396 Chicken/Australia/1992	8.5	8.7
33348851 Chicken/China	8.2	8.8
293933 Chicken/Australia/1932	9.2	8.8
7229463 Chicken (QV4)/Australia/1967	7.4	9.0
253317820 Cormorant/USA/1992	12.3	9.3
11527326 B1 vaccine	0.9	12.2
293927 LaSota vaccine	0.0	12.4

[a] The percentages of amino acid substitutions between sequences are shown. The numbers used to identify the viruses represent the GenBank GenInfo (GI) sequence identification numbers. Analyses were conducted using the JTT matrix-based model as implemented in MEGA5 (26). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated. There were a total of 551 positions in the final data set.

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TABLE 2 Serological response and protection conferred by the vaccine program in place against a genotype VII lethal challenge

Group	Vaccination protocol (genotype VII Venezuelan isolate challenge virus; $n = 21$ birds)	No. of birds with positive results/total no. of birds tested (% protection)	Mean ELISA titer[a]					
			NDV			IBDV		
			Day 14	Day 21	Day 28	Day 14	Day 21	Day 28
G01	Exptl farm (live/killed day 1 + live day 7 and 17)	2/21 (90.5)	856a	1,713a	2,384a	573a	10,506a	7,295a
G02	Commercial farm (live/killed day 1 + live day 7 and 17)	9/21 (57)	908a	89b	1,356b	160b	141b	1,489b
G03	Exptl farm (challenged, nonvaccinated)	21/21 (0)	140b	53b	22c	134b	77b	52c

[a] Serology data are presented as mean titers. Group means were analyzed by ANOVA with Tukey's multiple-comparison test. Different letters within columns indicate significant differences ($P < 0.05$) between antibody levels. ELISA antibody titers were 2,343 and 1,043 for experimental and commercial farms, respectively, indicating a significant ($P < 0.05$) decrease in the level of humoral immunity provided by the NDV vaccination program in the birds raised in the commercial set. For IBDV, the antibodies measured in the commercial farm suggest an early IBDV field infection with antibody levels of 10,506 and 7,295 at 21 and 28 days, respectively. In the group reared under controlled conditions, the IBDV antibody level was 1,489.

tein was up to 12.4% divergent from that of the LaSota vaccine strain that belongs to genotype II in class II and is commonly used in vaccination programs.

Regardless of the genotype differences between NDV strains circulating worldwide, all NDV isolates belong to the same serotype; if given correctly, ND vaccines prepared with any NDV should protect poultry from clinical disease and mortality in the event of a virulent challenge (21, 23, 27). Current vaccination strategies do protect against mortality; however, existing vaccines have proven to be incapable of completely stopping viral excretion after infection, leading to high recirculation of the virus in the environment and to a viral population suitable to mutations and adaptive changes (17, 18).

In Venezuela, a rigorous vaccination program is applied, including several live and killed boosts during a breeder's life to provide high maternal antibodies (ELISA titers above 13,000 at 1 day of age) and protect against egg production losses and clinical disease. Additionally, the offspring receives dual (live/killed) priming at 1 day of age, followed by two (days 7 and 17) field revaccinations. Nonetheless, outbreaks in vaccinated broilers reared under field conditions occur and are responsible for high mortalities and production losses in the broiler and layer industry.

The serological response and protection against a genotype VII lethal challenge conferred by the current vaccine program implemented in Venezuela were tested under experimental and commercial conditions; the results are summarized in Table 2. At day 28, the average NDV serological responses measured as ELISA antibody titers were 2,384 and 1,356 for experimental and commercial farms, respectively, indicating a significant ($P < 0.05$) decrease in the level of humoral immunity provided by the NDV vaccination program in the birds raised in the commercial set. The antibodies measured in the commercial farm suggest an early IBDV field infection, with antibody levels of 10,506 and 7,295 at 21 and 28 days, respectively. In the group reared under controlled conditions, the IBDV antibodies were at a level of 1,489 at 28 days, which is within the range of previously reported levels for challenge-free birds after administration of two live vaccines (4). The vaccine-challenge trial results showed that all the naïve controls died during the observation period, validating the challenge. Furthermore, only 57% of the birds from the commercial farm survived, differing ($P < 0.05$) from the 90.5% survival rate observed in the experimental farm (Table 2).

The differences in humoral immune response and protection observed are noteworthy (Fig. 2); additional results show that at the challenge age (28 days), the bursa/body weight ratios were 2.03 and 0.9 in the experimental set and the field, respectively, suggesting the presence of better-sized and healthier bursae in the experimental birds. The bursa of Fabricius is the main target organ for the IBDV. The virus replicates in actively dividing IgM-positive B cells (20). Infection results in lymphoid depletion and severe atrophy of the bursa as the predominant features of the pathogenesis of this disease, eliciting a suboptimal immune response to live attenuated vaccines (13).

In this trial, the histopathology evaluation of the bursa in the field group demonstrated severe lymphoid depletion of the follicles, an increased amount of stroma between follicles, and severe follicular atrophy consistent with grade 4 in the bursal lesion scores at 28 days; the experimental farm birds showed a bursal score of 1.5 at the same age. The grading system is a diagnostic tool for IBDV infection and provides valuable information on the predicted immune status of the flock (4, 13, 20). All together, these results suggest that, despite vaccination, the field birds developed IBD, compromising the ability of the birds to mount an adequate immune response to the NDV vaccination and challenge.

Therefore, and based on the differences between the experimental and the field sets, the extent of the vaccine failure in the commercial farm group may be explained by field environmental and/or immunosuppressive factors affecting the efficacy of the

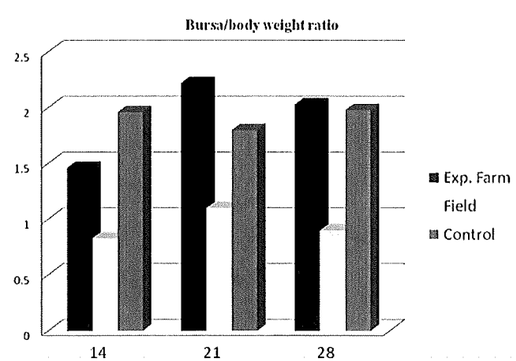

FIG 2 Bursa/body weight ratio. The bursal index was significantly ($P < 0.05$) higher for the experimental farm vaccinates than for the field vaccinates.

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vaccine (9). An additional plausible explanation for the significantly higher protection observed in the group subjected to experimental conditions is that the NDV and IBDV field vaccination procedures (implemented by field personnel in a population of 20,000 birds) could have been impaired, affecting vaccine efficacy and emphasizing the well-known fact that proper vaccination procedures are required for vaccination success (14, 27).

Additional experiments increasing the number of birds challenged are required to assess the cause of the lack of 100% protection in chickens reared under experimental conditions after the intensive vaccination program.

In summary, the presence of a velogenic NDV belonging to genotype VII has been confirmed in Venezuela. The differences in protection observed in the field-vaccinated birds suggest that management, environmental, and/or immunosuppressive factors are affecting ND control and vaccine efficacy in the country.

ACKNOWLEDGMENTS

This work was supported by USDA-ARS CRIS (6612-32000-049-00D).

We acknowledge Dawn Williams-Coplin for excellent technical assistance and the South Atlantic Area Sequencing Facility for nucleotide sequencing.

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.

REFERENCES

1. Aldous EW, Mynn JK, Banks J, Alexander DJ. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. 32:239–256.
2. Alexander DJ, Senne DA. 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections, p 75–98. *In* Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (ed), Diseases of poultry, 12th ed. Blackwell Publishing, Ames, IA.
3. Alexander DJ, Swayne DE. 1998. Newcastle disease virus and other avian paramyxoviruses, p 156–163. *In* Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM (ed), A laboratory manual for the isolation and identification of avian pathogens, vol 4. The American Association of Avian Pathologists, Kennett Square, PA.
4. Banda, Alejandro Villegas P, Purvis L, Perozo F. 2008. Protection conferred by coarse spray vaccination against the challenge with infectious bursal disease virus in commercial broilers. Avian Dis. 52:297–301.
5. Bermudez A. Principles of disease prevention: diagnosis and control, p 75–98. *In* Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (ed), Diseases of poultry, 12th ed. Blackwell Publishing, Ames, IA.
6. Czeglédi A, et al. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120:36–48.
7. Ding Z, et al. 2010. Genetic analysis of avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from swine populations in China related to commonly utilized commercial vaccine strains. Virus Genes 41:369–376.
8. Felsenstein J. 1992. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. Genet. Res. 60: 209–220.
9. Hoerr F. 2010. Clinical aspects of immunosuppression in poultry. Avian Dis. 54:2–15.
10. Khan ST, Rehmani, Rue C, Miller P, Afonso CL. 2010. Phylogenetic and pathological characterization of Newcastle disease virus isolates from Pakistan. J. Clin. Microbiol. 48:1892–1894.
11. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. 2007. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. J. Clin. Microbiol. 45:1310–1314.
12. Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB. 2003. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Arch. Virol. 148:1387–1403.
13. Lukert PD, Saif YM. 2003. Infectious bursal disease, p 161–179. *In* Saif YM, Barnes HJ, Fadly AM, Glisson JR, McDougald LR, Swayne DE (ed), Diseases of poultry, 11th ed. Iowa State University Press, Ames, IA.
14. Marangon S, Busani L. 2007. The use of vaccination in poultry production. Rev. Sci. Tech. 26:265–274.
15. Mayo MA. 2002. Virus taxonomy—Houston 2002. Arch. Virol. 147: 1071–1076.
16. Miller PJ, Decanini EL, Afonso CL. 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. 10: 26–35.
17. Miller PJ, Kim LM, Ip HS, Afonso CL. 2009. Evolutionary dynamics of Newcastle disease virus. Virology 391:64–72.
18. Miller PJ, King DJ, Afonso CL, Suarez DL. 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 25:7238–7246.
19. Moscoso H, Brett M, Fernandez R, Hofacre CL. 2007. Molecular analysis Newcastle disease virus in avian species from Venezuela, p 117. Proceedings of the 144th American Veterinary Medical Association National Convention, Washington, DC, 14 to 18 July 2007. American Veterinary Medical Association, Washington, DC.
20. Müller H, Islam MR, Raue R. 2003. Research on infectious bursal disease—the past, the present and the future. Vet. Microbiol. 97:153–165.
21. OIE. 2009. Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees, 5th ed, vol 1, part 2, chapter 2.3.14, p 576–589. Office international des elpizooties, Paris, France.
22. Perozo F, Villegas P, Estevez C, Alvarado I, Purvis LB. 2006. Use of FTA filter paper for the molecular detection of Newcastle disease virus. Avian Pathol. 35:93–98.
23. Perozo F, Villegas P, Dolz R, Afonso CL, Purvis L. 2008. The VG/GA strain of Newcastle disease virus: mucosal immunity, protection against lethal challenge and molecular analysis. Avian Pathol. 37:237–245.
24. Perozo F, Merino R, Afonso CL, Villegas P, Calderon N. 2008. Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. Avian Dis. 52:472–479.
25. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
26. Tamura K, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739.
27. Villegas P 1998. Viral diseases of the respiratory system. Poult. Sci. 77: 1143–1145.
28. Wang Z, et al. 2006. Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. Ann. N. Y. Acad. Sci. 1081:228–239.
29. Yu L, Wang Z, Jiang Y, Chang L, Kwang J. 2001. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. J. Clin. Microbiol. 39:3512–3519.

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AMERICAN ASSOCIATION SWINE VETERINARIANS

30 Swinepox Virus

Gustavo Delhon, Edan R. Tulman, Claudio L. Afonso, and Daniel L. Rock

RELEVANCE

Swinepox (SWP) is a mild, acute disease of swine characterized by typical poxviral lesions of the skin. SWP is distributed worldwide and is usually associated with poor sanitation. Morbidity may be high in individual herds where young pigs are most severely affected, but mortality is usually negligible. Clinical signs and epidemiology are usually sufficient for SWP diagnosis.

ETIOLOGY

SWP virus (SWPV) is the sole member of the genus *Suipoxvirus* in the family *Poxviridae* (Moyer et al. 2000). The SWPV virion is morphologically similar to vaccinia virus, exhibiting a brick-like structure approximately 320 × 240 nm in horizontal section (Figure 30.1) (Blakemore and Abdussalam 1956; Cheville 1966a; Teppema and De Boer 1975). The virion is composed of a central biconcave core or nucleoid bordered by two ellipsoid lateral bodies and at least two lipid membranes (Blakemore and Abdussalam 1956; Cheville 1966a; Conroy and Meyer 1971; Kim and Luong 1975; Smid et al. 1973; Teppema and De Boer 1975). SWPV is ether-sensitive.

The SWPV genome is a double-stranded DNA molecule of 146 kilobase pairs predicted to contain 150 genes. It shares features common with other members of the *Poxviridae*, including a conserved central genomic region containing genes necessary for intracytoplasmic replication and virion structure (Afonso et al. 2002). Although genetically related to viruses of other poxviral genera (*Capripoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*), SWPV represents a distinct poxvirus genus and contains in terminal genomic regions a unique complement of genes predicted to affect virus/host interaction and aspects of SWPV virulence and host range (Afonso et al. 2002; Massung et al. 1993).

Despite reports of limited antigenic cross-reactivity, SWPV is antigenically distinct, as evidenced by the failure of SWPV antibodies to cross-protect, cross-neutralize, or efficiently immunoprecipitate viral proteins of other poxviruses (De Boer 1975; Massung and Moyer 1991; Meyer and Conroy 1972; Ouchi et al. 1992; Shope 1940).

Although some isolates have been reported to induce first-passage cytopathic effect (CPE) on swine cells (Afonso et al. 2002; Paton et al. 1990), SWPV generally replicates poorly on initial isolation in swine cell cultures. It requires multiple passages before inducing CPE, but maintains pathogenicity for swine (Garg and Meyer 1972; Kasza and Griesemer 1962; Kasza et al. 1960; Meyer and Conroy 1972). CPE, characterized by cytoplasmic vacuoles and inclusion bodies, nuclear "vacuoles," and cell rounding and clumping (Borst et al. 1990; De Boer 1975; Kasza et al. 1960; Meyer and Conroy 1972), is seen 3–5 days postinoculation (PI), and isolated plaques remain relatively small in size (<1.5 mm) (Kasza et al. 1960; Massung and Moyer 1991; Meyer and Conroy 1972). Infection of cell cultures results in detectable expression of early and late SWPV mRNA by 4 and 8 hours PI, respectively, and viral protein synthesis by 4 hours PI (Massung and Moyer 1991).

Most attempts to grow or adapt SWPV for growth in nonswine cell cultures or on chicken chorioallantoic membranes have failed (Garg and Meyer 1972; Kasza et al. 1960; Meyer and Conroy 1972), indicating a host-range restriction for replication at the cellular level. Culture-adapted SWPV has been shown to replicate,

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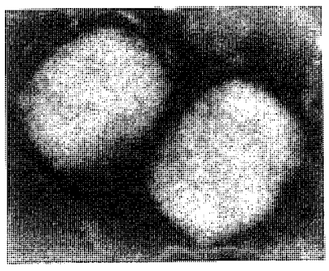


30.1. Negative stain of swinepox virus particles with characteristic brick shape and complex woven pattern of surface filaments (courtesy of D. A. Gregg).

albeit to lower titers, in nonswine cell lines (Barcena and Blasco 1998; Hahn et al. 2001).

Due to its restricted host range, SWPV has been proposed as a vaccine expression vector (Foley et al. 1991; Tripathy 1999). Genetically engineered SWPV vectors expressing pseudorabies (Aujeszky's disease) virus and classical swine fever virus antigens have been constructed and, in the case of pseudorabies, shown to induce immune responses in pigs (Hahn et al. 2001; van der Leek et al. 1994). In addition, SWPV is able to express antigens and nonchemical adjuvants in nonswine cells and may represent a safe, host-range restricted, vaccine vector for nonswine species (Barcena and Blasco 1998; Winslow et al. 2003, 2005). Availability of the complete genome sequence and identification of SWPV virulence and host-range genes should enable engineering of live-attenuated, host-range restricted, SWPV vaccine vectors with enhanced efficacy and greater versatility (Afonso et al. 2002).

ROLE IN PUBLIC HEALTH

There is no evidence that SWPV is infectious for humans or plays a role in public health.

EPIDEMIOLOGY

The pig is the only known host of SWPV. Unlike vaccinia virus, SWPV failed to experimentally infect or adapt to several mammalian and avian species (Schwarte and Biester 1941; Shope 1940), with only a single report of nonproductive SWPV infection following intradermal inoculation of rabbits (Datt 1964). This restricted host range suggests that swine represent the reservoir of SWPV in nature.

SWP is present worldwide. Limited serological survey data from Europe indicated that 8–19% of swine serum samples contained anti-SWPV antibodies (De Boer 1975; Paton et al. 1990). Young swine are most often affected, as adult swine rarely present with clinical disease (Kasza et al. 1960; McNutt et al. 1929). Morbidity rates can be high (up to 100%), but mortality is generally negligible (less than 5%) (De Boer 1975). Overall, SWP is of little economic consequence.

Natural transmission of SWP is not well understood, but is often associated with poor sanitation. SWP has been associated with louse (*Haematopinus suis*) infestation. Lice are able to mechanically transmit SWPV and are thought to affect the extent and distribution of cutaneous lesions, which often occur in less keratinized abdominal and inguinal regions (Kasza et al. 1960; Manninger et al. 1940; Shope 1940). However, SWP without evidence of louse involvement has been described, suggesting a role for other insect vectors or the possibility of horizontal transmission (De Boer 1975; Jubb et al. 1992; Paton et al. 1990; Schwarte and Biester 1941). Vertical SWPV transmission is indicated by sporadic cases of congenital infection resulting in stillborn fetuses with generalized lesions (Afonso et al. 2002; Borst et al. 1990; Paton et al. 1990).

PATHOGENESIS

SWPV may enter the host through preexisting skin abrasions and preferentially replicates in epidermal keratinocytes of the stratum spinosum (Meyer and Conroy 1972). Although mature viral particles have been observed in epidermal basal cells (Teppema and De Boer 1975) and viral antigen has been detected in dermal macrophages (Cheville 1966b), there is no indication that these cell types support virus replication. With the exception of moderate changes in superficial lymph nodes, tissues other than the skin are rarely affected. Infectious virus can be readily isolated from the skin of infected animals as early as 3 days post-intradermal inoculation (Kasza and Griesemer 1962) and from regional lymph nodes only if skin lesions are severe (Kasza and Griesemer 1962). A viremic stage has been suggested to account for virus spread from the primary to secondary sites of replication in the skin and for congenital infection, however, virus has not been isolated from the blood of infected animals (Borst et al. 1990; Kasza and Griesemer 1962; Paton et al. 1990; Shope 1940).

Functional studies on SWPV pathogenesis are lacking. The complement of virus genes with putative roles in virulence and host range suggests that modulation of host immune responses and inhibition of apoptosis may play a role in the course of the disease (Afonso

et al. 2002; Kawagishi-Kobayashi et al. 2000; Massung et al. 1993). SWPV contains genes with sequence similarity to cellular and viral genes encoding CD47, and proteins that bind interferon (IFN)-α/β, IFN-γ, tumor necrosis factor (TNF)-α, interleukin-18 (IL-18), and CC chemokines. Products of these genes potentially modulate host immune responses, including natural killer (NK) and T-cell responses, and may facilitate SWPV replication and dissemination. SWPV TNF-binding protein has been shown to interact with high affinity with porcine TNF but not with TNF from other species (Rahman et al. 2006). SWPV encodes proteins similar to host major histocompatibility complex (MHC)-I, NFκB activators, Bcl-2 family proteins, serine proteases, and PKR, some of which in other poxviruses are known to interfere with or delay inflammatory responses at the site of virus replication.

CLINICAL SIGNS

SWPV causes eruptive dermatitis in pigs. Animals up to 3 months of age are the most susceptible, with adults usually developing a mild, self-limiting form of the disease. Multiple cutaneous lesions are commonly found on the flanks, belly, inner side of the legs, ears, and less frequently, on the face of affected animals (De Boer 1975; Jubb et al. 1992; Kim and Luong 1975; McNutt et al. 1929; Olufemi et al. 1981; Schwarte and Biester 1941). Lesions can also be found in the teats of sows and on the face, lips, and tongue of suckling pigs (Olufemi et al. 1981). In congenital infections, lesions are observed over the entire body and in the oral cavity (Borst et al. 1990; Paton et al. 1990). When virus transmission is associated with mechanical vectors, the distribution of lesions tends to reflect the vector's preferred feeding areas.

The incubation period is thought to be 4–14 days under field conditions (De Boer 1975; McNutt et al. 1929) and 3–5 days after intradermal or intravenous virus inoculation (Kasza and Griesemer 1962; Schwarte and Biester 1941), although longer periods have been described (Shope 1940).

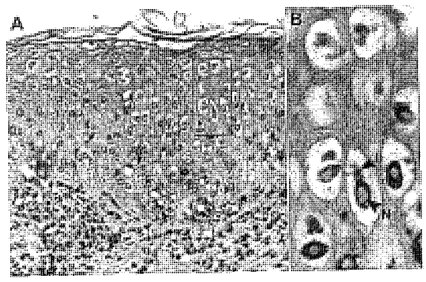
Initial lesions are flat, pale, rounded areas 3–5 mm in diameter (maculae). Over the course of 2 days, these progress to papulae 1–2 mm in height, 1–2 cm in diameter, and occasionally confluent (Figure 30.2). The appearance of papulae may be accompanied by a slight and transient increase in body temperature and loss of appetite (Kasza and Griesemer 1962; Kasza et al. 1960). A true vesicle stage is absent or transient (Borst et al. 1990; Datt 1964; Kasza and Griesemer 1962; Meyer and Conroy 1972). The lesions usually become umbilicated and shrink about a week after appearing. They are replaced by crusts, which are eventually shed, leaving discolored spots (Kasza and Griesemer 1962). Complete recovery is observed 15–30 days postexposure, but it may be delayed if secondary bacterial infection occurs

30.2. Swinepox lesions on the skin of a naturally infected pig (courtesy of B. Brodersen).

(De Boer 1975; McNutt et al. 1929; Miller and Olson 1980; Schwarte and Biester 1941).

LESIONS

The most conspicuous histological change caused by SWPV infection is hydropic degeneration of stratum spinosum keratinocytes (Figure 30.3A,B) (Borst et al. 1990; Cheville 1966a; Kasza and Griesemer 1962; McNutt et al. 1929; Meyer and Conroy 1972; Olufemi et al. 1981; Paton et al. 1990; Schwarte and Biester 1941; Teppema and De Boer 1975). Hyperplasia of epidermal cells is not as marked as in poxviral infections of other mammals, an observation that might be related to the lack of an SWPV-encoded homolog of the poxviral epidermal growth factor-like gene (Afonso et al. 2002; McNutt et al. 1929; Schwarte and Biester 1941). The cytoplasm of affected cells is brightened and enlarged, contains eosinophilic inclusion bodies resembling poxviral type B inclusion bodies (Teppema and De Boer 1975) (Figure 30.3B), and reacts strongly with antibodies against viral antigens (Cheville 1966b). Hydropic degeneration and inclusion bodies are also observed in the outer root sheaths of the hair follicles (Kasza and Griesemer 1962; Meyer and Conroy 1972). The nucleus of affected cells exhibits margination of chromatin and a large, central "vacuole" resembling the nuclear clearing observed in sheeppox virus-infected keratinocytes (Figure 30.3B) (Cheville 1966a; Kasza and Griesemer 1962; McNutt et al. 1929; Meyer and Conroy 1972; Plowright and Ferris 1958; Teppema and De Boer 1975). No significant fluid accumulation is observed between keratinocytes. Apical keratinocytes undergo necrosis at later stages of infection. Leukocyte



30.3. Histopathological changes caused by swinepox virus. (A) Skin section showing hydropic degeneration in the stratum spinosum of the epidermis and inflammatory cells in the dermis. (B) Magnification of the boxed area in A showing ballooned keratinocytes containing cytoplasmic inclusion bodies (I) and central nuclear clearing (N). Hematoxylin and eosin (courtesy of D. A. Gregg).

infiltration is observed in the underlying dermis and, to a lesser degree, the affected epidermis (Figure 30.3A), with few viral antigen-containing dermal macrophages (Cheville 1966b). When involved, the inguinal lymph nodes present edema, hyperemia, hyperplasia, and few virus antigen-containing cells (Cheville 1966a; Kasza and Griesemer 1962).

Ultrastructurally, infected cells exhibit a marked decrease in keratin precursors (tonofilaments) and loss of intercellular interdigitations characteristic of the stratum spinosum (Cheville 1966a; Teppema and De Boer 1975). Individual inclusion bodies consist of electrodense central cores surrounded by lamellar bodies and maturing viral particles (viroplasm) (Cheville 1966a; Conroy and Meyer 1971; Kim and Luong 1975; Smid et al. 1973; Teppema and De Boer 1975). The large, well-defined nuclear "vacuole" can be more accurately described as a region of low electron density, which lacks a surrounding membrane and contains cross-striated fibrils similar to those observed in the cytoplasm.

DIAGNOSIS

Presumptive SWP diagnosis is based on the observation of pox lesions on the skin of affected animals. Differential diagnosis includes vesicular diseases, allergic skin reactions, sunburn, bacterial dermatitis, ringworm, and skin parasitosis (*Acarus* (*Tyroglyphus*) spp., sarcoptic mange) (Blood and Radostits 1989; Yager and Scott 1985). SWPV involvement can be confirmed by electron microscopy and histopathology and includes pathognomonic epidermal changes: ballooned stratum spinosum keratinocytes containing cytoplasmic eosinophilic inclusion bodies and a "vacuolated" nucleus. SWPV involvement may be definitively confirmed using SWPV-specific antibodies to neutralize virus isolated on swine cell cultures (primary swine kidney cells, PK-15 cell line) (Borst et al. 1990; Meyer and Conroy 1972; Paton et al. 1990) or by performing immunocytochemistry on tissue samples or infected cell cultures (Garg and Meyer 1973; Mohanty et al. 1989; Paton et al. 1990). Papular/pustular exudate or crusted materials are clinical samples of choice for virus isolation. At least seven blind passages should be attempted before considering the sample negative (De Boer 1975).

Virus neutralization and precipitating antibody tests to detect SWPV-specific antibodies in convalescent serum have been described (De Boer 1975; Shope 1940). Since swine do not reliably develop high levels of neutralizing antibodies (Kasza et al. 1960; Shope 1940), negative test results should be interpreted with caution.

The availability of the SWPV genome sequence and the identification of unique SWPV gene sequences will permit the development of rapid, sensitive, and specific polymerase chain reaction-based detection and diagnostic assays (Afonso et al. 2002).

Although differential diagnosis of SWP from a vaccinia virus infection in pigs was important when human smallpox vaccination was practiced, this no longer represents a diagnostic concern (Shope 1940).

IMMUNITY

Convalescent swine are resistant to SWPV challenge, indicating that infection induces protective immunity (De Boer 1975; Garg and Meyer 1972; Kasza et al. 1960; Schwarte and Biester 1941; Shope 1940). However, the

immune mechanisms associated with protection are not known. SWPV neutralizing activity is present in swine sera as early as 7 days PI. However, low neutralizing titers, delayed kinetics of antibody response, and lack of neutralizing antibodies at 50 days PI have been reported (Kasza et al. 1960; Meyer and Conroy 1972; Shope 1940; Williams et al. 1989). Suckling pigs may also be protected by maternal antibody (Manninger et al. 1940), although high neonatal mortality rates have been observed (Olufemi et al. 1981). Decreased mitogen and SWPV-induced proliferative responses have been observed in peripheral blood mononuclear cells from experimentally infected swine (Williams et al. 1989).

PREVENTION AND CONTROL

No specific treatment for SWPV exists. Antibiotic regimens are recommended for the treatment of secondary bacterial infection. Given that SWP is of relatively low economic impact, no vaccine has been developed. Good animal husbandry, including ectoparasite control, should be practiced.

REFERENCES

Afonso CL, Tulman ER, Lu Z, et al. 2002. J Virol 76:783–790.
Barcena J, Blasco R. 1998. Virology 243:396–405.
Blakemore F, Abdussalam M. 1956. J Comp Pathol 66:373–377.
Blood DC, Radostits OM. 1989. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses, 7th ed. London: Bailliere Tindall, pp. 571–958.
Borst GH, Kimman TG, Gielkens AL, van der Kamp JS. 1990. Vet Rec 127:61–63.
Cheville NF. 1966a. Am J Pathol 49:339–352.
——. 1966b. Pathol Vet 3:556–564.
Conroy JD, Meyer RC. 1971. Am J Vet Res 32:2021–2032.
Datt NS. 1964. J Comp Pathol 74:62–69.
De Boer GF. 1975. Arch Virol 49:141–150.
Foley PL, Paul PS, Levings RL, et al. 1991. Ann N Y Acad Sci 646:220–222.
Garg SK, Meyer RC. 1972. Appl Microbiol 23:180–182.
——. 1973. Res Vet Sci 14:216–219.
Hahn J, Park S-H, Song J-Y, et al. 2001. J Virol Methods 93:49–56.
Jubb TF, Ellis TM, Peet RL, Parkinson J. 1992. Aust Vet J 69:99.
Kasza L, Bobl EH, Jones DO. 1960. Am J Vet Res 21:269–273.
Kasza L, Griessemer RA. 1962. Am J Vet Res 23:443–450.
Kawagishi-Kobayashi M, Cao C, Lu J, et al. 2000. Virology 276: 424–434.
Kim JCS, Luong LC. 1975. Vet Med Small Anim Clin 70: 1043–1045.
Manninger R, Csontos J, Salyi J. 1940. Uber die atiologie des pockenartigen ausschlages der ferkel. Archiv fur Tierheilkunde 75:159–179.
Massung RF, Jayarama V, Moyer RW. 1993. Virology 197: 511–528.
Massung RF, Moyer RW. 1991. Virology 180:355–364.
McNutt SH, Murray C, Purwin P. 1929. Am Vet Med Assoc 74: 752–761.
Meyer RC, Conroy JD. 1972. Res Vet Sci 13:334–338.
Miller RC, Olson LD. 1980. Am J Vet Res 41:341–347.
Mohanty PK, Verma PC, Rat A. 1989. Acta Virol 33:290–296.
Moyer RW, Arif B, Black DN, Boyle DB, et al. 2000. Family *Poxviridae*. In MHV van Regenmortel, CM Fauquet, DHL Bishop, EB Carstens, MH Estes, SM Lemon, J Maniloff, MA Mayo, DJ McGeoch, CR Pringle, RB Wickner, eds. Virus Taxonomy: Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses. San Diego, CA: Academic Press, pp. 137–157.
Olufemi BE, Ayoade GO, Ikede BO, et al. 1981. Vet Rec 109: 278–280.
Ouchi M, Fujiwara M, Hatano Y, et al. 1992. J Vet Med Sci 54: 731–737.
Paton DJ, Brown IH, Fitton J, Wrathall AE. 1990. Vet Rec 127:204.
Plowright W, Ferris RD. 1958. Br J Exp Pathol 39:424–435.
Rahman MM, Barrett JW, Brouckaert P, McFadden G. 2006. J Biol Chem 281:22517–22526.
Schwarte LH, Biester HE. 1941. Pox in swine. Am J Vet Res 2: 136–140.
Shope RE. 1940. Swine pox. Arch Gesamte Virusforsch 1: 457–467.
Smid B, Valicek L, Mensik J. 1973. Zentralbl Veterinarmed B 20: 603–612.
Teppema JS, De Boer GF. 1975. Arch Virol 49:151–163.
Tripathy DN. 1999. Adv Vet Med 41:463–480.
van der Leek ML, Feller JA, Sorensen G, et al. 1994. Vet Rec 134: 13–16.
Williams PP, Hall MR, McFarland MD. 1989. Vet Immunol Immunopathol 23:149–159.
Winslow BJ, Cochran MD, Holzenburg A, et al. 2003. Virus Res 98:1–15.
Winslow BJ, Kalabat DY, Brown SM, et al. 2005. Vet Microbiol 111:1–13.
Yager JA, Scott DW. 1985. The skin and appendages. In KVF Jubb, JA Yager, DW Scott, eds. Pathology of Domestic Animals, Vol. 1, 3rd ed. Orlando, FL: Academic Press, pp. 407–549.

31 Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus)

Jeffrey J. Zimmerman, David A. Benfield, Scott A. Dee, Michael P. Murtaugh, Tomasz Stadejek, Gregory W. Stevenson, and Montserrat Torremorell

RELEVANCE

In the late 1980s, severe outbreaks characterized by severe reproductive losses, respiratory disease, reduction in growth rate, and increased mortality were reported in the United States (Hill 1990; Keffaber 1989; Loula 1991). Initial efforts to identify the etiology of the outbreaks were unsuccessful. Clinically similar outbreaks occurred in Germany in November 1990 (OIE 1992). No common link was found between the outbreaks in Germany and the United States (Anonymous 1991). Spreading rapidly, over 3000 outbreaks were documented in Germany in May 1991 and across Europe in the following 4 years (Baron et al. 1992; Bøtner et al. 1994; Edwards et al. 1992; OIE 1992; Pejsak and Markowska-Daniel 1996; Plana Duran et al. 1992a; Valíček et al. 1997). In Asia, outbreaks occurred in Japan in 1988 (Hirose et al. 1995) and in Taiwan in 1991 (Chang et al. 1993).

The etiology of the disease was established in 1991 when Koch's postulates were fulfilled with a previously unrecognized RNA virus (Terpstra et al. 1991a; Wensvoort et al. 1991). Shortly thereafter, the virus was isolated in the United States (Collins 1991; Collins et al. 1992) and Canada (Dea et al. 1992a,b). The first virus isolates in The Netherlands and the United States were designated Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus (BIAH-001), respectively. European researchers introduced the term "porcine reproductive and respiratory syndrome" (PRRS) in 1991 (Terpstra et al. 1991b).

The origin of porcine reproductive and respiratory syndrome virus (PRRSV) remains unknown. Regardless, the virus has become endemic in most swine-producing regions of the world and control remains problematic. Neumann et al. (2005) estimated annual PRRS losses to U.S. pig producers at $560.32 million annually. A more recent study estimated losses in the United States at $668.58 million annually, exclusive of costs related to PRRSV vaccination, treatment, diagnostics, and biosecurity (Holtkamp et al. 2011). For this reason, elimination of PRRSV from herds, regions, and countries is considered the best solution.

ETIOLOGY

Taxonomy and Classification

PRRSV is classified with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus in the family *Arteriviridae*. The families *Arteriviridae*, *Coronaviridae*, and *Roniviridae* comprise the order *Nidovirales*. The PRRSV species forms two major genetic lineages represented by type 1 and type 2 genotypes. The two prototype genomes, Lelystad virus (type 1) and VR-2332 (type 2), vary by ~44% in nucleotide sequence. Both types were discovered around 1990: type 1 viruses in Europe and type 2 viruses in North America. Today, both types share worldwide distributions, with type 1 predominant in Europe and type 2 predominant in North America and Asia.

