ANTIGENIC AND BIOLOGICAL

CHARACTERIZATION OF H5 AVIAN INFLUENZA VIRUSES

by

JENNIFER PFEIFFER

(Under the Direction of David L. Suarez)

ABSTRACT

Avian influenza (AI) undergoes antigenic drift, enabling it to evade the host immune response, allowing higher replication and enhanced transmission. Ongoing surveillance and biological characterization is necessary to monitor currently circulating viruses, in part to allow the optimal use of vaccines to protect vaccinated birds from disease and to reduce the amount of virus that is shed. Current methods of characterization include sequence analysis, antigenic characterization, and vaccine efficacy studies, but a simpler and more reliable method for characterization is needed.

Nineteen highly pathogenic H5N1 AI isolates from poultry in northern Vietnam in 2005 were characterized, and commercial AI vaccines were evaluated in their abilities to prevent disease and to reduce viral shedding. The H5N1 viruses have drifted, not only from other Asian H5N1s, but also amongst themselves; the Vietnamese viruses were in two genetic and antigenic subgroups, clades 2.3.2 and 2.3.4. These H5N1 viruses were exceptionally virulent in both chickens and Pekin ducks. When two representative strains were tested in a vaccine efficacy study, current vaccines protected against disease and reduced viral shedding. However, the vaccine produced from an older virus provided marginal protection and should be replaced.

In analysis of another H5 influenza lineage, the Mexican H5N2, previous studies had characterized antigenic drift in the face of vaccination, and had identified potential antigenic sites important for virus neutralization. Using DNA vaccines and reverse genetics, point mutations in the hemagglutinin (HA) protein were introduced to determine the influence that particular amino acids have on the antigenicity of this H5 lineage. Based on data obtained from cross-HI tests, virus neutralizations in embryonating chicken eggs, and an *in vivo* study comparing levels of virus shed between groups of chickens, differences were detected which corresponded to overall sequence similarity of the HAs used. However, it appears that none of these differences could be attributed to the point mutations alone.

These studies emphasize the importance of continuous monitoring of currently circulating HP AI H5N1 viruses. Until an alternative, simplified method is developed, the best way to assess vaccine efficacy and seed strain selection is by direct, *in vivo* testing.

INDEX WORDS: Hemagglutinin, Avian influenza, H5N1, Highly pathogenic, Antigenic mapping

ANTIGENIC AND BIOLOGICAL CHARACTERIZATION OF H5 AVIAN INFLUENZA VIRUSES

by

JENNIFER PFEIFFER

B.S., North Georgia College and State University, 2002

M.S., The University of Georgia, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

ANTIGENIC AND BIOLOGICAL

CHARACTERIZATION OF H5 AVIAN INFLUENZA VIRUSES

by

JENNIFER PFEIFFER

Major Professor: David L. Suarez

Committee: David E. Swayne
Mark W. Jackwood
Maricarmen Garcia
R. Jeff Hogan

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

DEDICATION

To Mom and Dad, for your continuous support. I am extremely lucky and will forever be grateful.

To Grandma Faye for all of the thoughtful and sweet things you have done for me. And in memory of Beepa, Grandma, and Grandpa.

DR. Chicken, J-Mack, Erin, Andrea, Amy, and Erin, you've all been wonderful friends. I'm so thankful to have each one of you. Thank you for all of your love and support these past 12+ years.

TROY, we may've shaved ~20 years off our lives, but we've gotten through it and will hopefully see that it will all work out for the best and the best of both worlds. Thanks for all of your sarcasm and silly ideas.

Sco-tA and Jamie-Burnett, I couldn't have asked to live within 90s (with or without an 8-lb. hand-bag!) of two better people, these past few years. From slug-feeding, green-turned-orange peppers, watching you dancing on your front porch, Scot-tA, summer afternoons at the pool (no tanning beds, Burnett!), snowy nights by candle light, 'black night', and the priceless investment of turning 86 cents of street/driveway change into ongoing entertainment and laughter, to a raspy-voiced lady answering my phone, you never cease to amuse me or make me laugh!! Thanks for looking out for me. "I'll remember you." And that's NOT made up!!…dot-com…

Cardee, it's been a joy seeing you first thing every morning, for our daily swims. Thank you for always sending me good vibes on test days and remembering the important dates. Your post cards have allowed me to travel vicariously through you!

And of course, to Risi and Charlie

ACKNOWLEDGMENTS

Dr. Suarez, ‘the pinball wizard’, thank you for your patience and for maintaining your sense of humor during this learning process of mine. You had more confidence in me than I did in myself. I appreciate your willingness to always help me or answer any questions (which were often delayed, due to my slow-processing brain) that I may have had. Thanks, Dog.

Chang-Won, thank you for all of your pointers, starting nearly six years ago when I began my master’s degree, and continuing even after you moved on from SEPRL.

Suzanne, thank you for all of your help with the bird work (the Billions and Billions of times!) and for helping me to adopt a *slightly* more laid back attitude.

Aniko, thank you for accompanying and assisting me with my numerous RNA extractions and real-time runs and swabbing. It was very comforting to hear your words of positive affirmation when I started getting stressed. Keep collecting the label sheets!

My committee members, Drs Jackwood, Hogan, Swayne, and Garcia, thank you for your guidance and support over the past few years. I appreciate all of your help.

To everyone at SEPRL who has ever given me their advice, escorted me in high containment, checked my eggs or put them in the cooler for me, shared their reagents or protocols, or made a joke to cheer me up when Science was testing the strength of my will, I want to say thank you.

Toni, thank you for your positive attitude and comforting reinforcement. You truly are my angel - I don’t know what I would’ve done if you hadn’t visited our lab and enlightened me with your TOPO-cloning knowledge. I am so glad that we have kept in touch since then. You have been a great friend.

Patti, my faithful friend/office companion/confidant. …I just don’t know where to even begin….I am so lucky to have you...thank you for your camaraderie not only at SEPRL, but everywhere else we’ve gone/ been these past 4.5 yrs. We have had lots of fun – from ‘summer vacations’ to shopping sprees. Thank you for always looking out for me and holding my hand during the more difficult endeavors. You’re awesome.

To Mary Pantin-Jackwood, for telling me, “I know you can do it” and Troy West, for asking me, “Why the hell not?” I thank you for these remarks when I was questioning whether to pursue my goal of obtaining this degree. Your words were what ‘sealed the deal’ for me.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Background of the study

Objective #1

In 1997, a highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype that spread through live bird markets in Hong Kong first received international recognition. The outbreak resulted in at least 18 human cases. To control the outbreak, most all poultry in Hong Kong were culled. Although the outbreak in Hong Kong was contained, similar HPAI viruses continued to circulate in the region, but were contained primarily in Southeast Asia until 2005 (1, 9). However, a major change was observed in the virus in April 2005 that resulted in a widespread infection of wild waterfowl that over-wintered at Qinghai Lake, in western China (4). It has been speculated that for the first time, wild waterfowl were important vectors for the spread of HPAI viruses, and that a combination of infected wild birds and infected poultry movements contributed to the unprecedented westward spread of a HPAI virus to Europe and Africa, (3, 4, 6). Today, these H5N1 viruses have become endemic in poultry in some countries in Asia and Africa.

When genetically analyzed, Asian H5N1 viruses that were isolated over the past 13 years, beginning in 1996, can be divided into multiple clades (clade 0, clade 1, or clade 2 being the most widespread), based on their hemagglutinin (HA) sequences (11, 12). Clade 1 viruses circulating in Cambodia, Thailand, and Vietnam caused human

infections in these countries from 2004 to 2005 and in Thailand in 2006 (13). Since 2003, clade 2 viruses circulated in birds in China and Indonesia and spread westward during 2005-2006, to the Middle East, Europe, and Africa (13). Clade 2 viruses have been the main cause of human infections since late 2005 (13). Recently, a distinct clade 2 sublineage of viruses from southern China, referred to as the Fujian-like sublineage, appears to have become the most prevalent lineage in Southeast Asia (7).

Due to the fact that H5N1 AI viruses continue to circulate and have become endemic in poultry, the potential threat of a future pandemic in humans is not going away. Therefore, it is extremely important to continue characterizing these Asian H5N1 viruses as they continue to drift. By doing so, any changes in their genetic make up or other potential factors altering their virulence could be detected in time for response measures to be implemented, to contain and prevent further spread of these viruses. All knowledge gained from studies with these viruses will provide a better understanding of them and allow for wiser decisions to be made, such as how to most effectively control future outbreaks or which viral vaccine seed strain should be selected for optimal protection.

Because of the great predisposition that avian influenza viruses have to antigenic drift, H5N1 viruses have acquired many mutations since they began circulating. Some of these changes may enable the viruses to evade the host immune response by allowing them to escape neutralizing antibodies induced by vaccination or natural infection. This phenomenon could result in a decreased ability of a vaccine to induce protection against viral disease and/or shedding in poultry. Vaccinates subsequently infected with virus

may not display signs, but still shed high levels of virus, allowing greater transmission of the virus within a flock or between flocks before the virus was detected.

A positive correlation exists in that the closer a vaccine and a challenge strain are related in amino acid sequence similarity, the better the protection that is observed against viral shedding in vaccinated chickens (8). Therefore, it is important that the vaccines used in poultry be routinely evaluated for their protective efficacy, to determine if there is a need to update the vaccines that are currently in use.

The main objective of part 1 of this research project was to characterize, molecularly and antigenically, 19 HPAI H5N1 viruses isolated from ducks and chickens in northern Vietnam in late 2005 and to evaluate commercially produced oil emulsion vaccines currently used in Vietnam in their abilities to protect chickens against disease and shedding of two representative viruses. Ultimately, the information gained from these studies can be applied when making future selections for a vaccine virus seed strain.

Objective #2

In 1994, an outbreak of a low pathogenic (LP) H5N2 AI began in Mexico. Highly pathogenic strains, which emerged between 1994 and 1995, were eradicated. However, LP viruses continued to circulate among chickens in Mexico and eventually spread to El Salvador and Guatemala, and have been isolated in the US, from parrots which were most likely smuggled from Mexico (2) (5). A vaccination program in Mexico, which was implemented in 1995 as part of a control strategy, continues to be used (5).

Unlike flu viruses that circulate among humans, AI viruses were thought to remain antigenically stable (10). However, Lee *et al.* (2004) analyzed viral strains isolated in Mexico and other neighboring regions including Guatemala between 1994 and 2002, and found this is not necessarily true. It was shown that over a long period of time, in a region where the outbreak had persisted, antigenic drift had occurred. In addition to the natural host antibody response to multiple infections with LP viruses, this may also have been caused in part by vaccination-induced pressure, and resulted in large differences in HI titers between vaccine viruses and antibodies against more recent isolates such as CK/Guatemala/194573/02 (CK/Guat). Multiple changes were detected in the HA1 protein regions of these isolates, some of which occurred at the proposed antigenic sites.

In 2004, an H5N2 was isolated from a pet parrot in California. The HA of this virus clustered with the Mexican lineage of viruses, indicating that the parrot was most likely illegally imported from Mexico (2). The HA gene of this isolate was even more genetically distant from the vaccine virus strain than was the HA gene of the CK/Guat isolate. Some of the differences between the CK/Guat and the Parrot/CA isolates were located at the proposed antigenic sites. These changes in the Parrot/CA isolate indicate that the virus had since 2002, continued to drift from the vaccine seed strain virus.

Mutations acquired by circulating viruses may allow for the viruses to evade antibodies induced in the host by vaccination or natural infection. As a result, the circulating virus may be better able to replicate and hence more likely to be shed by infected birds, ultimately spreading from infected flocks to uninfected, neighboring flocks. It is important to genetically match a vaccine virus with the currently circulating

viruses so that the utmost protection will be induced by the vaccine and minimal levels of virus shedding will occur. If one were able to pinpoint particular amino acids of the HA protein involved in evasion of the antibody response, vaccine seed viruses could more appropriately and more easily be chosen based on sequence information only. The vaccine seed strain candidate HA sequences, particularly the amino acid sequences involved in antibody neutralization, could be compared to those sites within the HAs of circulating viruses. Theoretically speaking, the most effective vaccine seed strain virus could be selected based on which or how many amino acids are similar at the proposed antigenic sites in comparison with the circulating field strains.

Site-directed mutagenesis involves the introduction of nucleotide mutations within specific codons with the goal of altering the amino acid sequence of a protein. This approach is useful for studying the role of those particular amino acids in the function of the protein. By changing the amino acids in the proposed antigenic regions of the HA protein of an avian influenza isolate, it may be possible to specifically locate those amino acids involved in escaping the host antibody response.

The main objective of part two of this research project was to determine how particular amino acids of the proposed antigenic sites were involved in formation of drift variants originating from Mexican isolate CK/Hidalgo/232/94, that were able to escape the antibody response induced by vaccination. Theoretically, it would allow for more accurate and easier selection of vaccine seed strains in the future.

Information gained from these studies will help to pinpoint amino acids that affect HI titers and that are involved in protection against viral disease and shedding. By being able to pinpoint the involved amino acids, one could evaluate the sequences of potential

vaccine strain candidates and make decisions based on these observations, as to which virus strain to select to use in a vaccine. Collectively, the knowledge gained from all aforementioned projects will lend insight into vaccine seed strain selection, whether it be Eurasian or North American viruses.

REFERENCES

1. Cauthen, A. N., D. E. Swayne, S. Schultz-Cherry, M. L. Perdue, and D. L. Suarez. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J. Virol. 74:6592-6599. 2000.

2. Hawkins, M. G., Crossley, B. M., Osofsky, A, Webby, R. J., Lee, C. W., Suarez, D. L., and Hietala, S. K. Avian influenza A virus subtype H5N2 in a red-lored amazon parrot. J Am Vet Med Asoc 228:236-241. 2006.

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

4. Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. W. Zhang, X. L. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. F. Gao. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309:1206. 2005.

5. Senne, D. A. Avian influenza in North and South America, 2002-2005. Avian Dis. 50:167-173. 2007.

6. Sims, L. D. Lessons learned from Asian H5N1 outbreak control. Avian Dis. 50:174-181. 2007.

7. Smith, G. J., X. H. Fan, J. Wang, K. S. Li, K. Qin, J. X. Zhang, D. Vijaykrishna, C. L. Cheung, K. Huang, J. M. Rayner, J. S. Peiris, H. Chen, R. G. Webster, and Y. Guan. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U S A 103:16936-16941. 2006.

8. Swayne, D. E., and D. L. Suarez. Highly pathogenic avian influenza. Rev. Sci. Tech. 19:463-482. 2000.

9. Webster, R. G., Y. Guan, M. Peiris, D. Walker, S. Krauss, N. N. Zhou, E. A. Govorkova, T. M. Ellis, K. C. Dyrting, T. Sit, D. R. Perez, and K. F. Shortridge. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J. Virol. 76:118-126. 2002.

10. 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.

11. WHO H5N1 nomenclature. In. World Health Organization H5N1 Evolution Working Group. Geneva, Switzerland. http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html. 2007.

12. WHO Evolution of H5N1 avian influenza viruses in Asia. Emerg. Infect. Dis. . In. Global Influenza Program Surveillance Network. pp 1515-1521. . 2005.

13. WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines. 2007.

CHAPTER 2

LITERATURE REVIEW

Overview

Avian influenza virus (AIV) is a member of the family *Orthomyxoviridae* and of the genus Influenza virus type A (28, 40, 59, 89). Four other genera within this family include Influenza virus types B, C, Thogotovirus, and Isavirus (40, 59, 89, 105). Influenza types B and C infect primarily humans, but type A influenza infects not only humans but many different species of birds, swine, and horses as well (104).

Avian influenza (AI) is a disease in poultry, which phenotypically presents in either a low pathogenic form (LP) or a highly pathogenic (HP) phenotype and is caused by *avian influenza* virus (89). The signs associated with LPAI range from asymptomatic infection, respiratory disease, or decreases in egg production (3, 89). LPAI is usually associated with low mortality, but in cases of concurrent bacterial or viral infection or poor environmental conditions, a mortality rate of 30% or higher has been observed (89). Highly pathogenic AI (HPAI) can cause a mucosal as well as a systemic disease with up to 100% mortality in chickens (3, 89). Avian influenza is of great economic importance to the poultry industry (13, 89). For example, the most severe economic losses are a result of HPAI outbreaks on commercial farms with intensive poultry production (89). Direct losses of such outbreaks include costs of depopulation and disposal, losses due to high morbidity and mortality, quarantine and surveillance costs, indemnities to compensate for the elimination of marketing birds, and loss of export markets for trade

(13, 89). LPAI outbreaks have also resulted in great economic losses for chicken, turkey, and duck producers, particularly when opportunistic secondary bacterial and viral pathogens are present (1, 13, 104). However, these losses are less significant than those resulting from HPAI because of lower mortality rates and fewer interruptions to national or international trade (89). Avian influenza was originally thought to be associated exclusively with chickens, but further research showed it also affects turkeys, ducks, quail, and various other domestically raised avian species (13, 79, 89).

History

Highly pathogenic AI was likely first described in 1878, when Perroncito reported 'fowl plague' in chickens in Italy (13, 28, 89). Rivolta and Delprato further described that 'fowl plague' was different from fowl cholera in 1880, which they called *typhus exudatious gallinarum* (79). Centanni and Savonuzzi, in 1901, next determined that the cause of 'fowl plague' was a filterable agent (79, 89) isolated from an infected chicken, suggesting that its etiology was viral (1, 28). Hirst then characterized the hemagglutination activity of influenza virus in 1941, and Schafer further characterized 'fowl plague' as a member of the influenza A virus group in 1955 (28).

HPAI outbreaks have been known to spread widely since being first described in Italy. For example, during 1894, a severe outbreak of HPAI in Northern Italy spread to chickens in various parts of Europe (28, 79, 89) where it remained endemic until the mid-1930s (89). HPAI was first discovered in the United States in 1924-25, and then again in 1929 (31). In both instances, it was fully eradicated (31). By the middle of the

twentieth century, AI had been diagnosed in most of Europe, Russia, Netherlands, Hungary, Great Britain, Egypt, China, Japan, Brazil, and Argentina (89).

In 1949, the first isolate of LPAI, an H10, was obtained from chickens in Germany (89). During the first half of the twentieth century, it had been assumed that all H5 and H7 hemagglutinin subtypes of AI were HP (89). This idea was changed with the isolation of a HP H5 influenza virus in Scotland in 1959 (89). The false notion that all H5 and H7 subtypes of AIV were HP was reversed after 1971, when many LP-causing H5 and H7 AIVs were isolated (28, 89).

The first isolation of a HPAI H5N3 influenza virus from wild birds was in 1961, found in common terns in South Africa (1, 11). The next discovery in the history of AI was made in the 1970s during surveillance for exotic Newcastle disease in California, when AIV was isolated from migratory waterfowl (28, 89). The data gained from these surveys helped to determine that AIV causes asymptomatic infection in wild birds (28, 89). Since then, healthy wild birds, particularly Anseriformes (shorebirds) and Charadniiformes (waterfowl) have been found to be asymptomatic reservoirs of AIV (1, 28, 89). Avian influenza virus is most commonly introduced into domestic poultry by wild migratory waterfowl (31). Fortunately, most AI from these wild birds is LP (28, 89), and the prevailing understanding is that HPAI emerges only after the virus has been introduced to poultry from wild birds (1).

Until recently, the presence of HPAI was rare in domestic poultry and it is considered to be a foreign animal disease in the U.S (3, 42). A mere 17 episodes were reported worldwide from 1959-1998 (3). The last major outbreak of HPAI in the U.S. occurred in 1983, when an H5N2 outbreak occurred in Pennsylvania, costing nearly 60

million dollars just for the control effort and resulted in the death or destruction of nearly 17 million birds (89). The Pennsylvania outbreak was the first documented case of LPAI virus mutating to the highly pathogenic form of the virus (42). The source of the LPAI virus associated with the outbreak was not determined, but one speculation is that the LPAI had been endemic in live bird markets (LBM) in this region (28, 42, 84). The role of LBMs in avian influenza epidemiology was not recognized until 1986 when LP H5N2 that was related to the HPAI outbreak AI was first isolated, and the H5N2 Pennsylvania lineage of virus was not completely eradicated until 1989 (84). Since 1997, eight more episodes of HPAI have occurred over a seven year period (3), including an unusual H5N2 outbreak in Texas in 2004 (3, 45). Interestingly, the virus isolated from the Texas 2004 outbreak contained a cleavage site identical to the HP isolate CK/Scotland/59, but did not cause disease when experimentally inoculated into chickens (45). There has been an increase in LPAI outbreaks, particularly of the H5 and H7 subtypes, reported in various other countries (3). These incidences have occurred in Pakistan, Mexico, Central America, Australia, Italy, Ireland, parts of Europe, North America, and in Southeast Asia (3), causing it to be an international disease of economic concern (89).

For a long time, interspecies transmission of AIV from birds to humans was considered to be highly unlikely and seldom occurred (28, 79, 89). However, in Hong Kong in 1997, 18 people became infected with a HPAI strain of H5N1 containing a genome entirely of avian origin (28, 41). Of these 18 infected humans, six people died (28). New human cases of lethal H5N1 AI are currently being reported in Indonesia, Egypt, Vietnam, and China, as well as various other countries worldwide, nearly every week (WHO). This phenomenon has raised fears that the H5 strain could cause the next

global influenza pandemic among humans, should it become better adapted for direct human-to- human transmission (28).

Etiology

Classification

Avian influenza is a member of the family *Orthomyxoviridae* (40, 59). "Ortho" means "other" and "myxo" means "mucous" in Greek (40). This family was so named because of its ability to bind to mucous and also as a means of differentiating it from other negative sense RNA viruses, such as members of the *Paramyxoviridae* family (40). The orthomyxovirus family is comprised of influenza A, B, and C, thogotovirus, and isavirus, of which influenza A is the only member of the family known to infect birds (59). The name influenza is an Italian word derived from the Latin word "influentia" meaning "epidemic", so chosen because disease epidemics were thought to occur as a result of occult influences (40).

Orthomyxoviridae viruses are enveloped, segmented, single-stranded, negative sense RNA viruses (40, 59). The term negative sense refers to the fact that the viral RNA lacks a 7-methyl guanosine cap and can not be directly translated into viral proteins and is complementary to the coding sequence (40). Thus, each of the eight viral RNA segments must be transcribed into positive sense RNA (40). Influenza viral RNA serves two purposes: 1) template for mRNA synthesis; proteins comprising the virus particles are manufactured from these and 2) template or cRNA production; the negative sense viral RNA serves as a template for the anti-genome (+) strand that serves as template for making more viral RNA (40).

Unique characteristics of influenza viruses that separate them from other RNA viruses are that both transcription and replication occur in the nucleus of the infected cell (40). For initiation of mRNA synthesis, an influenza virus-encoded cap-dependent endonuclease snatches the 7-methyl guanosine-caps of cellular mRNA to use as primers for mRNA synthesis (40). Influenza also takes advantage of the cellular splicing machinery (40). In doing so, it also utilizes its compact genome to great capacity; its replication involves some spliced mRNAs with overlapping reading frames, bicistronic mRNAs with overlapping reading frames, and finally, coupled translation of tandem repeats (40).

Differentiating the various types of influenza viruses (A, B, or C) is based on antigenic differences between the nucleocapsid (NP) and matrix (M) proteins (40). Type A influenza viruses naturally infect various avian species, humans, and other mammalian species (*i.e.* swine and horses) (40, 58). Further subtyping of type A influenza is carried out based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (58, 89). These two glycoproteins show greater variability in their amino acid sequences compared to those of the other types of influenza (40).

Virion morphology

The typical shape of virions is spherical to pleomorphic but may also be filamentous (40, 58, 89). The size ranges from 80-120 nm in diameter (40, 89). Each particle is surrounded by a lipid envelope derived from the plasma membrane of the infected host cell from whence the viral particle budded (40, 89). Protruding from the envelope are two different types of spikes (40, 89). The HA proteins are rod-shaped

trimers and are approximately four to five times more abundant than the mushroom-shaped neuraminidase tetramer spikes (40, 89). Nestled within the lipid envelope layer is a third integral membrane protein, the ion channel (M_2) (40).

Just beneath the envelope lies the matrix (M_1) protein (40). Associated with the matrix are the various-sized ribonucleoprotein (RNP) complexes (40). Each RNP complex is comprised of nucleocapsid proteins (NP), which are helical-shaped and are intertwined closely with one of the eight segments of ss viral RNA (40). In addition to the NP and viral RNA, the RNA-dependent RNA polymerase complex composed of polymerase basic (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA) also takes part in forming the RNP complexes (40). One RNP complex exists per virus RNA segment (40). The eight segments of genomic viral RNA encode for at least 10 proteins (7); nine comprise the virus particle itself (HA, NA, NP, PB2, PB1, PA, M_1, M_2, and NS_2), while the NS_1 is found exclusively in the cytoplasm of infected cells. (40). The PB1-F2 accessory protein is found in some human and animal viruses (59).

Virus genome and proteins

As previously mentioned, AI has eight RNA segments which are single-stranded and negative sense. Each segment has been designated a number according to its size in nucleotides (40). In order from largest to smallest, the segments are as follows: 1 is PB2, 2 is PB1, 3 is PA, 4 is HA, 5 is NP, 6 is NA, 7 is M, and 8 is NS (40). The size of the entire genome is approximately 13, 588 nucleotides, but it varies according to each particular virus strain (40).

Polymerase Proteins (PB2, PB1, and PA)

The polymerase proteins are encoded by the three largest viral RNA segments (40). Their molecular weights range from 85-96 kDa (40). They were named according to their behavior on isoelectric focusing gels; PB1 and PB2 have a basic net charge of +28 at pH 6.5 while the PA has an acidic charge of –13.5 at pH 6.5 (40).

RNA segments 1 and 2, PB2 and PB1, are each 2,341 nucleotides and code for proteins of 759 and 757 amino acids (aa), respectively (40). The third segment, PA, is 2,233 nucleotides long and encodes for a protein of 716 aa (40).

Together, these three polymerase proteins form a complex in the cytoplasm and nucleus of the host cell (40). The complex sedimentation values on sucrose gradients are 11S-22S (40). These proteins are synthesized in the cytoplasm and due to their intrinsic karyophilic properties, are transported to the nucleus; each of the proteins contains a nuclear localization signal (40).

The PB1 protein is involved in the recognition and snatching of the 7-methyl-guanosine cap of the 5' end of host cell mRNA (16, 39). It is also part of the transcriptase complex (39, 40). PB2 has endonuclease activity and catalyzes nucleotide addition (40, 89). It also is part of the transcriptase and replication complex (40, 89). Lastly, the PA protein is involved in viral genomic RNA replication and has proteolytic activity (16, 40, 89).

Nucleocapsid Protein (NP)

The NP is the primary structural protein that interacts with each viral RNA segment to form the RNPs (40). It is a type-specific antigen in that the different types (A,

B, or C) of influenza virus can be distinguished based on this protein (40). It is viral RNA segment five, which is 1,565 nucleotides long and encodes the NP, which is 498 aa in length (40). The protein has a molecular weight of approximately 56 kDa (40). NP is rich in arginine residues and has a basic charge of +14 at pH 6.5 (40). It has no particular clusters of basic residues, which is an implication that multiple regions of NP may participate in its binding to the genomic RNA (40). Like the polymerase proteins, NP is synthesized in the cytoplasm and then transported to the nucleus, due to its two nuclear localization signals (40). During late infection, a cellular protease involved in apoptosis, named caspase, cleaves NP (40). This cleavage may have a function in virus – host interaction or may merely be a marker for apoptosis; this is currently unclear (40). NP molecules carry out various functions in infected cells (89). They are involved in transporting vRNPs from the cytoplasm to the nucleus of an infected cell and are necessary for the synthesis of full-length vRNA (40). NP molecules are one of the major targets for cytotoxic T lymphocytes (40, 98). DNA vaccine studies involving NP DNA have shown to induce both $CD4^+$ and $CD8^+$ T cells and elicit limited heterosubtypic protection against influenza challenge (98).

Hemagglutinin (HA)

Hemagglutinin (HA) is a very important AI protein. While it is the means by which the virus first initiates propagation in the host, it also serves as a major target by which the host keeps this virus in check and from being further being transmitted. It was so named because of its ability to agglutinate erythrocytes (40). The HA has three major roles (40). First, it provides the virus attachment to host receptors containing sialic acid

residues, which are found lining the upper respiratory and intestinal tracts (29, 73, 89). This is important in that if the virus is not able to attach to the cell, it is unable to infect the host, and can not replicate. Second, HA mediates fusion between the virus and endosomal membranes, which ultimately results in the release of viral RNA into the cytoplasm (40). Finally, HA is the major antigenic protein against which neutralizing antibodies are produced (40, 89). It is against this protein that vaccines are targeted. Both antigenic drift and shift promote epidemics to occur (40, 104).

Epidemic patterns of influenza viruses occur in humans (104) as a result of two types of processes (16, 104). Antigenic drift occurs in both the HA and neuraminidase (NA) proteins when point mutations arise (16, 104). These mutations are found predominantly in the globular heads of each HA monomer and flanking the sialic acid-binding site of NA tetramers (105). These mutations enable the virus to evade host immunity against previously circulating viruses in a single person or an entire population (16, 104). The resulting new variants of influenza are the cause of yearly epidemics (104).

Antigenic shift occurs only in segmented viruses (16, 104). This phenomenon arises when a HA, not previously circulating in the population, emerges (104). It can occur by direct transmission from hosts of other species or during coinfection by two distinct subtypes of influenza A (16). During coinfection, the viruses exchange segments, resulting in the emergence of reassortant viruses with different antigenic properties (16). Antigenic shift has been the notorious cause of pandemics associated with worldwide morbidity and mortality (16, 104).

Viral RNA segment four encodes for the HA (40, 59). It is synthesized on membrane-bound ribosomes and then translocated to the lumen of the endoplasmic reticulum (ER) as a precursor polypeptide, HA_0 (40). Its N-terminal signal sequence containing approximately 16 amino acid residues is cleaved by signal peptidase in the ER (39). Being a type I membrane glycoprotein, its amino-terminus (N-terminus) is in the ectodomain while its carboxy-terminus (C-terminus) is found in the proximal transmembrane region (40). It is cotranslationally modified in that its ectodomain acquires up to seven oligosaccharide chains (of which one is needed for proper folding in the ER) and proximal cysteines at its C-terminus acquire three thioether-linked palmitate residues (40, 52, 53).

The HA protein exists as the HA_0 precursor with a molecular weight of 76 kDa or as a cleaved protein made up of HA_1 and HA_2 subunits (47 kDa and 29 kDa, respectively) linked together by disulfide chains (40, 73). Cleavage of HA_0 to HA_1 and HA_2 is important (40, 73) because it is a necessity for virus infectivity and thus determines pathogenicity and spread of infection (40, 77). The newly exposed N-terminus of the HA_2 formed upon cleavage of the molecule, referred to as the fusion peptide, is more conserved among different strains of influenza and is required for HA endosomal fusion activity (40, 77).

In intracellular environments with low pHs, such as the endosome, cleaved HAs undergo irreversible conformational changes which move the fusion peptide toward the endosomal membrane, ultimately resulting in fusion of the viral and endosomal membranes (16, 40). Cleaved HAs at a neutral pH are considered to be in a metastable form (40). The low pH enables them to take on a more stable form, which renders the

HAs more susceptible to digestion by proteases found within the host (40). At this time, some antigenic sites are lost while others are created (40).

The HA gene on RNA segment four was the first influenza gene to be fully sequenced and it encodes for 16 different known subtypes (17, 40). A 16^{th} subtype was described in 2005 (17). The HA protein is approximately 1,750 nucleotides in length and encodes a protein of about 564 amino acids (40). In the mature virus, each HA is a homotrimer composed of three monomers (40, 59). Following cleavage, the fusion peptide is buried in the interior of the trimer (40). The trimeric structure is divided into two regions: the stalk and the globular head (40). The stalk contains the HA_2 part and is proximal to the membrane and the membrane-distal head contains the HA_1 part (40).

The HA glycoprotein is the viral component that binds to the cell receptor sialic acid (15). A receptor-binding pocket, inaccessible to antibodies, is located in the distal head of each monomer (40). The amino acid sequences forming this pocket are conserved among various subtypes, resulting in different binding specificities to host cells (25, 40). Attachment of the virus to host cells requires sialic acid, although strains can vary in their affinities for different sialyloligosaccharides (15). In human trachea, the sialic acid receptor is primarily linked to galactose via α2-6 linkages, while the receptors in avian species contain primarily α2-3 linkages and swine trachea contain both types (25, 40). The differences in receptor specificity are thought to contribute to host specificity; human viruses preferentially bind to the former while avian viruses preferentially bind to the latter (15). However, it is not the only determinant (25) . Most neutralizing antibodies attach to epitopes near the receptor binding sites, which for H3

influenza viruses are characterized into five antigenic binding sites located on the globular heads (40, 59).

The hemagglutinin cleavage site is the primary virulence determinant for avian influenza, with the type and the number of aa at cleavage sites affecting virus virulence (3). Cleavage sites containing two basic aa (arginine or lysine), at positions -1 (which is always an arginine) and -3 or -4, for H7 and H5 subtypes, respectively, are viruses of low virulence (3, 40, 73). These cleavage sites require exogenous proteases such as trypsin, which restricts virus replication to occur only on areas along the respiratory and intestinal tracts (40). Conversely, HPAI viruses typically possess multiple basic aa adjacent to the cleavage site or they contain an insert of aa (3). These multiple basic aa can result from insertion, substitution, or duplication (3, 22) . The presence of multiple basic aa result in motifs that are targets for ubiquitous intracellular proteases such as furin (3, 40, 73). This concept explains why LPAI is localized to mucosal regions while HPAI is mucosal and systemic.

Another factor thought to be involved in determining virulence for some viruses is the presence or absence of a sugar chain near the cleavage site (40). The idea has been proposed that this sugar would block furin from accessing the cleavage site and disease of low virulence would result (28, 40). However, when no sugar is present, furin has access to the cleavage site and disease of the highly pathogenic form may result (40).

Neuraminidase (NA)

The NA is another integral membrane protein found on the influenza virus particle that is involved in subtyping the virus (40, 58, 59). This protein is a

homotetramer of 220 kDa (40). The molecule contains a distal head region with enzymatic activity and a stalk region that is proximal to the virus particle membrane (40). NA has two important purposes: 1) it removes sialic acid from glycoproteins (*i.e.* HA, NA, and cell surfaces), preventing influenza viruses from attaching to each other or remaining bound to cells, which would effectively reduce the level of infectious viral particles and 2) it is an antigenic determinant and undergoes antigenic variation (40). These proteins may also allow the virus to move through the respiratory tract's mucin layer and find the target epithelial cells (25, 40). Finally, some AI NAs have receptor-binding sites which cause hemagglutination (40).

Segment six of the genomic RNA encodes for NA and nine protein subtypes have been identified (40). The gene is approximately 1,400 nucleotides in length and the protein contains about 450 aa (40). The NA protein contains one hydrophobic region, which is located in the N-terminus and spans the lipid bilayer (40). This region serves as both an uncleaved signal and an anchor domain by targeting NA to the ER membrane and allowing for stable attachment in the membrane, respectively (40). The NA's amino-terminus is in the cytoplasm, which puts it in the type II class of membrane glycoproteins (40). There are five possible N-linked carbohydrate sites for most neuraminidase proteins (40, 70).

The head of NA is box-shaped, with large pockets on the surfaces of each monomer for ligand-binding (40). Hemagglutinating activity associated with NA occurs at a sialic acid binding site separate from the enzyme active site (40). On each NA molecule there are four main antibody-binding domains which cluster in regions distal to the membrane but are not neutralizing (40). As a result, antibodies against NA prevent

viral spread from infected cells but do not prevent infection (40). It has been demonstrated through the use of NA subunit vaccines that antibodies against the NA are capable of providing neuraminidase inhibition and partial protection from HPAI challenge (92).

Neuraminidase inhibitors such as oseltamivir and zanamivir can be effective both therapeutically and prophylactically in humans (15, 27). These drugs function by binding to the NA active site with higher binding affinity than does the hydroxyl at the 4' position on the sugar ring of sialic acid (15, 74, 102). Essentially, the neurminidase inhibitors closely mimic the natural subsrate, fitting into the active site pocket and engaging the protein in the most energetically favorable interaction (50). As a result, the NA works to cleave the drug instead of the cellular target (74). Ultimately, if the NA binds to the drug, it is prevented from releasing the progeny virus particle from the infected host cell (50). Infection of new host cells is prevented and spread of infection is halted (50). Recent findings indicate that neuraminidase inhibitors may not be effective against avian influenza due in part to considerably higher viral loads and virulence (33).