Phylogenetic analyses, based largely on open reading frame (ORF) 5, which encodes the major envelope glycoprotein (GP5), have showed that both type 1 and type 2 PRRSVs are remarkably diverse. Intratype pairwise nucleotide sequence variation up to 30% is present in type 1 viruses, and it exceeds 21% in type 2 viruses. Although the origin of PRRSV is not known, the extensive differences between type 1 and type 2 PRRSVs

Diseases of Swine, Tenth Edition. Edited by Jeffrey J. Zimmerman, Locke A. Karriker, Alejandro Ramirez, Kent J. Schwartz, Gregory W. Stevenson.
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AVIAN DISEASES 56:836–844, 2012

Invited Review—

DIVA Vaccination Strategies for Avian Influenza Virus

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Received 16 April 2012; Accepted 14 July 2012; Published ahead of print 14 July 2012

SUMMARY. Vaccination for both low pathogenicity avian influenza and highly pathogenic avian influenza is commonly used by countries that have become endemic for avian influenza virus, but stamping-out policies are still common for countries with recently introduced disease. Stamping-out policies of euthanatizing infected and at-risk flocks has been an effective control tool, but it comes at a high social and economic cost. Efforts to identify alternative ways to respond to outbreaks without widespread stamping out has become a goal for organizations like the World Organisation for Animal Health. A major issue with vaccination for avian influenza is trade considerations because countries that vaccinate are often considered to be endemic for the disease and they typically lose their export markets. Primarily as a tool to promote trade, the concept of DIVA (differentiate infected from vaccinated animals) has been considered for avian influenza, but the goal for trade is to differentiate vaccinated and not-infected from vaccinated and infected animals because trading partners are unwilling to accept infected birds. Several different strategies have been investigated for a DIVA strategy, but each has advantages and disadvantages. A review of current knowledge on the research and implementation of the DIVA strategy will be discussed with possible ways to implement this strategy in the field. The increased desire for a workable DIVA strategy may lead to one of these ideas moving from the experimental to the practical.

RESUMEN. *Estudio Recapitulativo*—Estrategias de vacunación con la diferenciación entre animales infectados y vacunados (DIVA) para el virus de la influenza aviar.

La vacunación para la influenza aviar de baja y alta patogenicidad se utiliza comúnmente por los países que se han convertido en endémicos del virus de la influenza aviar, pero las políticas de sacrificio y eliminación masivos siguen siendo comunes en los países en donde la enfermedad se han introducido recientemente. Las políticas de eutanasia de parvadas infectadas o en riesgo ha sido una herramienta efectiva de control, pero representa un alto costo social y económico. Los esfuerzos para identificar formas alternativas de respuesta a los brotes sin recurrir al sacrificio masivo se ha convertido en un objetivo para organizaciones como la Organización Mundial de Sanidad Animal [OIE]. Un problema importante con la vacunación contra la influenza aviar son las consideraciones comerciales, ya que los países que vacunan a menudo se consideran endémicos de la enfermedad y por lo general pierden sus mercados de exportación. Principalmente como una herramienta para promover el comercio, el concepto de vacunación DIVA (diferenciación entre animales infectados y vacunados) ha sido considerado para aplicarse contra la influenza aviar, pero el objetivo para el comercio es el de diferenciar animales vacunados y no infectados de los animales vacunados e infectados ya que los socios comerciales no están dispuestos a aceptar la presencia de aves infectadas. Varias estrategias han sido investigadas por una estrategia DIVA, pero cada una tiene ventajas y desventajas. Se discute una revisión de los conocimientos actuales en la investigación y aplicación de la estrategia DIVA, con las posibles formas de aplicar esta estrategia en el campo. El deseo cada vez mayor de tener disponible una estrategia DIVA factible puede dar lugar a que una de estas ideas se traslade de lo experimental a lo práctico.

Key words: avian influenza, DIVA, vaccination, marker vaccine, trade, highly pathogenic

Abbreviations: aa = amino acid; AGID = agar gel immunodiffusion; AI = avian influenza; AIV = avian influenza virus; CSF = classical swine fever; DIVA = differentiate infected from vaccinated animals; EID_{50} = egg infectious dose; ELISA = enzyme-linked immunosorbent assay; FMD = foot-and-mouth disease; HA = hemagglutinin; HI = hemagglutinin inhibition; hNA = heterologous neuraminidase; HPAI = highly pathogenic avian influenza; iIFAT = immunofluorescent antibody test (iIFAT); LPAI = low pathogenicity avian influenza; M2 = matrix 2 protein; M2e = external 18 aa of the M2 protein; NA = neuraminidase; NI = neuraminidase inhibition; NSP = nonstructural protein; OIE = World Organisation for Animal Health; rfowlpox = fowlpox-vectored vaccine; rNDV = Newcastle disease virus-vectored vaccine

The DIVA principal, which stands for 'differentiate infected from vaccinated animals' for vaccination, is not a new concept and has been used as part of eradication programs for pseudorabies in swine, classical swine fever, and for avian influenza (AI). Interest in the use of a DIVA vaccination strategy as a possible tool for the control of avian influenza virus (AIV) has increased as both low pathogenicity avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) has become endemic in many countries.

The use of the DIVA principal for vaccination is designed to assist in the eradication of an infectious disease from a region or country. The principal has been applied primarily to disease outbreaks that are not normally endemic in a country and where eradication is the ultimate goal. The decision to eradicate by a stamping-out program, or to use other control measures that may include vaccination, is often a difficult decision that must consider multiple factors. This may include how many premises have infected birds, how widely distributed are the affected farms, the strength of the veterinary infrastructure, the impact on trade, the availability of appropriate vaccines, the availability of indemnity, and so on.

The principals of stamping out are clear: Diagnose when a disease is introduced into a country or region, identify farms with infected animals, and then destroy the infected animals and those animals that are likely to have been exposed to the infected animals as quickly as possible before they have a chance to spread the infection to naïve animals. If the index case and close contacts are identified quickly,

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the rapid destruction of the flock or herd can be the most cost-effective approach for control. For countries with a good veterinary infrastructure and an efficient veterinary diagnostic laboratory system, the use of stamping out has been shown to be an effective method for control of disease outbreaks (48). One of the primary alternatives to stamping-out programs is to vaccinate and try to protect uninfected animals. Effective vaccination programs can lead to a robust immune response that can reduce clinical disease symptoms, reduce the amount of virus that birds excrete if infected, and increase the resistance of the bird to being infected. All these factors can help break the transmission chain that can end an epidemic (30). However, vaccination if improperly applied, either by ineffective application, insufficient coverage, or through poor antigenic matching of vaccines to field strains may contribute to the persistence of infection and disease in the region (27). One argument against the use of vaccination is that vaccinated animals could not be easily differentiated from naturally infected animals. Most importing countries have viewed the presence of antibody as evidence of prior or active infection with the disease agent and will actively block the importation of live poultry and poultry products. This negative effect on trade is a major concern for exporting countries. The two major poultry transboundary diseases that affect trade are AI virus and Newcastle disease virus. For AI, any highly pathogenic virus or, alternatively, low pathogenic viruses of the H5 and H7 subtype are reportable to the World Organisation for Animal Health [OIE] and typically result in trade sanctions on the reporting country. The DIVA vaccination principal has been proposed as a potential tool to assure the safe movement of poultry and poultry products even when vaccination is used for the control of AI.

Animal welfare concerns of stamping-out programs. As previously described, stamping out programs are a reliable and proven method for disease control that has been utilized for over 2000 years (7). However, in contemporary times the use of stamping out has included not only the mass depopulation of diseased animals but also includes the mass depopulation of animals that are likely to have been exposed to infection through close proximity to infected farms or through likely fomite transmission by contaminated equipment, clothing, or people. The animals on the farms are considered "dangerous contacts" and are preemptively culled. Although the principal is sound to destroy apparently healthy looking animals to break the transmission chain because these animals may be latently infected and shedding virus, the practicalities of this policy result in the killing of large numbers of uninfected animals (50). The use of stamping out has also been used to control outbreaks of low pathogenicity H5 and H7 viruses in poultry because of the requirement to report these viruses to the OIE because of the concern that these viruses will mutate to the highly pathogenic form of the virus. Therefore, outbreaks of LPAIV have also become barriers to trade. Infection with LPAIV often will cause only minor, self-limiting respiratory disease and, therefore, stamping-out programs may result in the euthanasia of large numbers of healthy birds (16). Historically, LPAI-infected flocks were quarantined until the birds were thought to have recovered and were no longer shedding virus, and then the birds were sent to slaughter under increased biosecurity arrangements. This procedure was well accepted as a control tool to salvage as much value from the infected birds as possible, which was important because farmers were not typically compensated for any disease losses that occurred (16). However, the paradigm shifted in the United States when federal support was authorized to support farmers after an LPAI H7N2 outbreak that occurred in Virginia and West Virginia in 2002, with the primary justification that intervention and eradication would prevent the virus from mutating to the virulent form (1). The philosophy of aggressive control of H5 and H7 LPAI became international policy when the OIE made these viruses notifiable disease agents (62). Alternative control measures like vaccination were rarely considered for exporting countries because a country was usually not considered as being free of disease by trading partners until the last vaccinated animal was slaughtered.

Animal welfare concerns are beginning to come to the forefront, and this increased awareness results in the consideration for alternatives to the stamping approach that, by necessity, results in many clinically healthy animals being culled (61). One commonly cited example of deciding to use eradication over alternatives (including vaccination) was the foot-and-mouth disease (FMD) outbreak in England and other countries in Europe in 2001 (13). In this disease outbreak, the importance of eradication as the fastest way to open export markets took precedence over other concerns, although the costs of eradication were enormous and resulted in the destruction of over 4 million animals (13). Vaccination has often been considered the main alternative to stamping out, although it is still not routinely applied to foreign disease outbreaks for a variety of reasons (37).

Successful DIVA programs. The DIVA acronym was first coined in 1999 as an alternative and more-refined description of a marker vaccine (54). A marker vaccine can be either positive or negative in that it can include antigens not found in the wild-type virus, which would be a positive-marker vaccine, or it can contain fewer antigens than the wild-type virus and would be a negative-marker vaccine. In either case, a companion diagnostic test needs to be available to allow the differentiation of the vaccinated animal to the naturally infected animal. The term DIVA was specifically coined to refer to the negative-marker vaccine because, as an eradication tool, it was important to be able to differentiate infected animals from noninfected animals, whether vaccinated or not (54). The earliest DIVA vaccines to get widespread use were for pseudorabies, which is also known as Aujeszky's disease. During the study of this herpesvirus, an attenuated mutant was found that had a deletion that resulted in the virus not expressing the glycoprotein E. Alternative vaccines were developed to further attenuate the vaccine, including deleting the thymidine kinase gene or the gG or gC proteins. For all these vaccines, companion diagnostic tests were developed to the missing proteins to allow for a differentiation of vaccinated and naturally infected animals. Several countries are using this approach to eradicate the disease from the domestic swine population (39,53). Other diseases where DIVA vaccines are being used or considered are classical swine fever (CSF) and FMD virus, although using different strategies. For CSF, a subunit vaccine has been developed that expresses only the envelope glycoprotein E2 in a baculovirus system. This vaccine, although it offers less protection than the live attenuated CSF, can be used with the companion diagnostic test to evaluate virus spread as part of an emergency control program (55). A different approach has been proposed for FMD wherein killed adjuvanted vaccines are primarily used. The FMD virus has a nonstructural protein (NSP) that is not packaged in the virus virion and, in purified vaccines, animals will not develop antibody to this protein. However, in naturally infected animals, antibodies will develop to the NSP because it is produced in large amounts in infected cells. This differential antibody response to the NSP, as measured by an enzyme-linked immunosorbent assay (ELISA) test, provides the basis for a DIVA FMD vaccine (39). For both diseases, additional strategies continue to be developed using molecular biology techniques to create unique mutants, or to express key protective proteins in other virus vector systems, in order to continue to improve upon the original concept.

Common features for DIVA strategies for AIV. Many different DIVA strategies have been proposed for AIV, but they all rely upon

several key elements. First, all the vaccines express the hemagglutinin (HA) protein because antibodies to the HA protein are neutralizing and provide the best protection. Other influenza proteins can induce protective neutralizing antibody, including the neuraminidase (NA) protein, but it is difficult to induce high enough levels of NA antibody to provide complete protection (49). In addition, several studies have shown that when the HA and NA are co-expressed in the same system, and the HA is antigenically matched to the challenge virus, similar protection is observed whether or not the NA is antigenically matched (31). Cell-mediated immunity or mucosal immunity from live influenza or viral-vectored vaccines can increase protection from vaccines, but they cannot reliably induce heterosubtypic immunity for AI (45). Second, the vaccines must not only be of the same subtype as the challenge strain, but protection—particularly as measured by reduced virus shedding—improves when the vaccine and field strain are also antigenically closely matched (47). Third, vaccines to AI, particularly under field circumstances, will not provide sterilizing immunity to a high viral challenge. However, well-vaccinated birds will have an increased resistance to infection which will require a higher challenge dose as compared to unvaccinated animals (11).

A positive goal for vaccines as part of an eradication program is to reduce virus shedding from vaccinated, and then exposed, birds and to have increased resistance to infection in vaccinated birds, as was mentioned previously. However, all the different DIVA strategies, because they are negative-marker vaccines, rely upon an antibody response to the missing protein as the indication of viral infection. If a vaccine was to induce sterilizing immunity where the virus does not infect cells, then the virus will not induce an antibody response to the new antigen and a DIVA antibody response will not be detected. Because one of the primary goals of DIVA vaccination is to prevent infected poultry or poultry products from being exported to other countries, sterilizing immunity would be advantageous because no transmission could occur in this situation, although you would lose the surveillance potential if the exposed birds do not seroconvert. Current vaccines do not provide sterilizing immunity, but they can provide enough protection to significantly lower virus replication in the animal. This lower virus replication can affect the immune response because a minimum level of antigen is needed to stimulate a measurable antibody response. Experimentally, it has been observed that the minimum infectious dose can vary greatly between different influenza viruses, from as low as a single egg infectious dose 50 (EID_{50}) to greater than 10^6 EID_{50} in different bird species. The minimum level of infection may, therefore, not be sufficient to produce enough antigenic mass to induce a measurable antibody response in viruses that are poorly adapted to poultry, and the bird, although infected and shedding virus, may not seroconvert (43). The antibody response depends on a number of factors including the level of immunogenicity of the protein itself. The HA protein shows a wide range of immunogenicity between and within a subtype, and as little as a single amino acid (aa) change can greatly change the immunogenicity of a virus (18,29,58). Other factors can also influence the immune response for influenza viruses, including the size of the protein and the relative amount of protein that is produced during the virus infection. For example, in the influenza virus particle there is estimated to be four times more HA protein than NA protein, and the HA antibody response typically dwarfs the NA antibody response (60). All of these factors have to be taken into consideration for a successful DIVA strategy.

Goals for a successful DIVA strategy. The primary goal for a DIVA vaccination program is to be able to differentiate infected animals from either vaccinated only or uninfected animals. Most importantly, we need to differentiate vaccinated and not-infected from vaccinated and then infected animals because this provides the primary benefit to facilitate international trade. Importing countries, when free of a particular disease agent, clearly have a strong incentive to regulate the import of animals and animal products to reduce the risk of introducing the disease to their own animal population. The establishment of the OIE was to provide the standards so that countries can safely trade animals and animal products, with the goal of using science-based information to understand the risk. No trade, of course, is completely without risk, but through the use of international standards and transparent reporting, the risk of trading internationally can be managed. The ultimate goal of the DIVA vaccination program is to assure the trading partner that, although the animals have been vaccinated, they have not been infected with the target virus. The use of vaccination could be used as both a preventative measure or in response to a disease outbreak and, with the appropriate scheme in place, the exporting country can provide the information to the importing country that the product or animals are free of disease and present negligible risk of a spread of infection.

To achieve the goal of a working DIVA vaccination scheme, several objectives have to be satisfied. First, all DIVA vaccines require a sensitive, specific, cost-effective companion diagnostic antibody test that can be run on large numbers of samples. For the available DIVA strategies, most researchers have used ELISA tests as the primary detection system, although for proof of principal many different antibody detection systems have been used. If the companion diagnostic test is available, sufficient scientific research must be performed to show that the test has a "fitness for purpose." In this case, the test must reliably differentiate vaccinated from vaccinated and infected animals. A level of validation needs to be performed under different circumstances so that the parameters for proper interpretation of the results can be determined. For example, diagnostic tests need to be validated for different species because different species often respond differently to different viral infections (40). In addition, the immune response after infection needs to be characterized in both naïve and vaccinated birds to determine how early an antibody response can be detected, how long the antibody levels persist, and the seroconversion rate that will exist in similarly infected animals. As mentioned previously, because there are many types of vaccines available that could be given under a variety of circumstances, understanding how the diagnostic test works with animals that are well vaccinated *vs.* poorly vaccinated, antigenically well-matched *vs.* poorly matched vaccines, and different age of birds, etc. will help in understanding how to apply different DIVA methods. Although there is a wide possible variation to different vaccination protocols for age of bird, type of vaccine, and whether booster vaccines are used, a certain level of controlled comparisons are needed to assure the test will work under reasonable conditions. The importing country will want assurance that false negatives will not occur with any regularity. Field vaccination studies should also be conducted with birds being challenged in the laboratory to assess factors like co-infection with immunosuppressive viruses, additional vaccinations, and natural environmental conditions. As with any validation method, having outside laboratories also evaluate the test to make sure it is reproducible should also be done. This information can then be collected to produce a dossier which can then be used to establish a statistical basis on which to estimate the number of animals that need to be tested to determine the incidence of infection within a certain confidence level. A common target is to establish a minimum level of detection that will detect a 5% incidence of infection with a 95% confidence of detection. Because of the highly infectious nature of many influenza viruses, it may be

acceptable to target for a higher incidence level of 10% with a 95% confidence of detection, which would require the sampling of fewer birds.

Once the validation of a test has been completed and estimates for detection levels have been determined, the recognition by national or international groups will provide a level of oversight of the work. This national or international recognition can include the licensing of the diagnostic test for DIVA testing in a particular country or the submission of the dossier to groups like the OIE. It is impractical to license a vaccine or diagnostic test in every country in which it might be used, but it is common to license the vaccine or diagnostic test in a country with a well-defined regulatory procedure that will provide rigorous and transparent review of the product. If it is licensed under these conditions, other countries will often either accept the third-party review or provide a streamlined review to facilitate licensure.

The final step in the DIVA process is to have the importing country accept the method used to show freedom from disease and then to allow the birds or product into the country. Because trade between countries is primarily a bilateral agreement, the relationship between countries will often dictate how any changes will be received. Countries that are transparent and use recognized diagnostic procedures are more likely to receive approval for trade. Countries with a reputation or history of hiding disease are going to have a harder time in gaining, or regaining, the trust of their trading partners.

Proposed DIVA strategies. *Sentinel birds and flock status.* The use of sentinel birds and the close monitoring of flock status do not closely fit the definition of a DIVA strategy, but these methods can be used to monitor vaccinated flocks. The use of sentinel birds can have many roles diagnostically but, in the DIVA scheme, leaving a small percentage of unvaccinated birds, typically 1%, in the house can be a sensitive way of detecting exposure to infection (34). The use of sentinels also allows the use of all the traditional diagnostic tests, including serologic tests such as the Type A-specific ELISA test and the agar gel immunodiffusion (AGID) tests, both of which detect antibodies to the nucleoprotein. In addition, the direct detection diagnostic tests, including virus isolation and real-time reverse-transcription–PCR can be used, particularly when clinical disease is observed in the flock or the sentinels. The primary disadvantage to the use of sentinel birds is the difficulty in managing the birds. The birds need to be marked to indicate that they are sentinels and then they should be randomly spread in the poultry facility. Particularly if the birds are raised on the floor, it may be time consuming to identify and sample the sentinels in the house. The other concern that is often raised is that the naïve birds in the flock, if infected, can act as amplifiers of infection that could infect the rest of the flock or be transmitted to other flocks. Although this argument does have some validity, in practical terms, because vaccination procedures and response to immunization are not perfect, a certain percentage of birds in a flock will always be susceptible to infection. If a high percentage of the flock is immunized against AI, then the herd immunity will efficiently disrupt the transmission chain. Immunity levels of 80% within the flock are thought to be the minimum level to establish effective herd immunity, and leaving 1% of the birds purposefully unvaccinated is unlikely to affect the overall herd immunity (8).

Flock status is another way to monitor for early infection by monitoring key factors in production. These factors can include the monitoring for small increases in the daily mortality, decreased feed or water consumption, or drops in egg production. In particular, the monitoring of daily mortality for infection with LPAI has been shown to be a good way to identify infected flocks. The approach was used for the H7N2 outbreaks in Virginia in 2002 and for the H7N2 outbreak in Connecticut layers in 2003 (15,44). The close monitoring of egg production in layer flocks or breeder birds is also another marker for infection with both LPAI and HPAI viruses. In particular, LPAI viruses may cause a decrease in egg production as the primary clinical sign, and any abnormal drop in egg production should trigger increased diagnostic testing with an emphasis on sick or dead birds.

Heterologous neuraminidase DIVA Strategy. Avian influenza viruses are typically characterized by their two surface glycoproteins, the HA and NA proteins, because these proteins have considerable antigenic variation that has been divided into unique subtypes. There have been 16 HA and 9 NA antigenic subtypes isolated from birds. The definition of each subtype is that antibody to one subtype will neutralize viruses of the same subtype but not viruses of other subtypes. This lack of cross-reactivity between subtypes has provided the basis for a DIVA strategy. As previously mentioned, the antibodies to the HA protein are the key to solid protection against avian influenza, and antibodies to the NA protein provide only limited protection. The general idea of vaccinating with a killed vaccine, which has a matched HA subtype to the field strain but a mismatched or heterologous neuraminidase (hNA) subtype to the field strain, that could be used as a differential DIVA test was first suggested by C. W. Beard in 1986 (4), but it was not put into practice until almost 15 yr later.

Several manuscripts have shown experimentally that hNA DIVA strategies can be protective in turkeys and chickens against LPAI and HPAI (3,5,10,19,25,28,63). Several of these studies used reverse genetics to produce a vaccine seed strain with a rare NA subtype to reduce the possibility that the vaccine would be the same subtype as the field strain (5,19,25,28). This issue occurred in Italy during their second DIVA vaccination program, where the field strain was an H7N3 virus which overlapped with the only available H7 vaccine that was also an N3 subtype. The Italians had to identify and license an alternative vaccine, an H7N1 LPAI strain, which delayed the implementation of the vaccination program (34). The use of a reverse genetics system has allowed the use of site-directed mutagenesis on the HA cleavage site for HPAI viruses in order to convert them into LPAI viruses (19). All the studies showed that the killed vaccines used in the studies were protective and that the vaccinated animals could be serologically differentiated from the vaccinated and challenged animals, but few of the studies looked at how quickly the birds responded to the vaccine or how many of the birds responded to the vaccine. One paper tested an H7N1 vaccine with an H7N3 challenge and showed that some of the turkeys had an N3 antibody response as early as 7 days after challenge and that most of the birds had a detectable titer at 10 days postchallenge. In this example, the turkeys in the control group responded with detectable N3 antibodies before detectable H7 antibodies, but more unvaccinated birds responded at 7 days after challenge than did the vaccinated birds showing some delay in the antibody response. In two studies in chickens, detectable NA antibody titers were seen in very few birds at 1 wk postchallenge, but all had detectable titers at 2 wk postchallenge. In the H9N2 LPAI challenge, the unvaccinated controls also had more birds seroconvert at 1 wk postchallenge compared to the vaccinated birds (3,25). Based on limited longitudinal studies, it appears that both turkeys and chickens after challenge respond well to the NA antigen by at least 2 wk postchallenge, but the trend is that the response is slightly delayed compared to unvaccinated controls. This delayed response is likely because of the immunity from vaccination reduced viral replication providing a smaller amount of antigen to prime the

immune response. Only a single paper has looked at the field response. In turkeys, almost all birds in a flock that had been naturally exposed seroconverted to the NA protein. However, in chickens from vaccinated and then infected flocks, less than half of the birds seroconverted (12). Although there appears to be a large difference between chickens and turkeys, the number of chicken samples tested were small (24) and were from a single flock. Because there are so many variables to consider in evaluating the protection and susceptibility of vaccinated flocks, broad conclusions shouldn't be made at this point.

With the hNA DIVA strategy, the companion diagnostic test is a unique and critical piece of the puzzle that must be included for a successful test. Four different serologic tests have been described for the hNA DIVA strategy including the indirect immunofluorescent antibody test (iIFAT), a standard neuraminidase inhibition (NI) test, a modified NI test, and the ELISA test. The biggest impediment that remains with the hNA strategy is the theoretical need for nine different NA antibody detection tests to allow detection of all nine NA subtypes found in birds. For both the standard and modified NI tests, because they measure the reduction of NA activity they are designed to be used with any NA subtype. However, because of antigenic variation and the difficulty in running large numbers of samples with either test, both tests are used only experimentally, with little chance that they could be adapted to be a rapid, sensitive, and inexpensive test that can test large numbers of samples (3,19,57). The first field use of the hNA used an iIFAT that expressed the NA protein, either N1 or N3, in insect cells using a baculovirus expression system (10,12). This approach had an excellent correlation with the hemagglutinin inhibition (HI) test and had a low false-positive rate in field samples. This approach, however, has not been widely adopted because the iIFAT test is relatively labor intensive and the interpretation can be somewhat subjective.

In more-recent studies, the most common test system is the ELISA because of the ability to test large numbers of samples quickly. The ELISA, like the iIFAT, has to be targeted to the individual NA subtype and, therefore, nine different tests theoretically need to be developed. Currently, ELISA tests have been developed for four NA subtypes, N1, N2, N3, and N7 (17,23,25,32,36,52,63). Of the different published tests, three have done sufficient validation to show that the ELISA has enough sensitivity and specificity to be used as a screening test and could differentiate vaccinated from vaccinated and then infected animals (25,32,36). However, none of the current ELISA tests have been tested on large numbers of field-vaccinated and potentially exposed birds, and more work needs to be done to commercialize and license these tests. One commercial competitive ELISA test is available, but no published data is available yet on the performance of this test.

In Italy in 2000, the first application of DIVA vaccination for avian influenza was initiated. The Italian poultry industry had recently dealt with a costly and disruptive HPAI H7N1 outbreak, and when LPAI H7N1 emerged as a disease threat, the industry was restless for alternatives to stamping out—in part because of the lack of government compensation to deal with the outbreak. The DIVA strategy used a commercially available vaccine that was based on a Pakistan H7N3 virus isolate. A differential diagnostic test was developed using a baculovirus-expressed N1 protein in an iIFAT. The validation work demonstrated protection of the vaccine against the Italian field strain, and the iIFAT could differentiate vaccinated, and vaccinated and challenged, birds because the challenged birds developed antibodies to the N1 protein (10). This DIVA strategy was adopted as the official vaccination program to control the LPAI H7N1 outbreak, and surveillance was performed using both 1% sentinel birds in each vaccinated flock and the DIVA serologic test. The use of vaccination with some stamping out and controlled marketing eventually eliminated the virus from Italy. The DIVA strategy was accepted by the Italians' European Union trading partners, who eventually allowed the export of fresh turkey meat (9,34).

The Italians also used the DIVA strategy for an LPAI H7N3 outbreak that occurred in 2002–2004. In this outbreak, however, no appropriate heterologous H7 vaccine was available because the circulating field virus was an H7N3 virus that made the earlier vaccine and diagnostic test unusable. A LPAI H7N1 vaccine was licensed and, with the companion iIFAT N3 antibody test, DIVA vaccination was eventually employed in this outbreak. European trading partners again allowed the export of fresh poultry meat and table eggs during this outbreak after the DIVA strategy was in place (34).

Other examples of hNA DIVA vaccination include the H7N2 LPAI outbreak in the United States in 2003, where H7N3 vaccine was used, but because the outbreak was contained to just a few farms little additional validation data was produced (42).

Nonstructural protein. In infected cells, AIVs produce large amounts of the nonstructural protein 1 (NS1) that is essential for virus replication, but the NS1 protein is not packaged into the virion. Because most influenza vaccines are produced only from inactivated whole virus given with an adjuvant, vaccinated birds should not develop antibodies to the NS1 protein but birds infected with live influenza virus should produce NS1 antibody. This differential antibody response to the NS1 protein was first demonstrated in horses with equine influenza virus and suggested it could be a viable DIVA strategy in poultry (6,38). The primary advantage to the NS1 DIVA approach is that it can be used as a universal test for any killed influenza virus vaccines regardless of subtype. Several experimental studies showed that the approach could also be used in poultry and that vaccinated and not-infected, and vaccinated and then infected, birds could be differentiated based on the NS1 antibody response (51,64). Later manuscripts using either peptide- or baculovirus-expressed protein, designed to reduce background, looked at the longitudinal antibody response after natural infection (2,14,59). The paper by Dundon *et al.* (14) showed a major difference in the response of turkeys and chickens, with more turkeys responding to infection, but the antibody response was very short-lived. The Watson *et al.* (59) paper showed that most of the chickens responded to the NS1 protein but the antibody response was decreasing rapidly by 4 wk postchallenge. The Avellaneda *et al.* (2) paper had intermediate results in chickens, with only a few birds with a detectable NS1 antibody response after natural infection, and the antibody response also seemed to quickly decline. Avellaneda *et al.* (2) also looked at the immune response after vaccination and confirmed that the NS1 protein could be used as a DIVA test, but only 20–40% of chickens responded with an NS1 antibody response after an HPAI challenge. Similar DIVA vaccine results were seen by Wang *et al.* (57), showing that only a limited number of turkeys responded with an NS1 antibody response after challenge.

The NS1 DIVA strategy is still plausible, but because of the inconsistent antibody response to the NS1 protein after natural infection, it would be much more difficult to make it a practical DIVA strategy. Because of the lower immune response, a greater number of birds would need to be sampled to assure that the targeted level of infection could be identified. The reason for the poor immune response is not known, but it may be because of the relatively small size of the NS1 protein at 230 aa in length.

Matrix 2 protein. The matrix 2 (M2) protein is another proposed DIVA marker. The M2 protein is an ion channel protein that is important to trigger the release of viral RNA into the host cell. Although the protein is a structural protein, it is found in only small amounts in the virion and typically does not trigger an antibody response in killed, whole-virus vaccines. However, after natural infection an M2 antibody response can result. An ELISA test targeted to the external 18 aa of the M2 protein (M2e) was developed and shown to be able to differentiate vaccinated from vaccinated and infected birds (26). A follow-up report also showed that antibodies to the M2e could be detected after natural infection, but the immune response was smaller and declined more rapidly than did other influenza proteins (35). A second research group developed their own M2e ELISA test and found they could detect infected animals as early as 1 wk after infection, and the response lasted up to 24 wk. However, a high false-positive rate was observed in some flocks and, if flocks were vaccinated multiple times, then the higher level of protective antibodies inhibited the immune response to the M2e protein (24).

The M2e DIVA strategy also appears to be a viable approach, but more work needs to be done to define the immune response in naïve and vaccinated animals after infection. Conflicting results about length of antibody response also exist, and concerns about interpretation of test results may limit further interest in the test.

Positive-marker vaccine–tetanus toxoid. A positive-marker vaccine has been proposed to provide a differential test between vaccinated and naturally infected animals. The strategy is to include an additional antigen, tetanus toxoid, to killed, whole-virus vaccines as a convenient positive marker to identify vaccinated birds. The use of tetanus toxoid has the advantages that the protein is immunogenic and relatively inexpensive, serologic tests are readily available against tetanus toxoid antibody, and poultry should not normally be exposed to the protein. Experimental studies have shown that the addition of tetanus toxoid can act as a positive marker without affecting the immune response to the influenza proteins (20,21,22).

The positive-marker approach can provide a unique tool to help address vaccine efficacy, particularly in countries where vaccine administration and maintenance of the cold chain are serious impediments to effective immunization. Additionally, for countries that have concurrent infections with LPAI, it can help evaluate the immune response. However, it can't be used to differentiate vaccinated from vaccinated and infected animals because both groups should have similar antibody titers and, therefore, it is not a true DIVA strategy.

Subunit vaccines. A broad and diverse group of vaccines will be considered together because they all encompass the same DIVA strategy. Subunit vaccines all express just the HA protein and, occasionally, other proteins including the NA protein. This limits the immune response to just the proteins that elicit neutralizing antibody, and the animals remain naïve to the internal proteins that are typically used for serologic diagnostic tests. Specifically, the birds will be naïve to the nucleoprotein that is the antigen used in almost all the primary screening tests for avian influenza in poultry, including the commercially available ELISA tests and the AGID test. Birds naturally infected with AIV will typically develop high levels of antibody to the nucleoprotein that will persist for many months. Therefore, the strength of this DIVA strategy is that the commonly used diagnostic tests for AI can be used to identify naturally infected animals, which greatly simplifies the testing process. There are dozens of different subunit vaccines that can experimentally protect against AIV infection, but I will highlight two of the subunit vaccines that are commercially available; the Newcastle disease virus-vectored vaccine (rNDV), and the fowlpox-vectored vaccines (rfowlpox). Currently, only the H5 HA gene is available for most or all of the vaccines but, experimentally, other subtypes have also been developed.

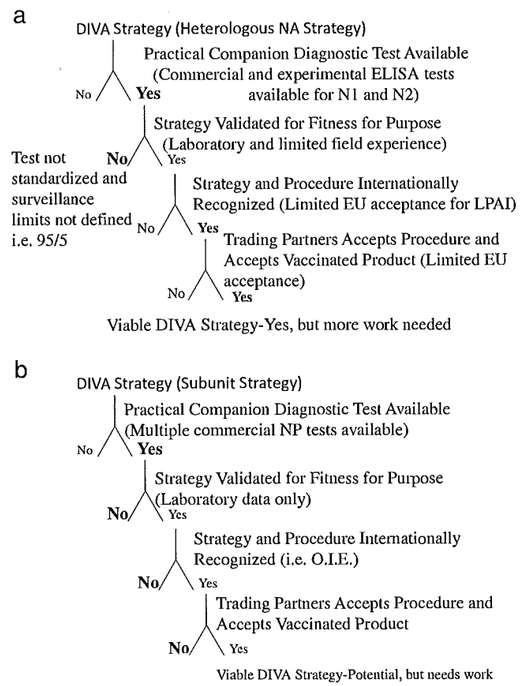

Fig. 1. For a DIVA strategy to be viable it must meet four general criteria. A decision tree was developed to determine the stage of development for two different DIVA strategies; a) the heterologous neuraminidase strategy, and b) the subunit vaccine strategy. Both tests meet some, but not all, of the four criteria for a successful DIVA strategy, and additional work needs to be completed before it can be practically implemented.

The rNDV expressing the H5 gene has been shown to produce protective antibodies to both AI and Newcastle disease. One of the first reports showed that after a single vaccination, all the birds were negative for antibodies to the nucleoprotein by ELISA on the day of challenge with an HPAI virus, and 90% of the birds had seroconverted at 7 days and 100% of the birds had seroconverted at 21 days after challenge (56). A different rNDV that was given as a killed vaccine was also able to differentiate vaccinated from vaccinated and infected birds after challenge, based on the NP antibody response, with 100% of chickens responding by 10 days after challenge by ELISA (33). Similar results can be observed with the rfowlpox, with vaccinated birds staying seronegative when tested with the AGID test but, after challenge, 100% of the birds seroconverted by 14 days with the AGID test (46).

The viral-vectored vaccines have great promise as an effective vaccine tool but also with the additional benefit of being an easily applied DIVA strategy. However, even though some of the viral-vectored vaccines have been commercially available for over 10 yr, little work has been done on applying the DIVA strategy

experimentally or in the field. Additional work needs to be done to evaluate how quickly the immune response occurs, and how long it lasts, with these different vectored vaccines; such work will provide data for how many birds need to be sampled to provide assurance of freedom from infection.