Matrix (M_1) and ion channel (M_2)

The matrix (M_1) protein is the most abundant protein in the virus particle and lies just beneath the virus envelope, providing structural support (40). The ion channel (M_2) protein is not as abundant in the virion but it serves an important function; it allows the influx of hydrogen ions (H^+) into the virus particle while the virus is in the endosome (40). This ultimately results in a drop in pH inside the virion that results in a conformational change in the HA (40).

Viral RNA segment seven encodes for both of these two proteins (40). The gene segment is typically 1027 nucleotides long (40). A colinear transcript mRNA codes for M_1 and a spliced mRNA encodes for M_2 protein, which overlaps M_1 by 14 residues (40).

The M_1 protein is typically 252 amino acids that is approximately 28 kDa (40). It, like NP, is a type-specific antigen and its aa sequence is highly conserved among influenza type A subtypes (40). M_1 is a peripheral membrane protein which interacts with the cytoplasmic tails of the three integral membrane proteins (40). If M_1 remains intact with RNPs, during uncoating, the RNPs are not transported to the nucleus; thence, viral replication does not ensue (40). Later on in the replication process of infection, M_1 must enter the nucleus in order for newly synthesized RNPs to exit the nucleus (40).

M_2 is abundant within the plasma membrane of the virus-infected cells, but only between 14-68 of these protein molecules are actually incorporated into the virus particle (40, 106). It spans the membrane only once and because it has an N-terminal ectodomain but no cleavable signal sequence, it is considered a type III integral membrane protein (40). Instead of a signal sequence, it depends on signal recognition particles in order to be cotranslationally inserted into the ER membrane (40). This protein is posttranslationally modified but these modifications are not required for its proper functioning (40). It is a homotetramer that forms a pore in the membrane ion channel (40). The influenza-specific anti-viral drug amantadine targets M_2 and blocks its ion channel activity which prevents the virus from uncoating (40).

M_2 has two important roles for the virus: 1) it permits the influx of hydrogen ions (H^+) into the virion during uncoating and 2) it regulates the pH of the Golgi apparatus (40). This ion channel is activated at low pH levels within endosomes and the trans golgi

network (40). An important characteristic pertinent to these functions is that M_2 is highly specific for H+ ions (40).

Nonstructural Proteins (NS_1 and NS_2)

The smallest segment of viral RNA, segment eight, encodes for two nonstructural proteins, NS_1 and the nuclear export protein (NEP), NS_2 (40, 60). The NS_2 mRNA was the first proof that splicing occurs with an RNA virus that lacks any DNA intermediates during replication (40).

Viral RNA segment eight is typically 890 nucleotides in length (40). The NS_1 protein for which it encodes has a molecular weight of approximately 26 kDa and is made up of 230 aa while the NS_2 protein is about 14 kDa and is typically 121 aa in length (40). The NS_1 is directly encoded by mRNA and the NS_2 mRNA is spliced (40). They both share a 56-nucleotide leader sequence which contains the codon involved in initiation of protein synthesis (40).

The NS_1 protein exists abundantly in the nuclei of infected cells (40, 97). However, none have been detected in virions (40). In the nuclei, they interact with polysomes (40). They contain two nuclear localization signals (NLS) and one nuclear export signal (NES) which regulate the export of proteins from the nucleus (40). This protein regulates many host cell functions such as suppression of innate immunity by preventing host cell mRNA processing (56)and preventing export of polyadenylated cellular transcripts from the nucleus(66, 71).The NS_1 protein is also important in endowing the virus with resistance to host cell interferon (21, 24, 40). Double stranded (ds) RNA usually triggers the Protein Kinase R (PKR) pathway, either directly or via

triggering the production of interferon (IFN) (26, 40, 47). The NS_1 protein binds to ds viral RNA, which blocks the PKR pathway (24, 40, 80). Hence, the PKR inhibition of protein synthesis is abrogated (40). The NS1 protein also attenuates human dendritic cell (DC) maturation and DCs' abilities to induce T-cell responses (14).

The NS_2 proteins do exist in virions in small numbers and are associated with the ribonucleoprotein (RNP) through interactions with M_1 (40, 60). They are located in the nucleus and cytoplasm and each protein contains an NES, which aids in transporting viral mRNAs to the cytoplasm for translation (16, 40).

Viral Replication and Assembly

The replication process of influenza virus begins with the attachment of the virus via HA to sialic acid residues found on cell surface glycoproteins and glycolipids (40). Though some avian viruses bind α2,6-linked sialic acid, AIV typically binds to mucoproteins with sialic acid α2,3 linkages (9, 28, 40, 73). Individual cleaved, native HAs have a low affinity for sialic acid but when multiple HAs are present, high avidity occurs, allowing for initiation of infection (9, 40).

The avian influenza virus enters the cell by receptor-mediated endocytosis (16, 40). When H^+ ions are pumped into the endosome, causing a drop in pH to 5-6, this triggers the HA to undergo a conformational change (40). The hydrophobic fusion peptide at the N-terminus of HA_2 becomes exposed and inserts into the endosomal membrane, bringing both endosomal and viral membranes into close proximity (9, 40, 73). A highly bent stalk structure forms, ultimately resulting in fusion pore formation (9, 40). Meanwhile, H^+ ions flow from the endosome into the virion via M_2 (40). This drop

in pH within the virus particle causes disruption of protein-protein interactions between M_1 and RNPs (40). M_1 dissociates from RNPs (40). The internal contents of the virus mix with the cytoplasm of the endosome by passing through multiple pores (9, 40). At this point, viral RNPs are able to be directed into the nucleus (9). Nuclear localization signals (NLS) found on RNP proteins enable the RNP to be actively transported through nuclear pores (40). Both old and freshly synthesized M_1 enter the nucleus via passive diffusion (40). Synthesis of viral mRNA (vmRNA) is catalyzed by PB2, involved in the cap binding, and PB1, involved in elongation (40). These proteins are functional only in the presence of vRNA (40). When the 5' end of vRNA binds to PB1, PB2's cellular mRNA cap binding activity is triggered (40). Next, the 3' end of vRNA binds to PB1 and activates PB1 to cleave the cap from cellular mRNAs, facilitating elongation of the vmRNA (40).

The next step to occur is virion RNA replication which involves two parts: 1) template (+ sense) RNA synthesis, followed by 2) vRNA (- sense) synthesis (40). The transition from vmRNA production to vRNA replication requires solitary NP molecules not associated with nucleocapsids (40). These NP molecules are important for cessation of cap snatching and antitermination at poly A sites (40). One theory is that NPs bind to the common 5' ends of nascent transcripts, followed by the addition of more NPs to the elongating chain of RNA (40). In this set up, the vmRNA is prevented from slipping backward along the vRNA template and the reiterative As are blocked (40). According to this theory, the number of NP molecules in infected cells regulates the amounts of vmRNA vs. genomic RNA produced because full-length RNAs are synthesized only when NP molecules are present (40). The requirements for (+) and (–) sense vRNA but

not vmRNA for NP help to explain why they occur encapsidated with NP subunits and do not involve cap-primed synthesis (40). Once vRNPs are assembled, each forms a complex with M_1 and NS_2 (40). The NES found on NS_2 overrides the NLSs found on the polymerase and NPs (40, 55). Hence, RNPs are exported from the nucleus (40, 55).

Infection with AI is divided into two phases, based on the quantities of the individual RNA segments transcribed: 1) Early phase and 2) Late phase (40). During the early phase, synthesis of particular vRNAs, vmRNAs, and viral proteins are all coupled (40). Following primary transcription, equimolar amounts of (+) sense template RNA is made at the start of this phase (40). With the exception of particular vRNAs, there occurs a sharp decline in their synthesis (40). Both NP and NS_1 are made early because they are involved in the initiation and synthesis of all template and genomic vRNA (40). M_1 vmRNA and protein synthesis are both delayed (40). Upon entry into the late phase, the synthesis of all vRNAs reaches a peak, but the rate drops drastically soon thereafter (40). Protein as well as genomic vRNA synthesis continues through the end of this stage (40). M_1 and HA proteins are produced in particularly large amounts in the late phase (40). This feature makes sense due to the fact that M_1 stops transcription of vRNA into vmRNA and is also involved in the transport of RNPs to the cytoplasm (40, 55).

As previously mentioned, posttranscriptional processing of NS_1 and M_1 vmRNAs occurs with the assistance of cellular machinery in the nucleus (40). Two smaller mRNAs encoding for NS_2 and M_2, respectively, are produced (40). Only about 10% of such vmRNA produced is spliced and the amount of splicing is determined by the rates of splicing and the rates of nuclear export of the vmRNA (40). All spliced and unspliced vmRNA are exported to the cytoplasm for translation (40).

During infection with influenza, the virus takes control over host cell translation by two main aspects (40). First, the NS_1 protein prevents type I IFN induction, which reduces the interferon cellular response of blocking protein synthesis, which ensures efficient translation of virus-specific proteins in infected cells (21, 24, 40). Additionally, translation of cellular mRNAs is inhibited and viral mRNA is preferentially translated (40).

Virus Assembly and Release

The HA, NA, and M_2 proteins are all synthesized on membrane-bound ribosomes and are translocated across the ER membrane in a signal recognition particle (SRP)-dependent manner (40). The HA's N-terminal signal sequence is cleaved in the ER by signal peptidase (40). The other two surface proteins do not contain such signal sequences (40). N-linked carbohydrate chains are transferred to HA and NA (40). Following folding and assembly of these three proteins, they are transported from the ER to the Golgi apparatus for further processing of the oligosaccharide chains (40). Interesting to note is that both HA and NA lack terminal sialic acids on their complex carbohydrate chains, most likely due to NA action (40). Removal of sialic acids would help to reduce the likelihood of virus particles binding to each other. Binding and clumping together would hinder the virus particles from further infecting more cells. HA, NA, and M_2 proteins are expressed at the plasma membrane with the HA dispersed over the surface while M_2 and NA occur in clusters (40).

Bud formation and release of virus particles are the final steps in viral replication and production of new infectious virions (40, 55). The precursor to the envelope of the

budding virion is a patch of cell membrane containing envelope proteins (40, 55). Typical budding sites are regions among the cell membrane where lipid rafts occur (40, 55). vRNA-M_1-NS_1 complexes exiting the nucleus are transported to these rafts containing HA and NA (16). Virus assembly and budding requires the interactions between the viral envelope, M_1, and vRNP, with M_1 serving as a bridge between the two (55). The M_1 may also serve as a bridge between the envelope proteins and the vRNP (55). Interactions between multiple M_1 proteins help to concentrate the viral components and to exclude cellular components from the virion (40, 55). Outward curvature of the cell plasma membrane at virus assembly sites leads to bud formation (55). Virus particles bud from the apical plasma membranes of polarized epithelial cells (40, 55). Fusion of the opposing membranes results in bud closure and the separation of the virus particle from the host plasma membrane (55). The virus particle is released into the extracellular environment to infect other cells (55). The complete release of virus particles requires NA activity to release them from the sialic acid on the cellular proteins which allows for spread from cell to cell (40, 55).

Clinical Signs

An important determinant in the clinical signs of AI is the pathotype – either HPAI or LPAI (89). The symptoms are variable, depending on the host species, sex, age, the simultaneous presence of any other infections, acquired immunity, and environmental conditions(89).

Until recently, wild birds have usually been infected only with LPAI and display no clinical signs of disease (89). In domestic poultry such as chickens and turkeys, the

most common signs of clinical disease with LPAI include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation (89). Increased broodiness and decreased egg production occurs in breeder and layer hens (89). In addition, huddling, ruffled feathers, depression, decreased activity, decreased intake of food and water, and some diarrhea may occur (89). A slight increase in daily mortality may also be observed (89). Many flocks infected with LPAI may seroconvert without evidence of clinical disease (89). In experimental inoculations of specific pathogen free chickens with LPAI, clinical disease is seldom observed (83, 89).

In domestic chickens, turkeys, and other galliformes infected with HPAI, clinical manifestations tend to be more systemic and vary, depending on the affected organs (83, 89). HPAI can replicate and cause damage directly to a number of visceral organs as well as the cardiovascular and nervous systems (89). Often, chickens and turkeys die before any clinical signs occur (89). If the birds survive for a few days, they may begin to exhibit nervous disorders including tremors of the head and neck, inability to stand, torticolis, and other uncommon positions of the head and appendages (89). Depression and decreased intake of food and water are also displayed (89). A drastic decrease or possibly complete cessation of egg laying within six days may occur in breeders and layers (89). Respiratory signs similar to those seen in LPAI may also occur (89). Hemorrhage or necrosis of the combs and wattles, legs, and serosal surfaces of internal organs may also be observed (89). Until recently, the general consensus has been that LPAI causes strictly localized infection without viremia (89). However, H9N2 strains have been isolated from chicken meat and bone marrow imported from China (36). It has more recently been demonstrated that a LPAI strain of H7N1 (TK/Italy/3675/99) was

able to cause viremia in some unvaccinated turkeys that were experimentally infected (93).

Morbidity and mortality rates in chickens and turkeys are as variable as the clinical signs (89). With LPAI, there is usually high morbidity, however, mortality can vary widely depending primarily on if secondary infections are present (89). Highly pathogenic avian influenza is used to describe disease in chickens and is based on clinical signs such as respiratory distress, sinusitis, and diarrhea, caused by virulent strains of AIV (57). Generally both morbidity and mortality rates are high with HPAI in gallinaceous birds, sometimes reaching up to 100% mortality in flocks (89). Classification of an AIV isolate as HP is assessed by pathogenicity testing in chickens (mentioned below). Clinical signs may vary greatly, depending on the host, age of the bird, presence of other organisms, and environmental conditions (57). In addition, because a particular strain is classified as HP in chickens does not mean that it will necessarily cause disease in other species (62). For ducks and geese, mortality is usually much lower for HPAI, but recent H5N1 isolates from Asia may be an exception to this rule (35, 45, 62) . Many strains isolated from ducks in China, between 1999 and 2002, caused infection with oropharyngeal and cloacal shedding, but did not cause illness or death (61, 88). New strains that have been isolated are able to produce illness and death along with replicating in internal organs including the brain in the two-week old duck model (61, 88). As more time goes by, these H5N1 viruses are continuing to evolve and show extremely high lethality in ducks (35).

Immunity

Infection and vaccination with AIV elicits both systemic and mucosal Ab responses (83, 89). The intensity and level of the antibody response varies from species to species, with a general trend of highest to lowest levels beginning with chickens > pheasants > turkeys > quail > ducks (83, 89). As previously mentioned, Abs against HA can be both neutralizing and protective while Abs against NA do not prevent infection, but can reduce clinical disease and viral shedding (40, 83, 89, 92). Furthermore, M2-based vaccination in mice has been found to induce an antibody response against the conserved M2e region and offer protection against challenge with lethal heterologous virus (94). The extracellular domain of M2 is 18-23 amino acids and can be removed from the surface of virions and infected cells by trypsin-like enzymes (106). This region serves as a target for antibodies, but acts as a 'dodging bullet' once it is cleaved and is no longer present to bind to the antibodies. The degree of protection against mucosal challenge and shedding of the virus depends on the sequence similarity between the vaccine and challenge virus antigens such as HA and NA (83, 89). Another factor influencing the level of protection rendered against challenge is the level of antibodies induced upon initial infection or vaccination (87, 100). The duration of protection against clinical signs and death has been shown to last up to 30 weeks in the field (89). Birds, which have recovered after field exposure, are normally protected against homologous HA and NA subtypes (89). Immune responses against the internal virus proteins have demonstrated no protection from clinical signs or death but may curtail the replication and shedding period, likely through cell mediated immunity (89).

Diagnostics

The most commonly used diagnostic test in the U.S. for type A influenza viruses is a double immunodiffusion test, also known as the agar gel immunodiffusion (AGID) or agar gel precipitation (AGP) test (57, 58, 89, 104). Concentrated virus and antiserum against the NP or M_1 proteins are used because these proteins are highly conserved among the type A viruses (89, 104). This test is relatively simple to perform and requires a minimum of equipment. However, one drawback is that not all avian species produce precipitating antibodies following infection with AI (58). An Enzyme-linked Immunosorbant Assay (ELISA) against the influenza type A-specific antigens is also available (58, 89, 104). Several commercial ELISA tests are available, and are commonly used as a screening test with confirmation of positives by the AGID test. Further serologic analysis for subtyping of the virus is carried out using the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests (57, 58, 89, 104). Both tests rely on a panel of viruses of all 16 known HA and nine known NA subtypes (57, 104). The drawback to the HI test is that non-specific inhibitors found in the serum sometimes have to be removed before running the test (57, 104).

While conventional methods of virus isolation and characterization techniques are the preferred means of diagnosing AI, molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) have also been tested and shown to be a useful tool for detecting and subtyping (H5 and H7) AI (58, 75, 76, 80). This technique is highly sensitive and allows for rapid detection of viral RNA (85). Primers specific for the matrix gene are used to first detect AI, then separate primers are used to determine if positive matrix samples are subtype H5 or H7 (58). It is likely that in the near future, this

molecular technique will have developed to a degree sufficient enough for it to be used for detecting AI and identifying specific subtypes and virulence markers (58).

Surveillance/Transmission/Spread

In addition to transmission by infected poultry, contaminated equipment, and people, wild birds have been implicated in the expansion of HPAI across Asia, the Middle East, Europe, and Africa (34, 38). Different duck species display different degrees of signs upon infection with HP H5N1s (34). It was suggested that mallards should be given priority for active surveillance (the sampling of apparently healthy birds), because they can become infected and excrete virus without showing signs (34). On the other hand, pochards and tufted ducks could serve as sentinels in a passive surveillance program because those birds, when infected, excreted the most virus also developed neurologic disease (34). It would be most likely that these birds would succumb to disease before having much opportunity to spread the virus (34).