CONCLUSIONS

In the last 5 yr, the interest in DIVA vaccination has increased considerably. This is in part because of animal welfare concerns, but also because of a greater understanding of how this technology can assist in disease control efforts. Although considerable progress has been made to evaluate which different DIVA strategies are most likely to be applicable in the field, considerably more work needs to be done (Figure 1). Almost all of the methods described need additional validation work, including longitudinal studies, to understand how quickly antibody can be detected and how long it persists. As has already been mentioned, the immune response is dependent on the amount of virus replication. It is possible that as vaccines improve or when multiple doses are given, the protection afforded will reduce or prevent an antibody response. A well-vaccinated flock, if exposed to a high viral challenge, may still become infected, but the reduced virus replication may result in only a small amount of virus being shed into the environment and a poor seroconversion to the DIVA antigen. However, it is unlikely that these well-vaccinated birds will transmit the virus to other flocks (8,41). Because of the changing nature of influenza, both in its adaptation to different hosts and in the antigenic drift that makes precise predictions of virus replication and antibody response impossible, more data need to be gathered to understand the parameters that will affect our surveillance policies.

Other factors will also affect the DIVA process. First, concurrent infection of multiple viral subtypes will confound all the different strategies. Co-circulation of different viral subtypes is a serious problem because most of the countries that are endemic for H5N1 HPAI are also endemic for H9N2 influenza. Second, the goal of the DIVA strategy is to assure trading partners of freedom from infection. Because most of the strategies rely upon a serologic response, it is possible that flocks infected shortly before slaughter or movement will be not be picked up by this type of surveillance. However, as the level of surveillance increases, the likelihood of something like this happening is remote. If properly applied, DIVA vaccination strategies promise to provide new tools for the control and eradication of avian influenza virus.

REFERENCES

1. Akey, B. L. Low-pathogenicity H7N2 avian influenza outbreak in Virginia during 2002. Avian Dis. 47:1099–1103. 2003.

2. Avellaneda, G., E. Mundt, C. W. Lee, S. Jadhao, and D. L. Suarez. Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of avian influenza virus. Avian Dis. 54:278–286. 2010.

3. Avellaneda, G., M. J. Sylte, C. W. Lee, and D. L. Suarez. A heterologous neuraminidase subtype strategy for the differentiation of infected and vaccinated animals (DIVA) for avian influenza virus using an alternative neuraminidase inhibition test. Avian Dis. 54:272–277. 2010.

4. Beard, C. W. To vaccinate or not to vaccinate. In: Second International Symposium on Avian Influenza. Extension Duplicating Services University of Wisconsin–Madison, Athens, GA. pp. 258–263. 1986.

5. Beato, M. S., M. Rigoni, A. Milani, and I. Capua. Generation of avian influenza reassortant viruses of the H7N5 subtype as potential vaccine candidates to be used in the framework of a "DIVA" vaccination strategy. Avian Dis. 51:479–480. 2007.

6. Birch-Machin, I., A. Rowan, J. Pick, J. Mumford, and M. Binns. Expression of the nonstructural protein NS1 of equine influenza A virus: detection of anti-NS1 antibody in post infection equine sera. J. Virol. Methods. 65:255–263. 1997.

7. Blancou, J. History of the surveillance and control of transmissible animal diseases. World Organisation for Animal Health, Office of International Des Epizooties, Paris, France. 2003.

8. Bouma, A., I. Claassen, K. Natih, D. Klinkenberg, C. A. Donnelly, G. Koch, and M. van Boven. Estimation of transmission parameters of H5N1 avian influenza virus in chickens. PLoS Pathog 5:e1000281. 2009.

9. Capua, I., G. Cattoli, and S. Marangon. DIVA—a vaccination strategy enabling the detection of field exposure to avian influenza. Dev. Biol. (Basel) 119:229–233. 2004.

10. Capua, I., C. Terregino, G. Cattoli, F. Mutinelli, and J. F. Rodriguez. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. 32:47–55. 2003.

11. Capua, I., C. Terregino, G. Cattoli, and A. Toffan. Increased resistance of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus. Avian Pathol. 33:158–163. 2004.

12. Cattoli, G., A. Milani, F. Bettini, M. Serena Beato, M. Mancin, C. Terregino, and I. Capua. Development and validation of an anti-N3 indirect immunofluorescent antibody test to be used as a companion diagnostic test in the framework of a "DIVA" vaccination strategy for avian influenza infections in poultry. Avian Pathol. 35:154–159. 2006.

13. Davies, G. The foot and mouth disease (FMD) epidemic in the United Kingdom 2001. Comp. Immunol. Microbiol. Infect. Dis. 25:331–343. 2002.

14. Dundon, W. G., S. Maniero, A. Toffan, I. Capua, and G. Cattoli. Appearance of serum antibodies against the avian influenza nonstructural 1 protein in experimentally infected chickens and turkeys. Avian Dis. 51:209–212. 2007.

15. Elvinger, F., B. L. Akey, D. A. Senne, F. W. Pierson, B. A. Porter-Spalding, E. Spackman, and D. L. Suarez. Characteristics of diagnostic tests used in the 2002 low-pathogenicity avian influenza H7N2 outbreak in Virginia. J. Vet. Diagn. Invest. 19:341–348. 2007.

16. Halvorson, D. A. Control of Low Pathogenicity Avian Influenza. In: Avian Influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA. pp. 513–536. 2008.

17. Ho, H. T., H. L. Qian, F. He, T. Meng, M. Szyporta, N. Prabhu, M. Prabakaran, K. P. Chan, and J. Kwang. Rapid detection of H5N1 subtype influenza viruses by antigen capture enzyme-linked immunosorbent assay using H5- and N1-specific monoclonal antibodies. Clin. Vaccine Immunol. 16:726–732. 2009.

18. Hoffmann, E., A. S. Lipatov, R. J. Webby, E. A. Govorkova, and R. G. Webster. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. Proc. Natl. Acad. Sci. U. S. A. 102:12915–12920. 2005.

19. Jadhao, S. J., C. W. Lee, M. Sylte, and D. L. Suarez. Comparative efficacy of North American and antigenically matched reverse genetics derived H5N9 DIVA marker vaccines against highly pathogenic Asian H5N1 avian influenza viruses in chickens. Vaccine 27:6247–6260. 2009.

20. James, C. M., Y. Y. Foong, J. P. Mansfield, S. G. Fenwick, and T. M. Ellis. Use of tetanus toxoid as a differentiating infected from vaccinated animals (DIVA) strategy for sero-surveillance of avian influenza virus vaccination in poultry. Vaccine 25:5892–5901. 2007.

21. James, C. M., Y. Y. Foong, J. P. Mansfield, A. R. Vind, S. G. Fenwick, and T. M. Ellis. Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance. Vaccine 26:5345–5351. 2008.

22. James-Berry, C. M., D. Middleton, J. P. Mansfield, S. G. Fenwick, and T. M. Ellis. Use of a tetanus toxoid marker to allow differentiation of infected from vaccinated poultry without affecting the efficacy of a H5N1 avian influenza virus vaccine. Vet. Rec. 167:695–699. 2010.

23. Kim, J. N., S. H. Byun, S. Y. Kang, and I. P. Mo. Evaluation of neuraminidase antigen-based competitive enzyme-linked immunosorbent

assay in chickens vaccinated with avian influenza inactivated vaccine. Avian Dis. 54:682–685. 2010.

24. Kim, M. C., J. G. Choi, J. S. Kwon, H. M. Kang, M. R. Paek, O. M. Jeong, J. H. Kwon, and Y. J. Lee. Field application of the H9M2e enzyme-linked immunosorbent assay for differentiation of H9N2 avian influenza virus-infected chickens from vaccinated chickens. Clin. Vaccine Immunol. 17:1977–1984. 2010.

25. Kwon, J. S., M. C. Kim, O. M. Jeong, H. M. Kang, C. S. Song, J. H. Kwon, and Y. J. Lee. Novel use of a N2-specific enzyme-linked immunosorbent assay for differentiation of infected from vaccinated animals (DIVA)-based identification of avian influenza. Vaccine 27:3189–3194. 2009.

26. Lambrecht, B., M. Steensels, S. Van Borm, G. Meulemans, and T. van den Berg. Development of an M2e-specific enzyme-linked immunosorbent assay for differentiating infected from vaccinated animals. Avian Dis. 51:221–226. 2007.

27. Lee, C. W., D. A. Senne, and D. L. Suarez. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol. 78:8372–8381. 2004.

28. Lee, C. W., D. A. Senne, and D. L. Suarez. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (differentiating infected from vaccinated animals) strategy for the control of avian influenza. Vaccine 22:3175–3181. 2004.

29. Lee, C. W., D. A. Senne, and D. L. Suarez. Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clin. Vaccine Immunol. 13:395–402. 2006.

30. Lee, C. W., and D. L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Anim. Health Res. Rev. 6:1–15. 2005.

31. Lee, Y. J., H. W. Sung, J. G. Choi, E. K. Lee, O. M. Jeong, Y. K. Kwon, J. H. Kwon, C. S. Song, and J. H. Kimd. Effects of homologous and heterologous neuraminidase vaccines in chickens against H5N1 highly pathogenic avian influenza. Avian Dis. 51:476–478. 2007.

32. Liu, Y., E. Mundt, A. Mundt, M. Sylte, D. L. Suarez, D. E. Swayne, and M. Garcia. 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. Avian Dis. 54:613–621. 2010.

33. Lozano-Dubernard, B., E. Soto-Priante, D. Sarfati-Mizrahi, F. Castro-Peralta, R. Flores-Castro, E. Loza-Rubio, and M. Gay-Gutierrez. Protection and differentiation of infected from vaccinated animals by an inactivated recombinant Newcastle disease virus/avian influenza H5 vaccine. Avian Dis. 54:242–245. 2010.

34. Marangon, S., I. Capua, G. Pozza, and U. Santucci. Field experiences in the control of avian influenza outbreaks in densely populated poultry areas. Dev. Biol. (Basel) 119:155–164. 2004.

35. Marche, S., B. Lambrecht, and T. van den Berg. Evaluation of different serologic markers for the early detection of avian influenza infection in chickens. Avian Dis. 54:690–698. 2010.

36. Moreno, A., E. Brocchi, D. Lelli, D. Gamba, M. Tranquillo, and P. Cordioli. Monoclonal antibody based ELISA tests to detect antibodies against neuraminidase subtypes 1, 2 and 3 of avian influenza viruses in avian sera. Vaccine 27:4967–4974. 2009.

37. Morton, D. B. Vaccines and animal welfare. Rev. Sci. Tech. 26:157–163. 2007.

38. Ozaki, H., T. Sugiura, S. Sugita, H. Imagawa, and H. Kida. Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. Vet. Microbiol. 82:111–119. 2001.

39. Pasick, J. Application of DIVA vaccines and their companion diagnostic tests to foreign animal disease eradication. Anim. Health Res. Rev. 5:257–262. 2004.

40. Perez-Ramirez, E., V. Rodriguez, D. Sommer, J. M. Blanco, P. Acevedo, U. Heffels-Redmann, and U. Hofle. Serologic testing for avian influenza viruses in wild birds: comparison of two commercial competition enzyme-linked immunosorbent assays. Avian Dis. 54:729–733. 2010.

41. Poetri, O. N., A. Bouma, S. Murtini, I. Claassen, G. Koch, R. D. Soejoedono, J. A. Stegeman, and M. van Boven. An inactivated H5N2 vaccine reduces transmission of highly pathogenic H5N1 avian influenza virus among native chickens. Vaccine 27:2864–2869. 2009.

42. Suarez, D. L. Overview of avian influenza DIVA test strategies. Biologicals 33:221–226. 2005.

43. Suarez, D. L. Avian influenza: our current understanding. Anim. Health. Res. Rev. 11:19–33. 2010.

44. Suarez, D. L., C. W. Lee, and D. E. Swayne. Avian influenza vaccination in North America: strategies and difficulties. Dev. Biol. (Basel) 124:117–124. 2006.

45. Suarez, D. L., and S. Schultz-Cherry. Immunology of avian influenza virus: a review. Dev. Comp. Immunol. 24:269–283. 2000.

46. Swayne, D. E., G. Avellaneda, T. R. Mickle, N. Pritchard, J. Cruz, and M. Bublot. Improvements to the hemagglutination inhibition test for serological assessment of recombinant fowlpox-H5-avian-influenza vaccination in chickens and its use along with an agar gel immunodiffusion test for differentiating infected from noninfected vaccinated animals. Avian Dis. 51:697–704. 2007.

47. Swayne, D. E., M. Garcia, J. R. Beck, N. Kinney, and D. L. Suarez. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 18:1088–1095. 2000.

48. Swayne, D. E., G. Pavade, K. Hamilton, B. Vallat, and K. Miyagishima. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev. Sci. Tech. 30:839–870. 2011.

49. Sylte, M. J., B. Hubby, and D. L. Suarez. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine 25:3763–3772. 2007.

50. Thrusfield, M., L. Mansley, P. Dunlop, A. Pawson, and J. Taylor. The foot-and-mouth disease epidemic in Dumfries and Galloway, 2001. 2: serosurveillance, and efficiency and effectiveness of control procedures after the national ban on animal movements. Vet. Rec. 156:269–278. 2005.

51. Tumpey, T. M., R. Alvarez, D. E. Swayne, and D. L. Suarez. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J. Clin. Microbiol. 43:676–683. 2005.

52. Upadhyay, C., A. Ammayappan, and V. N. Vakharia. Detection of NP, N3 and N7 antibodies to avian influenza virus by indirect ELISA using yeast-expressed antigens. Virol. J. 6:158. 2009.

53. Vannie, P., I. Capua, M. F. Le Potier, D. K. Mackay, B. Muylkens, S. Parida, D. J. Paton, and E. Thiry. Marker vaccines and the impact of their use on diagnosis and prophylactic measures. Rev. Sci. Tech. 26:351–372. 2007.

54. van Oirschot, J. T. Diva vaccines that reduce virus transmission. J. Biotechnol. 73:195–205. 1999.

55. van Oirschot, J. T. Vaccinology of classical swine fever: from lab to field. Vet. Microbiol. 96:367–384. 2003.

56. Veits, J., D. Wiesner, W. Fuchs, B. Hoffmann, H. Granzow, E. Starick, E. Mundt, H. Schirrmeier, T. Mebatsion, T. C. Mettenleiter, and A. Romer-Oberdorfer. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc. Natl. Acad. Sci. U. S. A. 103:8197–8202. 2006.

57. Wang, L., Z. Qin, M. Pantin-Jackwood, O. Faulkner, D. L. Suarez, M. Garcia, B. Lupiani, S. M. Reddy, Y. M. Saif, and C. W. Lee. Development of DIVA (differentiation of infected from vaccinated animals) vaccines utilizing heterologous NA and NS1 protein strategies for the control of triple reassortant H3N2 influenza in turkeys. Vaccine 29:7966–7974. 2011.

58. Wang, W., B. Lu, H. Zhou, A. L. Suguitan Jr, X. Cheng, K. Subbarao, G. Kemble, and H. Jin. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. J. Virol. 84:6570–6577. 2010.

59. Watson, D. S., S. M. Reddy, V. Brahmakshatriya, and B. Lupiani. A multiplexed immunoassay for detection of antibodies against avian influenza virus. J. Immunol. Methods 340:123–131. 2009.

60. Webster, R. G., W. G. Laver, and E. D. Kilbourne. Reactions of antibodies with surface antigens of influenza virus. J. Gen. Virol. 3:315–326. 1968.

61. Whiting, T. L. Foreign animal disease outbreaks, the animal welfare implications for Canada: risks apparent from international experience. Can. Vet. J. 44:805–815. 2003.

62. (OIE) World Organisation for Animal Health. Avian influenza. In: Terrestrial Animal Health Code–2011. World Organisation for Animal Health (Office International Epizooties), Paris, France. 2011.

63. Wu, R., Q. Chen, L. Zheng, J. Chen, Z. Sui, Y. Guan, and Z. Chen. Generation and evaluation of an H9N1 influenza vaccine derived by reverse genetics that allows utilization of a DIVA strategy for control of H9N2 avian influenza. Arch. Virol. 154:1203–1210. 2009.

64. Zhao, S., M. Jin, H. Li, Y. Tan, G. Wang, R. Zhang, and H. Chen. Detection of antibodies to the nonstructural protein (NS1) of avian influenza viruses allows distinction between vaccinated and infected chickens. Avian Dis. 49:488–493. 2005.

Arch Virol (2012) 157:1931–1947
DOI 10.1007/s00705-012-1385-9

ORIGINAL ARTICLE

Evolution of highly pathogenic avian influenza H5N1 viruses in Egypt indicating progressive adaptation

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Received: 30 January 2012 / Accepted: 13 May 2012 / Published online: 4 July 2012
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Abstract Highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype was first diagnosed in poultry in Egypt in 2006, and since then the disease became enzootic in poultry throughout the country, affecting the poultry industry and village poultry as well as infecting humans. Vaccination has been used as a part of the control strategy to help to control the disease. Epidemiological data with sequence analysis of H5N1 viruses is important to link the mechanism of virus evolution in Egypt. This study describes the evolutionary pattern of Egyptian H5N1 viruses based on molecular characterization for the isolates collected from commercial poultry farms and village poultry from 2006 to 2011. Genetic analysis of the hemagglutinin (HA) gene was done by sequencing of the full-length H5 gene. The epidemiological pattern of disease outbreaks in Egyptian poultry farms seems to be seasonal with no specific geographic distribution across the country. The molecular epidemiological data revealed that there are two major groups of viruses: the classic group of subclade 2.2.1 and a variant group of 2.2.1.1. The classic group is prevailing mainly in village poultry and had fewer mutations compared to the originally introduced virus in 2006. Since 2009, this group has started to be transmitted back to commercial sectors. The variant group emerged by late 2007, was prevalent mainly in vaccinated commercial poultry, mutated continuously at a higher rate until 2010, and started to decline in 2011. Genetic analysis of the neuraminidase (NA) gene and the other six internal genes indicates a grouping of the Egyptian viruses similar to that obtained using the HA gene, with no obvious reassortments. The results of this study indicate that HPAI-H5N1 viruses are progressively evolving and adapting in Egypt and continue to acquire new mutations every season.

Abbreviations

AIV	Avian influenza virus
HA	Hemagglutinin
HPAI	Highly pathogenic avian influenza
RBD	Receptor binding domain
AS	Antigenic site

Electronic supplementary material The online version of this article (doi:10.1007/s00705-012-1385-9) contains supplementary material, which is available to authorized users.

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Introduction

Highly pathogenic avian influenza virus of the H5N1 subtype (HPAI-H5N1) has disseminated widely across Asia, Europe, and Africa, infecting a wide range of domestic and wild avian species and sporadically infecting

humans and other mammals since its emergence in 1996 in Guangdong, China [9, 51]. Over time, the HPAI-H5N1 virus has diversified into multiple phylogenetically distinct lineages, classified as clades 0 to 9 according to the unified WHO/OIE/FAO nomenclature system [48, 50]. The H5N1 lineage currently circulating in central Asia, the Middle East, Europe, and Africa has been classified as clade 2.2 [48] and has also been described as "EMA" or Qinghai-like in previous publications [9, 31, 36]. The 2.2 clade was first recognized in April 2005 during a large outbreak of a phylogenetically distinct H5N1 virus among wild bird populations at Qinghai Lake in western China and rapidly spread west through central Asia and Europe, eventually reaching Africa in 2006 [36]. Clade 2.2 has further diversified, forming the genetic third-order clade 2.2.1 [49] including the Egyptian viruses and recently has been subgrouped to the fourth-order clade 2.2.1.1, which includes variant Egyptian isolates that emerged from 2.2.1 [50]. Formally, three genetically distinct sublineages (I, II, and III) [7, 32, 40], all of which are found in Africa. Since 2006, HPAI H5N1 viruses belonging to clade 2.2 have disseminated across multiple countries in Western, Eastern, and Northern Africa, including Nigeria, Egypt, Niger, Cameroon, Sudan, Burkina Faso, Djibouti, Ivory Coast, Ghana, Togo and Benin. These countries have experienced several major outbreaks of HPAI-H5N1 virus, posing a serious threat to food security and public health in Africa [7]. However, since 2008, Egypt has been the only country in Africa reporting H5N1 HPAI outbreaks [13].

HPAI virus of subtype H5N1 was first recognized in Egypt in 2006 [3], and since then, the disease has become endemic and still causes a significant threat to the poultry industry and humans in Egypt. Village poultry and household birds constitute one large sector of poultry production in Egypt, with approximately 4-5 million families who are closely associated with their birds, estimated at 250 million birds. Chickens, ducks, turkeys, and other poultry are usually kept together, providing a chance of interspecies transmission [20]. Different types of surveillance, including active, passive and targeted, have been established to monitor the spread of H5N1 HPAI virus in village poultry in backyards, in the commercial poultry sector, and in live-bird markets [2].

In March 2006, vaccination was implemented nationwide as part of the national control strategy. Around 20 inactivated H5N1 and H5N2 AIV vaccines with different seed viruses from many vaccine manufacturers were applied in the field. In late 2007, some poultry farms experienced vaccination failure outbreaks, and a variant H5N1 virus was identified. Sequencing of isolated viruses confirmed that this variant was an HPAI virus of the H5N1 subtype with marked increase in the number of amino acid substitutions in the HA1 protein [1, 4].

This work describes molecular epidemiological investigations including molecular and phylogenetic analysis of H5N1 avian viruses based on surveillance conducted by the General Organization of Veterinary Services (GOVs) in cooperation with National Laboratory for Veterinary Quality Control on Poultry Production (NLQP) in commercial poultry farms and village poultry in Egypt during 2006–2011.

Materials and methods

Oro-pharyngeal and cloacal swab samples were collected from suspected or clinically diseased birds from cases associated with high mortality rates during outbreaks recorded in Egypt from 2006 to 2011. The samples were collected in phosphate-buffered saline with antibiotics and delivered on ice to the central laboratory or its satellites, which are distributed in five governorates in Egypt. Samples from 2579 outbreaks were analyzed. The analysis was done on 236 isolates representing six successive seasons, starting November 2005 to May 2006 and ending November 2010 to May 2011. The data were collected seasonally for the last six years, with the season extending from November to May. It was clearly noticed that the number of outbreaks starts to increase in November, reaches peaks in January to March, and declines in May. Occasionally, viruses were isolated in the period from June to October, outside of the typical avian influenza season. The number of isolates analyzed for the six different successive seasons was 20, 36, 37, 38, 47 and 58, respectively (Fig.3; Table 1).

Virus monitoring was conducted from 2006 to 2011 in most Egyptian provinces. These provinces are located in different regions, designated in this study as D (Delta region) in the northern part of Egypt, C (Greater Cairo) in the middle, U (Upper Egypt) in south, and O (other provinces) as indicated in Fig. 1. The epidemiological data were collected from the NLQP database of official outbreaks reported to GOVs.

All of the 2579 samples were tested for the presence of influenza A viruses by detection of the matrix (M) gene [39] using a viral RNA extraction kit (QIAamp, QIAGEN, Germany) and a one-step real-time RT-PCR kit (Quantitect, QIAGEN, Germany). Samples were subtyped using specific H5 gene primers [38]. The selected 236 samples from AI outbreaks were isolated in 9- to 11-day-old specific-pathogen-free chicken embryos and then analyzed by sequencing the full-length HA gene. This selection was based on the distribution of outbreaks with respect to season and region (Tables 2, 3).

Determination of nucleotide sequences of the HA genes of the Egyptian viruses was carried out for the H5

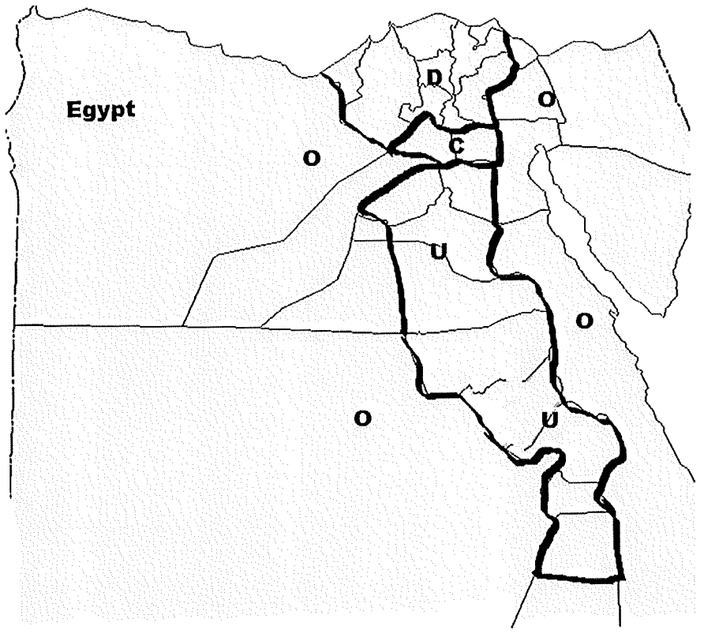


Fig. 1 Map of Egypt indicating the different regions included in the study, where D=Delta, C=Cairo, U= Upper Egypt and O=other far governorates

Table 1 Outbreaks of HPAI-H5N1 recorded in different regions in Egypt from 2006 to 2011

Region	2005/ 2006	2006/ 2007	2007/ 2008	2008/ 2009	2009/ 2010	2010/ 2011	Total
Delta	708	143	80	161	302	201	1595
Cairo	198	24	8	23	51	26	330
Upper Egypt	125	77	31	88	104	88	513
Other	40	38	8	15	19	21	141
Total	1071	282	127	287	476	336	2579

Data collected from NLQP database of official outbreaks reported to the General Organization of Veterinary Services

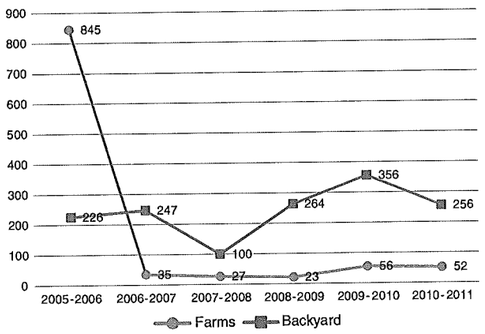


Fig. 2 Seasonal incidence of outbreaks in poultry from 2006 to 2011. The seasonal pattern of outbreaks reported from 2006 to 2011. Data were collected from the NLQP database of official outbreaks reported to the General Organization of Veterinary Services

amplified gene using a BigDye Terminator Kit (version 3.1; Applied Biosystems, Foster City, CA) on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA), using specific internal primers for sequencing of full-length coding genes. Primer sequences are available upon request.

For some selected isolates, the NA gene and six other internal genes were also sequenced, and all of these sequences have been submitted to the GenBank database. (Supplementary Table 1). Comparative analysis of 236 HA gene sequences of the H5N1 viruses was carried out and compared with the available sequences using the National Center for Biotechnology Information (NCBI) influenza virus resources database for avian H5N1 viruses. All sequences were generated at NLQP and are present in the GenBank database. The data from full-length HA and NA genes and other internal gene sequences were retrieved from the GenBank database for the sequences that were selected for analysis in this work. Besides other reference

Table 2 Evolution of the HA protein of Egyptian viruses from 2006 to 2011

Strain	Epidem. unit /sector	Geographic location[1]	HA Genotype[2]	Season	HA Substit.[3]
A/ Chicken /Egypt/2253-1/2006	Farm	C	C	05/06	0
A/Chicken/Egypt/07118-NLQP/2007	Farm	D	C	06/07	3
A/geese/Egypt/07364S-NLQP/2007	Backyard	U	C		7
A/ Chicken /Egypt/07201-NLQP/2007	Farm	D	V	07/08	11
A/ Chicken /Egypt/07665S-NLQP/2007	Farm	D	C		4
A/ Peacock /Egypt/07667s-NLQP/2007	Backyard	C	C		5
A/Chicken/Egypt/0811-NLQP/2008	Backyard	U	C		5
A/Duck/Egypt/0821S-NLQP/2008	Backyard	U	C		4
A/ Chicken /Egypt/0879-NLQP/2008	Farm	C	V		23
A/Chicken/Egypt/08139S-NLQP/2008	Farm	C	V	08/09	11
A/Duck/Egypt/093L-NLQP/2009	Backyard	U	C		4
A/Chicken/Egypt/0935-NLQP/2009	Backyard	C	C		8
A/Chicken/Egypt/0962S-NLQP/2009	Backyard	D	C		14
A/Chicken/Egypt/09359S-NLQP/2009	Farm	D	V		21
A/Turkey/Egypt/09534S-NLQP/2009	Farm	C	V	09/10	23
A/Chicken/Egypt/096767V-NLQP/2009	Farm	C	V		23
A/ Chicken /Egypt/1029/2010	Backyard	D	C		6
A/ Chicken /Egypt/1022L/2010	Backyard	U	C		2
A/ Chicken /Egypt/1031/2010	Backyard	D	V		31
A/Duck/Egypt/10118/2010	Backyard	D	C	10/11	10
A/ Chicken /Egypt/116AD/2011	Farm	D	V		27
A/ Chicken /Egypt/118AF/2011	Farm	U	C		10

[1] Geographic location, C= Greater Cairo, D= Delta, U= Upper Egypt, [2] HA Genotype, C= classic 2.2.1, V= Variant 2.2.1.1, [3] HA substitutions of amino acid residues in comparison to the original Egyptian 2006 strain A/ Chicken /Egypt/2253-1/2006

Chinese sequences of the strains A/Goose/Guangdong/1/1996 (Gs/GD/1/96) and A/Bar-headed goose/Qinghai/5/05, the sequences of other Egyptian and international strains were obtained from GenBank. The genetic data were assembled using BioEdit software, V.7 [21].

The evolutionary history was inferred using the neighbor-joining method [35]. The bootstrap consensus trees inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to each branch [14]. Phylogenetic analysis was conducted for the full-length HA and NA genes and other internal genes from Egyptian strains in this study from 2006 to 2011 using MEGA4 software [45].

Results

The epidemiological pattern of disease outbreaks in Egyptian poultry farms and village poultry indicates the persistence of an endemic situation in different localities with a seasonal pattern, mainly increasing in winter (Fig. 2). The outbreaks are not equally distributed across

Table 3 Incidence of the classic and variant H5N1 groups in commercial and backyard poultry in the 2010 and 2011 seasons

Group	Farm						Backyard						Total		
	Chicken		Duck		Turkey		Chicken		Duck		Goose				
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	Total
Classic 2.2.1	4	13	2	1	1	0	**24**	5	17	3	1	2	**49**	**24**	**73**
Variant 2.2.1.1	**15**	2	0	0	1	0	3	0	1	0	0	0	**20**	**2**	**21**
Total	**19**	**15**	**2**	**1**	**2**	**0**	**27**	**5**	**18**	**3**	**1**	**2**	**69**	**26**	**95**

Fig. 3 Phylogenetic analysis of HA genes of Egyptian viruses from 2006 to 2011 C= classic 2.2.1, V= variant 2.2.1.1

Egypt; most outbreaks (1595 out of 2579) were reported in the Delta region, followed by Upper Egypt (513 out of 2579), and Cairo (330 out of 2579). The other governorates reported only 141 outbreaks in the same period (Table 1; Fig. 2).

Molecular evolution of H5N1 viruses isolated in Egypt from 2006 to 2011

Phylogenetic analysis indicated that there was only one introduction into Egypt in 2006 from the Eurasian lineage

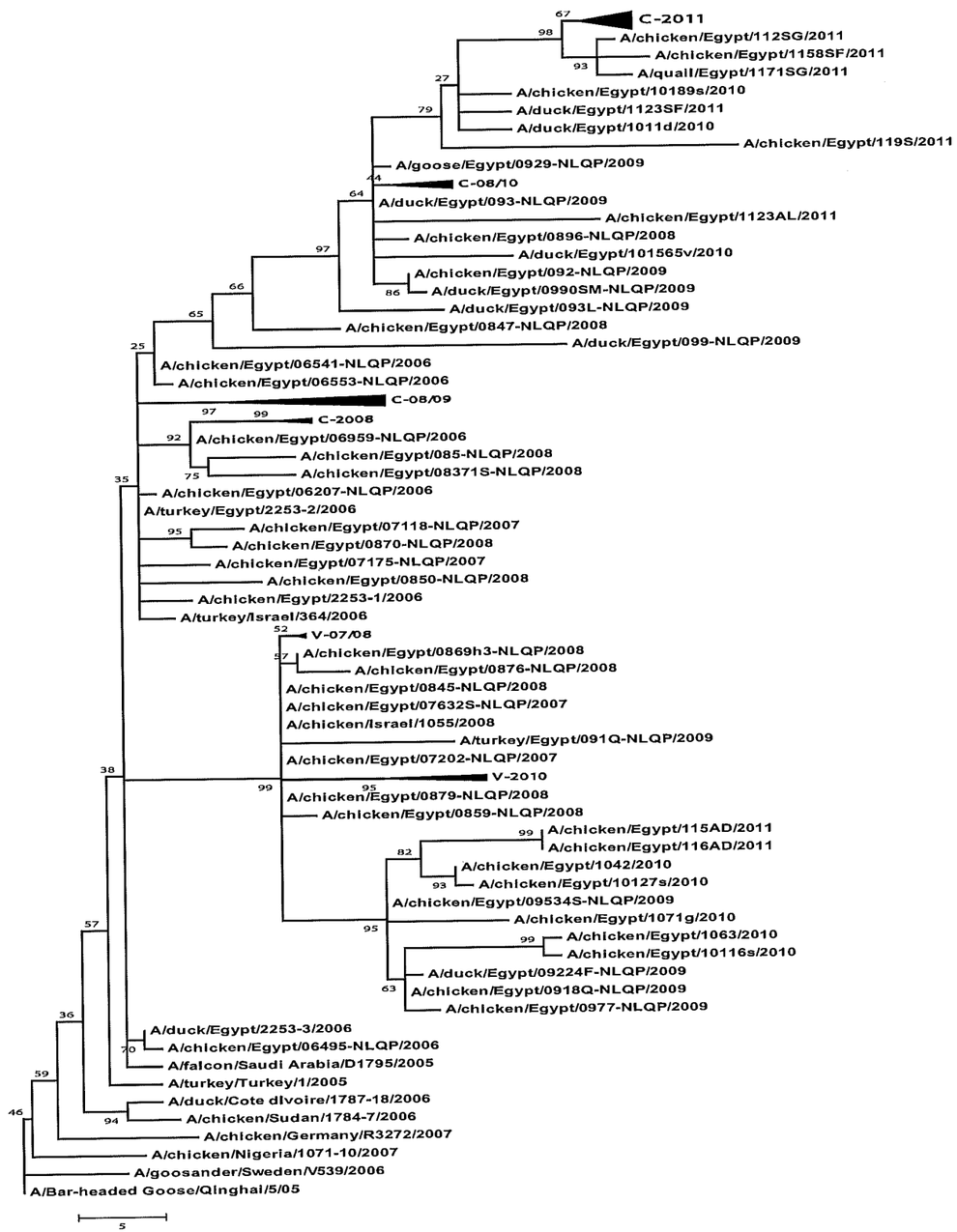


Fig. 4 Phylogenetic analysis of NA genes of Egyptian viruses from 2006 to 2011 C= classic 2.2.1, V= variant 2.2.1.1

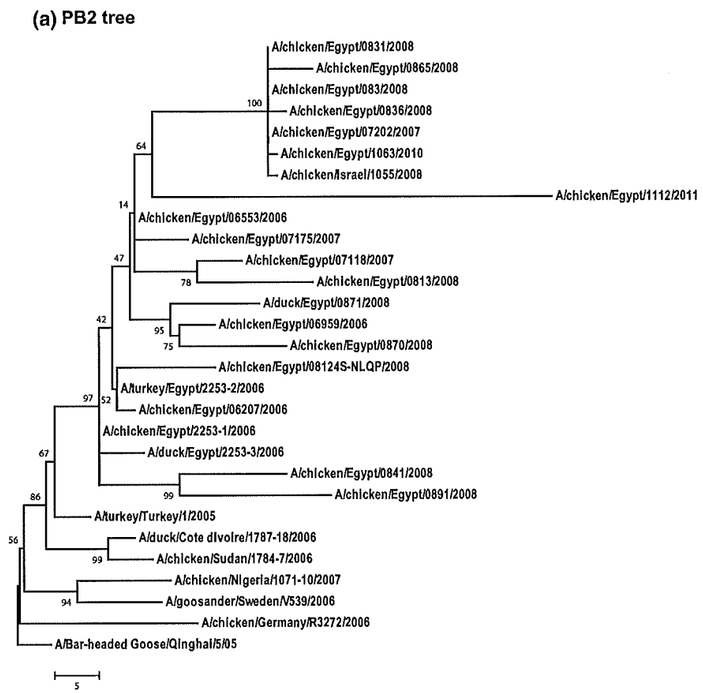


Fig. 5 (a–f)Phylogenetic analysis of six internal genes, PB2, PB1, PA, NP, M and NS, of Egyptian viruses from 2006 to 2011

of clade 2.2, which was widely distributed at that time (Figs. 3, 4, 5). In the 2006/2007 season, there was no obvious clustering observed, and the viruses continued to spread across the country. In 2007/2008, the most important feature was the emergence of a new variant sublineage that escaped vaccine immunity, and minor subgroups within the classic group, originating from the initial 2006 strains, started to appear. These can be defined by unique amino acid changes that are common to each subgroup (Table 4). The variant sublineage that evolved in the 2007/2008 season was reported mainly in vaccinated farms, while the old classic group was widely present in village poultry. In the backyard sector, the classic group was reported frequently in clinically healthy ducks and was sometimes associated with mortality in chickens reared in the vicinity. However, some nervous signs have been reported, and those may vary among different duck species and according to their health status and age at infection.

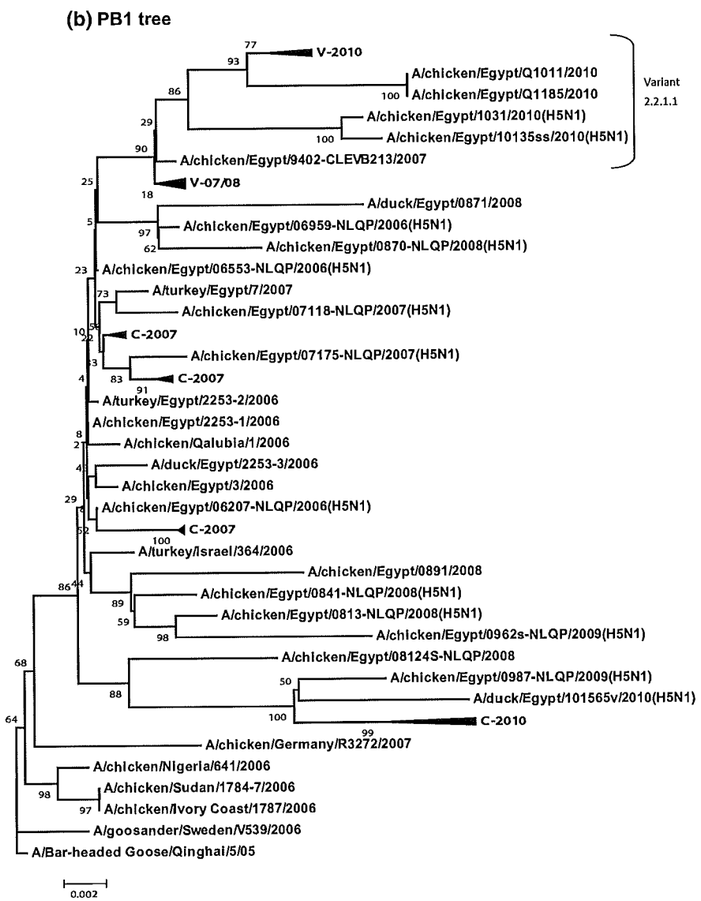
In 2008/2009, separate distinct sublineages were distributed regardless of the bird species, production sector, and locality. Two main clusters were prominent: the variant group and the classic group. Both of them were subdivided into multiple subgroups, reflecting extinction of some of the classic subgroups over time and the faster evolution of the variant group. The two main clusters (classic and variant) continued to circulate during the 2009/2010 season. They were grouped together into the same Egyptian subclade 2.2.1 [49]. There was only one classic group in that season without obvious subdivision, supporting the same observation as in the previous season, while there was a subdivision of the variants into two minor subgroups.

It was very clear in the two seasons from 2010 to 2011 that the classic group was prevailing in household and backyard birds, as there were 24 and 17 outbreaks reported in chickens and ducks, respectively, in 2010, while the variant viruses were prevailing in commercial vaccinated farms (Tables 2, 3).

During the 2010/2011 season, the classic sublineage appeared to be dominant with a marked decrease in variant viruses; only two cases from variant versus 13 classic from commercial chickens (Table 3). Recently, the variant group was grouped in 2.2.1.1, separating them from the classic viruses grouped in subclade 2.2.1 [50].

Phylogenetic analysis of the NA gene indicated a clustering of Egyptian viruses similar to that obtained using the HA gene, showing that there were two major groups (classic 2.2.1 and variant 2.2.1.1). The same findings have

Fig. 5 continued



also been observed for other internal gene trees with different minor genetic subgroups. The two major groups were easily distinguishable by specific amino acid mutations in different proteins, with the exception of PA, for which no specific markers were found (Table 4).

Molecular analysis of the Egyptian H5N1 viruses revealed continuous evolution, as was indicated by distinct mutations in the HA gene that affected different regions of the gene that could possibly affect the function of the hemagglutinin protein. The most characteristic mutation near the receptor-binding domain (RBD) is a deletion at position 129 of the amino acid serine. It was first detected in a classic group during the 2007/2008 season. It then became prevalent in 2008/2009 and became the dominated phenotype in the 2009/2010 and 2010/2011 seasons. A glycosylation site was lost at N165H in the variant sublineage, and one was gained at D154N-A156T. The antigenic sites (AS) showed multiple mutations, all of them originating sporadically during the 2006/2007 season, and some of them appeared to be specific to the variant group (A184E, S120N, R140G and N165H). The characteristic mutations in the antigenic sites found in the HA1 protein are described in Tables 5 and Supplementary Table 2 for positions 120, 140, 141, 151, 152, 154, 156, 162 and 185. Other changes have frequently been associated with most Egyptian viruses from the first introduction in 2006. These include P74, N94, D97, H110, S123, F144, A184, Q192, M226 and A238 (Table 6). Also, the substitution R323G found in the cleavage site in comparison to the strain A/Gs/Gd/1/96. The proteolytic cleavage site (PCS) showed frequent variations in relation to the first introduced phenotype of the strain (A/ Chicken /Egypt/2253-1/2006) that

Fig. 5 continued

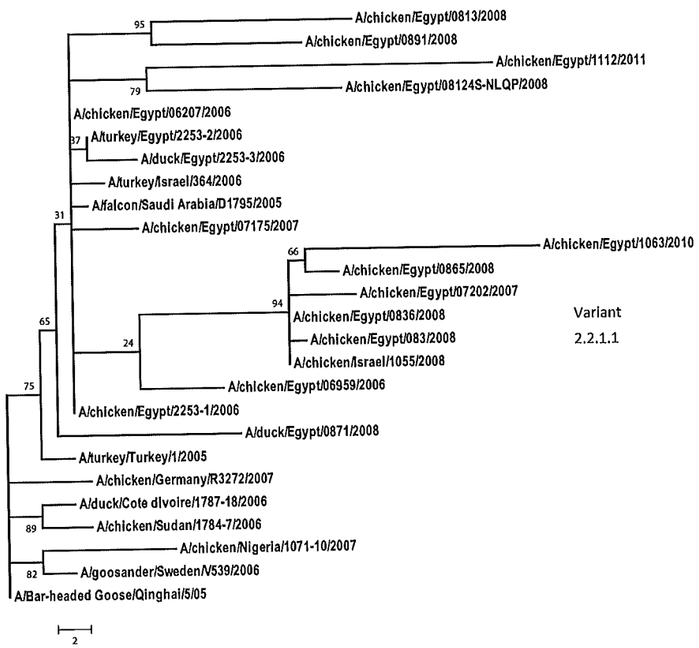
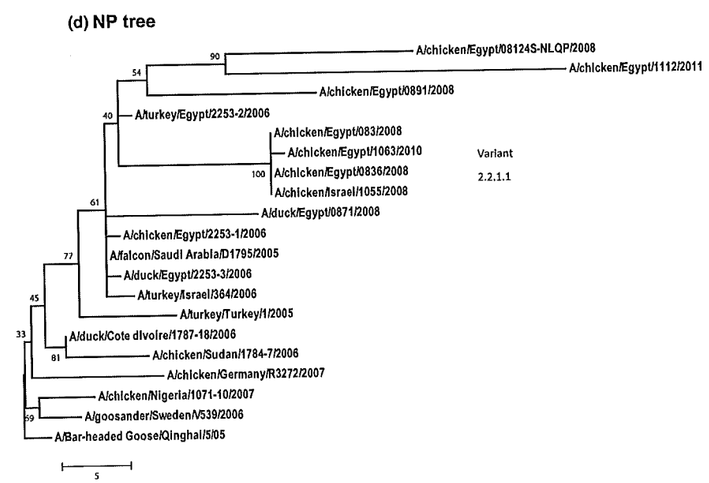


Fig. 5 continued

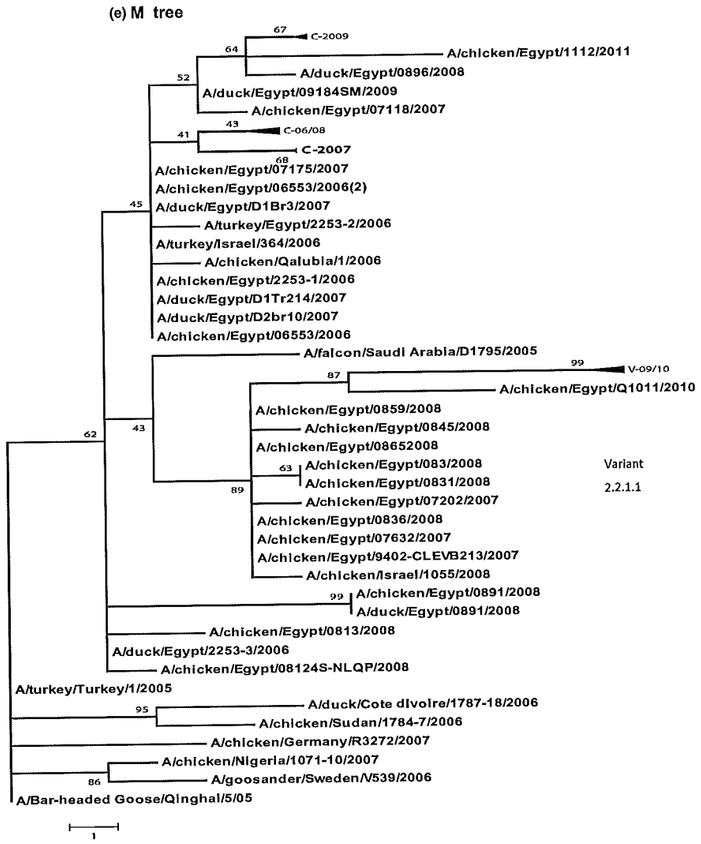


was still dominant in Egypt from 2006 until 2011 (PQGERRRKKRGLF). PCS patterns varied from year to year, with three unique patterns in the 2006/2007 season, seven patterns in 2009/2010, and one pattern in 2007/2008, although this season showed multiple heterogenic strains (Table 7).

Discussion

In this study, molecular epidemiological analysis was conducted for six successive influenza seasons from 2006 to 2011 in Egypt. The epidemiological pattern of the

Fig. 5 continued

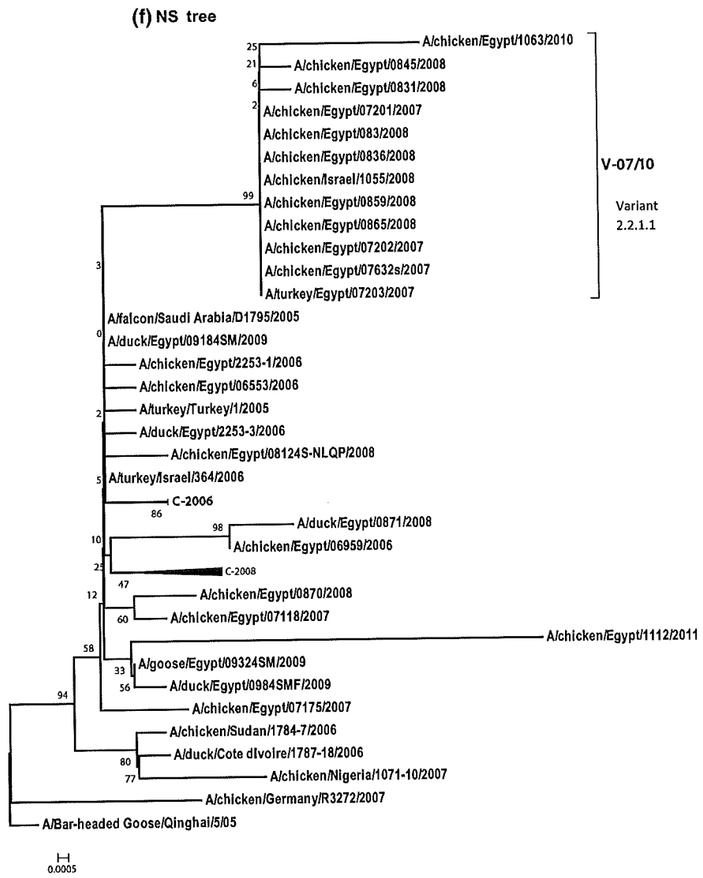


outbreaks in different poultry sectors, either commercial or household poultry, indicates the persistence of an endemic situation in different localities, with a seasonal peak during winter. There were no clear limitations for the spread of virus based on geographic distribution of the circulated viruses where outbreaks were reported every season in the Delta region, Cairo region, Upper Egypt, and sporadically in other regions in border governorates, but this could be due to low poultry density.

Overall, the Delta region had the highest number of positive cases (1595) due to intensive commercial poultry production together with dense breeding of backyard and household birds. There was a small number of cases (330 and 513, respectively) in the greater Cairo region, representing the link between the Delta and Upper Egypt in the south part of the country. A few sporadic cases were reported in other regions (141 cases). With regard to season, the highest number of cases (1071 cases) was reported during the first introduction in 2006, when the compensation strategy was applied to encourage disease reporting as a means of disease control. In the next three seasons, from 2007 to 2009, there was a marked decrease in the number of cases, most likely due to the lack of disease reporting. The reasons for this underreporting are related to unfair compensation in the commercial sector, lack of awareness of disease control, and an ineffective outbreak management response. In the seasons 2008/2009 and 2009/2010, there was a marked increase in the number of cases, which may have been due to an increase in surveillance activities over the whole year in all governorates as part from the national surveillance strategy and other projects supporting surveillance. However, there was insufficient surveillance conducted in the last season, 2010/2011, due to political changes in Egypt.

Egyptian H5N1 viruses continue to mutate and rapidly evolve over time [1, 4]. Phylogenetic analysis (Fig.3)

Fig. 5 continued



indicated that there has been only one introduction into Egypt, in 2006, from the Eurasian lineage of clade 2.2. Several reports have described later introductions of distinct sublineages of clade 2.2 HPAI H5N1 viruses into European countries through wild birds, for example, in Germany [40], Denmark [6], Hungary [44] and France [16].

In the 2007/2008 season, the viruses started to evolve into several distinct sublineages, which clustered mainly in two major groups (classic and variant) with unique amino acid markers distinguishing each group (Table 4). The available inactivated vaccines showed better protection against the classic strains when they were first introduced in 2006. These included a Mexican H5N2 vaccine and a reassortant virus seed strain from China based on the strain A/Gs/GD/1/96 [20]. The emergence of variant H5N1 viruses in Egypt, which often have been isolated from vaccinated flocks, was first observed in the 2007/2008 season, when there were 18 cases identified as variant, all of which were from commercial farms, versus 19 cases identified as classic, most of which were from household birds. The variant sublineage acquired 11 unique amino acid changes in the HA protein in comparison to the original 2006 strains (P74S, D97N, H110R, S123P, R140G, S141P, F144Y, R162K, N165H, A184E and M226V), with most of these amino acid substitutions being found in areas that are predicted to be antigenically important. The distinct sublineages were identified in both ducks and chickens, although viruses from the classic group were more often found in ducks and village poultry (with very rare exceptions, like strain A/duck/Egypt/0955-NLQP/2009, which was variant), while the viruses from the variant group were more often found in the commercial chicken sector (presumably mostly from vaccinated chickens).

Table 4 Amino acid signatnres of the Egyptian H5N1 proteins characterizing Egyptian sublineages:

	PB2	PB1	PB1-F2	NP	NA	M1	M2	NS1	NS2
C-2008-11	V338I	-	D40G	I109V	M15V M29I A46D P48S L204M S319F	-	-	R44K	-
C-2010-11	R369K	T182I K214R L384S	L43P I45T I55T	A129S	T56A R91K D378E S430G	R95K I168T N207S	C50F	V175I	-
V-2007-11	T559A	R584H N645S		S34N T350A I425V	T168I	-	S31N	T122I	S10Y A35V
V-2010-11	-	R430K	E107G	-	V20A V79A	-	S294F A313T N323D	-	-

Subisititutions were based on comparison with the original Egyptian strain (A/chicken/Egypt/2253-1/2006) using H5 numbering. C= classic 2.2.1, V= variant 2.2.1.1

However, care has to be taken in interpreting this result, since most surveillance activities were targeting the backyard system, and samples mainly originating from ducks. In addition, most ducks survive infection with H5N1 HPAI, while the other bird species usually die. In the commercial sector, the outbreaks were mainly in chicken farms, with easily detected signs in diseased birds. However, silent infection remains a major concern in vaccinated flocks.

The majority of the variant virus group originated from vaccinated farms from sectors 2 and 3, where severe outbreaks were recorded in the three successive seasons from 2007/2008 to 2009/2010 in broiler and layer farms and one case reported from sector 1 grandparents. The classic group was mostly linked to village poultry. In season 2009/2010, when viruses from both the variant and classical groups were circulating, the classic group was detected in backyard and village poultry and started to spread to commercial farms. This classic group was linked to all human cases reported during 2010, unlike the variant viruses, which have not been associated with human infections, with the exception of one human case in 2008 (out of 109 human cases reported by WHO between 2008 and November 2, 2011 [23, 47].

In the 2010/2011 season, most positive H5N1 HPAI outbreaks were reported from farms in Fayoum Governorate in Upper Egypt due to increased surveillance activities in this governorate. All of these farms were considered to be dense backyard settings not exceeding 10,000 birds per farm; all of these cases belonged to the classic 2.2.1 sublineage.

The co-circulation of multiple genetically distinct sublineages has been reported previously in Nigeria as a major new subclade that was phylogenetically distinguishable from all previously identified sublineages, as well as two novel reassortment events [15]. The co-circulation of multiple sublineages has also been recorded in many countries, such as China [46], Vietnam [34], Indonesia [29] and Thailand [43].

The most characteristic mutation in the receptor-binding domain (RBD) is the deletion of Serine 129, which first appeared in the classic group during the 2007/2008 season, (strain: A/chicken/Egypt/0847-NLQP/2008), continued in 2008/2009, and became the dominant genotype in the 2009/2010 season. The mutations in RBD play an important role in directing attachment of the virus to host-cell receptors, and they can alter the recognition from avian-type α-2,3-linked sialic acid to mammalian-like α-2,6-linked sialic acid, which can permit the binding of avian viruses to human receptors [5, 24]. As was also reported by Watanabe et al. [47], the Q192H mutation markedly increased viral binding to α-2,6-SA, and to a lesser extent, so did the S235P mutation in Egyptian viruses.

HA glycosylation sites were lost at N165H and N154D when the N165H mutation was first observed in the 2006/2007 season and remain linked to the classic group in the next seasons, while the N154D started at first introduction at season 2005/2006 and spread afterwards. Previous studies have shown that N-linked glycans close to the HA receptor-binding domain can affect receptor-binding preference through steric hindrance or other mechanisms

Table 5 Amino acid changes at different sites in the HA protein

Strain	RBD	GS	AS								
			120	140	141	151	152	154	156	162	185
A/ chicken /Egypt/2253-1/2006		N154D	S	R	S	I	K	D	A	R	A
A/chicken/Egypt/07118-NLQP/2007		N154D	S	R	S	I	K	D	A	R	A
A/geese/Egypt/07364S-NLQP/2007			S	R	S	I	K	N	A	R	A
A/ chicken /Egypt/07201-NLQP/2007		N154D N165H	S	**G**	**P**	I	K	D	A	K	E
A/ chicken /Egypt/07665S-NLQP/2007		N154D	S	R	S	I	K	D	A	I	A
A/ peacock/Egypt/07667S-NLQP/2007		N154D	S	R	S	I	K	D	A	R	A
A/chicken/Egypt/0811-NLQP/2008			S	R	S	I	K	N	A	I	A
A/duck/Egypt/0821S-NLQP/2008	Δ129	N154D	N	R	S	T	K	D	A	R	A
A/chicken/Egypt/0879-NLQP/2008	L190I S129L	N165, P194S	S	**G**	**P**	I	K	N	T	E	A
A/chicken/Egypt/08139S-NLQP/2008		N154, N165H	S	**G**	**P**	I	K	D	A	K	A
A/duck/Egypt/093L-NLQP/2009	Δ129	N154D	N	R	S	T	K	D	A	R	A
A/chicken/Egypt/0935-NLQP/2009		N154D	S	R	S	I	K	D	A	R	A
A/chicken/Egypt/0962S-NLQP/2009		N154D	S	S	S	V	K	D	A	K	A
A/chicken/Egypt/09359S-NLQP/2009	S129L	N165H	S	**G**	**L**	I	E	N	T	K	A
A/turkey/Egypt/09534S-NLQP/2009	L190I S129L	N165H	S	**G**	**L**	I	K	N	T	E	E
A/chicken/Egypt/096767V-NLQP/2009	L190I S129L	N165H	S	**G**	**P**	I	K	N	T	E	E
A/ chicken /Egypt/1029/2010	Δ129		N	R	S	T	K	N	A	K	A
A/ chicken /Egypt/1022L/2010	Δ129	N154D	S	R	S	T	K	D	A	R	A
A/ chicken /Egypt/1031/2010	S129L	N165, P194S	N	**G**	**L**	I	E	N	T	K	A
A/duck/Egypt/10118/2010	Δ129		D	R	S	T	E	N	A	K	A
A/ chicken /Egypt/116AD/2011	L190I S129L	N165H	S	**G**	**P**	T	K	N	T	K	E
A/ chicken /Egypt/118AF/2011	Δ129		D	R	S	T	K	N	A	K	A

The comparison of the amino acids changes with the strain (Gs/Gd/96) and its H5 numbering were used (DQI,...). RBD= receptor-binding domain, Δ = deletion, GS= glycosylation site and AS= antigenic site

[28]. The lack of the potential glycosylation site at HA amino acid positions 158–160 is critical for the H5N1 influenza viruses to bind α-2,6 glycans and to be transmitted in mammalian hosts [17].

The mutations in the antigenic sites and their related immunogenic epitopes play an important role in the evolution of H5N1 hemagglutinin. Analysis of the distribution, frequency and mutation of the positive selection (PS) sites led to the observation that known and newly identified PS sites are mostly distributed within or adjacent to epitope sites [11, 22, 25]. Also, some of the epitopes in the HA are targets for neutralizing antibodies. However, it is important to evaluate whether the antibodies targeting epitopes in other genes such as NA and M2 may facilitate clearance of H5N1 infection in human [52]. Also, the five amino acid substitutions at positions 74, 140, 141, 144 and 162 are primarily involved in antigenic drift, and these mutations alone can reproduce the antigenic vaccine drift observed for the variant 2008 field strain [8].

The proteolytic cleavage site (PCS) also showed marked variation in relation to the first introduction phenotype (PQGERRRKKRGLF) [7], which as still dominant in Egyptian viruses from 2006 to 2011. The most characteristic patterns included three patterns that were present in 2006/2007 season. The mutations in the cleavage site sequence started in some of the 2007 viruses with a change in one amino acid: K346R, E340K or R341K [23]. The presence of a polybasic HACS is the main characteristic of strains with high pathogenicity; however, this is not the sole determinant of virulence in chickens, which is also influenced by additional pathogenicity determinants within the HA itself or other viral proteins [41].

Progressive accumulation of mutations at multiple sites within the H5N1 HA1 molecule enhances antigenic drift and is necessary for increasing the ability of mutants to escape a polyclonal antibody response, as revealed by HI testing [8, 33]. Analysis of the amino acid sequence of the Egyptian 2008 variant H5N1 strain revealed eleven amino

Table 6 Amino acid mutations in HA of Egyptian H5N1 viruses

Strain	P74	D94	D97	H110	S123	F144	A184	Q192	M226	A238
A/goose/Guangdong/1/96										
A/Bar-headedGoose/Qinghai/5/05	P	N	D	H	S	F	A	Q	M	A
A/turkey/Turkey/1/2005	P	N	D	H	S	F	A	Q	M	A
A/chicken/Nigeria/SO227/2006	P	N	D	H	S	F	E	Q	M	A
A/mute swan/France/070203/2007	P	N	D	H	S	F	A	Q	M	A
A/chicken /Egypt/2253-1/2006	P	N	D	H	S	F	A	Q	M	A
A/chicken/Egypt/07118-NLQP/2007	P	N	D	H	S	F	A	Q	I	A
A/geese/Egypt/07364S-NLQP/2007	P	D	D	H	S	F	A	Q	M	A
A/ chicken /Egypt/07201-NLQP/2007	S	N	N	R	P	Y	E	Q	V	A
A/ chicken /Egypt/07665S-NLQP/2007	P	N	D	H	S	F	A	Q	M	A
A/peacock/Egypt/07667S-NLQP/2007	P	D	D	H	S	F	A	Q	M	A
A/chicken/Egypt/0811-NLQP/2008	P	N	D	H	S	F	A	Q	M	A
A/duck/Egypt/0821S-NLQP/2008	P	N	D	H	S	F	A	Q	M	A
A/chicken/Egypt/0879-NLQP/2008	S	N	N	R	P	Y	E	K	V	T
A/chicken/Egypt/08139S-NLQP/2008	S	N	N	R	P	Y	E	Q	V	A
A/duck/Egypt/093L-NLQP/2009	P	N	D	H	S	F	A	Q	M	A
A/turkey/Egypt/0935-NLQP/2009	P	D	D	H	S	F	A	H	M	A
A/chicken/Egypt/0962S-NLQP/2009	P	N	D	H	S	F	A	Q	I	V
A/chicken/Egypt/09359S-NLQP/2009	S	N	N	R	P	Y	E	K	V	V
A/chicken/Egypt/09534S-NLQP/2009	S	N	N	R	P	Y	E	K	V	T
A/chicken/Egypt/096767V-NLQP/2009	S	N	N	R	P	Y	E	K	V	T
A/ chicken /Egypt/1029/2010	P	N	D	H	S	F	A	Q	M	A
A/chicken/Egypt/1022L/2010	P	N	D	H	S	F	A	Q	M	A
A/chicken /Egypt/1031/2010	S	N	N	R	P	Y	E	K	V	V
A/duck/Egypt/10118/2010	P	N	D	H	S	F	A	Q	M	A
A/chicken /Egypt/116AD/2011	S	N	N	R	P	Y	E	K	V	T
A/chicken/Egypt/118AF-2011	P	N	D	H	S	F	A	Q	M	A

The comparison of the amino acids changes with the strain (Gs/Gd/1/96) and its H5 numbering were used (DQI...)

acid substitutions when compared to the extinct parent virus introduced into Egypt in early 2006, and many of these changes occurred in the immunogenic epitopes A, B and D, which are located on the proximal surface of the HA protein, and are therefore likely to provoke an extensive antigenic drift in the H5 protein [26]. The number of amino acid substitutions varied greatly among Egyptian viruses and ranged from 4 to 31 in the HA protein in comparison to the original Egyptian strain (A/chicken/Egypt/2253-1/2006). A higher rate was found in the variant strains, and the number of substitutions usually increases every season, starting with 11 substitutions in 2007/2008 and reaching 31 in the 2009/2010 season in strain A/chicken/Egypt/1031/2010, while the maximum number of substitutions in the classic strains was 14, as reported for strain A/chicken/Egypt/0962S-NLQP/2009 in the 2008/2009 season (Table 2). The mutations that accumulated in the HA antigenic sites will increase the tendency of the viruses to escape vaccine immunity and change the phenotypic character of the viruses. [18, 37], as has also been described for H3N2 human influenza [19, 24, 42]. The mutations present in the variant strains have had a significant impact on vaccine efficacy, as shown in various experimental infection studies [12, 30].

The amino acid mutations located in the globular head of the HA1 protein are close to functional binding sites, and some of these mutations correspond to the A and B sites of H3. These sites contain functional epitopes with high neutralizing efficiency [8]. Some of those mutations seem to be adaptive for the Egyptian environment, as they have not been recorded elsewhere for any H5N1 viruses.

The results from this study indicate that the Egyptian viruses acquired new mutations each season and that the viruses continually adapted to the Egyptian environment. Genetic drift accelerated, as can be clearly seen in Tables 2 and 5, and the number of amino acid substitutions increased each season, with a much higher rate observed in variant viruses [8]. The mutations at antigenic sites (S120N/D, R140G, S141P/L, I151T/V, K152E, N154D, A156T, R162K/I/E and A185E) can affect the immune

Table 7 Variation in the proteolytic cleavage site in Egyptian viruses from 2006 to 2011

A/Bar-headedGoose/Qinghai/5/2005(H5N1)	PQGERRRKKRGLF	No. of isolates
Egypt- 2005 /2006	**PQGERRRKKRGLF**	20
Egypt- 2006 /2007	**PQGERRRKKRGLF**	32
	PQGERRR**R**KRGLF	2
	PQGE**K**RRKKRGLF	2
Egypt- 2007 /2008	**PQGERRRKKRGLF**	37
Egypt- 2008 /2009	**PQGERRRKKRGLF**	27
	PQG**KS**RRKKRGLF	4
	PQGE**G**RRKKRGLF	6
	PQG**DG**RRKKRGLF	1
Egypt- 2009 /2010	**PQGERRRKKRGLF**	30
	PQGE**G**RRRKKRGLF	1
	PQGE**G**RRKKRGLF	11
	PQG**D**RRRKKRGLF	2
	PQGER**K**RKKRGLF	1
	PQ**R**ERRRKKRGLF	1
	PQ**REG**RRKKRGLF	1
Egypt- 2010 /2011	**PQGERRRKKRGLF**	21
	PQGE**K**RRKKRGLF	34
	PQGE**G**RRKKRGLF	2
	PQ**EEG**RRKKRGLF	1

Patterns of amino acids in the proteolytic cleavage site in Egyptian viruses compared with the original Chinese strain A/Bar-headedGoose/Qinghai/5/2005(H5N1) with the number of isolates in each pattern in this study

The amino acid substitutions are indicated in bold and underlined

response to vaccines [27]. One of the most important observations in the 2010/2011 season is that the classic group became widely distributed in the country, causing all human infections, and so became the predominant group. As this virus was always present in the backyard sector, humans were more likely to be exposed to this classic group. These viruses acquired frequent mutations in different parts of the HA protein that may have affected the mechanism of escaping vaccine immunity during the last two seasons (Table 4). However, it was also reported for clinically healthy vaccinated birds in the commercial sector applying multiple vaccination programs, especially in sector 2 of commercial layers and broiler breeders, where the mortality rates had reached an acceptable level for the farmer. This suggests silent spread of viruses in vaccinated birds. It is possible that avian influenza vaccination has resulted in accelerated genetic drift in the HA gene sequence, as has been suggested previously [12, 30].

Analysis of mutations in the NA protein indicates a grouping of Egyptian viruses like the H5 groups, with specific mutations differentiating each group (Table 4). Previous studies identified host-specific markers that discriminate between human influenza viruses and avian influenza viruses [10]. Previous molecular epidemiological studies also highlighted the association between genetic clustering and the area of origin [7]. Phylogenetic analysis of the NA gene and other internal genes gave results similar to those obtained with HA, and this indicates independent evolution of Egyptian viruses each year by acquiring frequent mutations that are very specific to Egypt (Tables 6, 4). Heterogenic strains of H5N1 in an endemic situation like that in Egypt have a very critical role in virus persistence in the country.

Although the variant group declined markedly during the 2010/2011 season, but is not considered extinct yet, the cause for this sudden decline and replacement by the widespread classic group in the commercial sector remains unclear. It may be related to a competitive advantage of the classic group with some adaptive mutations. This replacement is unlikely to be related to vaccine use or improvement in biosecurity in the field.

The recorded cases from the variant group in 2010/2011 were limited to the Delta region, as the three reported cases were from two different governorates (Behera and Dakahlia). However, factors related to a lower level of reporting and insufficient surveillance in the commercial sector should be considered as well as the fact that the unusual interior situation in Egypt during that season affected the normal sequence of the disease outbreak response.

The persistence of virus in the reservoir host plays a critical role in virus maintenance across seasons, especially in ducks, which usually do not show any obvious signs or increased mortality rates, and this allows transmission of the virus to poultry populations and humans.

This study reflects the importance of combining epidemiological data with molecular genetic data and analysis

for circulating viruses to better understand the evolution of H5N1 viruses in nature and to tailor more-effective vaccination and control strategies.

Acknowledgments This work is part of the output of the FAO project, "Vaccine Efficacy for the Control of Avian Influenza in Egypt" (OSRO/EGY/801/USA), implemented in partnership with National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), General Organization of Veterinary Services (GOVS) and South East Poultry Research Laboratory (SEPRL). Laboratory consumables used for the diagnosis of A/H5NI HPAI were obtained from another FAO project "Strengthening Avian Influenza Detection and Response (SAIDR)" (OSRO/EGY/701/USA). Both of these projects were funded by the United States Agency for International Development (USAID). The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the government of the United States of America. The authors would also like to thank Erica Spackman, Filip Claes, Toni Ettel and the OFFLU network through the Food and Agriculture Organization of the United Nations (FAO) for their diverse technical and administrative assistance during project implementation.