Assessing Pathogenicity

Once an isolate of avian influenza has been identified and subtyped, its pathogenicity is next evaluated (57). Eight 4- 6-week old susceptible chickens are inoculated with 0.2 ml of a 1/10 dilution of infective, bacteria-free allantoic fluid (57, 58). If six or more of these chickens die within 10 days, it is considered HPAI (57, 58). Alternatively, the Intravenous Pathogenicity Index (IVPI) is used, whereby fresh, infective allantoic fluid with a HA titer of greater than 1/16 is diluted 1/10 in sterile isotonic saline and 0.1 ml of the diluted virus is injected intravenously into each of 10

six-week-old SPF chickens. Birds are examined at 24-hour intervals for 10 days and ranked from 0 to 3 at each evaluation (57, 58). Normal behavior is scored 0, 1 is sick, 2 is severely sick, and 3 is dead (57, 58). The IVPI is the mean score per bird per observation over the 10-day period (57, 58). When all birds die within three days, the IVPI is 3, and when no bird shows any clinical signs during the observation period, the IVPI is 0 (57, 58). A virus is considered highly pathogenic if it has an IVPI greater than 1.2 (58). Finally, LP H5 and H7 viruses that have sequence similarity with other HPAI isolates at the HA cleavage site, particularly with multiple basic amino acids, are considered to be HPAI, even if they are later shown to be of low pathogenicity in chickens (57, 58). To date, all HPAIs identified have been of the H5 or H7 subtypes (40, 58).

Vaccines

Vaccination against AI is not authorized in most developed countries, primarily because vaccination can interfere with serological surveillance in domestic poultry (99). However, it has been used in some countries such as Mexico, Italy, Pakistan, and the USA, to control LPAI, with variable success (86, 99). Before the HPAI H5N1 outbreak in southeast Asia, only a few reports of controlling HPAI outbreaks through vaccination occurred: the H5N2 outbreak in Mexico (1994), the H7N1 outbreak in Italy (2000), and the H7N3 outbreak in Pakistan (2003) (49, 51, 99, 101). In the face of a HPAI H5N1 outbreak, several countires in Asia and Africa have been the only places where vaccination has been used (12). The vaccines currently used commercially are the whole

virus in oil emulsion adjuvant and fowlpox recombinant with an H5 insert (81-83, 90, 99).

Various types of vaccines have been developed and shown experimentally, to protect against AI challenge (83). Most of these vaccines have been evaluated for protection from HPAI (83). Vaccines can be divided into five groups: 1) conventional killed whole virus, 2) subunit vaccines which contain only a part of the entire virus, 3) live, 4) recombinant vectors expressing AI genes, and 5) DNA vaccines (90).

Conventional inactivated virus vaccines are produced from viruses that are grown in embryonating chicken eggs, followed by chemical inactivation (90). Typically, these vaccines are produced from LPAI viruses which grow to high titers, because this enhances yield for vaccine preparation (90). Poultry vaccines against AI are non-purified allantoic fluid containing whole AI virions that are administered with an oil adjuvant subcutaneously in the nape of the neck, or intramuscularly, in the thigh (90). Oil emulsion vaccines slowly release antigen over time, inducing a more robust immune response than would be produced from antigen alone (90).

Live virus vaccines provide superior protection, compared to inactivated vaccines, because they induce humoral, cellular, and mucosal immunity (90). However, due to their potential to reassort with the circulating viruses and increase in virulence (especially the H5 and H7 subtypes), they are not currently recommended for use (90). They also may cause respiratory disease or drops in egg production (43, 90). The technique of reverse genetics has aided in the progress of developing live, attenuated vaccines (43). Recent studies on the pathogenic effects and transmissabilities of viruses with truncated NS1 genes have been conducted on chickens (5, 103). The viruses containing truncated

NS1 genes did not transmit efficiently from bird to bird, were stronger inducers of IFN than the same strain containing the full-length NS gene, and also induced relatively high HI antibody titers (5, 103). These studies suggest that such viruses be considered as potential vaccines for the future (5, 103).

Subunit vaccines for AI are based on *in vitro* expression of the HA gene in animal or plant cells, bacteria, viruses, or yeast (6, 8, 10, 69, 90). The HA protein purified from cell extracts is quantified, oil emulsified, and administered parenterally (90). While this type of vaccine is safe and requires no handling of live virus, they are expensive, abrogating their use in the field (90).

Several viral and bacterial vectors have been studied for *in vitro* or *in vivo* expression of HA genes to be used in poultry (90). Some examples of the vectors studied include infectious layrngotracheitis virus, vaccinia virus, Venezuelan equine encephalitis virus, and retrovirus (6, 10, 30, 48, 65, 72, 90, 91, 100). More recently, adenovirus-based vaccines have been tested and shown to protect chickens against HPAI challenge (20, 95, 96). Another application for reverse genetics has been in the development of a recombinant Newcastle disease virus (rNDV) expressing the H5 or H7 HA (23, 54, 63, 90). In 2006, a commercially licensed rNDV vaccine containing an H5 HA gene insert was first used in poultry in China and recently, one has been licensed for use in Mexico (90, 99).

Plasmid DNA containing an HA gene insert have also been tested as vaccines in poultry (18, 19, 37, 68, 81). While these vaccines induce an immune response that closely mimics that of a live virus infection and are safe to manufacture, they require

multiple boosts to achieve protective antibody titers and are expensive (90). Optimization of these vaccines is needed before they will be useable in the field.

Improvements in technology have led to the generation of virus-like particles (VLP), which are morphologically similar to AI virus particles, but are nonviable and are often created only to contain the M1, HA, and sometimes the NA protein (64, 67). Studies conducted on mice have demonstrated that this type of vaccine, which are produced in insect cells, with further study, may have prophylactic potential in the future (67). With regards to the most attractive vaccine vector candidates for the future, live-virus-vectors including rNDV, rFP, AI-NDV chimeras, Marek's disease herpesviruses with AI H5 HA inserts, as well as gene-deletion attenuated Salmonella containing H5 HA genes certainly show great potential (90).

An important aspect to consider when choosing to use vaccination as part of a control strategy is being able to distinguish vaccinated from infected members of a flock (4, 43). One of the primary purposes for the DIVA strategy is to assure trading partners that the vaccinated product is free of infection (43). Various approaches for the differentiation of infected from vaccinated animals (DIVA) strategy have been developed; some are currently in use while others are being tested. Thus far, such approaches have included using an inactivated whole virus vaccine containing the same HA subtype as the virus causing an outbreak (autogenous vaccine), but a different NA subtype from the outbreak virus (2, 43, 78). Vaccinated birds are protected with the subtype-matched HA antibodies induced by the vaccine, but if the bird is infected with the virus, its NA antibodies will be differentiated from vaccine-derived NA antibodies (43). Alternatively, detection of an antibody response against the NS_1 protein can help

differentiate vaccinated from infected birds (43). This approach is based on the principle that the NS1 protein is produced in large quantities in infected cells, but is not packaged into the virion (43). Killed influenza vaccines are generated from whole virions, so an antibody response in infected birds can be differentiated from vaccinated birds (43). A third approach is the use of subunit or recombinant vaccines containing or encoding for (respectively) only the HA protein (82). Vaccinated birds would only have antibodies against the HA, and not other influenza proteins. Placing sentinel birds randomly throughout a flock that is vaccinated and periodically testing them for exposure to avian influenza is yet another means of determining whether a flock has been exposed to AI (82).

Recently, ducks have been included in the vaccine regimens of countries with large duck populations, such as Vietnam, China, and Indonesia (32). Yet another recently developed DIVA strategy tested is the use of exogenous tetanus toxoid (TT) coadministered with inactivated whole, killed oil emulsion vaccines (32). Through this approach, vaccinated birds would show up positive for having not only HA antibodies, but also antibodies against TT (32). Extending such a strategy to ducks is particularly important since ducks can become infected with HP H5N1 viruses without displaying clinical signs yet shedding large amounts of virus (32).

As mentioned earlier, due to the frequency that AI undergoes antigenic drift, it is important to routinely evaluate the currently used vaccines for their efficiency at protecting birds from disease and shed. In Asia, H5N1 AI viruses have become endemic and continue to circulate (46). The fact that these viruses have become a potential pandemic threat to humans underscores the importance of maintaining optimally effective

vaccines in poultry. By keeping the levels of virus shed among birds down to a minimum, these viruses will have a reduced chance to be passed both among poultry and to humans. Characterization of currently circulating viruses molecularly, antigenically, and biologically will continue to provide information about these viruses that can be applied when selecting viruses for updated vaccines.

With regards to human vaccines, when the predicted circulating strain of virus elicits an antibody titer with a four-fold reduction in cross-neutralization activity to the currently used vaccine, a different vaccine seed strain is chosen, and the vaccine is changed. Mexico currently vaccinates its poultry with both a whole-killed oil emulsion and an H5-encoding recombinant fowlpox vaccine. The whole-killed vaccine, which contains the CK/Hidalgo/232/94 (H5N2) virus, has been used since 1995, to control both LP and HP viruses. Up to 16-fold differences in HI activity have been seen between more recently circulating viruses isolated in 2002, and the vaccine strain, strongly indicating that an updated vaccine strain should be chosen (44).

Another major consideration taken when selecting the seed strain for the future pertains to the genetic similarities of the current vaccine with newly circulating viruses. In addition to information obtained through characterization of viruses, determining the amino acid(s) involved in HA-specific antibody binding will also provide insight when updating a vaccine seed strain. If these particular amino acids are known, a better fitting vaccine virus can be chosen by comparing the vaccine candidate with current strains at these specific amino acid sites.

REFERENCES

1. Alexander, D. J. A review of avian influenza in different bird species. Veterinary Microbiology 74:3-13. 2000.

2. Beard, C. W. To vaccinate or not to vaccinate? In: Proceedings of the second international symposium on avian influenza. C. W. a. E. Beard, B. C., ed. United States Animal Health Association, Richmond, VA. pp 258-263. 1986.

3. Capua, I., and Alexander, D. Avian influenza: recent developments. Avian Path. 3:393-404. 2004.

4. Capua, I., Marangon, S. Vaccination for avian influenza in Asia. Vaccine 22:4137-4138. 2004.

5. Cauthen, A. N., Swayne, D. E., Sekellick, M. J., Marcus, P. I., and Suarez, D. L. Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J Virol 81:1838-1847. 2007.

6. Chambers, T. M., Kawaoka, Y., Webster, R. G. Protection of chickens from lethal influenza infection by vaccine-expressed hemagglutinin. Virology 167:414-421. 1988.

7. Chen, W., Calvo, P. A., Malid, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J. R., and Yewdell, J. W. A novel influenza A virus mitochondrial protein that induces cell death. Nature medicine 7:1306-1312. 2001.

8. Crawford, J., Wilkinson, B., Vosnesensky, A., Smith, G., Garcia, M., Stone, H., and Perdue, M. L. . Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 17:2265-2274. 1999.

9. Cross, K. J., Burleigh, L. M., and Steinhauer, D. A. Mechanisms of cell entry by influenza virus. Cambridge University Press. 2001.

10. De, B. K., Shaw, M. W., Rota, P. A., Harmon, M. W., Esposito, J. J., Rott, R., Cox, N. J., and Kendal, A. P. Protection against virulent H5 avian influenza virus infection in chickens by an inactivated vaccine produced with recombinant vaccinia virus. Vaccine 6:257-261. 1988.

11. Easterday, B. C. The influenza viruses and influenza. Academic Press, New York. 1975.

12. Ellis, T. M., Leung, C. Y. H. C., Chow, M. K. W., Bissett, L. A., Wong, W., Guan, Y., and Peiris, J. S. M. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Path. 33:405-412. 2004.

13. Fenner, F., Bachman, P. A., Gibbs, E. P. J., Murphy, F. A., Studdert, M. J., and White, D. O. Orthomyxoviridae. In: Veterinary Virology. Academic Press, New York. pp 473-484. 1975.

14. Fernandez-Sesma, A., Marukian, S., Ebersole, B. J., Kaminski, D., Park, M. S., Yuen, T., et al. Influenza virus evades innate and adaptive immunity via the NS1 protein. J Virol 80:6295-6304. 2006.

15. Flint, S. J., Enquist, R. M., Racaniello, V. R., and Skalka, A. M. Principles of virology; molecular biology, pathogenesis, and control of animal viruses, 2nd ed. ASM Press, Washington D. C. 2004.

16. Flint, S. J., Enquist, L. W., Krug, R. M., Racaniello, V. R., and Skalka, A. M. Principles of virology, molecular biology, pathogenesis, and control. ASM Press, Washington D.C. 2000.

17. Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst, S., Smith, D., Rimmelzwaan, G. F., Olsen, B., and Osterhaus, A. D. M. E. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79:2814-2822. 2005.

18. Fynam, E. F., Robinson, H. L., and Webster, R. G. Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol 12:785-789. 1993.

19. Gall-Recule, G., Cherbonnel, M., Pelotte, N., Blanchard, P., Morin, Y., and Jestin, V. Importance of a prime-boost DNA/protein vaccination to protect chickens against low-pathogenic H7 avian influenza infection. Avian Dis. 51:490-494. 2007.

20. Gao, W., Soloff, A. C., Lu, X., Montecalvo, A., Nguyen, D. C., Matsuoka, Y., Robbins, P. D., Swayne, D. E., Donis, R. O., Katz, J. M., Varratt-Boyes, S. M., and Gambotto, A. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. Journal of Virology 80:1959-1964. 2006.

21. Garcia-Sastre, A. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand viruses. Virol 279:375-384. 2001.

22. Garcia, M., J. M. Crawford, J. W. Latimer, E. Rivera-Cruz, and M. L. Perdue. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J. Virol. 77:1493-1504. 1996.

23. Ge, J., Deng, G., Wen, Z., Tian, G., Wang, Y., Shi, J., Wang, X., Li, Y., Hu, S., Jiang, Y., Yang, C., Yu, K., Bu, Z., and Chen, H. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge for

homologous and heterologous H5N1 avian influenza viruses. Journal of Virology 81:150-158. 2007.

24. Haller, O., Kochs, G., and Weber, F. The interferon response circuit: induction and suppression by pathogenic viruses. Virol 344:119-130. 2006.

25. Harvey, R. A., Martin, A. C. R., Zambon, M., and Barclay, W. S. Restrictions to the adaptation of influenza A virus H5 hemagglutinin to the human host. J Virol 78:502-507. 2004.

26. Hatada, E., and R. F. Binding of influenza A virus NS1 protein to ds RNA in vitro. J Gen Virol 73:3325-3329. 1992.

27. Hayden, F. G., Osterhaus, A. D., Treanor, J. J., et al. Efficacy and safety of the neuraminidase inhibitor zanimivir in the treatment of influenzavirus infections. The New England journal of medicine 337:874-880. 1997.

28. Horimoto, T., and Kawaoka, Y. Pandemic threat posed by avian influenza A viruses. Clinical Microbiol. Rev. 14:129-149. 2001.

29. Humiston, S. G., and Pappano, D. A. Influenza and influenza vaccination. Emergency medicine reports 25:321-332. 2004.

30. Hunt, L., A., Brown, D. W., Robinson, H. L., Naeve, C. W., and Webster, R. G. Retrovirus-expressed hemagglutinin protects against lethal influenza virus infection. J Virol 62:3014-3019. 1988.

31. Jacob, J. P., Butchner, G. D., Mather, F. B, and Miles, R. D. Avian influenza in poultry. In: University of Florida Extension Service, Institute of Food and Agricultural Sciences. University of Florida. pp 1-4. 1998.

32. James, C. M., Foong, Y. Y., Mansfield, J. P., Vind, A. R., Fenwick, S. G., and Ellis, T. M. Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance. Vaccine 26:5345-5351. 2008.

33. Jefferson, T., Demicheli, V., Rivetti, D., Jones, M., Di Pietrantonj, C., and Rivetti, A. Antivirals for influenza in healthy adults: systematic review. Lancet 367:303-313. 2006.

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

35. Kim, J. K., Seiler, P., Forrest, H. L., Khalenkov, A. M., Franks, J., Kumar, M., Karesh, W. B., Gilbert, M., Sodnomdarjaa, R., Douangngeun, B., Govorkova, E. A., and Webster, R. G. Pathogenicity and vaccine efficacy of dfferent clades of Asian H5N1 avian influenza A viruses in domestic ducks. J Virol doi:10.1128/JVI.01176-08. 2008.

36. Kishida, N., Sakoda, Y., Eto, M., Sunaga, Y., and Kida, H. Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Archives of virology 149:2095-2104. 2004.

37. Kodihalli, S., Kobasa, D. L., and Webster, R. G. Strategies for inducing protection against aviain influenza A virus subtypes with DNA vaccines. Vaccine 18:2592-2599. 2000.

38. Koehler, A. V., Pearce, J. M., Flint, P. L., Franson, J. C., and Ip, H. S. Genetic evidence of intercontinental movement of avian influenza in a migratory bird: the northen pintail (Anas acuta). Molec. Ecol. 17:4754-4762. 2008.

39. Krieg, A., Yi, A. K., Schorr, J., and Davis, H. L. The role of CpG dinucleotides in DNA vaccines. Trends Microbiol. 6:23-27. 1998.

40. Lamb, R. A., and Krug, R. M. Orthomyxoviridae: the viruses and their replication. In: Fields Virology, third ed. D. M. Knipe, and P. M. Howley, ed. Lippincott Williams & Wilkins, Philadelphia. pp 1353-1395. 1998.

41. Lamb, R. A., and Krug, R. M. Orthomyxoviridae: the viruses and their replication. In: Fields Virology, 4th ed. D. M. a. H. Knipe, P. M., ed. Lippincott Williams & Wilkins, Philadelphia. 2001.

42. Lee, C. W., D. A. Senne, J. A. Linares, P. R. Woolcock, D. E. Stallknecht, E. Spackman, D. E. Swayne, and D. L. Suarez. Characterization of recent H5 subtype avian influenza viruses from US poultry. Avian Pathol. 33:288-297. 2004.

43. Lee, C. W., and D. L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Animal Health Research Reviews 6:1-15. 2005a.

44. Lee, C. W., Senne, D., and Suarez, D. L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78:8372-8381. 2004.

45. Lee, C. W., Swayne, D. E., Linares, J. A., Senne, D. A., and Suarez, D. L. H5N2 avian influenza outbreak in Texas in 2004: the first highly pathogenic strain in the United States in 20 years? J Virol 17:11412-11421. 2005.

46. 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.

47. Lu, Y., Wambach, M., Katze, M. G., and Krug, R. M. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylated the ilf-2 translation initiation factor. Virol 214:222-228. 1995.

48. Luschow, D. W., O., Mettenleiter, T. C., and Fuchs, W. Protection of chckens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine 19:4249-4259. 2001.

49. Marangon, S., Capua, I. Control of avian influenza in Italy: from stamping out to emergency and prophylactic vaccination. Dev Biol (Basel) 124:109-115. 2006.

50. Moscona, A. Neuraminidase inhibitors for influenza. The New England journal of medicine 353:1363-1373. 2005.

51. Naeem, K. a. S., N. Use of strategic vaccination for the control of avian influenza in Pakistan. Dev Biol (Basel) 124:145-150. 2006.

52. Naeve, C. W., and Williams, D. Fatty acids on the A/Japan/305/57 influenza virus hemagglutinin have a role in membrane fusion. EMBO 9:3857-3866. 1990.

53. Naim, H. Y., and Roth, M. G. Basis for selective incorporation of glycoproteins into the influenza virus envelope. J Virol 67:4831-4841. 1993.

54. Nakaya, T., Cros, J., Park, M. S., Nakaya, Y., Zheng, H., Sagrera, A., Villar, E., Garcia-Sastre, A., and Palese, P. Recombinant Newcastle disease virus as a vaccine vector. Journal of Virology 75:11868-11873. 2001.

55. Nayak, D. P., Hui, E. K., and Barman, S. Assembly and budding of influenza virus. Virus Res. 106:147-165. 2004.

56. Nemeroff, M. E., Barabino, S. M., Li, Y., Keller, W., and Krug, R. M. Infuenza 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. 1998.

57. OIE Highly pathogenic avian influenza In: Manual of diagnostic tests and vaccines for terrestrial animals. 2004.

58. OIE Avian Influenza. In: Terrestrial Manual. pp 465-481. 2008.

59. Palese, P., and M. Shaw *Orthomyxoviridae:* The Viruses and Their Replication. In: Fields Virology

5ed. D. M. Knipe, and P. M. Howley, ed. Lippincott Williams & Wilkins. 2006.

60. Palese, P. a. S., M. L. Orthomyxoviridae: the viruses and their replication. In: Fields virology, 5th ed. D. M. a. H. Knipe, P. M., ed. Lippincot Williams & Wilkins, Philadelphia. pp 1647-1689. 2007.

61. Pantin-Jackwood, M., 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. 2007b.

62. 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. 2007a.

63. Park, M. S., Steel, J., Garcia-Sastre, A., Swayne, D., and Palese, P. Engineered viral vaccine constructs with dual specificity: avian influenza and newcastle disease.

Proceedings of the National Academy of Sciences of the United States of America 103:8203-8208. 2006.

64. Prel, A., Le Gall-Recule, G., and Jestin, V. Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low-pathogenic avian influenza strains in ducks. Avian Path. 37:513-520. 2008.

65. Qiao, C., Yu, K., Jiang, Y., Jia, Y., Tian, G., Liu, M., Deng, G., Wang, X., Meng, Q., and Tang, X. . Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 hemagglutinin and N1 neuraminidase genes. Avian Path. 32:25-31. 2003.

66. Qiu, Y. a. K., R. M. The influenza virus NS1 protein is a poly(A) -binding protein that inhibits nuclear export of mRNAs containing poly(A). J Virol 4:2425-2432. 1994.

67. Quan, F. S., Huang, C., Compans, R. W., and Kang, S. M. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 81:3514-3524. 2007.

68. Robinson, H. L., and Torres, C. A. T. DNA vaccines. Seminars in Immunology. 1997.

69. Saelens, X., Vanlandschoot, P., Martinet, W., Maras, M., Neirynck, S., Contreras, R., Fiers, W., and Jou, W. M. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. Eur. J. Biochem. 260:166-175. 1999.

70. Saito, T. a. Y., I. Effect of glycosylation and glucose trimming inhibitors on the influenza A virus glycoproteins. J. Vet. Med. Sci. 62:575-581. 2000.

71. Satterly, N., Tsai, P. L., van Deursen, J., Nussenzveig, D. R., Wang, Y., Faria, P.A., et al. Influenza virus targets the mRNA export machinery and the nuclear pore complex. Proc Natl Acad Sci USA 104:1853-1858. 2007.

72. Schultz-Cherry S., D., J. K., Davis, N. L., Williamson, C., Suarez, D. L., Johnston, R., and Perdue, M. L. Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. Virology 278:55-59. 2000.

73. Skehel, J. J., and Wiley, D. C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. 69:531-569. 2000.

74. Sompayrac, L. How Pathogenic Viruses Work. Jones and Bartlett Publishers, Sudbury, MA. 2002.

75. 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. Journal of clinical microbiology 40:3256-3260. 2002.

76. Stark, E., Romer-Oberdorfer, A., and Werner, O. Type- and subtype-specific RT-PCR assays for avian influenza viruses. J. Vet. Med. 47:295-301. 2000.

77. Steinhauer, D. A. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virol 258:1-20. 1999.

78. Stone, H. D. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. Avian Dis. 31:483-490. 1987.

79. Stubbs, E. L. Fowl Pest. In: Diseases of Poultry. H. E. a. S. Biester, L. H., ed. The Iowa State University Press, Ames. pp 603-614. 1948.

80. Suarez, D. L. Molecular diagnostic techniques: can we identify influenza viruses to differentiate subtypes and determine pathogenicity potential of viruses by RT-PCR? In: Proceedings o fhte Fourth International Symposium on Avian Influenza. US Animal Health Association, Pennsylvania, Athens, GA. pp 318-325. 1998.

81. Suarez, D. L., and S. Schultz-Cherry. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis. 44:861-868. 2000.

82. 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.

83. Suarez, D. L., and Schultz-Cherry, S. Immunology of avian influenza virus: a review. Developmental and Comparative immunology 24:269-283. 2000.

84. Suarez, D. L., and Senne, D. A. Sequence analysis of related low-pathogenic and highly pathogenic H5N2 avian influenza isolates from United States live bird markets and poultry farms from 1983-1989. Avian Dis. 44:356-364. 2000.

85. Suarez, D. L., Das, A.l, Ellis, E. . Review of rapid molecular diagnostic tools for avian influenza virus. Avian Dis. 51:201-208. 2007.

86. Swayne, D. E. Vaccines for list A poultry diseases: emphasis on avian influenza Dev Biol (Basel) 114:201-212. 2003.

87. Swayne, D. E., C. W. Lee, and E. Spackman. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Path. 35:141-146. 2006.

88. Swayne, D. E. Understanding the Complex Pathobiology of High Pathogenicity Avian Influenza Viruses in Birds. Avian Dis. 50:242-249. 2007.

89. Swayne, D. E., and Halvorson, D. A. Influenza. In: Disease of Poultry. Y. M. Said, Barnes, H. J., Glisson, J. R., Fadly A. M., McDougald, L. R., and Swayne, D. E., ed. Iowa State University Press, Ames. pp 135-160. 2003.

90. Swayne, D. E., and Kapczynski, D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunological Reviews 225:314-331. 2008.

91. Swayne, D. E., Garcia, M., Beck, J. R., Kinney, N., and Suarez, D. L. 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.

92. Sylte, M. J., Hubby, B., and Suarez, D. L. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine 25:3763-3772. 2007.

93. Toffan, A., Beato, M. S., De Nardi, R., Bertoli, E., Salviato, A., Cattoli, G., Terregino, C., and Capua, I. Conventional inactivated bivalent H5/H7 vaccine prevents viral localization in muscles of turkeys infected experimentally with low pathogenic avian influenza and highly pathogenic avian influenza H7N1 isolates. Avian Path. 37:407-412. 2008.

94. Tompkins, S. M., Zhao, Z., Lo, C., Misplon, J. A., Liu, T., Ye, Z., Hogan, R. J., Wu, Z., Benton, K. A., Tumpey, T. M., and Epstein, S. L. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg. Infect. Dis. 13:426-435. 2007.

95. Toro, H., Tang, D. C., Suarez, D. L., Sylte, M. J., Pfeiffer, J., and Van Kampen, K. R. Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector. Vaccne 25:2886-2891. 2007.

96. Toro, H., Tang, D. C., Suarez, D. L., Zhang, J., and Shi, Z. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. Vaccine 26:2640-2646. 2008.

97. Tumpey, T. M., Alvarez, R., Swayne, D. E., and Suarez, D. L. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. Journal of clinical microbiology 43:676-683. 2005.

98. Ulmer, J. B., Fu, T. M., Deck, R. R., Friedman, A., Guan, L., DeWitt, C. , et al. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. 72:5648-5653. 1998.

99. van den Berg, T., B. Lambrecht, S. Marche, M. Steensels, S. Van Borm, and M. Bublot. Influenza vaccines and vaccination strategies in birds. Comp. Immunol. Microbiol. Infect. Dis. 2007.

100. Veits, J., A. Romer-Oberdorfer, D. Helferich, M. Durban, Y. Suezer, G. Sutter, and T. C. Mettenleiter. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine 26:1688-1696. 2008.

101. Villareal, D. L. Control and eradication strategies of avian influenza in Mexico. Dev. Biol (Basel) 124:125-126. 2006.

102. von Itzstein, M. The war against influenza: discovery and development of sialidase inhibitors. Nature Reviews 6:967-974. 2007.

103. Wang, L., Suarez, D. L., Pantin-Jackwood, M., Mibayashi, M., Garcia-Sastre, A., Saif, Y. M., and Lee, C. W. Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine poultry. Vaccine 26:3580-3586. 2008.

104. WHO Surveillance for Influenza. In: WHO manual on animal influenza diagnosis and surveillance. 2002.

105. Wright, P. F., G. Neumann, and Y. Kawaoka Orthomyxoviruses. In: Fields Virology. D. M. Knipe, and P. M. Howley, ed. Lippincott Williams & Wilkins. pp 1691-1740. 2006.

106. Zebedee, S. L. a. L., R. A. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. Journal of Virology 62:2762-2772. 1988.

CHAPTER 3

Phylogenetic and Biological Characterization of Highly Pathogenic Avian Influenza Viruses (H5N1) in Chickens and Ducks[1]

[1]Pfeiffer, J., M. Pantin-Jackwood, T. L. To, T. Nguyen, and D. L. Suarez. Accepted by Virus Research. Reprinted here with permission of publisher

ABSTRACT

Analysis of Asian H5N1 avian influenza (AI) virus hemagglutinin (HA) genes shows a common origin, but the virus has evolved into at least three major clades (clades 0, 1, and 2) over the last 13 years. Previous reports of Vietnam viruses have documented predominantly clade 1 viruses. Unexpectedly, 19 viruses from northern Vietnam isolated in December 2005 fell into clade 2. These viruses further clustered into two distinct sublineages. Representative viruses from each sublineage were chosen for antigenic and pathogenic evaluation. Two distinct antigenic groups correlating with the genetic information were present when comparing hemagglutination inhibition (HI) titers. All viruses were highly virulent not only in chickens, killing them within two days of experimental inoculation, but also in two- and five-week-old Pekin ducks, causing 100% mortality within four days of challenge. The information gained about these viruses provides insight with regards to implementing control programs, including vaccine seed strain selection.

Asian avian influenza, characterization, ducks, pathotyping, highly pathogenic

INTRODUCTION

The H5N1 Asian lineage highly pathogenic avian influenza (AI) viruses that have spread to multiple countries in Asia, Europe, and Africa appear to have originated in southern China (82, 89). The first report of viruses of this lineage, A/Goose/Guangdong/1/96, was from an outbreak with moderate mortality in geese in 1996. It was not until 1997, when a virus with a highly similar hemagglutinin (HA) gene but different internal genes infected poultry in live bird markets in Hong Kong and began infecting humans, that the virus was recognized as a serious veterinary and public health threat (82). Successful culling of all poultry in Hong Kong seemed to have controlled the outbreak, but additional isolations of H5N1 in 1999 and 2001 demonstrated that variants of the virus continued to circulate in the region (12) (78) (60). In late 2003 and early 2004, the H5N1 lineage of viruses, for unexplained reasons, began to rapidly spread among countries in Southeast Asia, and eventually spread to Europe and Africa. The Asian H5N1 lineage of viruses have now become endemic in poultry in several countries of Asia and Africa, and new introductions of virus to countries on all three continents are occurring with increasing frequency (38, 60).

When genetically analyzed, the Asian H5N1 lineage viruses can be divided into multiple clades (clade 0, clade 1, and clade 2 being the most common) based on the amino acid sequences of their HA surface glycoprotein, which is the only protein with a common lineage for all of the viruses (87) (86). Clade 0 viruses are comprised of the originally circulating viruses, and no longer appear to circulate. From the original clade 0 viruses, two separate clades, 1 and 2, have emerged, and both lineages of viruses continue to circulate widely, but several other minor clades have also been described (86) (87)

(46). The geographic origins of clade 1 and 2 lineages are likely to have come from Southern China (80). Prior to isolation of the viruses used in this study, all but one isolate reported from Vietnam were clade 1 viruses. Viruses from clade 2 have in recent years spread widely in Asia, Europe, and Africa, including the Fujian-like lineage (Clade 2.3.4) and the Qinghai Lake-like lineage (Clade 2.2) (13, 61) (60, 85).

Asian H5N1 HPAIs typically produce systemic disease and cause high mortality in chickens, and more recent H5 HPAIs have caused increased virulence in chickens, as indicated by shorter mean death times (MDT) (75). However, virulence varies among species (70), and infection of domestic ducks with highly pathogenic avian influenza isolated before 2002 generally was asymptomatic or caused only mild clinical disease (35, 45, 70, 75, 77). However, some H5N1 HPAI viruses isolated in 2002 and later have shown great variation in terms of virulence in domestic ducks (27, 31, 49, 50, 65, 70, 75). Some isolates caused infection restricted to the respiratory tract while other recent isolates produced severe systemic infection and lesions in multiple organs (75). In 2002, both free and captive wild birds were infected with H5N1 HPAI and died (18), and reports of wild migratory birds becoming infected, exhibiting severe clinical disease with mortality have become more common (23), and it has been speculated that they have contributed to the spreading of disease to poultry (40, 60).

Vietnam has been one of the countries most affected by AIV in Southeast Asia, and three main waves of outbreaks occurred between 2004 and 2005, with 30 931 840 chickens and 14 339 788 ducks reported being infected in that period (17). In this study, a total of 19 H5N1 viruses isolated in December 2005, from either ducks or chickens from northern Vietnam were genetically and antigenically characterized (Table 1).

Selected isolates were also biologically characterized in both chickens and ducks. The analysis showed a marked difference in these viruses, as compared to earlier reported isolates from Vietnam, which can have important implications on the epidemiology of the virus and control methods.

MATERIALS & METHODS

Viruses

The H5N1 influenza viruses used in this present study are listed in Table 3.1, and were obtained from the National Center for Veterinary Diagnosis**,** Hanoi, Vietnam**.** GenBank accession numbers for each segment of each virus are **EU930876** through **EU931027**. Virus stocks were propagated as previously described (50). Subsequently, allantoic fluid from eggs was harvested and 50% egg infectious dose (EID_{50}) titers were determined by testing hemagglutination activity (71). Titration endpoints were calculated by the method of Reed and Muench (54). All H5N1 AI viruses had high infectivity titers in eggs ($\geq 10^{6.0}$ EID_{50}/ml). All experiments using HPAI H5N1 viruses, including work with animals, were conducted using biosafety level 3 enhanced containment procedures (5), and all personnel were required to wear a powered air protection respirator with HEPA-filtered air supply (3M^{TM}, St. Paul, MN).

Sequencing and phylogenetic analysis of influenza virus genes

Viral RNA was extracted from infectious allantoic fluid from embryonating chicken eggs, using Trizol LS reagent (Life Technologies, Rockville, MD) or using MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX). Virus gene

sequences were obtained by reverse transcription (RT)-PCR using QIAGEN One-Step RT-PCR kit (Valencia, CA) and primers specific for influenza virus genes. (listed in Supplementary Table 1). Following electrophoresis, PCR products of the HA genes were extracted from agarose gels, using QIAquick Gel Extraction Kit (Qiagen, CA).

ABI Big Dye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) run on 3730 XL DNA Analyzer (Applied Biosystems) sequencer was used for sequencing HA PCR products. The remaining seven genes were sequenced by SeqWright DNA technology services (Houston, TX), using primers specific for these genes (Supplementary Table 1). The MegAlign program (DNASTAR, Madison, WI) was initially used to compare nucleotide sequences, using the Clustal V alignment algorithm. The method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc, Sunderland, MA) was used for phylogenetic comparisons of the aligned sequences, using bootstrap resampling method with a heuristic search algorithm.

Cloning of HA genes

Primers specific for the open reading frames of the HA gene were designed to contain the *Mlu* I and *Sal* I restriction sites flanking the 5' and 3' ends, respectively. Reverse-transcription-PCR (QIAGEN) was used to amplify the HA genes. The PCR products were digested with *Mlu* I and *Sal* I restriction enzymes (New England BioLabs, Beverly, MA) and then cloned into pCI eukaryotic expression vector containing *Mlu* I and *Sal* I ends. Proper insertion into the plasmid was confirmed by sequence analysis. The TK/WI/68, CK/Hidalgo/232/94, and Swan/Mongolia/244/05 virus HA genes had

also been cloned or subcloned into pCI (by David Suarez, Chang-Won Lee, and Mia Kim, respectively), previously.

DNA vaccination preparation and hyperimmune antisera production

Six Asian isolates and two North American isolates were chosen, based on phylogenetic analysis of the hemagglutinin genes, for HA-specific antisera production. The Asian H5s were representative of various H5 clades/subclades; clades 2.2, 2.3.2, and 2.3.4. The North American H5s were from viruses isolated 26 years apart. The HAs of each of these viruses were cloned into pCI eukaryotic expression plasmid (mentioned above), which were used as DNA vaccines. For every 100 μg of DNA injected into birds, 20 μl Lipofectin (Life Technologies, Gaithersburg, MD) transfection reagent was used (37, 67). Specific pathogen free (SPF) white Leghorn chickens between the ages of three and 10 weeks of age were divided into multiple groups. Each group received 100 μg plasmid DNA encoding for one of the HA genes, via intramuscular injection. Each vaccine was administered a total of three or four times at monthly intervals. All birds were sedated by ketamine/xylazine (66 mg/ml ketamine, 6.6 mg/ml xylazine) and bled by cardiac puncture at the final sampling time and then euthanized. Sera were harvested and used in the cross-HI test.

Cross-HI tests

Hemagglutination inhibition titers were determined by using the HI test (71). The HI test method was a standard beta test, whereby four HA units of β-propiolactone-inactivated Ag in 96-well plates and two-fold serially diluted test sera were used (37).

Virus isolates and antisera used are listed in Table 3.2. Antigenic relatedness (R) between HAs of particular isolates was calculated using HI titers (4) (Table 3.3).