References

1. Abdel-Moneim AS, Shany SA, Fereidouni SR, Eid BT, el-Kady MF, Starick E, Harder T, Keil GM (2009) Sequence diversity of the haemagglutinin open reading frame of recent highly pathogenic avian influenza H5N1 isolates from Egypt. Arch Virol 154:1559–1562
2. Abdelwhab EM, Selim AA, Arafa A, Galal S, Kilany WH, Hassan MK, Aly MM, Hafez MH (2010) Circulation of avian influenza H5N1 in live bird markets in Egypt. Avian Dis 54(2):911–914. doi:10.1637/9099-100809
3. Aly MM, Arafa A, Hassan MK (2008) Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. Avian Dis 52:269–277
4. Arafa A, Suarez DL, Hassan MK, Aly MM (2010) Phylogenetic analysis of HA and NA genes of HPAI-H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct sublineages. Avian Dis 54:345–349
5. Auewarakul P, Suptawiwat O, Kongchanagul A, Sangma C, Suzuki Y, Ungchusak K, Louisirirotchanakul S, Lerdsamran H, Pooruk P et al (2007) An avian influenza H5N1 virus that binds to a human-type receptor. J Virol 81:9950–9955
6. Bragstad K, Jørgensen PH, Handberg K, Hammer AS, Kabell S, Fomsgaard A (2007) First introduction of highly pathogenic H5N1 avian influenza a viruses in wild and domestic birds in Denmark, Northern Europe. Virol J 11(4):43
7. Cattoli G, Monne I, Fusaro A, Joannis TM, Lombin LH, Aly MM, Arafa AS, Sturm-Ramirez KM, Couacy-Hymann E, Awuni JA, Batawui KB, Awoume KA, Aplogan GL, Sow A, Ngangnou AC, El Nasri Hamza IM, Gamatie D, Dauphin G, Domenech JM, Capua I (2009) Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. PLoS One 4:e4842
8. Cattoli G, Milani A, Temperton N, Zecchin B, Buratin A, Molesti E, Aly MM, Arafa A, Capua I (2011) Antigenic drift in H5N1 avian influenza in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to human influenza. JVI Accepts, published online ahead of print on 6 July 2011 J. Virol. doi:10.1128/JVI.02403-10
9. Chen H, Smith GJD, Zhang SY, Qn K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y (2005) Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436:191–192
10. Chen GW, Chang SC, Mok CK, Lo YL, Kung YN et al (2006) Genomic signatures of human versus avian influenza a viruses. Emerg Infect Dis 12:1353–1360
11. Duvvuri VR, Duvvuri B, Cuff WR, Wu GE, Wu J (2009) Role of positive selection pressure on the evolution of H5N1 hemagglutinin. Genomics Proteomics Bioinformatics 2009(7):47–56
12. Escorcia M, Vázquez L, Méndez ST, Rodríguez-Ropón A, Lucio E, Nava GM (2008) Avian influenza: genetic evolution under vaccination pressure. Virol J 2008(5):15
13. Fao (2011) EMPRES transboundary. Anim Dis Bull No. 39, p 39–43 Retrieved from. http://www.fao.org/docrep/015/i2530e/i2530e00.pdf
14. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
15. Fusaro A, Nelson MI, Joannis T, Bertolotti L, Monne I, Salviato A, Olaleye O, Shittu I, Sulaiman L, Lombin LH, Capua I, Holmes EC, Cattoli G (2010) Evolutionary dynamics of multiple sublineages of H5N1 influenza viruses in Nigeria from 2006 to 2008. J Virol 84(7):3239–3247
16. Gall-Recule GL, Briand FX, Schmitz A, Guionie O, Massin P, Jestin V (2008) Double introduction of highly pathogenic H5N1 avian influenza virus into France in early 2006. Avian Pathol 37:15–23
17. Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y et al (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog 5:e1000709
18. Grund C, Abdelwhab EM, Arafa A, Ziller M, Hassan MK, Aly MM, Hafez MH, Harder TC, Beer M (2011) Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. Vaccine 29:5567–5573
19. Ha Y, Stevens DJ, Skehel JJ, Wiley DC (2002) H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 21:865–875
20. Hafez MH, Arafa A, Abdelwhab EM, Selim A, Khoulosy SG, Hassan MK, Aly MM (2010) Avian influenza H5N1 virus infections in vaccinated commercial and backyard poultry in Egypt. Poult Sci 89:1609–1613. doi:10.3382/ps.2010-00708
21. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98
22. Hensley SE, Das SR, Bailey AL, Schmidt LM, Hickman HD, Jayaraman A, Viswanathan K, Raman R, Sasisekharan R, Bennink JR, Yewdell JW (2009) Hemagglutinin receptor binding avidity drives influenza a virus antigenic drift. Science 326:734–736
23. Kayali G, Webby RJ, Ducatez MF, El Shesheny RA, Kandeil AM et al (2011) The epidemiological and molecular aspects of influenza H5N1 viruses at the human-animal interface in Egypt. PLoS One 6(3):e17730. doi:10.1371/journal.pone.0017730
24. Kaverin NV, Rudneva IA, Ilyushina NA, Varich NL, Lipatov AS, Smirnov YA, Govorkova EA, Gitelman AK, Lvov DK, Webster RG (2002) Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. J Gen Virol 83:2497–2505
25. Kaverin NV, Rudneva IA, Govorkova EA, Timofeeva TA, Shilov AA, Kochergin-Nikitsky KS, Krylov PS, Webster RG (2007) Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J Virol 81:12911–12917
26. Kilany WH, Abdelwhab EM, Arafa A-S, Selim A, Safwat M, Nawar AA, Erfan AM, Hassan MK, Aly MM, Hafez HM (2010)

Protective efficacy of H5 inactivated vaccines in meat turkey poults after challenge with Egyptian variant highly pathogenic avian influenza H5N1 virus. Vet Microbiol 150(1–2):28–34
27. Kim J, Kayali G, Walker D et al (2010) Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. Proc Natl Acad Sci. doi:10.1073/pnas.1006419107
28. Klenk HD, Wagner R, Heuer D, Wolff T (2002) Importance of hemagglutinin glycosylation for the biological functions of influenza virus. Virus Res 82:73–75
29. Lam TT, Hon CC, Pybus OG, Kosakovsky Pond SL, Wong RT, Yip CW, Zeng F, Leung FC (2008) Evolutionary and transmission dynamics or reassortant H5N1 influenza virus in Indonesia. PLoS Pathog 4:e1000130
30. Lee CW, Senne DA, Suarez DL (2004) Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78:8372–8381
31. Liu J, Xiao H, Lei F, Qin K, Zhang XW, Zhang XL, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang J, Gao GF (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309:1206
32. Monne I, Joannis TM, Fusaro A, De Benedictis P, Lombin LH, Ularamu H, Egbuji A, Solomon P, Obi TU, Cattoli G, Capua I (2008) Reassortant avian influenza virus (H5N1) in poultry, Nigeria, 2007. Emerg Infect Dis 14:637–640
33. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K (1991) Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza a viruses. Virology 182:475–485
34. Pfeiffer J, Pantin-Jackwood M, To TL, Nguyen T, Suarez DL (2009) Phylogenetic and biological characterization of highly pathogenic H5N1 avian influenza viruses (Vietnam 2005) in chickens and ducks. Virus Res 142:108–120
35. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
36. Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Aly MM, Brown IH, Couacy-Hymann E, De Mia GM, Dung do H, Guercio A, Joannis T, Maken Ali AS, Osmani A, Padalino I, Saad MD, Savic V, Sengamalay NA, Yingst S, Zaborsky J, Zorman-Rojs O, Ghedin E, Capua I (2007) Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg Infect Dis 13:713–718
37. Shih AC, Hsiao TC, Ho MS, Li WH (2007) Simultaneous 414 amino acid substitutions at antigenic sites drive influenza a hemagglutinin evolution. Proc Natl Acad Sci USA 104:6283–6288
38. Slomka MJ, Pavlidis T, Banks J, Shell W, McNally A, Essen S, Brown IH (2007) Validated H5 Eurasian real-time reverse transcriptase– polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. Avian Dis 51:373–377
39. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL (2002) Development of a real time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40:3256–3260
40. Starick E, Beer M, Hoffmann B, Staubach C, Werner O, Globig A, Strebelow G, Grund C, Durban M, Conraths FJ, Mettenleiter T, Harder T (2008) Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus. Vet Microbiol 128:243–252
41. Stech O, Veits J, Weber S, Deckers D, Schröer D, Vahlenkamp W, Breithaupt A, Teifke J, Thomas C, Stech J (2009) Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. J Virol 83(11)
42. Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA (2006) Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 312:404–410
43. Suwannakarn K, Amonsin A, Sasipreeyajan J, Kitikoon P, Tantilertcharoen R, Parchariyanon S, Chaisingh A, Nuansrichay B, Songserm T, Theamboonlers A, Poovorawan Y (2009) Molecular evolution of H5N1 in Thailand between 2004 and 2008. Infect Genet Evol 9:896–902
44. Szeleczky Z, Da′n A, Ursu K, Ivanics E, Kiss I, Erde′lyi K, Bela′k S, Muller CP, Brown IH, Ba′lint A (2009) Four different sublineages of highly pathogenic avian influenza H5N1 introduced in Hungary in 2006–2007. Vet Microbiol 139:24–33
45. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
46. Vijaykrishna D, Bahl J, Riley S, Duan L, Zhang JX, Chen H, Peiris JS, Smith GJ, Guan Y (2008) Evolutionary dynamics and emergence of panzootic H5N1 influenza viruses. PLoS Pathog 4:10
47. Watanabe Y, Ibrahim MS, Ellakany HF, Kawashita N, Mizuike R, Hiramatsu H, Sriwilaijaroen N, Takagi T, Suzuki Y, Ikuta K (2011) Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. PLoS Pathog 7:19
48. WHO/OIE/FAO H5N1 Evolution Working Group. (2008) Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg Infect Dis 14:e1
49. WHO/OIE/FAO H5N1 Evolution Working Group (2009) Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. Influenza Other Respir Viruses 3:59–62
50. WHO/OIE/FAO H5N1 Evolution Working Group (2012) World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization. H5N1 Evolution Working Group. 2011. Letter to the editor: continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza Other Respir Viruses 6:1–5
51. Xu X, Subbarao K, Cox NJ, Guo Y (1996) Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 224:175–183
52. Yen H-L, Peiris JSM (2009) Mapping antibody epitopes of the avian H5N1 influenza virus. PLoS Med 6(4):3

Vaccine 30 (2012) 3097–3105

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Vaccination and acute phase mediator production in chickens challenged with low pathogenic avian influenza virus; novel markers for vaccine efficacy?

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ARTICLE INFO

Article history:
Received 1 November 2011
Received in revised form 7 February 2012
Accepted 20 February 2012
Available online 3 March 2012

Keywords:
Avian influenza
Low pathogenic
Vaccine efficacy
Alpha-1 acid glycoprotein
Prostaglandin E_2 and ovotransferrin

ABSTRACT

Methods to determine vaccine efficacy of low pathogenic avian influenza (LPAI) isolates are limited in poultry because experimental infections with LPAI virus in specific pathogen free chickens rarely causes clinical disease. The most commonly used method to compare LPAI vaccine efficacy is to quantify viral shedding after challenge, but it is time and labor intensive. Therefore, we sought alternative methods to demonstrate vaccine efficacy, and examined whether vaccination of chickens affected the production of acute phase mediators in serum (e.g., alpha-1 acid glycoprotein, ovotransferrin and prostaglandin E_2) following challenge with homologous (A/Chicken/Hidalgo/232/94 H5N2) or heterologous (A/Chicken/167280-4/02 H5N3) LPAI isolates. Vaccination significantly reduced oropharyngeal viral shedding, and serum concentration of acute phase mediators, regardless of whether homologous or heterologous challenge viruses were used. Examining the expression of acute phase mediators post-challenge may serve as additional markers to determine LPAI vaccine efficacy in chickens.

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1. Introduction

Avian influenza viruses are typically defined by the pathology they cause after an intravenous challenge of 4–6 week old specific pathogen free (SPF) chickens [1]. Highly pathogenic avian influenza (HPAI) viruses, by definition, kill 75% or more of chickens challenged with virus within 10 days, and low pathogenic viruses kill fewer birds. Typically a stark contrast is seen between low pathogenic avian influenza (LPAI) and HPAI viruses, because most LPAI viruses cause mild or no clinical disease experimentally in SPF chickens. Despite the lack of mortality experimentally, LPAI viruses can be important pathogens causing diseases of economic importance including respiratory infections, sinusitis, drops in egg production, and in the field when co-infection with other pathogens, poor environmental conditions, or secondary bacterial infections occur, high mortality may result [1].

The control of LPAI uses methods similar for the control of HPAI, such as increased biosecurity, quarantines and limiting animal movement, but can include stamping out procedures by depopulating flocks infected or exposed to the virus. The use of killed whole virus oil adjuvanted LPAI vaccines has also been used as a control strategy, but vaccination for avian influenza is more difficult than for most other viruses because of the antigenic variation that requires the knowledge of the circulating subtype and the consideration for issues of antigenic drift. Determining the efficacy of LPAI vaccines in chickens is enigmatic, because most LPAI isolates cause mild clinical disease in experimentally infected SPF chickens. Thus, the industry standards for vaccine efficacy with HPAI, morbidity and mortality, cannot be used when testing LPAI vaccines. The most common approach for comparing LPAI vaccines is to quantify oropharyngeal and/or cloacal shedding of LPAI isolates which requires the inoculating of embryonating chicken eggs and calculating of the egg infectious 50% dose (EID_{50}) or the use of quantitative real-time RT-PCR. The former uses large numbers of embryonating eggs and is time consuming, and the latter requires costly specialized technology and skilled labor to perform. Although quantitation of viral shedding provides an indication of protection and histological or immunohistochemical examination of tissues may provide some indication of vaccine efficacy [2], additional methods are needed to evaluate LPAI vaccine efficacy for use in the poultry industry. One potential approach is the measurement of acute phase mediators.

Soon after being infected by a virus, host cells respond by producing inflammatory mediators such as cytokines, prostaglandin metabolites (e.g., eicosanoids), nitric oxide and others [3]. Some of these mediators, especially pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and TNFα [4–8], act upon the hepatocytes and induce production of a diverse group of serum proteins called acute phase proteins (APP) [9–11]. Through generally thought of as non-specific markers of inflammation, serum APP levels are used as an indicator of health of humans [12] and veterinary species [13]. Acute phase proteins are divided into negative or positive groups

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2012.02.055

based on their relative level of production during or after acute inflammation. Negative APPs are expressed as basal levels in serum in the absence of inflammation, and their protein serum levels are decreased after producing inflammation in the host. Transferrin, transthyretin, retinol binding protein, albumin and cotrisol binding globulin are negative APPs in mammals [14,15]. In contrast, positive APP serum protein levels rapidly increase after exposure to an inflammatory agent [16]. Serum amyloid A, C reactive protein (CRP), alpha-1 acid glycoprotein (AGP), haptoglobin, cerumoplasmin and fibrinogen are positive APPs [15]. Ovotransferrin (OVT), the avian homologue of transferrin, is an exception and behaves as a positive APP in chickens [17]. During influenza infection, prostaglandin metabolites, such as PGE_2, are released from infected cells [18–20], which may have immunomodulatory effects on the acquired immune response [21]. Pro-inflammatory cytokines IL-1β and TNFα also act upon the hypothalamus, which causes an increase in PGE_2 synthesis and pyrexia [22,23]. Thus, PGE_2 is linked to the production of pro-inflammatory cytokines and APP during acute inflammation.

The goal for vaccines is induce specific protection in the host that prevents clinical disease and ideally prevents or at least greatly reduces pathogen replication to help block the transmission chain. A protective immune response from an efficacious vaccine would likely reduce the host inflammatory response after challenge, which likely affects the production of acute phase mediators. In chickens, previous reports document that positive APP increase in concentration acutely after causing inflammation [24,25]; the serum concentration of AGP or OVT were acutely elevated in chickens treated with LPS, croton oil or a variety of bacteria and viruses [17,26–31]. Administration of pro-inflammatory compounds to chickens (e.g., LPS) caused production of PGE_2 and pyrexia [32,33].

In these experiments, we sought to determine if vaccination affected serum levels of acute phase mediators (e.g., AGP, OVT or the eicosanoid metabolite PGE_2) following challenge by homologous or heterologous LPAI isolates. Results of the present study indicate that vaccination of chickens with LPAI significantly reduced the production of acute phase mediators following homologous or heterologous challenge, which correlated with viral shedding. Monitoring the production of acute phase mediators may be an additional tool to help define an efficacious LPAI vaccine in chickens.

2. Materials and methods

2.1. Animals

Four week old white leghorn chickens from the Southeast Poultry Research Laboratory (SEPRL) SPF flocks were housed in a biosafety level 3+ Ag animal facilities prior to challenge with different LPAI viruses. Care for chickens was provided, as required by the SEPRL institutional animal care and use committee.

2.2. Viruses

Stock cultures of LPAI A/chicken/Hidalgo/232/94 H5N2 (Ck/Hidalgo) [34] and LPAI A/chicken/Texas/167280-4/02 (Ck/Tx) H5N3 [35] were maintained in the virus repository at SEPRL. The 50% egg infectious (EID_{50}) titer of the challenge inocula was calculated using the method of Reed and Muench [36].

2.3. Vaccination and challenge

Twenty 4 week old white leghorn SPF chickens were injected subcutaneously once under the left wing with 0.5 mL of a commercially available inactivated oil emulsion Ck/Hidalgo vaccine (Volvac AI KV; Boheringer Ingelheim Vetmedica, Guadalajara, Mexico). In contrast, twenty 4 week old unvaccinated white leghorn SPF chickens served as negative controls. One day prior to challenge, chickens were bled via the brachial vein and serum was harvested and stored at −70 °C prior to analysis of hemagglutinin inhibition (HI) activity against vaccine virus and acute phase mediators described below. At 6 weeks of age, chickens were inoculated mucosally via the choanal slit with 10^6 EID_{50}/0.2 mL BHI broth of Ck/Hidalgo or Ck/Tx virus. Afterwards, chickens were bled at 12, 24, 48 and 96 h post-infection and blood was allowed to clot for 30 min at room temperature in serum separation tubes. Tubes were centrifuged at 1500 × *g* for 15 min at 4 °C, and serum was stored at −70 °C. For PGE_2 analysis, aliquots of serum were treated with acetylsalicylic acid (final concentration 10 μg/mL) before storage at −70 °C. Chickens were monitored daily for morbidity and mortality for 14 days post-infection. At the termination of the study, chickens were euthanized via cervical dislocation.

2.4. Real time RT-PCR

Oropharyngeal (OP) swabs were sampled from infected birds at days 3, 5 and 7 post-infection and suspended in 1 mL of BHI broth. Samples were stored at −70 °C before total RNA was extracted by using Trizol LS reagent (Invitrogen, Carlsbad, CA). Briefly, 250 μL of OP swab sample, 750 μL of Trizol LS reagent and 200 μL of chloroform (Sigma Chemical Co., St. Louis, MO) were added, briefly vortexed and centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase, containing total RNA, was harvested and viral RNA (vRNA) was purified using the MagMax 96 AI/ND viral RNA isolation kit (Ambion Inc., Austin, TX) with the KingFisher magnetic particle processor equipment (Thermo Fisher Scientific, Inc., Waltham, MA). Quantitative real-time RT-PCR (rRT-PCR) was performed with primers and probe specific for type A AI matrix RNA [37] with the addition of an AI internal positive control [38]. The following PCR cycling protocol was used; 45 cycles of denaturation at 94 °C for 1 s and annealing at 60 °C for 20 s with fluorescence data acquired at the end of each annealing step using Smart Cycler II (Cepheid, Sunnyvale, CA) real time-PCR equipment. Quantitative viral shedding (e.g., number of copies of vRNA recovered from swab/mL of BHI broth) was interpolated from the sample cycle thresholds by using a standard curve generated from known amounts of control Ck/Hidalgo or Ck/Tx RNA (e.g., titers were exactly 10^1 to 10^6 EID_{50}/mL), as described previously [39].

2.5. Hemagglutination inhibition (HI) assay

The HI test was performed to determine the pre- and post-challenge immune response to vaccine and challenge viruses. Briefly, 4 HA units of Ck/Hidalgo or Ck/Tx virus were incubated with twofold diluted serum samples, ranging from 1:2 to 1:4096, prior to addition of 0.5% chicken RBCs [40]. Endpoint HI titers were determined as the reciprocal of the highest serum dilution that produced complete inhibition of hemagglutination activity.

2.6. Determination of acute phase mediator production in serum

2.6.1. Alpha-1 acid glycoprotein (AGP)

Serum concentration of AGP was determined using a commercially available chicken-specific simple radial immunodiffusion (SRID) assay (Cardiotech Services Inc., Louisville, KY). The manufacturer's recommended protocol was followed with the following modifications. Briefly, chicken AGP standards were diluted from 62.5 to 1000 μg/mL. Five μL of standard or serum sample was loaded per well, in duplicate, and incubated 48 h at room temperature in a humidified chamber. The diameter (mm) of the precipitin rings were measured and mean serum AGP values (μg/mL) were interpolated from the standard curve using four parameter logistic

curve fit non-linear regression (GraphPad Prism software v4.03, GraphPad Software Inc., San Diego, CA).

2.6.2. Prostaglandin E_2 (PGE_2)

Serum concentration of PGE_2 was determined using a commercially available competitive enzyme linked immunosorbant assay (ELISA) (Prostaglandin E_2 Parameter Assay Kit; R&D Systems, Minneapolis, MN). The manufacturer's recommended protocol was followed with slight modifications, as indicated below. Aliquots of serum samples stored were treated with acetylsalicylic acid (Sigma; final concentration 10 μg/mL) prior to storage at −70 °C. Samples were thawed diluted 1:10 in the provided buffer. Unknown samples or a standard curve of PGE_2 (39–2500 pg/mL) were analyzed in triplicate. Absorbance at 450 nm corrected for 540 nm was measured using a Synergy HT plate reader (BioTek Instruments Inc.; Winooski, VT). Mean serum PGE_2 concentration (pg/mL) was interpolated from a PGE_2 standard curve, using a four parameter logistic curve fit non-linear regression (GraphPad Prism software v4.03).

2.6.3. Ovotransferrin (OVT)

Serum concentration of OVT was determined using a competitive ELISA, as described previously [17]. Absorbance at 450 nm was measured using a Synergy HT plate reader (BioTek Instruments Inc.). One hundred μL of substrate solution in a blank well was used to determine background and was subtracted from sample values prior to analysis. Mean serum OVT concentration (μg/mL) was interpolated from a chicken OVT standard curve (0–64 μg/mL), using a four parameter logistic curve fit non-linear regression (GraphPad Prism software v4.03).

2.7. Sequence analysis

A comparison of amino acid sequence identity of different HA1 antigens was determined using Megalign v5.08 software (DNA Star, Madison, WI) with a Clustal W algorithm. Accession numbers and sequence data for HA1 genes are available upon request.

2.8. Statistical analysis

Data were analyzed using Prism v4.03, and are expressed as arithmetic mean ± standard deviation (S.D.) from ten chickens per experimental group. One way analysis of variance (ANOVA) with Tukey post-test was used to analyze acute phase mediator production and viral shedding data. A paired *t*-test was used to analyze HI data. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Immune response to vaccination

Twenty 4 week-old SPF chickens were administered a single dose of inactivated oil emulsified Ck/Hidalgo vaccine subcutaneously and their HI immune response 2 weeks later was evaluated using two different antigens, the homologous vaccine strain and heterologous virus, Ck/Tx, that was also used as one of the challenge viruses. The arithmetic mean titer was highest using the CK/Hidalgo virus as antigen, 85 ± 9 (arithmetic mean ± S.D.), and 34 ± 5 with the CK/TX antigen (Table 1A).

3.2. Viral challenge and shedding

Groups of 10 vaccinated or unvaccinated birds were mucosally challenged with 10^6 EID_{50} of either Ck/Hidalgo or Ck/Tx LPAI viruses, which are homologous and heterologous in reference to the vaccine strain. The amino acid sequence identity of the HA protein from homologous or heterologous challenge viruses was 90.4%. As expected no morbidity or mortality was seen in vaccinated or control birds with either challenge virus up to 14 days post-challenge. Sampling the oropharynx for AI matrix RNA post-challenge demonstrated that vaccination with Ck/Hidalgo significantly reduced ($P \leq 0.01$) virus shedding at days 3 and 5 post-infection from homologous challenged chickens as compared to unvaccinated controls (Fig. 1). No AI matrix vRNA was detected from vaccinated chickens challenged with homologous virus at day 7 post-infection, suggesting that the infection was cleared by this date. Likewise, vaccination significantly reduced ($P \leq 0.01$) shedding of heterologous Ck/Tx vRNA at days 3, 5 and 7 post-infection, as compared to unvaccinated controls (Fig. 1).

3.3. Post-challenge HI titers

At the end of the study, day 14 post-infection, serum was harvested and post-infection HI titers were again performed using homologous and heterologous antigens. Vaccinated and homologously challenged chickens had significantly different HI titers, as compared to unvaccinated and challenged chickens, and the same was true for HI titers when cross titering with heterologous antigen (Ck/Tx H5) (Table 1B). Vaccinated and heterologously challenged chickens had significantly different HI titers as compared to chickens vaccinated with Ck/Hidalgo H5, and the same was true for HI titers when cross titering with homologous antigen (Table 1C). A statistical analysis of HI titers from vaccinated or unvaccinated chickens that were homologously or heterologously challenged,

Table 1
Serum HI titers from groups of chickens with or without vaccination with Ck/Hidalgo and prior to challenge (A), and after challenge with Ck/Hildalgo virus (B) or Ck/TX virus (C). Serum samples were titered using homologous (H5N2 Ck/Hidalgo) or heterologous (H5N3 Ck/TX) antigens. Data are expressed as arithmetic mean HI titer ± S.D. from groups of chickens. Student's *t*-test was used to evaluate statistical significance ($P < 0.05$) of HI response between vaccinated and unvaccinated groups.

	Challenge Virus	HI titer Antigen	
		H5N2 (Ck/Hidalgo)	H5N3 (Ck/Tx)
A			
Ck/Hidalgo vaccine	None (pre-challenge)	85 ± 9	34 ± 5
No vaccine control	None (pre-challenge)	4 ± 1	3 ± 2
P value		<0.0001	<0.0001
B			
Ck/Hidalgo vaccine	Ck/Hidalgo	269 ± 45	51 ± 5
No vaccine control	Ck/Hidalgo	461 ± 34	102 ± 20
P value		0.0341	0.0308
C			
Ck/Hidalgo vaccine	Ck/Tx	93 ± 12	166 ± 26
No vaccine control	Ck/Tx	230 ± 17	435 ± 39
P value		0.0001	0.0002

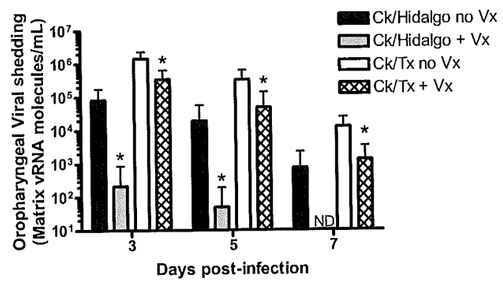

Fig. 1. Vaccination significantly reduces oropharyngeal shedding of low pathogenic avian influenza virus. Chickens were challenged with 10^6 EID_{50}/0.2 mL A/chicken/Hidalgo/232/94 (Ck/Hidalgo) or A/chicken/Texas/167280-4/02 (Ck/Tx) with or without vaccination (Vx) with inactivated Ck/Hidalgo virus. Viral shedding from the oropharynx was determined at days 3, 5 and 7 post-infection using rRT-PCR specific for avian influenza matrix vRNA. Data represent the mean ± S.D. AI matrix vRNA molecules from ten chickens per group. * = $P \leq 0.01$ when comparing no Vx and + Vx data per challenge virus group (ND = none detected).

comparing the two antigens, revealed significant ($P \leq 0.01$) differences in all examples (Table 1B and C). These data confirm the specificity and sensitivity of the HA antigens used in this study.

3.4. Production of acute phase mediator – alpha-1 acid-glycoprotein (AGP)

The serum production of AGP in chickens with or without vaccination was evaluated before challenge, and showed no significant change (Fig. 2A). Infection with either LPAI virus caused serum levels of AGP to increase up to 48 h post-infection, where vaccination significantly reduced ($P < 0.001$) serum levels of AGP, as compared to unvaccinated controls for both challenge viruses (Fig. 2D). Regardless of challenge virus, the serum level of AGP did not significantly differ. By 96 h post-infection, serum levels of AGP had reduced almost to baseline levels seen pre-infection.

3.5. Production of acute phase mediator – ovotransferrin (OVT)

The serum production of OVT in chickens with or without vaccination was evaluated before challenge, and showed no significant change (Fig. 3A). Vaccination and challenge with homologous virus significantly reduced the serum level of OVT at 48 ($P < 0.01$) and 96 h ($P < 0.001$) post-infection (Fig. 3D and E), as compared to unvaccinated and challenged chickens. Vaccination and challenge with heterologous virus significantly reduced the serum concentration of OVT at 48 h ($P < 0.05$) and at 96 h ($P < 0.001$) post-infection (Fig. 3D and E), as compared to unvaccinated and challenged chickens. At the end of the study, serum levels of OVT remained elevated above the concentration detected prior to challenge (Fig. 3E).

3.6. Production of acute phase mediator – prostaglandin E2 (PGE_2)

The serum production of PGE_2 in chickens with or without vaccination was evaluated before challenge, and showed no significant change (Fig. 4A). Serum levels of PGE_2 in chickens challenged with Ck/Hidalgo or Ck/TX peaked at 24 h post-infection. However, chickens vaccinated and challenged with homologous virus (Ck/Hidalgo) showed significantly reduced ($P < 0.001$) serum levels of PGE_2 from 12 to 96 h post-infection, as compared to unvaccinated controls (Fig. 4B–E). Vaccination and challenge with heterologous LPAI virus (Ch/TX) significantly reduced ($P < 0.001$) serum levels of PGE_2 only at 24 and 96 h post-infection (Fig. 4C and E), but not at other time points. Vaccination caused serum levels of PGE_2 to drop to levels seen pre-challenge at 96 h, regardless of challenge virus used (Fig. 4E).

4. Discussion

Determination of vaccine efficacy is difficult for LPAI isolates, because little morbidity and no mortality are typically observed in experimentally challenged SPF chickens. Quantifying viral shedding from the oropharynx of chickens is routinely how efficacy has been determined for vaccines against LPAI isolates. Alternative and preferable simpler methods of determining vaccine efficacy are needed to evaluate LPAI vaccines. The production of acute phase mediators (e.g., APPs and eicosanoid metabolites) in chickens is well established in response to viral, bacterial and other inflammatory stimuli [17,26–31,41,42]. We hypothesized that their production may be affected by an efficacious vaccine, and they may serve as additional markers to evaluate protection.

There are limited studies which have examined whether serum levels of acute phase mediators are affected in human or animal vaccine trials. For example, protective vaccination of cattle with attenuated *Pasteurella multocida* significantly reduced serum amyloid A levels, as compared to control cattle [43]. Recently it was suggested that monitoring production of the APP C reactive protein (CRP), may be beneficial to determine pneumococcal vaccine efficacy in humans [44]. However, a field vaccine trial measuring CRP in Gambia failed to consistently identify pneumococcal vaccinated humans that were protected against clinical disease [45]. The data from that study might be best explained because of the presence of endemic diseases, such as malaria, which caused elevated levels of CRP and lessened the sensitivity of their results. The inclusion of acute phase mediators as additional markers of vaccine efficacy is poorly understood, and appears to be most appropriate in specific pathogen free laboratory animals, such as chickens.

Our studies are the first to indicate that vaccination of chickens with a commercially available avian influenza vaccine significantly reduced serum levels of acute phase mediators following challenge with homologous or heterologous LPAI virus. Overall, their production peaked at different times and PGE_2 was first detected, followed by AGP and OVT. The production of AGP was similar to that from influenza infected mice, whose serum concentration peaked at days 3 and 4 post-infection, respectively [46,47], and rapidly returned to basal levels. In the present studies, AGP peak serum concentration was detected at 48 h post-infection. At the same time, vaccinated chickens had significantly reduced serum AGP compared to unvaccinated controls although the levels were higher than the pre-challenge sample. By 96 h post-infection, chickens vaccinated and homologously challenged with Ck/Hidalgo showed serum AGP that nearly returned to pre-challenge levels. In contrast, vaccinated chickens challenged with heterologous virus had significantly elevated serum AGP levels at 96 h post-infection. It is not clear from these studies how long AGP would remain in the heterologous challenged chickens, but AGP concentration returned to pre-challenge levels from select chickens prior to the termination of the study (data not shown). The amount of viral replication in these different groups may explain these challenge groups. Chickens infected with Ck/Tx were shedding higher levels of virus in the oropharyngeal at day 4 post-infection than those infected with Ck/Hidalgo. This data suggests that elevated AGP in Ck/Tx infected chickens are due to an overall increase in viral replication resulting in more tissue damage.