Pathogenesis studies in chickens and ducks

An intranasal (IN) inoculation study was conducted to determine the pathogenic phenotypes of the viruses in chickens and ducks. Two-and four-week-old SPF White Leghorn chickens (*G. gallus domesticus*) (from the SEPRL flock) and two- and five-week-old Pekin white ducks (*Anas platyrhynchus*) (obtained from a commercial farm) were inoculated IN with one of several viruses representing each group, A or B. Birds were evaluated for signs of illness for up to 5 days. Serum samples were collected from a representative number of ducks prior to inoculation to ensure that they were serologically negative for AIV, as determined with the HI test (71). The birds were housed in self-contained isolation cabinets that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. Feed and water were provided *ad libitum*. General care was provided, as required by the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (16). Each group contained 10 birds, which were inoculated IN with 0.1 ml of an inoculum containing 10^5 EID_{50} of one of the viruses. Two birds from selected groups were euthanized and necropsied at 2 days post infection (dpi). Gross lesions were recorded, and tissues collected separately from each bird for virus isolation. Portions of the brain, lung, spleen, heart and muscle tissue were collected in BHI and stored frozen at -70°C and titers of infectious virus (EID_{50}) subsequently

determined as previously described (71). Oropharyngeal and cloacal swabs were also collected from all ducks at 3 dpi and titered the same way. Lungs, bursa, kidneys, adrenal gland, thymus, brain, liver, heart, ventriculus, pancreas, intestine, spleen, trachea, and thigh tissue were collected from necropsied ducks. Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin (HE). Sample birds, moribund birds, and all birds remaining at the end of a 5-day period were euthanized by the intravenous (20) administration of sodium pentobarbital (100 mg/kg body weight).

Histopathology and immunohistochemistry (IHC)

Samples were prepared as previously described (50). Briefly, collected tissues were fixed by submersion in 10% neutral buffered formalin and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (HE). A duplicate 4-μm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2,000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein (developed at Southeast Poultry Research Laboratory, USDA) was applied and allowed to incubate for 2 hours at 37° C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using a biotin–streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex). Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain. All tissues were

systematically screened for microscopic lesions. Lesions were scored as follows: - = no lesions; + = mild; ++ = moderate; +++ = severe lesions. The intensity of viral antigen staining in each section was scored as follows: - = no antigen staining; + = infrequent; ++ = common; +++ = widespread staining.

RESULTS

Phylogenetic Analysis

HA gene

All eight gene segments of each of the 19 viruses were sequenced and compared to sequences available in GenBank. All of the viruses were genetically clade 2, which classifies only the hemagglutinin gene (87). The 19 isolates further separated into two distinct groups, which we designated as groups A and B (Fig. 3.1). One virus appeared to be an outlier, but was not different enough to be considered a separate sublineage. The viruses are distinct from most previously reported clade 1 Vietnamese viruses, with the single exception of a reported clade 2 virus, Duck/Vietnam/568/05, isolated in early 2005. The group A viruses were most closely related to viruses reported from Southern China, Laos, and Malaysia, and are part of the Fujian-like lineage (clade 2.3.4) that appears to be emerging as a dominant isolate in the region (Fig. 3.1, Table 3.4). One unique feature of Fujian-like viruses is the change from Q to L at position -9 from the HA cleavage site (61). Group B viruses most closely clustered with viruses reported from Southern China, as well as the other clade 2 Vietnamese virus previously isolated (Fig. 3.1, Table 3.4), and fell into clade 2.3.2 (46). All 19 isolates contain multiple basic amino

acids at the HA cleavage site (Table 3.5), indicative of a highly pathogenic phenotype (26). However, the HA1-HA2 connecting peptide amino acid sequence of these 19 isolates (RRRKR/G) differed from most previous isolates in the Goose/Guangdong/1/96 lineage (RRRKKR/G) by having a deletion of a lysine (Table 3.5). Glycosylation at residues 154-156 may alter the receptor binding profile and also help the virus to evade the host antibody response (38, 64). The Vietnamese isolates have a potential N-glycosylation site at residues 154 and 155 of the HA1 protein (H5 numbering). While most of the group A viruses have threonine at position 156, group B viruses have serine. All of the viruses have potential glycosylation sites at amino acid positions 10, 11, 23, 165, 286, 484, and 543 (H5 numbering of the HA1) (7). Most of the isolates have isoleucine at residue 151 of the HA1 protein, proposed to be involved in receptor binding (14). Similar to other H5N1 viruses isolated in Vietnam between 2005 and 2007, these 19 isolates contain K at amino acid 212 (H5 numbering of HA1) (46). Finally, there are glutamine and glycine at amino acids 222 and 224 (H5 numbering), respectively, of the HA1 proteins for all the isolates (Table 5), indicating a putative affinity for avian alpha 2, 3-linked sialic acid receptors (22). There appears to have been reassortment between the two groups of viruses, particularly in isolate VN/200 (Fig. 3.1, Fig.3.2, Fig.3.3). Based on its HA, it is an outlier from either group A or B. However, it falls distinctly into group B, based on its neuraminidase (NA) and NP sequences (Fig. 3.2, Fig. 3.3), and group A for the other internal proteins.

NA gene

The *NA* genes, like the *HA* genes, also most closely clustered with Chinese isolates, but into two definable groups. Again, the group A viruses were most closely associated with the Fujian-like lineage isolate (DK/Fujian/1734/05) (Fig. 3.1, Fig. 3.2, Table 3.5). Sequence analysis demonstrated that the proposed amino acid sequence of the NA protein of each of these viruses has a 20 amino acid deletion in the stalk region (residues 49 through 68) (Table 5), characteristic of viruses adapted in land-based poultry (43, 88). These Vietnamese isolates also contain isoleucine at position 223, a molecular marker for high pathogenicity in mice and a conserved framework residue in the NA molecule's active site that is located in the head (28).

NS gene

All the Vietnamese isolates belong to *NS* gene subtype A (41, 76). At amino acid 92, all of the Vietnamese isolates contain aspartic acid (Table 3.5), which is in contrast to the presence of a glutamic acid at this site which was reported to be important for cytokine resistance of a 1997 H5N1 isolate (57). They also contain a five amino acid deletion (positions 80 to 84) that was first observed in 2001 in poultry in Hong Kong, and has since become the most common sequence found in the HP H5N1 viruses (Table 3.5).

Internal protein genes

All the Vietnamese isolates contain glutamic acid at position 627 of the *PB2* gene (Table 3.4), unlike isolates which demonstrated high virulence in mammals and contained lysine at this location, (21) (19) (30, 42, 69). In the PB1 protein, they also

contained a lysine and methionine at residues 198 and 317, respectively, which correspond to high and low pathogenic phenotypes, respectively, in mice (28). As mentioned above, the NP sequences of the Vietnamese viruses subdivided amongst themselves into two groups (Fig. 3.3). When analyzing the proposed M1 proteins of all 19 viruses, an isoleucine was present at amino acid 15, which has been found in isolates that had high pathogenicity in mice (28). In the M2 protein sequences, a leucine and a serine were at sites 26 and 31, respectively, which are genotypes associated with sensitivity to adamantine antivirals (56) (53).

Antigenic characterization

Hyperimmune antisera against the HAs of selected isolates from each Vietnamese group, A or B, were produced in chickens and used in the HI test against either homologous or heterologous antigen. Regardless of the virus isolate, when homologous antisera and antigen were used, HI titers were the highest (Table 3.2). Approximately a one-$\log_2$ difference in HI titer was detected when comparing group B sera with group A antigen. However, a difference in titer of at least two $\log_2$s was detected when group A sera was used against group B antigen. When heterologous antigen from any of the three H5N1 clades was used against Vietnamese group A or B antisera, a drop in titers of between one and two $\log_2$s was seen, compared to homologous antigen. Differences of one to two-log2s were seen when serum HI titers of Whooper Swan/Mongolia/244/05, a clade 2 virus, were evaluated against the Vietnamese antigens (Table 3.2). Up to a three $\log_2$ difference was seen with group A or B sera and viral antigen produced from a

Mexican isolate from 1995. Similarly, when an older North American isolate, TK/WI/68, served as viral antigen, three $\log_2$ differences or greater were also seen.

A comparison of the antigenic relatedness using the HI data was performed using the method of Archetti and Horsfall (4) (Table 3.3). The clade 2.2, 2.3.2, and 2.3.4 H5N1 viruses used for antisera production have the closest HA sequence similarities to the Vietnam strains in this study, and this correlated to closer antigenic similarities (18-35%). In contrast, the antigenic relatedness of the Vietnam viruses and either North American isolate was lower (1-18%) (Table 3.3). The antigenic distances reflected the differences in HI titers seen between isolates.

Pathogenicity of Vietnam H5N1 2005 viruses for chickens and ducks

To evaluate the virulence of selected Vietnam H5N1 viruses, representatives of groups A and B were used to challenge both chickens and ducks by the IN route. Mortality in chickens was 100% and the mean death time (MDT) was less than 48 hours (Table 3.6). There was no difference in the range of MDTs caused by viruses in either group. As with any HPAI virus infection, chickens showed signs of depression, anorexia, and had edema and hemorrhages in comb and shanks.

The viruses were highly lethal for both 2-week-old and 5-week-old Pekin ducks (Table 3.6). The MDT was between 2.7 and 4.4 days. All ducks presented severe depression and anorexia as early as 1 dpi. Most ducks displayed mild to severe neurological signs beginning at 2 dpi, which were characterized by tremors, uncontrollable shaking, marked loss of balance, lack of coordination, tilted head, seizures, and paralysis. Some ducks died presenting only severe depression.

Virus replication was examined at 2 dpi in lung, spleen, brain, heart, and skeletal muscle tissue following the intranasal infection of chickens and ducks with VN/203 virus. As shown in Table 3.7, infection of both chickens and ducks with the virus resulted in detectable viral titers in all organs examined. Virus titers in the chicken tissues were lower than that observed for the ducks. Virus titers in the lungs and spleen were similar for both older and younger ducks; however, virus titers were higher in the rest of the tissues in the older ducks.

Infectious virus could be recovered from oropharyngeal and cloacal swabs collected from inoculated 2-week-old ducks at 3 dpi, which is consistent with previous reports in which virus shedding was detected from both routes in H5N1 AI virus-infected ducks (Table 3.8). Similar pathogenicity studies conducted at the Southeast Poultry Research Laboratory (SEPRL-USDA, Athens, Georgia) in ducks using clade 1 Vietnam H5N1 AI viruses show that the mortality produced by Vietnam H5N1 viruses in ducks has increased since 2002 (45, 49, 50) (Table 3.7).

Gross and microscopic lesions and AI viral antigen distribution in tissues

In chickens, enlarged and mottled spleen, pulmonary consolidation with edema, congestion and hemorrhage, hemorrhages in Peyer's patches and cecal tonsils, and petechial hemorrhages on the epicardium, were commonly observed. In ducks, the gross lesions observed were similar among all virus-inoculated groups, with dehydration, flaccid proventriculus, empty intestines, splenomegaly, pulmonary edema, and thymus atrophy present in most birds. Also commonly observed were a nasal yellowish discharge that could be expressed from the nostrils, 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, proventriculus full of feed, and gizzards with intense bile staining of the mucosa, and yellowish pancreas with petechia.

In the chickens inoculated with VN/203, the most severe microscopic lesions were found in the lung, heart, and spleen (Table 3.9). In the lung, moderate exudative interstitial pneumonia with congestion to hemorrhage was observed. Mild to moderate lymphohystiocytic infiltration and also mild necrotizing bronchitis with cellular debris in the lumen was present. In the heart, random multifocal to confluent myocardial degeneration to necrosis with minimal to mild lymphohystiocytic inflammation was observed. The spleen, thymus, bursa, and mucosa-associated lymphoid tissue had mild to moderate lymphoid depletion. In the ducks inoculated with VN/203, the most severe lesions were found in the lung, heart, brain, adrenal gland, pancreas, thymus and proventriculus (Table 3.9 and Figure 3.4). Lesions in the heart, lung, and spleen were similar but more severe than those described in chickens. In the brain, randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema were observed. Severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium occurred in the sampled ducks. Multifocal to confluent areas of vacuolar degeneration to necrosis of the adrenal glands, degeneration to necrosis of individual myofibers in skeletal muscle, and mild to moderate necrotizing tracheitis were also observed. Moderate to severe proventriculitis with diffuse lymphoid infiltration and moderate to severe lymphoid depletion was present in the bursa and thymus. The intestinal epithelium was mildly affected, with mild inflammatory changes in the lamina propria.

Immunohistochemistry

The tissue tropism in chickens and ducks of VN/203 was evaluated by immunohistochemical (IHC) analysis using an antiserum against the AI virus nucleoprotein. In both chickens and ducks, viral antigen staining was present in multiple tissues, indicating a systemic infection (Table 3.9). There was a strong correlation between the demonstration of viral antigen in a tissue and the identification of histological lesions in the same tissue from the same bird. However, viral antigen distribution was more widespread than the associated histopathologic lesions (Table 3.9). Viral antigen was closely associated with the observed lesions in the pancreatic acinar epithelium, neurons and glial cells of the brain, trachea epithelium, alveolar epithelium, fragmented cardiac and skeletal myofibers, and adrenal corticotrophic cells. In lymphoid organs, viral antigen was only identified in resident and infiltrating phagocytes but not in apoptotic lymphocytes. In ducks, different from what is observed in chickens, vascular endothelium was consistently negative for the presence of viral antigen. Viral antigen was also identified in the glandular epithelium of the proventriculus, in hepatocytes and Kupfer cells in the liver, smooth muscle of the gizzard, and in the epithelium and autonomic ganglia of the enteric tract.

DISCUSSION

In the winter of 2003- 2004, the first of five waves of outbreaks of H5N1 viruses in Vietnam were reported to be causing disease in poultry (60, 83) (http://www.fao.org/avianflu/news/vietnam.html). Further outbreaks were reported throughout 2004, including some fatal human cases (http://www.who.int) (83). Vietnam

became one of the countries hit especially hard by these viruses (http://www.who.int) (47). A campaign to vaccinate over two million chickens and ducks was begun in July of 2005 (47). Initially, this vaccination program appeared to be successful, with no new poultry outbreaks reported again until December of 2005 (http://web.worldbank.org). However, the virus continued to be sporadically isolated in unvaccinated chickens and ducks from markets and flocks throughout the country during routine surveillance (83). In mid-2007, human infections were again reported and cases of H5N1 in unvaccinated poultry, including ducks, continue to occur, as of January 2009 (84).

Phylogenetic characterization of the 19 Vietnamese isolates indicated that these viruses are members of the Goose/Guangdong H5N1 lineage of viruses, and are classified genetically as clade 2 viruses, but they form two genetically distinct sublineages. The clustering of these Vietnamese viruses into clade 2 was unexpected, since most other Vietnamese viruses isolated prior were in clade 1 (87). The group A viruses are similar to the Fujian-like sublineage (clade 2.3.4), which belongs to the H5N1 genotype Z while the group B viruses belong to clade 2.3.2. Isolates collected from Vietnam at time points following 2005 also were found to belong to clades 2.3.4 and 2.3.2 (46, 79).

The viruses were, however, distinct from the clade 2.2 Qinghai Lake-like lineage viruses that spread westwards to the Middle East, Europe, and Africa, during 2005-2006 (85) (55). Vietnamese viruses circulating before the westward spread of the H5N1s fall into two clusters. The larger of the two clusters, V2, is the one responsible for multiple cases in Southeast Asia since 2004, especially in Vietnam and Thailand (55). The second cluster, V1, contains five samples (55), of which only one was isolated prior to ours, DK/Vietnam/568/05 (55). It is into the V1 cluster that our Vietnamese group B isolates

fall. Other than the DK/Vietnam/568/05 virus, our viruses cluster most closely with Chinese viruses, similar to the findings of Nguyen *et al.* (46).

Despite the degree of virulence that these Vietnamese isolates demonstrated in both chickens and Pekin ducks, they contained only some of the amino acids that are associated with a virulent phenotype in mammals. Glutamic acid at residue 92 of the NS1 protein has been associated with increased virulence in pigs upon reassortment of the NS gene of A/HK/156/97 (H5N1) with the other gene segments of A/Puerto Rico/8/34 (57). Likewise, the presence of lysine at position 627 of the PB2 in H5N1 and H7N7 viruses has been correlated with high virulence and host range in mammals, including humans (21) (19) (30, 42, 69). Neither of these particular amino acids was found to exist in the Vietnamese viruses. Interestingly, the Vietnamese isolates contained a methionine at position 317 of the PB1 polymerase protein, which was found in isolates from Hong Kong in 1997 that were considered low pathogenic isolates for mice (28). None of the Vietnamese viruses contained the mutations at positions 26 and 31 of the M2 protein that are associated with resistance to the adamantine class of antivirals (53, 56) (6, 8) (24). Based on our evidence, the H5N1 viruses circulating in the northern provinces of Vietnam in 2005 have a binding preference for avian receptors, which was supported by the exceptionally high pathogenicity that they displayed in both chickens and ducks. Though they contain molecular markers of high virulence in their antigenic surface glycoproteins, they do not appear to pose an increased risk to the human population based on known markers in the internal genes. It also appears that the currently available antivirals would suffice as an effective means of prophylaxis against these viruses.

The HI test is a standard method for assessing the protective efficacy of vaccine seed viruses against circulating viruses with regards to protection from clinical disease (68). We vaccinated chickens with DNA vaccines instead of the customary whole, killed virus vaccine so that the sera would be HA-specific and prevent steric hindrance caused by antibodies against other viral proteins from interfering with the HI titers. Overall, we were able to see a one-$\log_2$ difference in HI titers when group B sera was used against group A antigen (Table 3.2) and at least a 2-$\log_2$ difference in titers was detected when group A sera was used against group B antigen (Table 3.2). It is possible that these antigenic differences were caused by amino acid differences found between the isolates at two previously identified potential antigenic sites, 181 and 188 of the HA1 subunit (based on H5 numbering) (11). Interestingly, the VN/200 isolate is nearly equal in nucleotide sequence similarity to group A viruses as it is to group B viruses (98.6% and 98.9%, respectively). Likewise, no difference in HI titer was seen when serum against VN/200 was used with viral antigen from either of the two groups. It contains amino acids at positions 180 and 181 that were identical to the subgroup A viruses, although we have found in other studies (unpublished data) that the antigenicity of viruses is not necessarily solely defined by amino acids found at proposed antigenic sites of the HA molecule. The antigenic differences seen support the finding of two distinct groups in the phylogenetic analysis of these isolates. From this information, we can infer that these groups of viruses within clade 2 are continuing to drift further away from each other, phylogenetically, as well as antigenically. If these viruses remain endemic, it is not unlikely that they will continue to differentiate into even more defined phylogenetic groups. Furthermore, the larger differences seen in HI titers when heterologous North

American antigens were used emphasize the diligence needed for continuing the monitoring of the antigenic relatedness between vaccine and circulating viruses. This would help to ensure that vaccines currently used against these viruses are antigenically closely enough related to confer protection against not only clinical disease but also viral shedding in vaccinated birds (68) (36).