In our studies, serum concentration of OVT in vaccinated chickens at 96 h post-infection remained significantly elevated above pre-challenge concentration, which differs from the AGP data. In

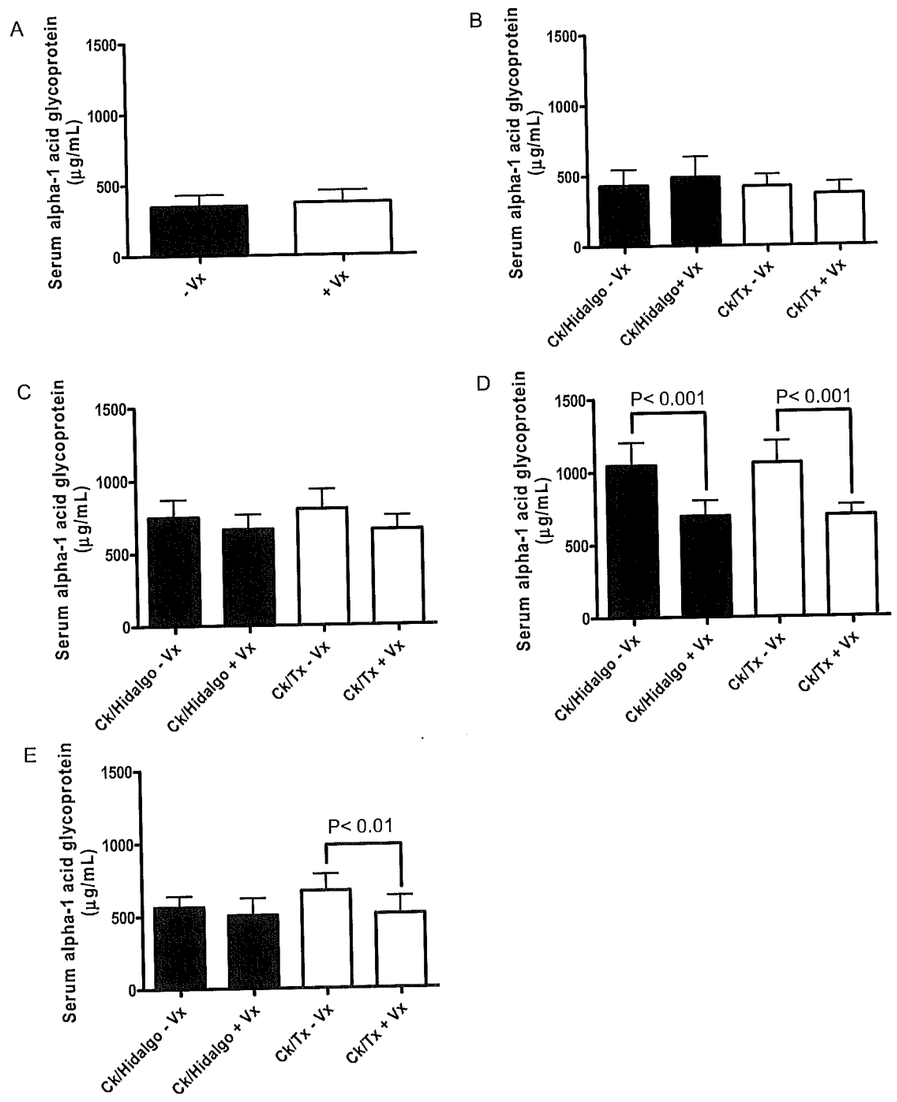

Fig. 2. Temporal changes in serum alpha-1 acid glycoprotein expression following LPAI challenge, with or without vaccination. Chickens were challenged with 10^6 EID_{50}/0.2 mL A/chicken/Hidalgo/232/94 (Ck/Hidalgo; black bars) or A/chicken/Texas/167280-4/02 (Ck/Tx; white bars) with or without vaccination (Vx) with inactivated Ck/Hidalgo virus. Serum was analyzed pre-challenge (A), 12 h (B), 24 h (C), 48 h (D) and 96 h (E) post-infection and analyzed for AGP concentration as described in the materials and methods. Data represent the mean ± S.D. AGP concentration (mg/mL) from ten chickens per group, performed in duplicate.

chickens, it is not known whether the pro-inflammatory stimuli required for the production of OVT differs from AGP. Perhaps cytokines that promote OVT synthesis are more readily produced during LPAI infection. Previous studies examining OVT expression in inflamed chickens were similar to our results, and showed peak serum concentration of OVT 72 h, which remained elevated for up to 120 h [17]. Although we did not determine peak serum concentration on our studies, it is apparent that LPAI-induced OVT expression behaves similar to previous studies in chickens. Peak serum PGE_2 was detected 24 h post-infection and vaccination significantly reduced its production. Because multiple cell types during influenza infection can produce PGE_2, it makes interpretation less straightforward than AGP or OVT data. Peak serum concentration of PGE_2 failed to correspond with peak oropharyngeal viral shedding (day 3 post-infection). Testing for AGP and OVT showed some promise in discriminating between homologous and heterologous challenge viruses. This difference in APP response provides valuable insight as to the level of protection that might be

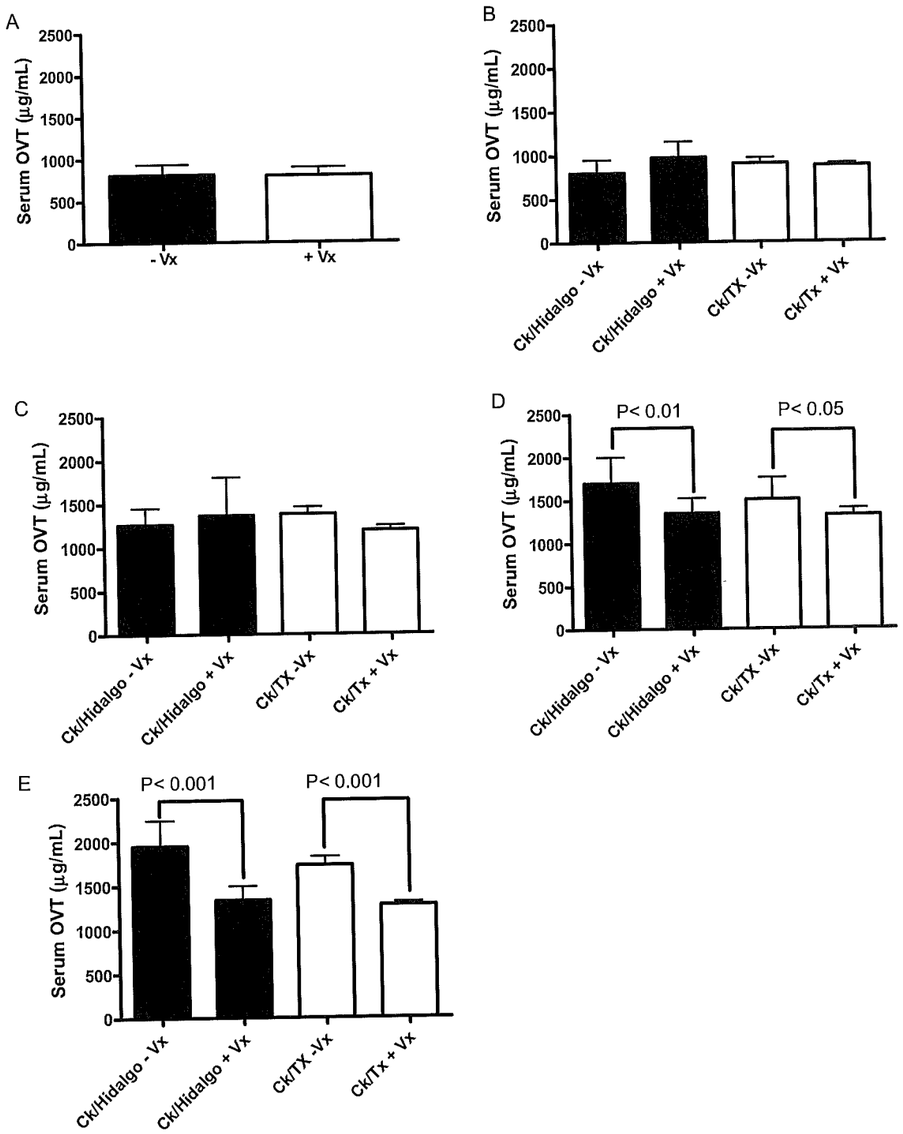

Fig. 3. Temporal changes in serum OVT expression following LPAI challenge, with or without vaccination. Chickens were challenged with 10^6 EID_{50}/0.2 mL A/chicken/Hidalgo/232/94 (Ck/Hidalgo; black bars) or A/chicken/Texas/167280-4/02 (Ck/Tx; white bars) with or without vaccination (Vx) with inactivated Ck/Hidalgo virus. Serum was analyzed pre-challenge (A), 12 h (B), 24 h (C), 48 h (D) and 96 h (E) post-infection and analyzed for OVT concentration as described in the materials and methods. Data represent the mean ± S.D. OVT concentration (µg/mL) from ten chickens per group, performed in triplicate.

observed in the field, and these tests may provide a useful marker to evaluate different levels of protection or decide on the best level of protection.

Although all three acute phase mediators were positively affected by LPAI vaccination, as compared to the challenge control birds, OVT or AGP appear superior to PGE_2 for several reasons. During acute inflammation, pro-inflammatory cytokines act upon the liver to cause rapid increases or decreases in serum APP concentration. Depending on the APP, serum levels may remain elevated or decreased for up to a week, and they are stable molecules that can resist freezing and thawing of serum. In contrast, eicosanoid metabolites are produced by many cells in response to inflammation or viral infection, but localized and intense production may be localized to the hypothalamus or other organs such as

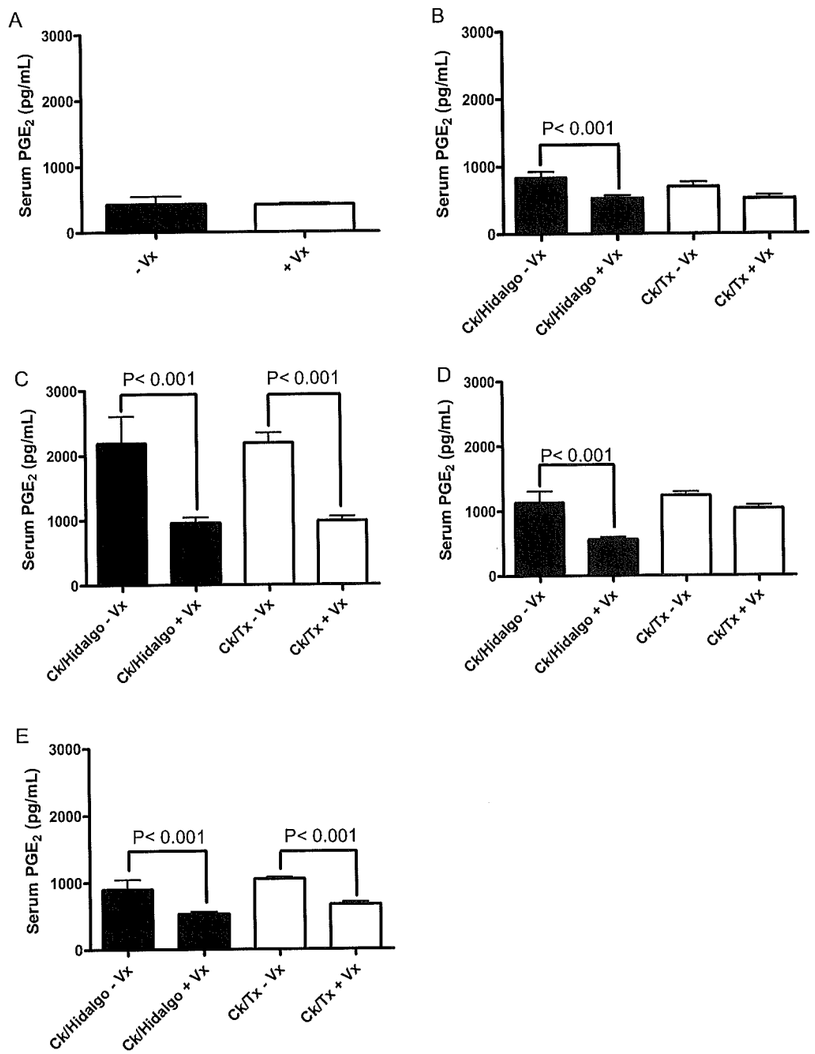

Fig. 4. Temporal changes in serum PGE_2 expression following LPAI challenge, with or without vaccination. Chickens were challenged with 10^6 EID_{50}/0.2 mL A/chicken/Hidalgo/232/94 (Ck/Hidalgo; black bars) or A/chicken/Texas/167280-4/02 (Ck/Tx; white bars) with or without vaccination (Vx) with inactivated Ck/Hidalgo virus. Serum was analyzed pre-challenge (A), 12 h (B), 24 h (C), 48 h (D) and 96 h (E) post-infection and analyzed for PGE_2 concentration as described in the materials and methods. Data represent the mean ± S.D. PGE_2 concentration (pg/mL) from ten chickens per group, performed in triplicate.

lymph nodes. Metabolites produced from these tissues may not be detected from serum samples. To detect eicosanoid metabolites such as PGE_2, serum samples require immediate treatment with a pan-cyclooxygenase inhibitor to prevent its degradation.

In the U.S., veterinary vaccines need to be licensed by the Center for Veterinary Biologics, USDA, and must document safety as well as efficacy. Vaccination for LPAI with killed whole virus adjuvanted vaccines has been used routinely for many years in both chickens and turkeys, however little empirical data are available to support the value of these vaccines. Although considered to be a reliable way to evaluate vaccine efficacy, demonstration of a reduction in virus shedding is not required as part of the licensure procedure. This is due, in part, to the technical requirements needed to obtain reliable virus shedding data from challenge experiments in outbred animals. However, the biggest issue is the lack of identifying a set amount in reduction of virus replication to consider a vaccine efficacious. A proposed goal for HPAI vaccines in chickens is that they produce a minimum a 2 $\log_{10}$ reduction in virus shedding [48]. However, this goal has not been clearly demonstrated as a marker for protection or to significantly reduce viral transmission.

An equally important consideration, particularly based on experiences with HPAI vaccines, is that antigenic drift of challenge strain

from the vaccine has a direct effect on virus shedding, although in many cases this difference in virus shedding does not reflect on morbidity and mortality. Because the vaccines currently available for poultry do not provide sterilizing immunity, a goal of the reducing virus shedding to the greatest extent possible should be the goal so the vaccination can be an effective control tool. The LPAI challenge results from the present study demonstrate that homologous vaccination reduces virus shedding more than the heterologous challenge. Attempts should be made to closely match the amino acid identity of the vaccine HA subtype with circulating LPAI isolates to produce maximal reduction in oropharyngeal viral shedding. In conclusion, we demonstrated that vaccination and homologous or heterologous challenge with LPAI significantly reduced the serum concentration of acute phase mediators, as well as decreasing the amount and duration of oropharyngeal viral shedding. These data suggest that combining detection of serum levels of acute phase mediators after challenge with measuring LPAI viral shedding may be a better method to determine LPAI vaccine efficacy. However, additional acute phase mediators, especially negative APPs, should be investigated to determine whether they are beneficial to help determine efficacy.

Acknowledgements

We thank Suzanne DeBlois, Aniko Zsak, Joan Beck and Roger Brock at SEPRL for their technical assistance. Mention of trade names or commercial products is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References

[1] Swayne DE, Halvorson DA. Influenza. In: Saif YM, editor. Diseases of poultry. 11th ed. Iowa State Press; 2003.
[2] Lierz M, Hafez HM, Klopfleisch R, Luschow D, Prusas C, Teifke JP, et al. Protection and virus shedding of falcons vaccinated against highly pathogenic avian influenza A virus (H5N1). Emerg Infect Dis 2007;13(Nov (11)): 1667–74.
[3] Gruys E, Snel FW. Animal models for reactive amyloidosis. Baillieres Clin Rheumatol 1994;8(Aug (3)):599–611.
[4] Baumann H, Gauldie J. Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. Mol Biol Med 1990;7(Apr (2)):147–59.
[5] Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J 1990;265(Feb 1 (3)):621–36.
[6] Klasing KC. Effect of inflammatory agents and interleukin 1 on iron and zinc metabolism. Am J Physiol 1984;247(Nov (5 Pt 2)):R901–4.
[7] Marinkovic S, Jahreis GP, Wong GG, Baumann H. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins in vivo. J Immunol 1989;142(Feb 1 (3)):808–12.
[8] Dinarello CA. Interleukin-1 and its biologically related cytokines. Adv Immunol 1989;44:153–205.
[9] Thomas T, Schreiber G. Acute-phase response of plasma protein synthesis during experimental inflammation in neonatal rats. Inflammation 1985;9(Mar (1)):1–7.
[10] Dinarello CA. Pathogenesis of fever during hemodialysis. Contrib Nephrol 1983;36:90–9.
[11] Fleck A. Clinical and nutritional aspects of changes in acute-phase proteins during inflammation. Proc Nutr Soc 1989;48(Sep (3)):347–54.
[12] Blackburn Jr WD. Validity of acute phase proteins as markers of disease activity. J Rheumatol 1994;42(Oct):9–13.
[13] Gruys E, Toussaint MJ, Niewold TA, Koopmans SJ, van Dijk E, Meloen RH. Monitoring health by values of acute phase proteins. Acta Histochem 2006;108(3):229–32.
[14] Ingenbleek Y, Young V. Transthyretin (prealbumin) in health and disease: nutritional implications. Annu Rev Nutr 1994;14:495–533.
[15] Gruys E, Toussaint MJ, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B 2005;6(Nov (11)):1045–56.
[16] Kushner I, Gewurz H, Benson MD. C-reactive protein and the acute-phase response. J Lab Clin Med 1981;97(Jun (6)):739–49.
[17] Xie H, Newberry L, Clark FD, Huff WE, Huff GR, Balog JM, et al. Changes in serum ovotransferrin levels in chickens with experimentally induced inflammation and diseases. Avian Dis 2002;46(Jan–Mar (1)):122–31.
[18] Kurokawa M, Imakita M, Kumeda CA, Shiraki K. Cascade of fever production in mice infected with influenza virus. J Med Virol 1996;50(Oct (2)):152–8.
[19] Mizumura K, Hashimoto S, Maruoka S, Gon Y, Kitamura N, Matsumoto K, et al. Role of mitogen-activated protein kinases in influenza virus induction of prostaglandin E2 from arachidonic acid in bronchial epithelial cells. Clin Exp Allergy 2003;33(Sep (9)):1244–51.
[20] Carey MA, Bradbury JA, Seubert JM, Langenbach R, Zeldin DC, Germolec DR. Contrasting effects of cyclooxygenase-1 (COX-1) and COX-2 deficiency on the host response to influenza A viral infection. J Immunol 2005;175(Nov 15 (10)):6878–84.
[21] Sijben JW, Schrama JW, Nieuwland MG, Parmentier HK. Immunomodulatory effects of indomethacin and prostaglandin E2 on primary and secondary antibody response in growing layer hens. Poult Sci 2000;79(Jul (7)):949–55.
[22] Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 2004;10(4):201–22.
[23] Dinarello CA, Gatti S, Bartfai T. Fever: links with an ancient receptor. Curr Biol 1999;9(Feb 25 (4)):R147–50.
[24] Gruys E, Toussaint MJ, Upragarin N, Van EA, Adewuyi AA, Candiani D, et al. Acute phase reactants, challenge in the near future of animal production and veterinary medicine. J Zhejiang Univ Sci B 2005;6(Oct (10)):941–7.
[25] Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet J 2004;168(Jul (1)):28–40.
[26] Takahashi K, Miyake N, Ohta T, Akiba Y, Tamura K. Changes in plasma alpha 1-acid glycoprotein concentration and selected immune response in broiler chickens injected with *Escherichia coli* lipopolysaccharide. Br Poult Sci 1998;39(Mar (1)):152–5.
[27] Xie H, Huff GR, Huff WE, Balog JM, Holt P, Rath NC. Identification of ovotransferrin as an acute phase protein in chickens. Poult Sci 2002;81(Jan (1)): 112–20.
[28] Nakamura K, Imai K, Tanimura N. Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. J Comp Pathol 1996;114(Jan (1)):11–21.
[29] Nakamura K, Mitarai Y, Yoshioka M, Koizumi N, Shibahara T, Nakajima Y. Serum levels of interleukin-6, alpha1-acid glycoprotein, and corticosterone in two-week-old chickens inoculated with *Escherichia coli* lipopolysaccharide. Poult Sci 1998;77(Jun (6)):908–11.
[30] Inoue M, Satoh W, Murakami H. Plasma alpha 1-acid glycoprotein in chickens infected with infectious bursal disease virus. Avian Dis 1997;41(Jan–Mar (1)):164–70.
[31] Holt PS, Gast RK. Comparison of the effects of infection with *Salmonella enteritidis*, in combination with an induced molt, on serum levels of the acute phase protein, alpha1 acid glycoprotein, in hens. Poult Sci 2002;81(Sep (9)):1295–300.
[32] Macari M, Furlan RL, Gregorut FP, Secato ER, Guerreiro JR. Effects of endotoxin, interleukin-1 beta and prostaglandin injections on fever response in broilers. Br Poult Sci 1993;34(Dec (5)):1035–42.
[33] Fraifeld V, Blaicher-Kulick R, Degen AA, Kaplanski J. Is hypothalamic prostaglandin E2 involved in avian fever? Life Sci 1995;56(Mar 10 (16)):1343–6.
[34] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 2004;78(Aug (15)):8372–81.
[35] Lee CW, Senne DA, Linares JA, Woolcock PR, Stallknecht DE, Spackman E, et al. Characterization of recent H5 subtype avian influenza viruses from US poultry. Avian Pathol 2004;33(Jun (3)):288–97.
[36] Pizzi M. Sampling variation of the fifty percent end-point, determined by the Reed-Muench (Behrens) method. Hum Biol: Int Rec Res 1950;22(Sep (3)):151–90.
[37] Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 2002;40(Sep (9)):3256–60.
[38] Das A, Spackman E, Senne D, Pedersen J, Suarez DL. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. J Clin Microbiol 2006;44(Sep (9)):3065–73.
[39] Sylte MJ, Hubby B, Suarez DL. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine 2007;25(May 10 (19)):3763–72.
[40] Swayne DE, Senne DA, Beard CW. In: Swayne DE, editor. A laboratory manual for the isolation and identification of avian pathogens. Kennett Square, PA: American Association of Avian Pathologists; 1998. p. 150–5.
[41] Adler KL, Peng PH, Peng RK, Klasing KC. The kinetics of hemopexin and alpha1-acid glycoprotein levels induced by injection of inflammatory agents in chickens. Avian Dis 2001;45(Apr–Jun (2)):289–96.
[42] Takahashi K, Orihashi M, Akiba Y. Dietary L-arginine level alters plasma nitric oxide and apha-1 acid glycoprotein concentrations, and splenocyte proliferation in male broiler chickens following *Escherichia coli* lipopolysaccharide injection. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1999;124(Nov (3)):309–14.
[43] Hodgson JC, Finucane A, Dagleish MP, Ataei S, Parton R, Coote JG. Efficacy of vaccination of calves against hemorrhagic septicemia with a live aroA derivative of *Pasteurella multocida* B:2 by two different routes of administration. Infect Immun 2005;73(Mar (3)):1475–81.
[44] Madhi SA, Heera JR, Kuwanda L, Klugman KP. Use of procalcitonin and C-reactive protein to evaluate vaccine efficacy against pneumonia. PLoS Med 2005;2(2).
[45] Cheung YB, Zaman SM, Ruopuro ML, Enwere G, Adegbola RA, Greenwood B, et al. C-reactive protein and procalcitonin in the evaluation of the efficacy of

a pneumococcal conjugate vaccine in Gambian children. Trop Med Int Health 2008;13(May (5)):603–11.
[46] Sidwell RW, Wong MH, Bailey KW, Barnard DL, Jackson MK, Smee DF. Utilization of alpha-1-acid glycoprotein levels in the serum as a parameter for in vivo assay of influenza virus inhibitors. Antivir Chem Chemother 2001;12(Nov (6)):359–65.
[47] Wong W, Aoki FY, Friesen AD, Sitar DS, Weselake RJ. Effect of acute experimental influenza A virus pneumonia on concentration of alpha 1-acid glycoprotein in mouse serum. Inflammation 1989;13(Dec (6)):659–72.
[48] Suarez DL, Lee CW, Swayne DE. Avian influenza vaccination in North America: strategies and difficulties. Dev Biol (Basel) 2006;124:117–24.

AVIAN DISEASES 56:555–560, 2012

Protection from Clinical Disease Against Three Highly Virulent Strains of Newcastle Disease Virus After *In Ovo* Application of an Antibody–Antigen Complex Vaccine in Maternal Antibody–Positive Chickens

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Received 11 January 2012; Accepted 6 May 2012; Published ahead of print 7 May 2012

SUMMARY. Worldwide, Newcastle disease (ND) remains one of the most economically important diseases of poultry. Current vaccination strategies for commercial poultry include the use of inactivated and live ND vaccines that typically induce protection against virulent field viruses. Here, we tested the efficacy of an antigen–antibody complex (AAC) ND vaccine delivered *in ovo*. Commercial maternal antibody–positive broiler chickens (*Gallus domesticus*) were vaccinated *in ovo* with an AAC vaccine composed of live B1-LaSota Newcastle disease virus (NDV) complexed with NDV-specific antiserum, and then they were challenged at weekly intervals after hatch. Challenge viruses included three exotic ND disease (END) viruses: the neurotropic strain Texas GB NDV-92-01 (TxGB) and two viscerotropic isolates, one isolate from the 2002–2003 outbreak in California (California 2002 isolate S212676 [CA]) and the other isolate from a 1997 END outbreak in South Korea (South Korea 94-147 [SK]). Results demonstrate that maternal antibody was able to provide approximately 50% protection in either vaccinated or control chickens at 7 days of age after TxGB challenge. However, with challenge at ≥14 days, most control birds died, whereas all AAC-vaccinated birds were protected. Challenge with the CA or SK viruses in chickens at 28 days of age resulted in 100% protection of vaccinated birds, whereas all control birds died. In addition, AAC-vaccinated birds displayed decreased incidence of viral shedding in oral and cloacal swabs than control birds. Antibody titers were significantly ($P < 0.05$) higher in vaccinated chickens, as determined by enzyme-linked immunosorbent assay and hemagglutinin-inhibition tests, than in nonvaccinated controls. Together, these results demonstrate the efficacy of AAC vaccines delivered *in ovo* to protect commercial poultry.

RESUMEN. Protección contra la enfermedad clínica inducida por tres cepas altamente virulentas del virus de la enfermedad de Newcastle después de la aplicación *in ovo* de una vacuna con base en complejos antígeno-anticuerpo en pollos con inmunidad materna.

La enfermedad de Newcastle sigue siendo una de las enfermedades económicamente más importantes en la avicultura a nivel mundial. Las estrategias actuales de vacunación para las aves comerciales incluyen el uso de vacunas inactivadas y vivas que normalmente inducen protección contra los virus de campo virulentos. En este trabajo, se analizó la eficacia de una vacuna aplicada *in ovo* basada en complejos antígeno-anticuerpo contra la enfermedad de Newcastle. Se vacunaron pollos de engorde (*Gallus domesticus*) por vía *in ovo* y que tenían anticuerpos maternos con una vacuna compuesta de complejos inmunes conteniendo un virus vivo de la enfermedad de Newcastle cepa B1-Sota adicionada con un antisuero específico, y luego fueron desafiados a intervalos semanales después de la eclosión. El desafío de los virus incluyó tres virus de la enfermedad de Newcastle exóticos: la cepa neurotrópica Texas GB NDV-92-01 (TxGB) y dos cepas viscerotrópicas, un aislamiento del brote en California durante loa años 2002–2003 (aislamiento de California 2002, S212676 [CA]) y otro aislamiento de un brote a finales de 1997 en Corea del Sur (Corea del Sur 94-147 [SK]). Los resultados demuestran que los anticuerpos maternos fueron capaces de proporcionar aproximadamente el 50% de protección, ya sea en los pollos vacunados o en los controles a los 7 días de edad después de la exposición con la cepa TxGB. Sin embargo, con los desafíos los ≥ 14 días, la mayoría de las aves del grupo control murieron, mientras que todas las aves vacunadas con la vacuna con complejos inmunes estuvieron protegidas. El desafío con las cepas CA o SK en los pollos a los 28 días de edad indujo una protección del 100% en las aves vacunadas, mientras que todas las aves de control murieron. Además de las aves vacunadas con la vacuna de complejos inmunes mostraron menor incidencia para la excreción del virus en las muestras orales y cloacales en comparación con las aves del grupo control. Los títulos de anticuerpos fueron significativamente ($P < 0.05$) mayores en los pollos vacunados, según lo determinado por el ensayo de inmunoabsorción con enzimas ligadas y por las pruebas de inhibición de la hemaglutinación, en comparación con los controles no vacunados. En conjunto, estos resultados demuestran la eficacia de las vacunas por complejos inmunes aplicadas *in ovo* para proteger aves de corral comerciales.

Key words: Newcastle disease, antigen–antibody complex, vaccine

Abbreviations: AAC = antigen–antibody complex; Ag = agriculture; BSL = biosafety level; CA = California 2002 isolate S212676; EID = egg infective dose; ELISA = enzyme-linked immunosorbent assay; END = exotic Newcastle disease; HI = hemagglutination inhibition; ICPI = intracerebral pathogenicity index; ND = Newcastle disease; NDV = Newcastle disease virus; pc = postchallenge; SK = South Korea 94-147; TxGB = Texas GB NDV-92-01

Newcastle disease (ND) is a disease of chickens (*Gallus domesticus*) of economic importance throughout the world, impacting foreign trade. The highly virulent form of the virus is endemic in numerous countries and produces a lethal disease characterized by severe respiratory illness, conjunctivitis, neuropathy, enteritis, or a

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combination. Although ND is readily controlled by low-virulence live vaccines in countries where the field viruses are mildly virulent, these live vaccines are often not sufficient to induce full protection against a highly virulent challenge (15).

ND virus (NDV) belongs in the *Avulavirus* genus, within the *Paramyxoviridae* family. NDV isolates can be classified by pathogenicity as lentogenic, mesogenic, or velogenic, depending on the severity of disease produced by the isolate in chickens (1). The occurrence of virulent NDV infections because of mesogenic or velogenic strains is recognized as ND, a notifiable disease reportable to the Office of International Epizooties (21). The term exotic ND (END) has been used to describe the notifiable disease in the United States. Virulent NDV isolates have entered the United States via illegal importation of birds, including psittacine birds (9,22,28), the causal agents of the last two major outbreaks in the United States in southern California during 1971–1973 and 2002–2003 (18,19,20,23,26,29). Outbreaks of virulent NDV have been reported from turkeys (*Meleagris gallopavo*) in North Dakota during 1992 (16), cormorants (*Phalacrocorax* spp.) in the north central United States (3,25,27), and game birds in California during 1998, underscoring the threat of virulent NDV to commercial U.S. poultry production (6).

Commercial vaccination programs for NDV in North America and western Europe include the use of low-virulent live-virus vaccines and inactivated vaccines designed to control against endemic, low-virulence field strains. For broilers, the goal of these vaccination programs is to induce protective immunity without affecting production efficacy. Immunization of poultry breeders is used to elicit maternal antibodies in the offspring. Although protecting chicks during the crucial early weeks of life, maternal antibodies also may interfere with acquisition of immunity to vaccination (7,8,15,30).

In countries where chickens are highly likely to encounter the virulent forms of NDV, it is not uncommon for poultry producers to vaccinate each chick with a killed vaccine, often in combination with a live vaccine either in the hatchery or as a boost in the field (5). Such vaccination programs can induce high antibody titers and lasting protection. The disadvantage of this type of vaccination regimen is the cost of the vaccine and the effort required to handle each chick and ensure it receives its vaccine dose. Thus, it would be preferred if a highly effective vaccine could be easily mass delivered at the hatchery.

The Embrex® Inovoject® delivery device (Pfizer Animal Health, Durham, NC) offers a method to administer vaccines to the chicken egg before hatch, ensuring that each egg receives a uniform dose of vaccine. Marek's disease vaccine, infectious bursal disease vaccine, and fowlpox vaccines are routinely administered via the Embrex Inovoject device. NDV, however, is highly lethal when administered into the egg; therefore, conventional live ND vaccines cannot be administered via the Embrex Inovoject device. The vaccine used in this study uses antibody–antigen complex (AAC) technology (12,13,31) to delay the release of virus until after hatch when the immune system is sufficiently developed to keep the virus in check, thus allowing *in ovo* administration.

In ND outbreak situations, controlling clinical disease and reducing the shed of virus from infected birds to susceptible cohorts are critical to controlling spread of disease. The objectives of the present study were to extend the knowledge of protection against highly virulent NDV by *in ovo* vaccination of commercial maternal antibody–positive chickens. An important factor for any vaccine is its ability to provide protection against a variety of challenge strains to which the bird may be exposed in the field. This study evaluated protection against three strains of highly virulent NDV in broilers vaccinated *in ovo* with an AAC NDV.

MATERIALS AND METHODS

Vaccine. Newplex™ vaccine (Lohmann Animal Health International, Enterprise, AL) is a live ND vaccine composed of a B1-LaSota NDV complexed with NDV-specific hyperimmune serum (note that this vaccine is no longer commercially manufactured). The lyophilized vaccine was reconstituted with sterile Marek's vaccine diluent (Merial Select, Duluth, GA) to give 1 dose/100 µl. The reconstituted vaccine was administered *in ovo* on day 18 of incubation at 100 µl/egg. Control eggs were injected with 100 µL of Marek's vaccine diluent.

Challenge viruses. Challenge viruses were the virulent NDV strains Texas GB NDV-92-01 (TxGB), California 2002 isolate S212676 (CA), and South Korea 94-147 (SK). The TxGB virus was obtained from the U.S. Department of Agriculture Center for Veterinary Biologics (Ames, IA). It has an intracerebral pathogenicity index (ICPI) of 1.75 and was used at a challenge dose of $10^{4.3}$ egg infective dose $(EID)_{50}$ per bird, applied by intramuscular injection in 0.1 ml. The CA virus was a chicken isolate from the California END outbreak of 2002 with an ICPI of 1.79. The CA challenge dose used was $10^{5.7}$ EID_{50}/bird. The SK isolate was a 1997 isolate from chickens in South Korea. It has an ICPI of 1.78 and was administered at $10^{5.9}$ EID_{50}/bird. Dilutions of CA and SK challenge virus were made in tryptose phosphate broth (Sigma Chemical Co., St. Louis, MO), with challenges administered intraocularly in 100-µl volumes split into each eye (50 µl each).

Study design and birds. Ross × Hubbard fertile broiler eggs (175) were inoculated *in ovo* at 18 days of embryonation with AAC vaccine. Matched diluent-injected eggs served as controls. Hatched chicks of each group were placed in isolated floor pens for rearing, with feed and water provided *ad libitum*. At 1, 7, 14, 21, 28, and 35 days of age, birds were taken from the floor pens and bled for antibody titers against NDV. At 7, 14, 21, 28, and 35 days of age, 11 control and 22 vaccinated birds were placed into isolator cages and challenged as described above with virulent TxGB in biosafety level (BSL)-2 plus Agriculture (Ag) conditions. Clinical signs and mortality of challenged birds were monitored daily for 14 days. Surviving birds were bled and euthanatized by CO_2 inhalation at 14 days postchallenge (pc). Oral and cloacal swabs were collected before challenge (day 0) and at 4 days pc for NDV reisolation.

At 26 days of age, 25 control (sham-vaccinated) birds and 40 vaccinated (Newplex) birds were randomly selected and shipped by ground to Southeast Poultry Research Laboratory (Athens, GA) for challenge in BSL-3 Ag facilities with the CA and SK virulent NDV isolates. Upon arrival, birds were assigned to treatment groups as follows: control unchallenged (five birds), control SK challenged (10 birds), control CA challenged (10 birds), vaccinated SK challenged (20 birds), and vaccinated CA challenged (20 birds). Birds were housed in isolation units (five birds/unit) and allowed to rest and acclimate for 2 days. Challenges were then administered on day 28 of age.

Serology. Both hemagglutination inhibition (HI) assays and enzyme-linked immunosorbent assay (ELISA) were performed on individual serum samples. HI was performed by standard microtiter plate methods (2) using four HA units per well of a LaSota strain NDV. The HI geometric mean titers were expressed as reciprocal $\log_2$. Antibody to NDV also was measured by ELISA using a commercial test kit (IDEXX FlockChek®; IDEXX Laboratories, Westbrook, ME) in accordance with the manufacturer's directions.

Incidence of virus shedding. Cloacal and pharyngeal swabs were used for detection of challenge virus at day 4 pc. Swabs were hydrated in tryptose phosphate broth and inoculated into specific-pathogen-free eggs at 9–11 days of incubation and then tested for NDV as described previously (15).

Statistical analysis. Data were analyzed with SigmaStat 2.0.3 (SPSS Inc., Chicago, IL). ANOVA using pairwise comparisons with Tukey's method was used to compare HI and ELISA values. Mortality and frequency of virus isolation were analyzed for significance by Fisher exact test. All tests were performed with a 5% level of significance.

RESULTS

Maternal antibody and onset of immunity. Because commercial layers were the source of birds used in these studies, maternal antibody

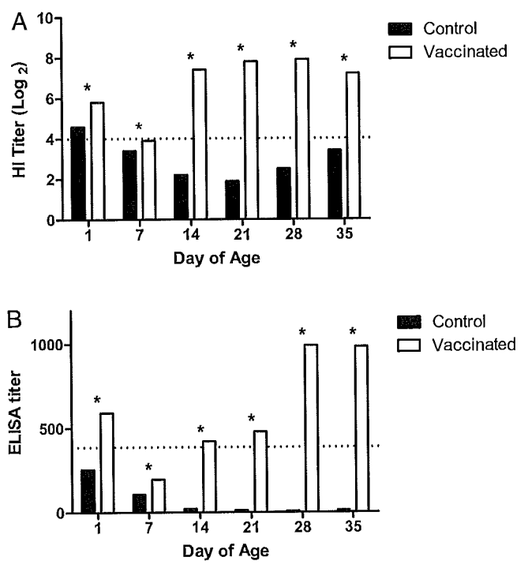

Fig. 1. Onset of antibody responses in maternal antibody–positive chickens receiving *in ovo* NDV vaccination determined by HI (A) and ELISA titer (B) at different days of age. Groups of chickens received mock (control) or antibody-complexed NDV (vaccinated) *in ovo* vaccination at 18 days of embryonation. At 1, 7, 14, 21, 28, and 35 days posthatch, serum was collected from birds in each group for testing. ELISA titers >396 are considered positive. Asterisk (*) indicates vaccinated birds significantly different from controls on that day by ANOVA ($P \leq 0.05$).

levels against NDV were expected to be present. Both groups of birds had maternal antibodies to NDV at 1 day of age (Fig. 1). The HI and ELISA titers for both treatment groups dropped from day 1 to day 7 of age, indicating a decrease in maternal antibody. After day of hatch, the anti-NDV serum antibody levels in unchallenged control birds declined to <4 ($\log_2$) as measured by HI, demonstrating a typical antibody decay pattern, and levels remained there throughout the study. In contrast, HI titers in vaccinated birds rose to >6 ($\log_2$) between 14 and 35 days of age, demonstrating a strong humoral immune response after *in ovo* vaccination. ELISA titers of control birds displayed similar antibody decay as observed in HI titers, with the highest ELISA titers found immediately after hatch and dropping thereafter (Fig. 1B). As stated by the manufacturer, titers >396 are considered positive. Birds in the vaccinated group demonstrated increasing ELISA titers from day 14 (405) through day 28, reaching a titer of approximately 1000 (Fig. 1B).

Response to TxGB challenge. When challenged with TxGB at 7 days of age, 64% of control birds survived challenge, indicating the protective potential of maternal antibody (Fig. 2A). Among the ACC-vaccinated birds, only 50% were protected from clinical signs and mortality, despite significantly higher antibody levels (Fig. 1A). At 14 and 21 days of age, only 18% and 20% of control birds survived challenge without clinical symptoms, probably due to waning maternal antibody. After 21 days of age, all control birds succumbed to challenge. In contrast, 100% of vaccinated birds challenged at 14, 21, 28, or 35 days of age survived challenge, a level that was statistically different from that of the control group.

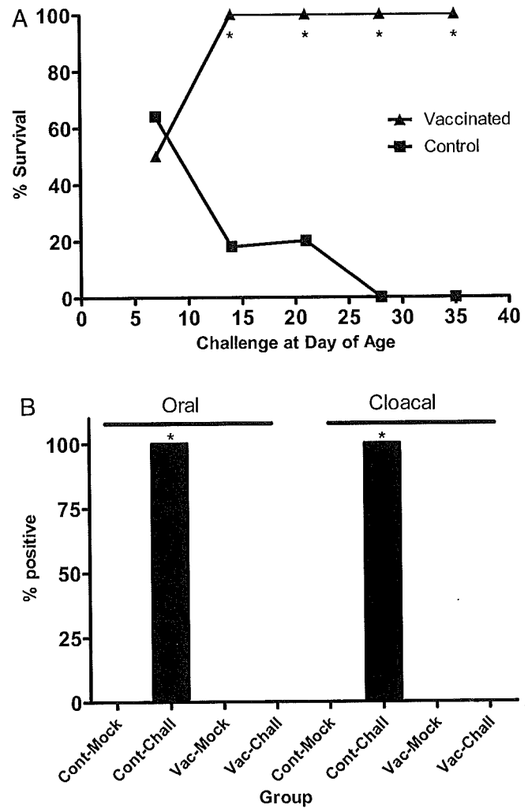

Fig. 2. Protection of maternally antibody–positive chickens after *in ovo* NDV vaccination against NDV TxGB challenge at different days of age. Groups of chickens received mock (control) or AAC NDV (vaccinated) by *in ovo* vaccination at 18 days of embryonation. (A) At 7, 14, 21, 28, and 35 days posthatch, birds (n = 11 and 22 for control and vaccinated groups, respectively) were challenged with $10^{4.3}$ EID_{50} TxGB via intramuscular route, and survival was monitored over a 14-day period. (B) Incidence of viral shedding in birds challenged at day 28 posthatch. Results shown are from oral and cloacal swabs taken 4 days pc. Asterisk (*) indicates vaccinated birds significantly different from controls on that day by Fisher exact test ($P \leq 0.05$).

Experiments to determine incidence of viral shedding at 4 days pc demonstrated that 100% of control birds were shedding virus in both oral and cloacal swabs (Fig. 2B). In contrast, no NDV-positive swabs were recovered from vaccinated or unchallenged groups.

Response to END challenge with isolates from California and South Korea. To determine cross-protection against other END isolates, birds were challenged at 28 days of age with either isolates from outbreaks in California (2002) or Korea (1997). All unvaccinated control birds succumbed to challenge after inoculation with CA and SK strains of NDV (Fig. 3A). In contrast, *in ovo*–vaccinated birds were 100% protected against both CA and SK virus challenges.

Comparison of the prechallenge (day 0) and pc (day 14) HI titers demonstrated that vaccinated birds had protective antibody titers ($\geq$4) before challenge (Fig. 3B). An anamnestic response was observed in these groups to END challenge at day 14. No detectable

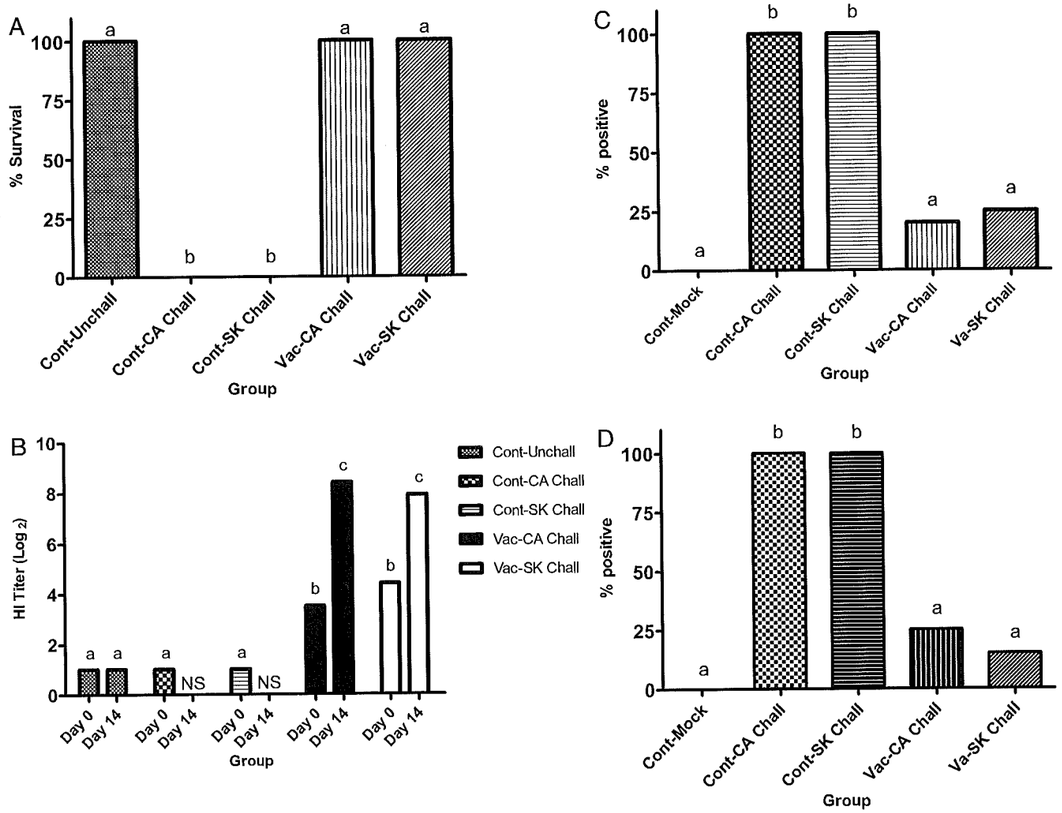

Fig. 3. Response of vaccinated birds to challenge with virulent NDV strain CA or SK at 28 days of age. Groups of maternal antibody–positive chickens received mock (control) or antibody-complexed NDV (vaccinated) *in ovo* vaccination at 18 days of embryonation. At 28 days posthatch, birds were challenged with approximately 10^6 EID_{50} CA or SK via intraocular route, and survival was monitored over 14-day period. (A) Protection after END challenge. (B) At 0 and 14 days pc, serum was collected from birds in each group for HI testing. Incidence of viral shedding from oral (C) and cloacal (D) swabs in groups of birds at day 4 pc. NS = no surviving birds. Differences in lowercase letters indicates significant difference ($P \leq 0.05$) between groups by Fisher exact test for survival and ANOVA for HI and viral shedding.

antibody titers were observed in unvaccinated birds before challenge, indicating maternal antibody was no longer present in these groups.

No virus was recovered from oral or cloacal swabs before challenge (data not shown). After challenge, virus was recovered from 100% of nonvaccinated control birds in both oral and cloacal swabs at 4 days pc (Fig. 3C, D). In contrast, virus was identified in 15%–25% of swabs from vaccinated birds on day 4 pc.

DISCUSSION

Overall, the *in ovo*–administered ND vaccine provided full protection against all three virulent isolates of the virus when challenged at 28 days of age. Maternal antibodies alone were able to provide partial protection against TxGB up to 21 days of age, although *in ovo* vaccination clearly enhanced protection. We have demonstrated previously the protection achieved from TxGB challenge after *in ovo* vaccination with an AAC vaccine (10). Because TxGB is the virulent reference challenge strain for licensing vaccines in the United States, all commercially licensed NDV vaccines are expected to provide protection against it. As new viral strains emerge, however, it is important to determine vaccine protection against them. The END challenge strains CA and SK are not closely related phylogenetically to TxGB and offered an opportunity to determine efficacy against distant END isolates (17,23). As demonstrated here, *in ovo* vaccination with AAC vaccines can induce protective levels of immunity in commercial chickens containing maternal antibody against more phylogenetically distant strains.

The rapid decline in antibody titers between 1 and 7 days of age indicates only a modest amount of maternal antibody at hatch. Nevertheless, survival of 64% of control birds to TxGB challenge at day 7 is indicative of the protection afforded by maternal antibody. Unlike inactivated or live NDV vaccines applied at day of age, the maternal antibody did not interfere with acquisition of protective immunity in *in ovo*–vaccinated birds. The HI titers of vaccinated birds reached fully protective levels by day 14, corresponding with 100% protection to TxGB challenge. Protection remained constant through 35 days of age.

An interesting observation was that ELISA titers developed more slowly than HI titers. This is in contrast with ELISA profiles for broilers vaccinated with conventional live virus vaccines at hatch and may be a feature of the immune-complexed ND vaccine (15). The earlier development of HI than ELISA titers also was observed under field conditions (11). HI titers, however, are more predictive of protection to virulent challenge (24).

Although *in ovo*–vaccinated birds were fully protected from challenge against all three virulent NDV strains, viral shedding was not eliminated. Although no virus was recovered from TxGB-challenged birds, virus was isolated from both oral and cloacal swabs of a few of the birds in the CA and SK groups 4 days pc, and this isolation may indicate decreased antigenic homology of the vaccine virus (B1-LaSota) to the challenge viruses. Decreased suppression of the challenge virus after CA and SK challenge also may be due to the slightly higher pathogenicity, as indicated by higher ICPI values of 1.79 and 1.78, respectively, than for TxGB strain (1.75).

Viral shedding after challenge may indicate the degree of local immunity in the respiratory system and digestive tract. Kapczynski and King (15) demonstrated that viral shedding in chickens challenged with CA strain virulent NDV was lower in birds vaccinated with a live vaccine by intraocular inoculation than in those vaccinated subcutaneously with an inactivated oil adjuvant ND vaccine (15). It is worth noting that the live vaccines used in our previous study (NEWHATCH-C2®; Merck Animal Health, Summit, NJ) differed from the vaccine used in these studies (B1-LaSota). The frequency of viral reisolation after CA challenge in the present study using *in ovo* vaccination is comparable to the published results for the live vaccine and is consistent with the presence of live virus in the AAC vaccine and indicates that the mechanism of protection may not be affected by complexing the virus with antibody. The role of local immunity to NDV in the respiratory tract has been demonstrated and is important for field protection against aerosol challenge (4). The challenges used in this study also mimic this route (administered intraocular) so as to match the desired conditions as well as possible. The *in ovo* route of vaccination presents antigen to the mucosal surfaces of the respiratory tract and digestive tract (14). This route favors development of mucosal immunity. *In ovo* vaccination for ND also elicits sufficient humoral immunity to afford protection against intramuscular challenge with TxGB as early as day 14 (10).

In summary, *in ovo* vaccination for ND broadens the options available to poultry producers and may offer a more efficient, cost-effective vaccination strategy. Whether *in ovo* NDV vaccination affects growth of broilers remains to be determined. Because full immunity against phylogenetically distant virulent viruses can develop at an early age in maternal antibody–positive chickens, this type of vaccine has an advantage when applied to commercial poultry. Finally, *in ovo* vaccination seems to stimulate both local and humoral immunity to NDV and may potentially enhance cell-mediated immunity, although that is the subject of future research.

REFERENCES

1. Alexander, D. J. Newcastle disease and other Paramyxoviradae infections. In: Diseases of poultry, 10th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, L. McDougald, and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 541–569. 1997.

2. Alexander, D. J. Newcastle disease virus and other avian Paramyxoviruses. In: Isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 156–163. 1998.

3. Banerjee, M., W. M. Reed, S. D. Fitzgerald, and B. Panigrahy. Neurotropic velogenic Newcastle disease in cormorants in Michigan: pathology and virus characterization. Avian Dis. 38:873–878. 1994.

4. Beard, C. W., and B. C. Easterday. The influence of the route of administration of Newcastle disease virus on host response. I. Serological and virus isolation studies. J. Infect. Dis. 117:55–61. 1967.

5. Chansiripornchai, N., and J. Sasipreeyajan. Efficacy of live B1 or Ulster 2C Newcastle disease vaccines simultaneously vaccinated with inactivated oil adjuvant vaccine for protection of Newcastle disease virus in broiler chickens. Acta Vet. Scand. 48:2. 2006.

6. Crespo, R., H. L. Shivaprasad, P. R. Woolcock, R. P. Chin, D. Davidson-York, and R. Tarbell. Exotic Newcastle disease in a game chicken flock. Avian Dis. 43:349–355. 1999.

7. Czifra, G., J. Meszaros, E. Horvath, V. Moving, and B. E. Engstrom. Detection of NDV-specific antibodies and the level of protection provided by a single vaccination in young chickens. Avian Pathol. 27:562–565. 1998.

8. Elazab, M. F., Y. Fukushima, Y. Fujita, H. Horiuchi, H. Matsuda, and S. Furusawa. Induction of immune suppression in the chick by an optimal dose of an immunizing antigen in the presence of its specific maternal antibody. J. Vet. Med. Sci. 72:257–262. 2010.

9. Francis, D. W. Newcastle and psittacines. Poult. Dig. 32:16–19. 1973.

10. Haddad, E. E., A. Avakian, C. E. Whitfill, D. E. Gross, D. B. Link, C. S. Blackmore, and P. S. Wakenell. Immunological evaluation of a novel Newcastle disease vaccine administered in ovo on day 18 of embryonic age to specific pathogen free and commercial broiler birds. In: Proc. XXII World's Poultry Congress, Istanbul, Turkey. 2004.

11. Haddad, E. E., M. Martin, J. Schaeffer, K. Burnes, and C. E. Whitfill. In ovo vaccination against Newcastle disease: field safety evaluation. In: Proc. 52nd Western Poultry Disease Conference, Sacramento, CA. pp. 34–36. 2003.

12. Haddad, E. E., C. E. Whitfill, A. P. Avakian, C. A. Ricks, P. D. Andrews, J. A. Thoma, and P. S. Wakenell. Efficacy of a novel infectious bursal disease virus immune complex vaccine in broiler chickens. Avian Dis. 41:882–889. 1997.

13. Jeurissen, S. H., E. M. Janse, P. R. Lehrbach, E. E. Haddad, A. Avakian, and C. E. Whitfill. The working mechanism of an immune complex vaccine that protects chickens against infectious bursal disease. Immunology 95:494–500. 1998.

14. Jochemsen, P., and S. H. Jeurissen. The localization and uptake of in ovo injected soluble and particulate substances in the chicken. Poult. Sci. 81:1811–1817. 2002.

15. Kapczynski, D. R., and D. J. King. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 23:3424–3433. 2005.

16. King, D. J. Influence of chicken breed on pathogenicity evaluation of velogenic neurotropic Newcastle disease virus isolates from cormorants and turkeys. Avian Dis. 40:210–217. 1996.

17. Lee, Y. J., H. W. Sung, J. G. Choi, J. H. Kim, and C. S. Song. Molecular epidemiology of Newcastle disease viruses isolated in South Korea using sequencing of the fusion protein cleavage site region and phylogenetic relationships. Avian Pathol. 33:482–491. 2004.

18. Nolen, R. S. Exotic Newcastle disease strikes game birds in California. J. Am. Vet. Med. Assoc. 221:1369–1370. 2002.

19. Nolen, R. S. Additional commercial flocks in California stricken by Newcastle disease. A Vet. Med. Assoc. 222:929–930. 2003.

20. Nolen, R. S. Emergency declared: exotic Newcastle disease found in commercial poultry farms. J. Am. Vet. Med. Assoc. 222:411. 2003.

21. Office International des Epizooties. Terrestrial Animal Health Code (2011). Organisation Mondiale de la Santé Animale World Organisation for Animal Health Organización Mundial de Sanidad Animal, Paris, France. 2011.

22. Panigrahy, B., D. A. Senne, J. E. Pearson, M. A. Mixson, and D. R. Cassidy. Occurrence of velogenic viscerotropic Newcastle disease in pet and exotic birds in 1991. Avian Dis. 37:254–258. 1993.

23. Pedersen, J. C., D. A. Senne, P. R. Woolcock, H. Kinde, D. J. King, M. G. Wise, B. Panigrahy, and B. S. Seal. Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002–2003 outbreak in

California and other recent outbreaks in North America. J. Clin. Microbiol. 42:2329–2334. 2004.

24. Reynolds, D. L., and A. D. Maraqa. Protective immunity against Newcastle disease: the role of antibodies specific to Newcastle disease virus polypeptides. Avian Dis. 44:138–144. 2000.

25. Rue, C. A., L. Susta, C. C. Brown, J. M. Pasick, S. R. Swafford, P. C. Wolf, M. L. Killian, J. C. Pedersen, P. J. Miller, and C. L. Afonso. Evolutionary changes affecting rapid identification of 2008 Newcastle disease viruses isolated from double-crested cormorants. J. Clin. Microbiol. 48:2440–2448. 2010.

26. Schloer, G. Antigenic relationships among Newcastle disease virus mutants obtained from laboratory strains and from recent California isolates. Infect. Immun. 10:724–732. 1974.

27. Seal, B. S., D. J. King, and J. D. Bennett. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. J. Clin. Microbiol. 33:2624–2630. 1995.

28. Senne, D. A., J. E. Pearson, L. D. Miller, and G. A. Gustafson. Virus isolations from pet birds submitted for importation into the United States. Avian Dis. 27:731–744. 1983.

29. Utterback, W. W., and J. H. Schwartz. Epizootiology of velogenic viscerotropic Newcastle disease in southern California, 1971–1973. J. Am. Vet. Med. Assoc. 163:1080–1088. 1973.

30. Westbury, H. A., G. Parsons, and W. H. Allan. Comparison of the immunogenicity of Newcastle disease virus strains V4, Hitchner B1 and La Sota in chickens. 2. Tests in chickens with maternal antibody to the virus. Aust. Vet. J. 61:10–13. 1984.

31. Whitfill, C. E., E. E. Haddad, C. A. Ricks, J. K. Skeeles, L. A. Newberry, J. N. Beasley, P. D. Andrews, J. A. Thoma, and P. S. Wakenell. Determination of optimum formulation of a novel infectious bursal disease virus (IBDV) vaccine constructed by mixing bursal disease antibody with IBDV. Avian Dis. 39:687–699. 1995.

ACKNOWLEDGMENTS

We thank Tracy Smith-Faulkner and Phillip Curry for outstanding technical assistance and Roger Brock for animal care assistance. This research was supported by a research agreement with Embrex (58-6612-4-250) and USDA–ARS CRIS projects 6612-32000-062 and 6612-32000-049.

Virus Genes (2012) 45:526–536
DOI 10.1007/s11262-012-0792-z

Dynamic equilibrium of Marek's disease genomes during in vitro serial passage

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Received: 28 March 2012 / Accepted: 14 July 2012 / Published online: 26 August 2012
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Abstract Attenuation of Gallid herpesvirus-2 (GaHV-2), the causative agent of Marek's disease, can occur through serial passage of a virulent field isolate in avian embryo fibroblasts. In order to gain a better understanding of the genes involved in attenuation and associate observed changes in phenotype with specific genetic variations, the genomic DNA sequence of a single GaHV-2 virulent strain (648A) was determined at defined passage intervals. Biological characterization of these "interval-isolates" in chickens previously indicated that the ability to induce transient paralysis was lost by passages 40 and the ability to induce persistent neurological disease was lost after passage 80, coincident with the loss of neoplastic lesion formation. Deep sequencing of the interval-isolates allowed for a detailed cataloguing of the mutations that exist within a single passage population and the frequency with which a given mutation occurs across passages. Gross genetic alterations were identified in both novel and well-characterized genes and cis-acting regions involved in replication and cleavage/packaging. Deletions in genes encoding the virulence factors vLipase, vIL8, and RLORF4, as well as a deletion in the promoter of ICP4, appeared between passages 61 and 101. Three mutations in the virus-encoded telomerase which predominated in late passages were also identified. Overall, the frequency of mutations fluctuated greatly during serial passage and few genetic changes were absolute. This indicates that serial passage of GaHV-2 results in the generation of a collection of genomes with limited sequence heterogeneity.

Keywords Marek's disease · Marek's disease virus · Sequencing · Polymorphisms · Serial passage · Gallid herpesvirus 2 · Pyrosequencing · Virulence

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Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens responsible for great economic losses to the poultry industry [1, 2]. The causative agent of the disease is a *Mardivirus*, *Gallid herpesvirus* type 2 (GaHV-2), a member of the *Alphaherpesvirinae* subfamily in the family *Herpesviridae* [3]. GaHV-2 is the most oncogenic herpesvirus known and represents the first of four neoplastic diseases (including hepatocellular carcinoma: *hepatitis B virus*; cervical carcinoma: *human papilloma virus* and leukemia: *feline leukemia virus*) for which an effective vaccine has been developed [4–6]. Many GaHV-2 vaccines have been developed through extensive serial passage (35–100 passages) of virulent field isolates in avian embryo fibroblasts [7–10], but in vaccine trials all pale in comparison to the protection elicited by the avirulent strain

CVI988 isolated from birds by Rispens et al. [11, 12]. To determine the genetic changes responsible for attenuation we have embarked on a study to identify the mutations that accumulate during extensive serial passage of the very virulent plus (vv+) strain of GaHV-2 known as 648A. Previously, the pathogenesis of regular passage levels (between 10 and 100) of this strain, propagated in avian embryo fibroblasts, was characterized in 15 × 7 cross-bred chickens [13]. These studies showed that the ability to induce transient paralysis (TP) was dramatically reduced between passages 30 and 40. However, persistent neurological disease (PND) was only reduced after an additional ten passages (p50) and disappeared between passages 80 and 90. The incidence of visceral tumors was also investigated and nearly 100 % of the chickens inoculated with passages 10 through 50 of 648A developed tumors. This frequency, however, dropped significantly between passages 50 and 60, and by passage 80 no tumors were observed in inoculated chickens for the period of the trial (12 weeks).

We have reported the genomic changes that occur during low (p11) and high (p101) in vitro passages of virulent plus strain 648A [14]. As a complementary study the sequentially passaged virus preparations between p11 and p101 were sequenced in a hope that a correlation between phenotype (e.g., PND, tumor formation, etc.) and genotype could be established. We have discovered that varying populations of polymorphisms (point mutations, deletions, and insertions) exist within the genomes at each passage interval. In addition to describing the mutations that occurs during serial-passage-induced attenuation of "interval-isolates", this paper describes the fluctuations in the mutation frequencies for 14 single-nucleotide polymorphisms (SNPs) and details the nucleotide sequencing evidence for intra-strain diversity within populations of GaHV-2.

Methods

Virus and cells

Very virulent plus strain 648A of GaHV-2 isolated by Witter [15] was used in these experiments. Serial passage of this strain to generate virus stocks at passages 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 in line 0 chicken embryo fibroblast (CEF) culture was described previously by Gimeno and co-workers [13]. Secondary CEFs were used for a single round of virus propagation prior to nucleocapsid DNA isolation from passages 31, 41, 61, and 81 and were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 8 % fetal bovine serum as previously described [14].

Isolation of DNA

Nucleocapsid DNA for each passage was isolated when the cytopathic effect (CPE) reached >80 % with a protocol originally described by Sinzger et al. [16] and modified by Volkening and Spatz [17]. GaHV-2 infected cells were trypsinized, centrifuged, and suspended in 320 mM sucrose, 5 mM $MgCl_2$, 10 mM TrisHCl (pH 7.5), and 1 % Triton X-100. Nuclei were pelleted by centrifugation at 1,300×*g* and suspended in 10 mM Tris HCl (pH 7.5), 2 mM $MgCl_2$, and 10 % sucrose and mixed with an equal volume of 2× nuclease buffer [40 mM PIPES (pH 7.0), 7 % sucrose, 20 mM NaCl, 2 mM $CaCl_2$, 10 mM 2-mercaptoethanol, and 200 μM PMSF]-containing 150 U of micrococcal nuclease and 1.0 μl of RNase A (20 mg/ml). Cellular and unpackaged viral nucleic acids were degraded by incubation at 37 °C. After stopping the nuclease reaction with EDTA, viral DNA was released from nucleocapsids by incubation at 50 °C in a buffer-containing 100 mM NaCl, 10 mM TrisHCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5 % SDS and 50 μg of proteinase K. The DNA was extracted with phenol/chloroform and precipitated with 6.5 % (w/v) PEG-8000 and 10 mM $MgCl_2$ at room temperature for 20 min. After centrifugation at 16,000×*g* for 40 min, the pellets containing the high MW viral DNA were washed with 500 μl 70 % ethanol and re-centrifuged at 16,000×*g* for an additional 20 min. High MW DNA pellets were air-dried and dissolved in 1X TE buffer, pH 7.5.

DNA sequencing

Sequencing of 5.0 μg DNA from 648A passages 11, 31, 41, 61, 81, and 101 was carried out commercially on a Genome Sequencer 20 (GS20) System (454 Life Science Corporation). DNA from each passage was contained within its own section of the sequencing chip using an eight-lane gasket. Genomic nucleotide consensus sequences were assembled using the DS De Novo Assembler (454 Life Sciences, Branford, CT) and the Sequencher program (Gene Codes, Ann Arbor, MI). The average length of each read was 179 nucleotides. The final sequences on average represented a 47-fold coverage at each base pair. Problematic regions containing mononucleotide reiterations were sequenced from PCR products generated in reactions containing Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) under standard PCR conditions. Sanger-based DNA sequencing of these PCR products 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. Origin of replication hairpins were analyzed using the mfold WebServer at the Rensselaer Polytechnic Institute, Troy, NY, USA [18]. Nucleotide sequence data reported in this paper have been submitted to GenBank and the accession numbers are as follows: 648Ap11-JQ806361; 648Ap31-JQ806362; 648Ap41-JQ809691; 648Ap61-JQ809692; 648Ap81-JQ820250, and 648Ap101-JQ836662.

Polymerase chain reaction (PCR) conditions

Deletions in the GaHV-2 genomes first identified through sequence analysis were verified using flanking oligonucleotides and PCR. A 632 base pair deletion within the ICP4 promoter (Md5 coordinates 151212–151845 and 166599–167232) was confirmed using 100 ng of nucleocapsid DNA from the six passages, one unit of Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) and the primers ir_94 (AACCGGATGGCGAACTTGC) and gap45-r (GAAATCAGGCGGGTTGTC) at 400 nM. The reaction contained 1× high fidelity buffer, 200 μM dNTP, 2 mM $MgSO_4$, and 5 % dimethylsufoxide. The cycling conditions were as follows: templates were initially denatured at 94 °C for 2 min and then for 30 s at 94 °C at the beginning of each cycle. Oligonucleotides were allowed to anneal for 30 s at 60 °C and nucleotides were incorporated at 68 °C for 90 s. This cycle was repeated 34 additional times. PCR products were resolved by electrophoresis in a 0.6 % agarose gel with 1× TAE.

Results

DNA sequencing of attenuated passages

Since 454 pyrosequencing does not rely on cloned DNA, microgram quantities of nucleocapsid DNA were needed. To achieve this goal, between 8 and 16 flasks (75 cm^2) of chick embryo fibroblasts were infected at an m.o.i. of ~1.0 with individual passage level stocks. The hypotonic lysis followed by micrococcal nuclease degradation provided a clear improvement over classical hypotonic lysis followed by sucrose gradient purification as reported previously [19]. Depending on the passage level and the number of cells infected, this protocol yielded 5–10 μg of DNA for most passages. The quality of the DNA was assessed both before and after PEG precipitation by separating restriction endonuclease-digested aliquots (500 ng) on 0.6 % agarose gels (data not shown).

Table 1 contains a summary of the pyrosequencing data for the 648A passages. The average read length was 179 nucleotides with an average of 45,663 reads per GaHV-2 genome. The number of reads excludes those that were avian specific (contaminating low MW host DNA). The fold-coverage varied over a wide range from 25 to 75 fold depending on the amount of contaminating avian DNA and the number of successful reads. This latter parameter is independent of the purity of the DNA and influenced by the chemistries involved in pyrosequencing. For example, p11 contained only 2.32 % contaminating DNA but only produced 43,601 GaHV-2-specific reads for a 44-fold coverage while p101-contained 20 % contaminating avian DNA but generated 84,144 GaHV-2-specific reads for a 73-fold coverage. Likewise p31 contained a lower percentage of contaminating DNA than p61 but sequenced poorly with only a 32.8 fold coverage relative to the 39.2 fold coverage obtained with p61. In general, however, DNA preparations with lower amounts of host DNA contamination yielded higher fold coverage thus making contig assembly easier.

Accumulation of mutations during serial passage

Deletions

Mutations were identified in each passage by comparison of DNA sequences to each other and the reference strain

Table 1 454 Life sciences pyrosequencing results

Sample	p11	p31	p41	p61	p81	p101
GaHV-2 reads #	43,601	55,550	75,127	67,898	78,813	84,144
Avian reads #	1,012	21,633	46,733	27,945	16,994	16,837
% GaHV-2 DNA	97.68 %	61.07 %	37.79 %	58.84 %	78.44 %	79.99 %
% Avian DNA	2.32 %	38.94 %	62.21 %	41.16 %	21.56 %	20.01 %
Number of nucleotides	7,793,787	5,816,766	4,514,646	6,951,822	11,961,977	13,057,558
Average read length	183	171.5	159	174	193.5	194
Fold coverage	43.9	32.8	25.4	39.2	67.4	73.6

Host cellular DNA contamination was present in the six samples (p11, p31, p41, p61, p81, and p101) due to incomplete removal of the low molecular weight DNA during 6.5 % PEG precipitation. All reads were mapped against *Gallus gallus* genome sequences with BLAST software and contaminating sequences (non-GaHV-2 sequences) were removed prior to contig assembly. Fold coverage was calculated as the number of GaHV-2 reads multiplied by the average read length and divided by the average length (177,500 bp) of the GaHV-2 genome

Md5 [20]. These mutations were catalogued as deletions, insertions, and single-nucleotide substitutions. Deletions were identified in the unique long, repeat long regions and the repeat short/unique short junctions (Fig. 1). As expected, deletions that occurred in genomes of early passages were fairly well maintained in later passages (Fig. 2). A 632 base pair deletion at the repeat short/unique short junction (Md5 coordinates 151,212–151,845 and 166,599–167,232) was identified in the genomes of passages 41 through 101. This deletion mapped 444 bp upstream of the ICP4 start codon within the 3′ terminus of the overlapping 10 Kb LAT [21, 22]. Five short-novel open reading frames (MDV084.5, 085, 085.3, 085.6 and 085.9) also mapped within the deleted region [23]. As shown in Fig. 1a, this deletion encompasses the Meq oncoprotein binding site [24] and MDV085.3 and alters the reading frames of the novel short ORFs MDV084.5, 085, 085.6, and 085.9.

To investigate the proportion of genomes containing this deletion within each individual passage, flanking oligonucleotides were synthesized and used in *Taq* polymerase amplification reactions with DNA isolated from each interval-isolate. As shown in Fig. 3, a 1.1 Kb PCR product was generated in reactions containing DNA from early passages. As the passage number increased beyond 41, two PCR products (1.1 and 0.5 Kb) were generated. At passage 61, the predominating PCR product was the smaller 0.5 Kb product and by passage 101 only trace amounts of the larger 1.1 Kb (non-deleted) product were detected. The intensity of the PCR bands correlated well with the frequency of individual sequences calculated from aligned reads. Based on analysis of the sequencing data, the full-length

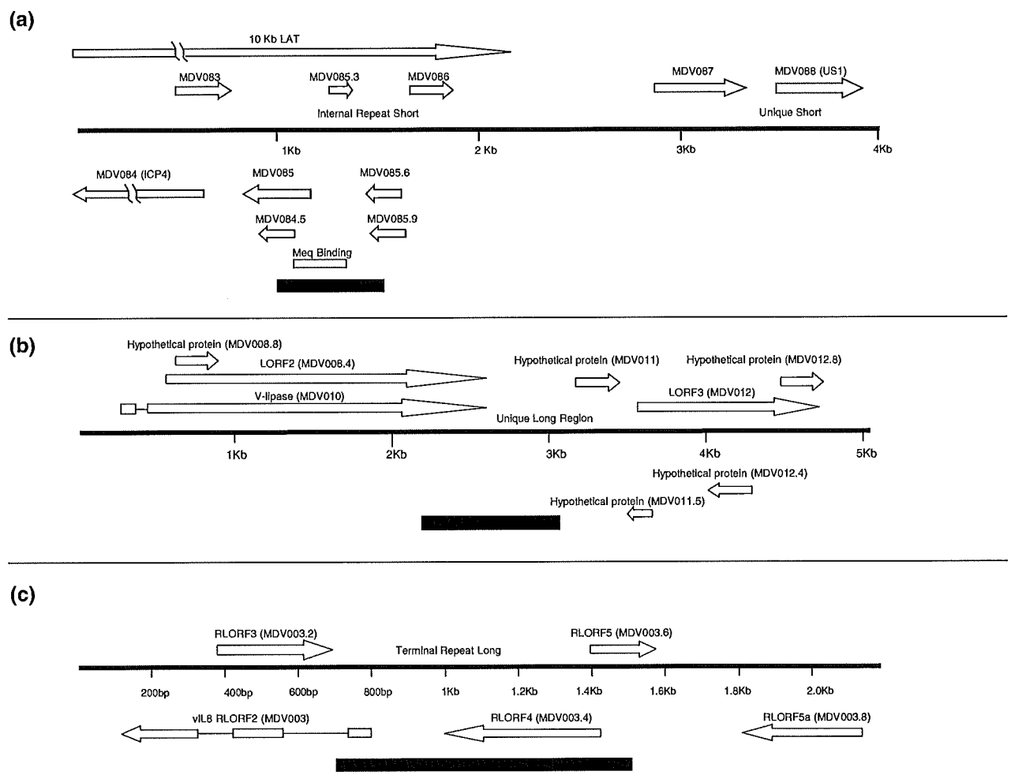

Fig. 1 Diagram of the locations of large deletions (>500 bp) in the repeat short, unique long, and repeat long regions of the 648A genome. Open reading frames are indicated by *white arrowheads*. The *black boxes* denoted the position of the deletions. (a) The 3′ terminus of the internal repeat short region showing the overlapping ICP4 and 10 Kb LAT genes and novel genes within the promoter of ICP4. The 632 bp deletion encompassing the Meq binding site (*open box*). **b** The 948 bp deletion at the 3′ termini of the overlapping LORF2 and V-lipase genes within the unique long region. **c** The 820 bp deletion within the terminal repeat long region encompassing vIL8, RLORF4, and RLORF5

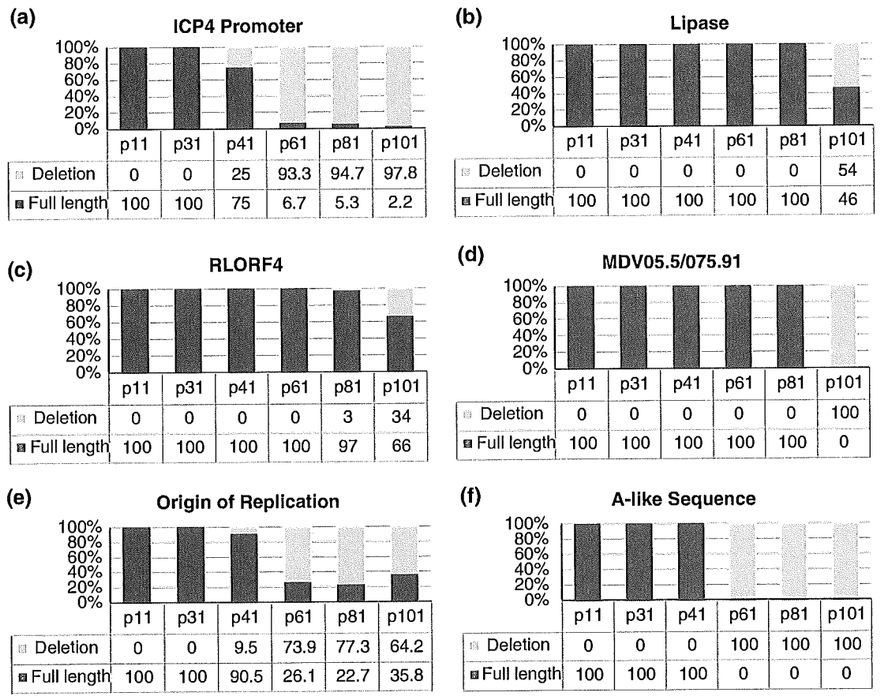

Fig. 2 Histograms showing the percentage of the 648A passages-containing deletions. **a** ICP4 promoters. **b** Lipase (MDV010). **c** vIL8, RLORF4, and overlapping RLORF5 genes. **d** diploid genes MDV05.5/075.91. **e** Origin of lytic replication and **f** A-like sequence involved in packaging. A 34 bp deletion was identified in all of the 648Ap101 populations. *Black* denotes the percentage of the population containing the full-length sequence and gray denotes the percentage of the population containing the deletion

sequences versus deleted sequences were present in the following proportions: p11–100/0 %: p31–100/0 %: p41–75/25 %: p61–6.7/93.3 %: p81–5.3/94.7 %: and p101–2.2/97.8 %.