Wild ducks are natural reservoirs of low pathogenic AI viruses (29, 63, 74) and epidemiologic evidence and experimental infections show that domestic ducks are also susceptible to AI viruses (13, 15, 59). Until recently, most AI viruses, including the highly pathogenic H5 and H7 strains did not cause disease or death in ducks (1, 2, 13, 51, 81). A number of more recent Asian HPAI H5N1 viruses have been found to be pathogenic to ducks (3, 18, 27, 31, 34, 35, 39, 49, 58, 62, 65, 66, 75, 90), however, this pathogenicity is not consistently observed among different strains (27, 49, 50, 58, 65, 66) and the age of the ducks appears to influence the outcome of the infection (34, 49).

In experiments with a HPAI H5N1 virus in gallinaceous birds, the virus produced a fulminating and rapidly fatal systemic disease (52). The results from experimental studies we performed using IN inoculation in chickens were similar to those found in previous studies, with regards to systemic replication and rapid mortality. We also evaluated the virulence and replication of representative viruses from both genetic and antigenic groups in 2-week-old Pekin ducks given a standard dose, a model that we consistently use in our laboratory to allow comparison between viruses. The microscopic lesions observed in the tissues collected from ducks infected at two weeks of age with VN/203 were similar to those of chickens, with the respiratory tract, brain, heart, pancreas and adrenal glands being the organs most consistently affected, and in this,

similar also to what is found in other avian species (3, 9, 10, 25, 32, 33, 35, 44, 51, 52, 72). Contrary to what has been described in chickens infected with HPAI, where edema and hemorrhage resulting from vascular damage and the resulting most obvious and consistent external clinical sign such as reddening and swelling of heads and legs (33, 52, 73), no severe hemorrhage or external swelling was observed in the ducks examined.

One difference observed between infection in ducks with VN/203 and previous H5N1 viruses that are also highly pathogenic for ducks, is the increase in the numbers of tissues affected, lesion severity and viral replication with this virus. Organs previously not affected by earlier viruses including proventriculus, gizzard, kidney, and enteric tract, presented lesions and/or viral staining. Furthermore, lesions in the lung, spleen, heart, thymus and liver were more severe than previously reported for other H5N1 viruses (49). This explains the increased and more rapid mortality observed in ducks with the 2005 Vietnam viruses. When comparing oral route of viral shedding, higher titers of these Vietnamese viruses were shed than other strains found to be pathogenic in ducks (27, 49, 65, 75). Another difference observed with these more recent Vietnam viruses is that lethality is not age dependent in ducks as seen with other strains; the Vietnamese viruses cause the same high mortality in 2-week-old and in 5-week-old ducks.

In conclusion, viruses isolated in Vietnam in 2005 have shown an additional increase in pathogenicity in ducks, compared to the previous studied viruses. This increase in pathogenicity is the consequence of an increase in viral replication in tissues and an expanded tissue tropism. Field observations coincide with these results, with increased numbers of cases of HPAI H5N1 among domestic ducks, with high mortality, reported in Vietnam during the last year (48). Through sequence analysis, it was

determined that there was not only genetic drift occurring between these viruses and others previously isolated, but also within this small group, as demonstrated by the existence of two distinct subgroups. Control strategies such as vaccination will only be effective if preventative measures utilized are of the appropriate degree, such as updating a vaccine seed strain with one that will be a suitable antigenic match to current viruses. This further underscores that constant and vigilant surveillance of the circulating viruses is needed to ensure that they are kept in check and do not spread any further than they already have, wreaking havoc among not only poultry, but humans as well.

Acknowledgements

The authors wish to thank Suzanne DeBlois and Diane Smith for excellent technical support and also Ronald Graham and Roger Brock for assistance with the animal caretaking. Appreciation is also extended to SAA Sequencing facility. This study was supported by a Specific Cooperative Agreement with Foreign Agriculture Service of the USDA and Agriculture Research Service CRIS Project 6612-32000-048.

TABLES

Table 3.1. Origin of viruses used.

Virus	Abbreviation	Province
Chicken/Vietnam/200/05	VN/200	Lang son
Duck/Vietnam/201/05	VN/201	Lang son
Chicken/Vietnam/202/05	VN/202	Cao Bang
Duck/Vietnam/203/05	VN/203	Ninh Binh
Duck/Vietnam/204/05	VN/204	Nghe An
Duck/Vietnam/205/05	VN/205	Nghe An
Duck/Vietnam/206/05	VN/206	Nghe An
Duck/Vietnam/207/05	VN/207	Quang Nam
Duck/Vietnam/208/05	VN/208	Hai Phong
Chicken/Vietnam/209/05	VN/209	Ninh Binh
Duck/Vietnam/210/05	VN/210	Son La
M Duck/Vietnam/211/05[a]	VN/211	Son La
Chicken/Vietnam/212/05	VN/212	Hai Duong
M Duck/Vietnam/213/05	VN/213	Cao Bang
Duck/Vietnam/215/05	VN/215	Cao Bang
Chicken/Vietnam/216/05	VN/216	Cao Bang
M Duck/Vietnam/217/05	VN/217	Cao Bang
Duck/Vietnam/218/05	VN/218	Nam Dinh
Duck/Vietnam/219/05	VN/219	Nam Dinh

[a]Abbreviation: M Duck, Muscovy Duck

Table 3.2. Hemagglutination inhibition titers using cross-HI test to evaluate antigenic divergence among H5 viruses.

	Viral Antigen[b]						
Antisera[a]	CK/VN/209/05	DK/VN/201/05	Whooper Swan/Mongolia/244/05	CK/HK/220/97	VN/1203/04	CK/Hdlgo/232/94	TK/WI/68
Amino acid similarity between antigen and (VN/201, VN/209)	(95.8%, 100%)[c]	(100%, 95.8%)	(93.4%, 94%)	(92.5%, 92.8%)	(94%, 93.7%)	(83.4%, 84.9%)	(83.1%,84%)
Clade 2 Eurasian							
Group A							
DK/VN/207/05	256	64	256	256	64	128	64
CK/VN/209/05	**2048**	256	512	1024	512	256	64
Group B							
CK/VN/200/05	128	128	64	256	128	64	16
DK/VN/201/05	128	**256**	32	128	128	32	32
DK/VN/206/05	128	256	128	128	64	128	16
Whooper Swan/ Mongolia/244/05	256	128	**512**	256	256	128	256
North American							
CK/Hidalgo/232/94	8	2	8	64	4	**128**	128
TK/WI/68	4	2	16	128	16	64	**512**

[a] serum was produced by DNA vaccination of chickens with plasmids encoding for the HA gene of the corresponding virus

[b] 4 HA units of β-propiolactone-inactivated virus were used as antigen

[c] HA1 amino acid similarity between antigen and (DK/VN/201/05, CK/VN/209/05)

Abbreviations: CK, chicken; DK, duck; Hdlgo, Hidalgo; HK, Hong Kong; TK, turkey; VN, Vietnam; WI, Wisconsin

Numbers in bold represent HI titers when homologous antigen and serum were used

Table 3.3. Percent Antigenic Relatedness[A]

Serum	Virus				
	VN/209	VN/201	Swan/Mong[B]	CK/Hidalgo[C]	TK/WI/68
CK/VN/209/05	100%	25%	35%	9%	2%
DK/VN/201/05		100%	18%	1%	2%
Sw/Mong/244/05[B]			100%	13%	18%
CK/Hidalgo/232/94				100%	35%
TK/WI/68					100%

[A]Antigenic relatedness was calculated based on R values for HI titers, as determined using the method of Archetti and Horsfall (4).
[B]Swan/Mongolia/244/05
[C]CK/Hidalgo/232/94

Table 3.4. Comparing CK/Vietnam/209/05 or DK/Vietnam/203/05 and influenza A genes in GenBank having highest nucleotide sequence similarity.

	Group A **CK/Vietnam/209/05**		Group B **DK/Vietnam/203/05**	
Gene	**isolate**	**% similarity**	**isolate**	**% similarity**
PB2	GS/Guangxi/1633/06	99.8	DK/Guangxi/4184/05	99.8
PB1	GS/Guangxi/4289/05	99.9	GS/Guangxi/3714/05	99.8
PA	DK/Guangxi/5457/05	99.9	DK/Guangxi/4665/05	99.7
HA	Guangxi/1/05	99.7	GS/Guangxi/3714/05	99.8
NP	DK/Guangxi/5165/05	99.8	DK/Guangxi/4196/05	99.7
NA	DK/Guangxi/4830/05	99.9	DK/Guangxi/4830/05	99.6
MA	CK/Laos/Xaythiani/36/06	99.8	DK/Guagxi/4184/05	99.8
NS	CK/Hunan/999/05	99.5	DK/Guangxi/951/05	98.9

Table 3.5. Amino Acid sequence comparison between various Asian H5N1 viral gene segments.

Virus strain	HA[a] residues at			Stalk deletion in NA aa		M2 residue		NS segment		PB2 aa
	222	224	cleavage site	54-72	49-68	26	31	deletion of aa 80-84 (allele)	aa92	627
Goose/Guangdong/1/96	Q	G	RERRRKKR	NO	NO	L	S	NO (B)	D	E
CK/Hong Kong/220/97	Q	G	RERRRKKR	YES	NO	L	S	NO (A)	E	E
DK/Anyang/AVL-1/01	Q	G	RERRRKKR	NO	YES	L	S	NO (A)	D	E
DK/China/E319-2/03	Q	G	RERRRK-R	NO	YES	L	S	YES (A)	D	E
Vietnam/1203/04	Q	G	RERRRKKR	NO	YES	I	N	YES (A)	D	K
DK/Fujian/1734/05	Q	G	RERRRK-R	NO	YES	L	S	YES (A)	D	E
Swan/Mongolia/244/05	Q	G	GERRRRKR	NO	YES	L	S	YES (A)	D	K
Indonesia/5/05	Q	G	RESRRKKR	NO	YES	L	S	YES (A)	D	E
CK/Vietnam/209/05	Q	G	RERRRK-R	NO	YES	L	S	YES (A)	D	E
DK/Vietnam/203/05	Q	G	RERRRK-R	NO	YES	L	S	YES (A)	D	E

[a]H5 numbering

Table 3.6. Pathogenicity of Vietnam HPAI H5N1 viruses in chickens and ducks inoculated intranasally with 10^5 EID_{50} of virus.

Species	Virus Isolate[a] (HA lineage)	Mortality	MDT
			(hours)
WLH chickens (2-4 wks)	DK/Vietnam/201/05 (B)	8/8	<36.0
	DK/Vietnam/203/05 (B)	8/8	48.0
	DK/Vietnam/206/05 (B)	8/8	<36.0
	DK/Vietnam/207/05 (A)	8/8	<36.0
	Muscovy DK/Vietnam/213/05 (A)	8/8	<36.0
	DK/Vietnam/218/05 (A)	8/8	<48.0
			(days)
Pekin ducks (2 wk)	CK/Vietnam/200/05 (B)	8/8	3.1
	DK/Vietnam/203/05 (B)	8/8	3.4
	DK/Vietnam/204/05 (B)	7/8	3.8
	CK/Vietnam/209/05 (A)	8/8	3.3
	CK/Vietnam/216/05 (A)	8/8	3.5
	DK/Vietnam/218/05 (A)	8/8	2.7
			(days)
Pekin ducks (5 wks)	DK/Vietnam/203/05 (B)	8/8	4.4
	DK/Vietnam/218/05 (A)	8/8	2.9

[a] HA sequence genotype enclosed in parentheses

Table 3.7. Comparison of mean titers[a] of influenza virus recovered from tissues of birds infected with 10^5 EID_{50} of DK/Vietnam/203/05.

Species	Tissues[b]				
	Lung	Spleen	Brain	Heart	Muscle
Chickens (2 wk)	5.5	7.3	4.1	4.9	2.5
Ducks (2 wk)	8.0	5.8	3.5	3.3	2.5
Ducks (5 wk)	7.8	5.6	4.5	5.6	5.3

[a]Mean $\log_{10}$ titers expressed as EID_{50}/g from tissues collected from two individual birds. The limit of detection was $10^{1.97}$ EID_{50}/g.
[b]Tissues were collected from two 2-week-old chickens and two 2 and 5-week-old ducks on 2 dpi and titers determined in eggs.

Table 3.8. Comparison of mortality rates, mean death time and oropharyngeal and cloacal titers of 2-week-old ducks inoculated with Vietnam-origin H5N1 influenza viruses.

Virus[a]	Mortality[b]	MDT[c]	Virus Isolation[d]	
			Oral titers 3 dpi	Cloacal titers 3 dpi
DK/Vietnam/218/05 (Clade 2, group A)	8/8	2.7	6.5	3.3
DK/Vietnam/203/05 (Clade 2, group B)	8/8	3.4	4.8	1.5
Vietnam/1203/04 (Clade 1)	7/8	4.2	4.9	2.0
GS/Vietnam/113/01 (Clade 1)	0/8	-	1.8	<1.6

[a]Ducks were inoculated intranasally with 10^{5-6} EID_{50} of the viruses. Includes recent and previous viruses isolated in Vietnam (45, 49, 50)
[b]Number of dead ducks/number of inoculated or exposed ducks.
[c]Mean death time in days
[d] Mean $\log_{10}$ titers expressed as EID_{50}/milliliter from oropharyngeal and cloacal swabs were sampled from three individual ducks on the days indicated. The limit of detection was $10^{0.9}$ EID_{50}/ml.

Table 3.9. Distribution of histologic lesions and viral antigen resultant from intranasal inoculation of 2-week-old chickens and ducks with DK/Vietnam/203/05 virus at 2 days post inoculation.

TISSUE[a]	Chickens		Ducks	
	HE[b]	IHC[c]	HE	IHC
Trachea	+	+	++	++
Lung	++	++	+++	+++
Heart	++	+++	+++	+++
Brain	+	++	+++	+++
Adrenal gland	+	+	+++	+++
Enteric tract	+	++	+	++
Pancreas	+	++	+++	+++
Liver	+	++	++	+++
Kidney	-	-	+	+
Spleen	++	+++	++	++
Bursa	+	++	++	++
Thymus	+	++	+++	+++
Muscle	-	-	++	++
Gizzard	-	-	+	+
Proventriculus	+	+	+++	+++

[a]Tissues collected from 2 birds.
[b]HE, histologic lesions: - = no lesions; + = mild; ++ = moderate; +++ = severe.
[c]IHC, immunohistochemical staining: - = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

FIGURE LEGENDS

Fig. 3.1 Phylogenetic analysis using parsimony for the *HA* gene segment based on nucleotide sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method, with PAUP 4.0b10 program. Branch lengths are indicated in the tree. The outgroup used is CK/Scotland/59. The isolates in the trees are full-length or nearly full-length sequences. Abbreviations used for identifying isolates: CK (chicken), DK (duck). Isolates characterized in this study appear in bold face.

Fig. 3.2 Phylogenetic analysis using parsimony for the *NA* gene segment based on nucleotide sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method, with PAUP 4.0b10 program. Branch lengths are indicated in the tree. The outgroup used is CK/Scotland/59. The isolates in the trees are full-length or nearly full-length sequences. Abbreviations used for identifying isolates: CK (chicken), DK (duck), Ph (pheasant). Isolates characterized in this study appear in bold face.

Fig. 3.3 Phylogenetic analysis using parsimony for the *NP* gene segment based on nucleotide sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method, with PAUP 4.0b10 program. Branch lengths are indicated in the tree. The tree is rooted to Puerto Rico/8/34. The isolates in the trees are full-length or nearly full-length sequences. Abbreviations used for identifying isolates: CK (chicken), DK (duck). Isolates characterized in this study appear in bold face.

Figure 3.4

A. Photomicrograph of the lung of a 2-week-old duck intranasally inoculated with DK/Vietnam/203/05 and euthanized at 2 dpi. Edema, congestion, and hemorrhage are present. Widespread viral staining present in the phagocytic cells and alveolar epithelium (insert).

B. Photomicrograph of the heart of a 2-week-old duck intranasally inoculated with DK/Vietnam/203/05 and euthanized at 2 dpi. Edema and myocardial degeneration and necrosis with mononuclear cell infiltration. Widespread viral staining present in the myocardial fibers (insert).

C. Photomicrograph of the proventriculus of a 2-week-old duck intranasally inoculated with DK/Vietnam/203/05 and euthanized at 2 dpi. Diffuse lymphocytic infiltration and moderate necrosis of the glandular epithelium. Viral staining present in the cells of the glandular epithelium (insert).

D. Photomicrograph of the cerebrum of a 2-week-old duck intranasally inoculated with DK/Vietnam/203/05 and euthanized at 2 dpi. Strongly positive viral staining present in neurons (insert).