Another large deletion (948 bp: Md5 coordinates 16,401–17,348) was identified within the U_L region, at the MDV010 locus encoding the spliced virulence factor vLipase [25]. Based on analysis of the sequencing data, this deletion was found in 54 % of the p101 genomes. The deletion (Fig. 1b) removed 475 bp at the 3′ terminus of MDV010 and the resulting open reading frames codes for a fusion polypeptide of 609 aa. Two vLipase proteins (a full-length 756 aa protein and the COOH fusion form) are therefore predicted for the p101 genomic populations.

A less predominant 820 bp deletion mapping to the R_L regions (MD5 coordinates 3,455–4,274 and 137,346–138,165) was identified in 3 % of the p81 sequences and 34 % of the p101 sequences. As shown in Fig. 1c this deletion affects the genes MDV003/MDV078 and MDV003.4/078.3 encoding the virulence factors vIL8 (RLORF2) [26–28] and RLORF4 [29] as well as the 5′ terminus of MDV003.6/078.2 encoding RLORF5.

A small deletion was identified in the novel gene MDV05.5/075.91 within the R_L region of the p101 genome. A missing thymidine residue (after nucleotide 8,062 in the Md5 genome) is predicted to generate an 81 aa protein. A 99 aa protein is predicted from this gene within the genomes of earlier passages. The role of the gene in virulence is unknown.

In addition to deletions in protein coding regions, two *cis*-acting sites (the origin of replication and the a-like sequence) also contained deletions within the genomes of later serial passages. A TTA deletion in the origin of replication's hairpin structure was identified within the genomes of passages 41 through 101. At passage 41, 9.5 % percent of the sequences contained the deletion. By passages 61 and 81, 73.9 and 77.3 %, respectively, contained the deletion. However, this deletion was not strictly maintained and by passage 101 only 64.2 % of the aligned reads contained the deletion, suggesting that genomes containing

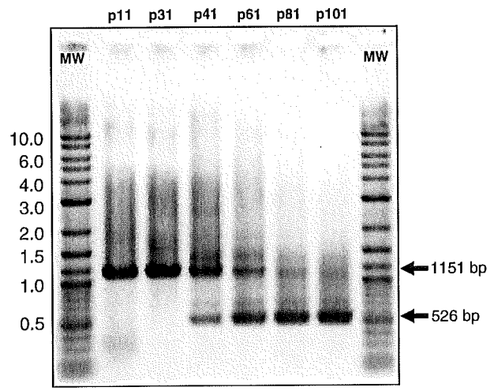

Fig. 3 Ethidium bromide-stained gel (0.6 % agarose) containing separated PCR products generated in reactions with primers that flank the 632 bp deletion found within the repeat short region of the 648A genomes. A 1,151 bp product was amplified in DNA extracted from early passages. The deletion, as evidenced by the appearance of the smaller 526 bp band, first appeared in DNA from p41 and predominated thereafter. The *arrows* indicate the positions of the 1.1 and 0.5 Kb products

the deletion are not strongly favored. This is in agreement with predicted thermostability data indicating that the shortened origin of replication hairpin has a slightly higher ΔG value (−22.46 vs −21.88 kcal/mol). Although the difference in ΔG values is only 0.58 kcal/mol, the loop structure at the tip of the hairpin varies greatly (Fig. 4).

Another region of sequence heterogeneity was identified in the a-like sequences [30]. A stretch of 89 nucleotides (Md5 coordinates 140,570–140,657; 177,787–177,874) between the *pac-1* site and the telomeric repeats was deleted in the genomes of passage 61 though 101. The genome of passage 101 also contained a 34 bp deletion downstream from the 3′ DR1 sequence and a 22 bp sequence (GTCATGTAGAGGGTCATGCGCG) repeated 14 times upstream of the 60 bp repeats. Deletions in this region downstream from *pac-2* have been reported in some attenuated GaHV-2 strains [30].

Insertions

Insertions accumulated less frequently than deletions in the serially passaged preparations. An insertion resulting in a reading frame shift was identified in the U_L gene encoding the U_L3 nuclear phosphoprotein (MDV015:Md5 coordinates 20,607–21,293). The mutation in this gene involved an additional adenosine residue in a stretch of mononucleotide reiterations (Md5 coordinates 21,039–21,046) that in most GaHV-2 genomes sequenced to date contain eight adenosine residues resulting in a 228 aa protein. Sixty-eight

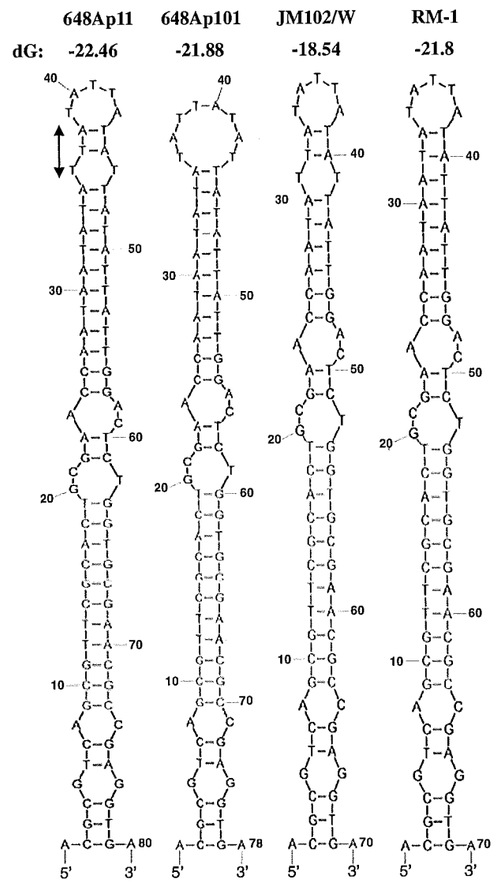

Fig. 4 Computer generated representations and ΔG values of folded hairpin structures at the origin of lytic replication within the genomes of 648Ap11, 648Ap101, JM/102W, and RM-1. The latter three exhibit attenuated phenotypes. A three nucleotide deletion (*arrow*) is present in the 648A genomes from passages 81 and 101. The *shaded boxes* indicate the U_L9 binding sites. A ten nucleotide deletion in the origin of lytic replication in the genomes of JM102/W (atatattata) and RM-1(tatattatat) maps to positions 30–40 on the 648Ap11 structure. The sequence of the origin of lytic replication of Md5 is the same as 648Ap11

percent of the genomes in passage 81 contained an extra adenosine residue resulting in a frameshift that is predicted to generate a 149 aa protein. This mutation was selected against during additional passages and by p101 all of the reads contained the eight adenosine residues within this region of the U_L3 gene. Interestingly, this gene was also mutated in the serially passaged virus 584Ap80 [9].

Variable thymidine reiterations of either seven (most common) or six residues were identified in U_L5 gene

encoding the DNA helicase–primase-associated protein). (MDV017:Md5 coordinates 22,669–25,245) of passages 11–101. The U_L5 gene within the genomes of passages 11–81-contained seven thymidine residues (Md5 coordinates 24,772–24,779), and was predicted to generate a 858 aa protein. At passage 101 43 % of the U_L5 genes contained seven thymidine residues while the majority contained a six thymidine stretch. It is predicted that genes with only six thymidines would generate a 29 aa peptide, but if a downstream start codon were used a 718 aa protein with a proper COOH terminus would be generated.

Insertions were also identified within the MDV002.6/078.5 diploid gene flanking the sequence CCCCCTTTTG CACGAAGAGT (Md5 coordinates 2,543–2,562 and 139,058–139,077). An additional cytosine residue at the 5′ end of the above sequence was present in the passage 81 genomes. This insertion would result is the production of a 51 aa protein, identical to the MDV002.6/078.5 protein of another attenuated strain (584Ap80) [9]. Two additional residues (cytosine and thymidine) at the 3′ end of the above sequence were identified within the genomes of passage 101. This would generate a 60 aa protein, in contrast to the 99 aa protein predicted from the translation of this gene within the genomes of the earlier passages as well as those of other sequenced GaHV-2 genomes annotated in GenBank.

Sequence variability was also identified in the MDV03.6/078.2 gene encoding RLORF5 (Md5 coordinates 4,154–4,327 and 13,7293–13,7466. Within the p101 genome, this gene contained extra guanosine and thymidine residues after nucleotide 4,301 (Md5 coordinates). This is predicted to generate a slightly larger protein (60 aa) than the RLORF5 homologues found within the genomes of earlier passages (57 aa). This ORF is not believed to be important in virulence since the region can be deleted without loss of virulence [29] and RLORF5 homologues in other sequenced genomes are predicted to encode proteins of varying lengths (57, 58, 59, 62, and 115 aa).

Single-nucleotide polymorphisms

With average fold coverage of 47, it was possible to identify a collection of SNPs that exist in differing proportions within a single-DNA preparation (Table 2). Fourteen SNPs were identified in the genomes of passages 11 through 101. The proportion of each SNPs within the genomes of each passage varied greatly. Only one G → A transition, within the promoter region of MDV006.1/075.1 encoding the hypothetical protein B68 [31, 32] transitioned completely by passage 101. The majority of the SNPs fluctuated to varying degrees but the proportion of the SNPs that predominated in the p11 genomes were largely inverted by passage 101. The changes were greatest for the SNPs found in MDV059 (U_L46 encoding the tegument protein VP11/12), the untranslated 3′ end of MDV082/102, the Val^{1871} to Ala^{1871} polymorphism of MDV84/100 encoding the immediate early protein ICP4, and the thymidine to cytosine switch in the promoter of MDV086.1/097.9. One polymorphism affecting three overlapping genes (MDV008/073, 009, and 009.5/073.4) within the repeat long region was present in roughly half the genomes within each passage.

Discussion

Serial passage of virulent field isolates of GaHV-2 in cell culture from various avian and non-avian species for the purpose of attenuation has been reported over the last 40 years [35–40]. However, defining the mutations involved in attenuation has only recently been attempted through the generation of knockout mutants, primarily in unique *Mardivirus* genes, and through whole genome sequencing of virulent and attenuated strains [41–43]. This study addresses the accumulation of attenuating mutations by determining the nucleotide sequences at defined intervals of the serially passaged very virulent plus virus 648A. This collection was extensively characterized in MD susceptible chickens in an earlier report [13] and it is reported that acute TP was lost after passage 30, classical TP was lost after passage 40, and the ability to form tumors was lost after passage 70. It was the hope of this study to also match genetic mutations occurring at a specific passage level with the loss of a virulent phenotype. The most obvious and perhaps the easiest phenotype to correlate with a genotype was the loss of tumor formation which occurred after passage 70. Of the 140 genes encoded by GaHV-2, at least three genes encoding the oncoprotein Meq, the viral encoded RNA component of telomerase (vTR), and the large tegument protein U_L36 have been identified as playing a role in tumor formation in chickens infected with GaHV-2 [44–46].

Analysis of the sequencing data for the Meq gene (MDV005/076) from each passage failed to identify a single mutation that differed among the passages. No mutations were identified in the ubiquitin-specific protease (USP) domain of U_L36. Differences were noted in the 3′ hypervariable domain of U_L36 [30], but nevertheless the genomes of each passage encoded a 3,327 aa U_L36 protein. Mutations were, however, identified in the diploid gene encoding the viral encoded telomerase RNA subunit. Three mutations within the pseudoknot, CR4 and CR7 domains were identified within passages 81 and 101 genomes, although at low frequencies. Two of these mutations within the CR4 and CR7 domains are likely to affect stem structures based on the vTR model [33, 47] Kaufer et al. [48]

Table 2 The identification of 14 SNPs and their proportions within each serial passage of 648A

Location	SNP		648 passage number						Common Name
			11	31	41	61	81	101	
MDV002/079 vTelomerase RNA	Arg^{180} G	Pro^{180} C	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	RLORF1 Arg-rich protein RNA subunit of telomerase
MDV003/078	Leu^{17}	Ser^{17}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Spliced gene encoding vIL8
MDV003/078	Gln^{96}	Arg^{96}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Spliced gene encoding vIL8
5'MDV006.6/075.1 3' MDV007/074	G	A	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	B68, 14 kDA protein RLORF12
MDV008/073 MDV009 MDV009.5/073.4	Gly^{9} Pro^{161} Arg^{45}	Arg^{9} Leu^{161} Lys^{45}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	RLORF14 LORF1 RLORF13
MDV054	Val^{359}	Ala^{359}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	U_L41, virion host shutoff (VHS)
MDV059	Glu^{59}	Lys^{59}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	U_L46, VP11/12
3' MDV082/102	A	G	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	RSORF1
MDV084/100	Val^{1871}	Ala^{1871}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Immediate Early ICP4
MDV084/100	Ser^{1641}	Pro^{1641}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Immediate Early ICP4
MDV084/100	G^{189}	A^{189}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Immediate Early ICP4
5' MDV086.1/097.9 3' MDV086.4/97.3	T	C	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	
MDV088	Asp^{133}	Gly^{133}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	Immediate Early ICP22
MDV096	Ser^{205}	Pro^{205}	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	100 50 0	US8, Glycoprotein E

The *vertical bars* represent the relative percentage of SNP or corresponding amino acids at each interval. The $G^{189} \rightarrow A^{189}$ transition in MDV084/100 is a silent mutation

have demonstrated that mutations of the template sequence of MDV-encoded vTR completely abrogated virus-induced tumor formation so it possible that the mutations we have identified may contribute to the lack of tumor formation as noted in the Gimeno study [13] after passage 70.

Based on the appearance of SNPs within the genomes of passages that failed to generate tumors and their generalized shift to nearly 100 % by passage 101 (Table 2), two candidates (U_L46 and a non-coding sequence 3′ of RSORF1) may be important in tumor formation. U_L46 encodes a tegument protein VP11/12 and it is highly unlikely that this well-conserved herpesviral protein plays a role in tumor formation. The point mutation downstream of RSORF1 is more attractive since it occurs in a region containing numerous microRNAs [49, 50]. However, it is far more likely that the 632 bp deletion identified within the promoter of ICP4 [51] plays a role in the lack of tumor formation by retarding the in vivo replication of the viral genome in passages p81 through p101 [52, 53]. A strong correlation between the ability of the virus to replicate and pathogenicity has been reported [54, 55] and the appearance of the deletion correlates well with the disappearance of tumor formation. However, since the deletion occurs in the region that Meq binds [24] and binding to the ICP4 promoter is thought to repress expression and maintain latency, it is counterintuitive to think that removal of a repressor-binding site would further suppress expression. This observation raises the possibility that the major role of Meq in GaHV-2 replication involves binding at the origin of replication [24].

Interestingly, mutations within the origin of replication were identified in the genomes of passages (p81 and p101) that reportedly were unable to generate tumors. These mutations may play a significant role in curtailing efficient replication by changing its tertiary structure to generate a bulky loop consisting of 8 mismatched adenosine and thymdine residues (Fig. 4) [56–59]. Similar positional mutations (Fig. 4 positions 30–40) were identified within

the origins of the attenuated GaHV-2 strains RM-1 and JM/102W [34]. Studies to measure the replicative ability of passages 81 and 101 in chickens are needed since the preceding clinical study characterizing these interval passages only measured the in vivo replicative ability of 648A in passages 10–50 [13].

Other virulent phenotypes that disappeared after passage 50 include acute transient and classical TP. Acute TP, the hallmark of vv+ strains, was lost after passage 30, and classical TP was lost after passage 40. Only one mutation within the promoter of the diploid gene MDV006.6/075.1 and the 3′ untranslated region of hypothetical protein MDV007/074 appeared exclusively after passage 31 (Table 2). This guanosine to adenosine switch was present in roughly 50 % of the genomes in passage 41, completely transitioned to adenosine by passage 61 and was maintained through passage 101. It is therefore possible that this mutation was responsible for the lack of acute TP in chickens receiving passage 40 and later. Mutations in three other proteins (VHS, RLORF1, and ICP4) appeared in the p31 genomes and persisted through p101. The Ala^{359} Val mutation in the virus host shutoff ORF (U_L41) appeared to fluctuate throughout later passages and it is unlikely to affect the tertiary structure of the VHS protein. However, the Arg^{180} Pro mutation which occurred at p31 in the Arg-rich protein (RLORF1) is more likely to affect its tertiary structure. Likewise, the Ser^{1641} Pro mutation in ICP4 which appeared at passage 31 and predominated throughout additional passages is also likely to affects its tertiary structure. It is therefore hypothesized that the loss of classical paralysis involved mutations in the Arg-rich protein RLORF1 and immediate early protein ICP4 which is essential for efficient replication.

One of the most interesting aspects of this study was the discovery that mutations (deletions, insertions, and SNPs) were present in differing proportions within individual passages. Polymorphisms within genes, whether synonymous or non-synonymous, tended to fluctuate throughout the passages (Table 2). Only one polymorphism (G → A) within the intergenic region between MDV006.6/075.1 and MDV007/074 transitioned completely during the course of serial passage. All others were in a state of dynamic equilibrium during passage.

The discovery that GaHV-2 exists as a collection of genomes with limited sequence heterogeneity has changed our views on the stability of the DNA genomes of herpesviruses. Recently it has been reported that other herpesviruses exist as a complex mixture of genome types [60, 61]. Mixed population of genome types or quasispecies has been described for RNA viruses since 1978 (RNA bacteriophage QB), [62] and later for most RNA viruses (e.g., HCV, HIV HBV, and influenza), but only within the last decade have viruses with small circular DNA genomes been reported to exist as quasispecies. Although most reports have involved geminiviruses and mosaic viruses that infect plants, some animal viruses within the family *Circoviridae* have also been reported to exist as a collection of genomes with great sequence heterogeneity [63, 64]. Our sequencing proof that an animal herpesvirus exhibits sequence heterogeneity within a single population has long been suspected by researchers in the field. Research by Witter [65] has demonstrated that upon back passage in vivo serially passaged attenuated viruses can regenerate virus "revertants" with virulent phenotype. Darwinian selection of minor virulent subpopulations was hypothesized to be the likely mechanism for the gain in virulence function. Silva et al. [66, 67] have shown that upon serial passage a region in the repeat long, known as the 132 bp repeat, is duplicated numerous times. When these viruses are back passaged in bird, this region reverts back to its low copy number. Preferential replication of subpopulations with high copy numbers of the 132 bp repeats also hinted at the existence of mixed genome populations [68].

The nucleotide sequencing of GaHV-2 genomes as bacterial artificial chromosomes (BACs) has also suggested the mixed nature of Marek's disease virus genomes. Petherbridge et al. [69] have reported that recombinant BACs generated from the virulent RB-1B strain exhibit distinctly differing phenotypes in vitro and in vivo. These differences were shown not to be due to cloning artefacts but rather due to random cloning of genomes from a single-parental virus stock [70]. One MDV-1 BAC (designated RB-1B-5) with frameshift mutations in RLORF1, U_L13, U_L44 (gC), and U_S6 (gD) when reconstituted, was unable to infect horizontally. Interestingly the parental stock, which was horizontal transmission competent, contained a mixed population of genomes with wild type and mutant RLORF1, U_L13, U_L44 (gC), and U_S6 (gD), genes [70, 71]. Barrow and Venugopal [72] have also noted that two BAC clones of the virulent European strain C12/130, when reconstituted, exhibit distinctly different phenotypes in birds. Nucleotide sequencing of these BACs have identified minor differences, [73] further supporting the hypothesis that GaHV-2 exists as a collection of mixed genome populations.

Because of the nature of the 454 pyrosequencing technology, linking mutations on the same genome molecule is not easily accomplished. Since it is widely believed that attenuation is multigenic, genetic linkage is very important. To accomplish this, future comparative genomic studies are needed that analyze viruses that are reconstituted from bacterial artificial chromosomes with minimal in vitro propagation. After serial passage of these reconstituted viruses in cell culture it should be possible to recapture the recombinant BACs from Hirt's extracts and stably

propagate them in *E. coli*. Determination of the nucleotide sequences of theses GaHV-2 BACs prior to assessment of their level of virulence upon transfection of isolated chicken macrophages and injection back into the chicken should allow for the identification of mutations, linked on the same genome—that are involved in attenuation.

Acknowledgments The authors would like to thank Barbara Riegle of the Avian Disease and Oncology Laboratory for her assistance in the propagation of the virus strains.

References

1. B.W. Calnek, Pathogenesis of Marek's disease virus infection, in *Current Topics in Microbiology and Immunology*, ed. by K. Hirai (Springer, Berlin, 2001), p. 25
2. R.L. Witter, Marek's disease virus vaccines-past, present and future (chicken vs. virus-a battle of the centuries), in *Current progression Marek's disease research. Proceedings of the 6th International Symposium on Marek's disease*, ed. by K.A. Schat et al. (American Association of Avian Pathologists, Kennett Square, 2001)
3. B. Roizman, A.E. Sears, in *Fields Virology*, ed by B.N. Fields, D.M. Knipe, P.M. Howley (Lippincott-Raven Press, New York, 1996), p. 2231
4. A.E. Churchill, L.N. Payne, R.C. Chubb, Marek's disease immunization against Marek's disease using a live attenuated virus. Lancet **1**(7595), 610–611 (1969)
5. S.R. Pagliusi, M. Teresa Aguado, Efficacy and other milestones for human papillomavirus vaccine introduction. Vaccine **23**(5), 569 (2004)
6. A.M. Prince, Prevention of liver cancer and cirrhosis by vaccines. Clin. Lab. Med. **16**(2), 493–505 (1996)
7. E. Dudnikova et al., Factors influencing the attenuation of serotype 1 Marek's disease virus by serial cell culture passage and evaluation of attenuated strains for protection and replication. Avian Dis. **53**(1), 63–72 (2009)
8. R.C. Karpathy, G.A. Firth, G.A. Tannock, Derivation, safety and efficacy of a Marek's disease vaccine developed from an Australian isolate of very virulent Marek's disease virus. Aust. Vet. J. **80**(1–2), 61–66 (2002)
9. S.J. Spatz et al., Clustering of mutations within the inverted repeat regions of a serially passaged attenuated Gallid herpesvirus type 2 strain. Virus Genes **37**(1), 69–80 (2008)
10. R.L. Witter, K.S. Kreager, Serotype 1 viruses modified by backpassage or insertional mutagenesis: approaching the threshold of vaccine efficacy in Marek's disease. Avian Dis. **48**(4), 768–782 (2004)
11. R.L. Witter, L.F. Lee, A.M. Fadly, Characteristics of CVI988/Rispens and R2/23, two prototype vaccine strains of serotype 1 Marek's disease virus. Avian Dis. **39**(2), 269–284 (1995)
12. B.H. Rispens et al., Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis. **16**(1), 108–125 (1972)
13. I. Gimeno et al., Differential attenuation of the induction by Marek's disease virus of transient paralysis and persistent neurological disease: a model for pathogenesis studies. Avian Pathol. **30**(4), 397–409 (2001)
14. S.J. Spatz, Accumulation of attenuating mutations in varying proportions within a high passage very virulent plus strain of Gallid herpesvirus type 2. Virus Res. **149**(2), 135–142 (2010)
15. R.L. Witter, Increased virulence of Marek's disease virus field isolates. Avian Dis. **41**(1), 149–163 (1997)
16. C. Sinzger et al., A simple and rapid method for preparation of viral DNA from cell associated cytomegalovirus. J. Virol. Methods **81**(1–2), 115–122 (1999)
17. J.D. Volkening, S.J. Spatz, Purification of DNA from the cell-associated herpesvirus Marek's disease virus for 454 pyrosequencing using micrococcal nuclease digestion and polyethylene glycol precipitation. J. Virol. Methods **157**(1), 55–61 (2009)
18. M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**(13), 3406–3415 (2003)
19. S.J. Spatz, C.A. Rue, Sequence determination of a mildly virulent strain (CU-2) of Gallid herpesvirus type 2 using 454 pyrosequencing. Virus Genes **36**, 478–489 (2008)
20. E.R. Tulman et al., The genome of a very virulent Marek's disease virus. J. Virol. **74**(17), 7980–7988 (2000)
21. D. Li et al., Further characterization of the latency-associated transcription unit of Marek's disease virus. Arch. Virol. **143**(2), 295–311 (1998)
22. R.W. Morgan et al., Marek's disease virus latency, in *Current Topics in Microbiology and Immunology*, ed. by K. Harai (Springer, Berlin, 2001), p. 223
23. S.J. Spatz et al., Comparative full-length sequence analysis of oncogenic and vaccine (Rispens) strains of Marek's disease virus. J. Gen. Virol. **88**(Pt 4), 1080–1096 (2007)
24. A.M. Levy et al., Characterization of the chromosomal binding sites and dimerization partners of the viral oncoprotein Meq in Marek's disease virus-transformed T cells. J. Virol. **77**(23), 12841–12851 (2003)
25. J.P. Kamil et al., vLIP, a viral lipase homologue, is a virulence factor of Marek's disease virus. J. Virol. **79**(11), 6984–6996 (2005)
26. P.L. Cortes, C.J. Cardona, Pathogenesis of a Marek's disease virus mutant lacking vIL-8 in resistant and susceptible chickens. Avian Dis. **48**(1), 50–60 (2004)
27. X. Cui et al., A Marek's disease virus vIL-8 deletion mutant has attenuated virulence and confers protection against challenge with a very virulent plus strain. Avian Dis. **49**(2), 199–206 (2005)
28. M.S. Parcells et al., Marek's disease virus (MDV) encodes an interleukin-8 homolog (vIL-8): characterization of the vIL-8 protein and a vIL-8 deletion mutant MDV. J. Virol. **75**(11), 5159–5173 (2001)
29. K.W. Jarosinski et al., Attenuation of Marek's disease virus by deletion of open reading frame RLORF4 but not RLORF5a. J. Virol. **79**(18), 11647–11659 (2005)
30. S.J. Spatz, R.F. Silva, Sequence determination of variable regions within the genomes of gallid herpesvirus-2 pathotypes. Arch. Virol. **152**(9), 1665–1678 (2007)
31. F. Peng et al., Isolation and characterization of cDNAs from BamHI-H gene family RNAs associated with the tumorigenicity of Marek's disease virus. J. Virol. **66**(12), 7389–7396 (1992)
32. L.J. Ross et al., Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. **70**(Pt 7), 1789–1804 (1989)
33. L. Fragnet et al., The RNA subunit of telomerase is encoded by Marek's disease virus. J. Virol. **77**(10), 5985–5996 (2003)
34. S.J. Spatz, R.F. Silva, Polymorphisms in the repeat long regions of oncogenic and attenuated pathotypes of Marek's disease virus 1. Virus Genes **35**(1), 41–53 (2007)
35. A. Abujoub, P.M. Coussens, Development of a sustainable chick cell line infected with Marek's disease virus. Virology **214**(2), 541–549 (1995)
36. D. Jaikumar, K.M. Read, G.A. Tannock, Adaptation of Marek's disease virus to the Vero continuous cell line. Vet. Microbiol. **79**(1), 75–82 (2001)

37. X. Li, K.A. Schat, Quail cell lines supporting replication of Marek's disease virus serotype 1 and 2 and herpesvirus of turkeys. Avian Dis. **48**(4), 803–812 (2004)
38. V. Majerciak et al., Increased virulence of Marek's disease virus type 1 vaccine strain CV1988 after adaptation to qt35 cells. Acta Virol. **45**(2), 101–108 (2001)
39. T. Onoda et al., Propagation of herpes type virus isolated from chickens with Marek's disease in Japanese quail embryo fibroblasts. Biken J **13**(3), 219–228 (1970)
40. D. Schumacher et al., Generation of a permanent cell line that supports efficient growth of Marek's disease virus (MDV) by constitutive expression of MDV glycoprotein E. J. Gen. Virol. **83**(Pt 8), 1987–1992 (2002)
41. K. Osterrieder, J.-F. Vautherot, The genome content of Marek's disease-like viruses, in *Marek's Disease: An Emerging Problem*, ed. by F. Davison, V. Nair (Elsevier, Oxford, 2004)
42. N. Osterrieder et al., Marek's disease virus: from miasma to model. Nat. Rev. Microbiol. **4**(4), 283–294 (2006)
43. L. Petherbridge et al., Replication-competent bacterial artificial chromosomes of Marek's disease virus: novel tools for generation of molecularly defined herpesvirus vaccines. J. Virol. **77**(16), 8712–8718 (2003)
44. K. Jarosinski et al., A herpesvirus ubiquitin-specific protease is critical for efficient T cell lymphoma formation. Proc. Natl. Acad. Sci. USA **104**(50), 20025–20030 (2007)
45. B. Lupiani et al., Marek's disease virus-encoded Meq gene is involved in transformation of lymphocytes but is dispensable for replication. Proc. Natl. Acad. Sci. USA **101**(32), 11815–11820 (2004)
46. S. Trapp et al., A virus-encoded telomerase RNA promotes malignant T cell lymphomagenesis. J. Exp. Med. **203**(5), 1307 (2006)
47. L. Fragnet, E. Kut, D. Rasschaert, Comparative functional study of the viral telomerase RNA based on natural mutations. J. Biol. Chem. **280**(25), 23502–23515 (2005)
48. B.B. Kaufer et al., Herpesvirus telomerase RNA(vTR)-dependent lymphoma formation does not require interaction of vTR with telomerase reverse transcriptase (TERT). PLoS Pathog. **6**(8), e1001073 (2010)
49. J. Burnside et al., Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. J. Virol. **80**(17), 8778–8786 (2006)
50. Y. Yao et al., MicroRNA profile of Marek's disease virus-transformed T-cell line MSB-1: predominance of virus-encoded microRNAs. J. Virol. **82**(8), 4007–4015 (2008)
51. A.S. Anderson, A. Francesconi, R.W. Morgan, Complete nucleotide sequence of the Marek's disease virus ICP4 gene. Virology **189**(2), 657–667 (1992)
52. P. O'Hare, G.S. Hayward, Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded trans-acting factors. J. Virol. **61**(1), 190–199 (1987)
53. P. O'Hare, G.S. Hayward, Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**(3), 751–760 (1985)
54. I.M. Gimeno et al., Biocharacteristics shared by highly protective vaccines against Marek's disease. Avian Pathol **33**(1), 59–68 (2004)
55. R. Yunis, K.W. Jarosinski, K.A. Schat, Association between rate of viral genome replication and virulence of Marek's disease herpesvirus strains. Virology **328**(1), 142–150 (2004)
56. J.W. Balliet et al., Site-directed mutagenesis of large DNA palindromes: construction and in vitro characterization of herpes simplex virus type 1 mutants containing point mutations that eliminate the oriL or oriS initiation function. J. Virol. **79**(20), 12783–12797 (2005)
57. J.W. Balliet, P.A. Schaffer, Point mutations in herpes simplex virus type 1 oriL, but not in oriS, reduce pathogenesis during acute infection of mice and impair reactivation from latency. J. Virol. **80**(1), 440–450 (2006)
58. T.R. Hernandez et al., Mutations in a herpes simplex virus type 1 origin that inhibit interaction with origin-binding protein also inhibit DNA replication. J. Virol. **65**(3), 1649–1652 (1991)
59. D.W. Martin et al., Analysis of the herpes simplex virus type 1 OriS sequence: mapping of functional domains. J. Virol. **65**(8), 4359–4369 (1991)
60. N. Renzette et al., Extensive genome-wide variability of human cytomegalovirus in congenitally infected infants. PLoS Pathog. **7**(5), e1001344 (2011)
61. M.L. Szpara et al., A wide extent of inter-strain diversity in virulent and vaccine strains of alphaherpesviruses. PLoS Pathog. **7**(10), e1002282 (2011)
62. E. Domingo et al., Nucleotide sequence heterogeneity of an RNA phage population. Cell **13**(4), 735–744 (1978)
63. T.F. Ng et al., Discovery of a novel single-stranded DNA virus from a sea turtle fibropapilloma by using viral metagenomics. J. Virol. **83**(6), 2500–2509 (2009)
64. T. Nishizawa et al., Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. J. Virol. **73**(11), 9604–9608 (1999)
65. R.L. Witter, Attenuated revertant serotype 1 Marek's disease viruses: safety and protective efficacy. Avian Dis. **35**(4), 877–891 (1991)
66. R.F. Silva, S.M. Reddy, B. Lupiani, Expansion of a unique region in the Marek's disease virus genome occurs concomitantly with attenuation but is not sufficient to cause attenuation. J. Virol. **78**(2), 733–740 (2004)
67. R.F. Silva, R.L. Witter, Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J. Virol. **54**(3), 690–696 (1985)
68. M. Niikura, J.B. Dodgson, H.H. Cheng, Stability of Marek's disease virus 132-bp repeats during serial in vitro passages. Arch. Virol. **151**(7), 1431–1438 (2006)
69. L. Petherbridge et al., Oncogenicity of virulent Marek's disease virus cloned as bacterial artificial chromosomes. J. Virol. **78**(23), 13376–13380 (2004)
70. S.J. Spatz et al., Comparative sequence analysis of a highly oncogenic but horizontal spread-defective clone of Marek's disease virus. Virus Genes **35**(3), 753–766 (2007)
71. K.W. Jarosinski et al., Horizontal transmission of Marek's disease virus requires US2, the UL13 protein kinase, and gC. J. Virol. **81**(19), 10575–10587 (2007)
72. A. Barrow, K. Venugopal, Molecular characteristics of very virulent European MDV isolates. Acta Virol. **43**(2–3), 90–93 (1999)
73. S.J. Spatz et al., Genotypic characterization of two bacterial artificial chromosome clones derived from a single DNA source of the very virulent gallid herpesvirus-2 strain C12/130. J. Gen. Virol. **92**(Pt 7), 1500–1507 (2011)