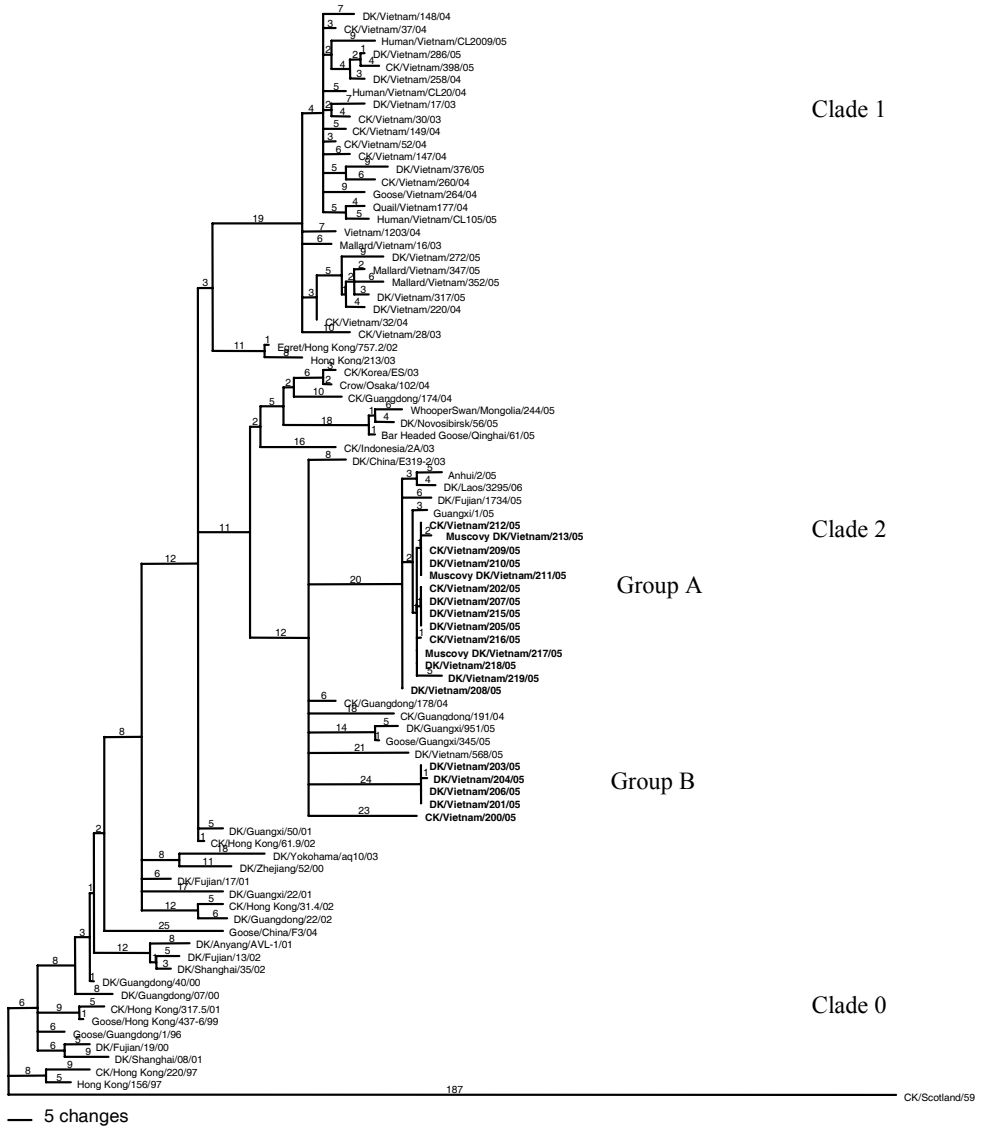
DK/Vietnam/148/04
CK/Vietnam/37/04
Human/Vietnam/CL2009/05
DK/Vietnam/286/05
CK/Vietnam/398/05
DK/Vietnam/258/04
Human/Vietnam/CL20/04
DK/Vietnam/17/03
CK/Vietnam/30/03
CK/Vietnam/149/04
CK/Vietnam/52/04
CK/Vietnam/147/04
DK/Vietnam/376/05
CK/Vietnam/260/04
Goose/Vietnam/264/04
Quail/Vietnam177/04
Human/Vietnam/CL105/05
Vietnam/1203/04
Mallard/Vietnam/16/03
DK/Vietnam/272/05
Mallard/Vietnam/347/05
Mallard/Vietnam/352/05
DK/Vietnam/317/05
DK/Vietnam/220/04
CK/Vietnam/32/04
CK/Vietnam/28/03
Egret/Hong Kong/757.2/02
Hong Kong/213/03
CK/Korea/ES/03
Crow/Osaka/102/04
CK/Guangdong/174/04
WhooperSwan/Mongolia/244/05
DK/Novosibirsk/56/05
Bar Headed Goose/Qinghai/61/05
CK/Indonesia/2A/03
DK/China/E319-2/03
Anhui/2/05
DK/Laos/3295/06
DK/Fujian/1734/05
Guangxi/1/05
CK/Vietnam/212/05
Muscovy DK/Vietnam/213/05
CK/Vietnam/209/05
DK/Vietnam/210/05
Muscovy DK/Vietnam/211/05
CK/Vietnam/202/05
DK/Vietnam/207/05
DK/Vietnam/215/05
DK/Vietnam/205/05
CK/Vietnam/216/05
Muscovy DK/Vietnam/217/05
DK/Vietnam/218/05
DK/Vietnam/219/05
DK/Vietnam/208/05
CK/Guangdong/178/04
CK/Guangdong/191/04
DK/Guangxi/951/05
Goose/Guangxi/345/05
DK/Vietnam/568/05
DK/Vietnam/203/05
DK/Vietnam/204/05
DK/Vietnam/206/05
DK/Vietnam/201/05
CK/Vietnam/200/05
DK/Guangxi/50/01
CK/Hong Kong/61.9/02
DK/Yokohama/aq10/03
DK/Zhejiang/52/00
DK/Fujian/17/01
DK/Guangxi/22/01
CK/Hong Kong/31.4/02
DK/Guangdong/22/02
Goose/China/F3/04
DK/Anyang/AVL-1/01
DK/Fujian/13/02
DK/Shanghai/35/02
DK/Guangdong/40/00
DK/Guangdong/07/00
CK/Hong Kong/317.5/01
Goose/Hong Kong/437-6/99
Goose/Guangdong/1/96
DK/Fujian/19/00
DK/Shanghai/08/01
CK/Hong Kong/220/97
Hong Kong/156/97
CK/Scotland/59
5 changes
Clade 1
Clade 2
Group A
Group B
Clade 0

Figure 3.1.

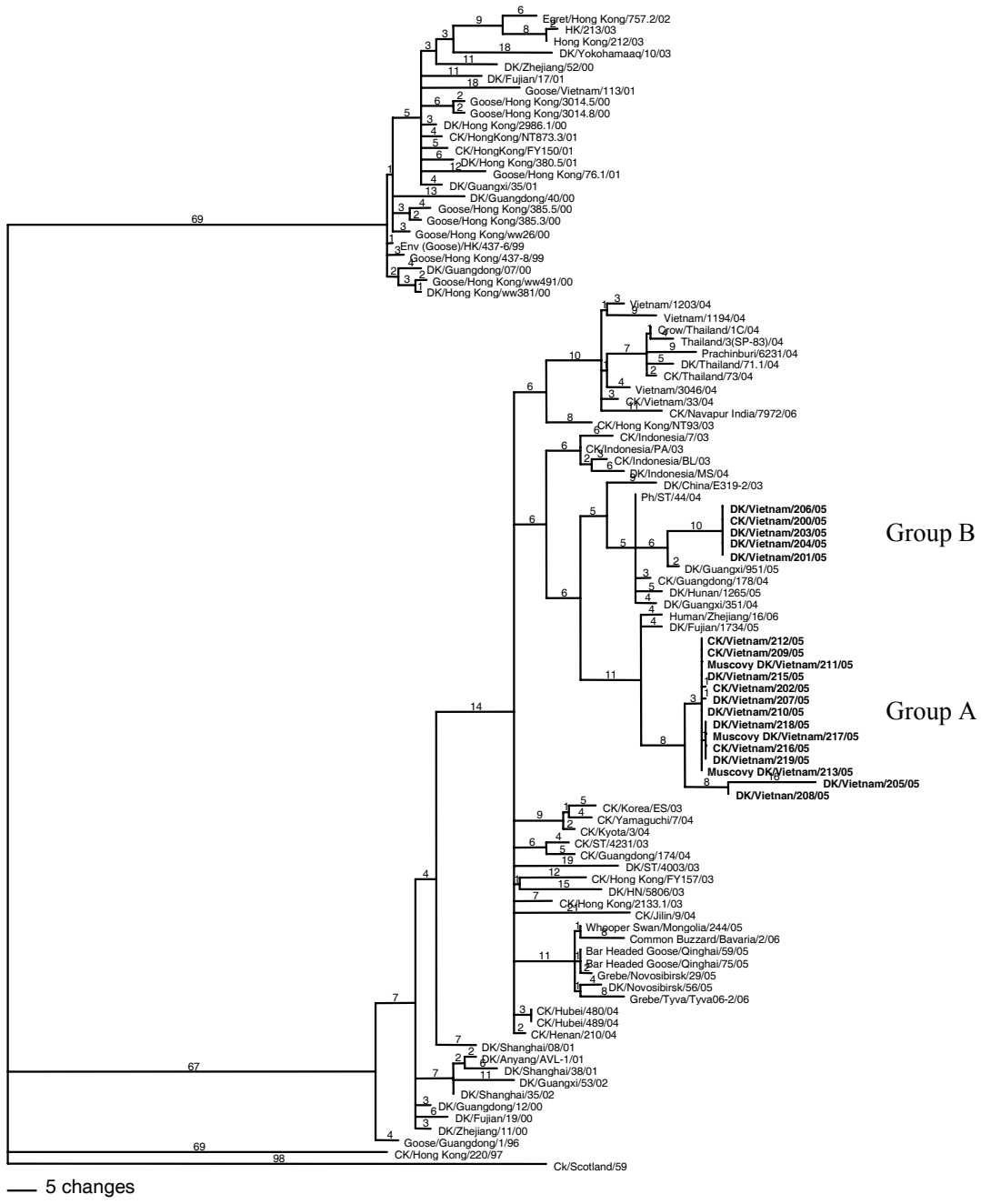
Egret/Hong Kong/757.2/02
HK/213/03
Hong Kong/212/03
DK/Yokohamaaq/10/03
DK/Zhejiang/52/00
DK/Fujian/17/01
Goose/Vietnam/113/01
Goose/Hong Kong/3014.5/00
Goose/Hong Kong/3014.8/00
DK/Hong Kong/2986.1/00
CK/HongKong/NT873.3/01
CK/HongKong/FY150/01
DK/Hong Kong/380.5/01
Goose/Hong Kong/76.1/01
DK/Guangxi/35/01
DK/Guangdong/40/00
Goose/Hong Kong/385.5/00
Goose/Hong Kong/385.3/00
Goose/Hong Kong/ww26/00
Env (Goose)/HK/437-6/99
Goose/Hong Kong/437-8/99
DK/Guangdong/07/00
Goose/Hong Kong/ww491/00
DK/Hong Kong/ww381/00
Vietnam/1203/04
Vietnam/1194/04
Crow/Thailand/1C/04
Thailand/3(SP-83)/04
Prachinburi/6231/04
DK/Thailand/71.1/04
CK/Thailand/73/04
Vietnam/3046/04
CK/Vietnam/33/04
CK/Navapur India/7972/06
CK/Hong Kong/NT93/03
CK/Indonesia/7/03
CK/Indonesia/PA/03
CK/Indonesia/BL/03
DK/Indonesia/MS/04
DK/China/E319-2/03
Ph/ST/44/04
DK/Vietnam/206/05
CK/Vietnam/200/05
DK/Vietnam/203/05
DK/Vietnam/204/05
DK/Vietnam/201/05
Group B
DK/Guangxi/951/05
CK/Guangdong/178/04
DK/Hunan/1265/05
DK/Guangxi/351/04
Human/Zhejiang/16/06
DK/Fujian/1734/05
CK/Vietnam/212/05
CK/Vietnam/209/05
Muscovy DK/Vietnam/211/05
DK/Vietnam/215/05
CK/Vietnam/202/05
DK/Vietnam/207/05
DK/Vietnam/210/05
DK/Vietnam/218/05
Muscovy DK/Vietnam/217/05
CK/Vietnam/216/05
DK/Vietnam/219/05
Muscovy DK/Vietnam/213/05
DK/Vietnam/205/05
DK/Vietnam/208/05
Group A
CK/Korea/ES/03
CK/Yamaguchi/7/04
CK/Kyota/3/04
CK/ST/4231/03
CK/Guangdong/174/04
DK/ST/4003/03
CK/Hong Kong/FY157/03
DK/HN/5806/03
CK/Hong Kong/2133.1/03
CK/Jilin/9/04
Whooper Swan/Mongolia/244/05
Common Buzzard/Bavaria/2/06
Bar Headed Goose/Qinghai/59/05
Bar Headed Goose/Qinghai/75/05
Grebe/Novosibirsk/29/05
DK/Novosibirsk/56/05
Grebe/Tyva/Tyva06-2/06
CK/Hubei/480/04
CK/Hubei/489/04
CK/Henan/210/04
DK/Shanghai/08/01
DK/Anyang/AVL-1/01
DK/Shanghai/38/01
DK/Guangxi/53/02
DK/Shanghai/35/02
DK/Guangdong/12/00
DK/Fujian/19/00
DK/Zhejiang/11/00
Goose/Guangdong/1/96
CK/Hong Kong/220/97
Ck/Scotland/59
5 changes

Figure 3.2.


Whooper Swan/Mongolia/244/05
Bar Headed Goose/Qunghai/67/05
Bar Headed Goose/Qinghai/60/05
Bar Headed Goose/Qinghai/62/05
Bar Headed Goose/Qinghai/65/05
Bar Headed Goose/Qinghai/5/05
Bar Headed Goose/Qinghai/12/05
Bar Headed Goose/Qinghai/68/05
Grebe/Novosibirsk/29/05
DK/Novosibirsk/56/05
CK/Korea/ES/03
CK/Yamaguchi/7/04
Hong Kong/739-2/02
Egret/Hong Kong/757.2/03
Hong Kong/213/03
Vietnam/1203/04
Crow/Thailand/1C/04
Quail/Thailand/57/04
Prachinbur
Bird/Thailand/3.1/04
DK/Thailand/71.1/04
Goose/Thailand/79/04
Vietnam/1194/04
CK/Indonesia/7/03
CK/Indonesia/BL/03
DK/Indonesia/MS/04
CK/Indonesia/PA/03
CK/Guangdong/178/04
CK/Hunan/1652/05
DK/Hunan/1608/05
DK/Guangxi/951/05
DK/Vietnam/203/05
DK/Vietnam/204/05
DK/Vietnam/201/05
DK/Vietnam/206/05
CK/Vietnam/200/05
CK/Vietnam/202/05
DK/Vietnam/207/05
DK/Vietnam/215/05
Muscovy DK/Vietnam/217/05
CK/Vietnam/216/05
DK/Vietnam/219/05
DK/Vietnam/218/05
Muscovy DK/Vietnam/211/05
CK/Vietnam/212/05
Muscovy DK/Vietnam/213/05
CK/Vietnam/209/05
DK/Vietnam/210/05
DK/Vietnam/205/05
DK/Vietnam/208/05
DK/China/E319-2/03
CK/Henan/01/04
CK/Henan/12/04
CK/Henan/16/04
CK/Hubei/327/04
Swan/Guangxi/307/04
DK/Shanghai/38/01
DK/Shanghai/35/02
CK/Jilin/9/04
CK/Hong Kong/37.4/02
Goose/Vietnam/113/01
DK/Shanghai/13/01
DK/Guangdong/01/01
Goose/Hong Kong/ww26/2000
Env (Goose)/HK/437-6/99
CK/Hong Kong/17-5/01
DK/Guangdong/07/00
Goose/Guangdong/1/96
DK/Zheijiang/11/00
DK/Guangdong/12/00
DK/Shanghai/08/01
DK/Anyang/AVL-1/01
DK/Fujian/01/02
DK/Guangxi/53/02
DK/Shanghai/37/02
DK/Fujian/19/00
DK/Fujian/17/01
DK/Guangdong/22/02
DK/Fujian/13/02
CK/Hong Kong/220/97
Hong Kong/481/97
Parakeet/Narita/92A/98
Hong Kong/1073/99
DK/Guangdong/40/00
Puerto Rico/8/34
5 changes

Figure 3.3.

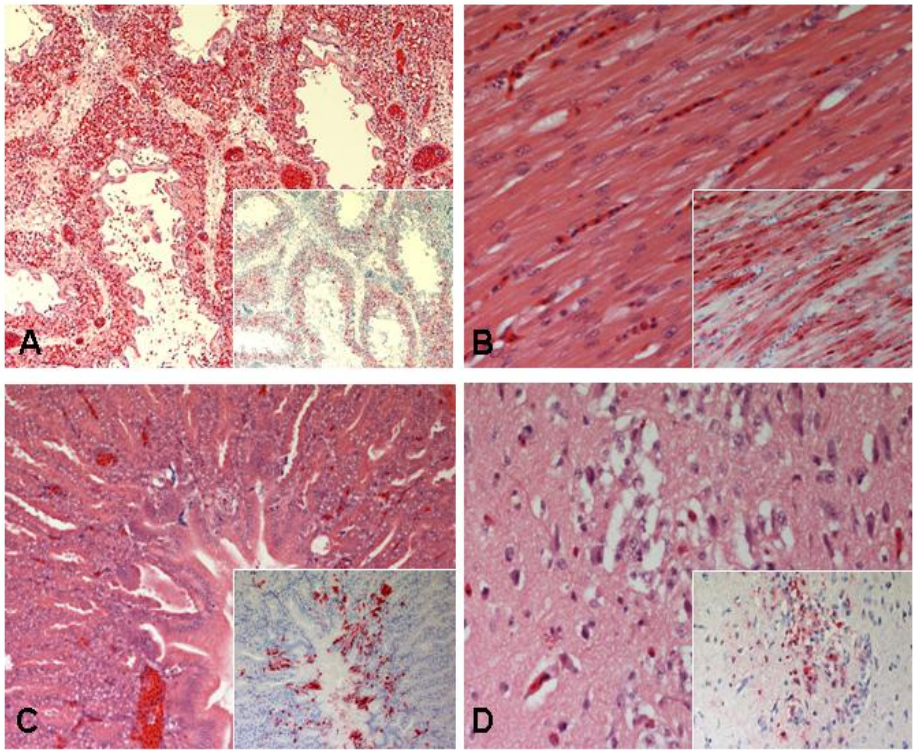

Fig. 3.4

REFERENCES

1. 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.

2. Alexander, D. J., W. H. Allan, D. Parsons, and G. Parsons. The pathogenicity of eight avian influenza A viruses for fowls, turkeys and ducks. Res. Vet. Sci. 24:242-247. 1978.

3. Antarasena, C., R. Sirimujalin, P. Prommuang, S. D. Blacksell, N. Promkuntod, and P. Prommuang. Tissue tropism of a Thailand strain of high-pathogenicity avian influenza virus (H5N1) in tissues of naturally infected native chickens (*Gallus gallus*), Japanases quail (*Coturnix coturnix japonica)* and ducks (*Anas* spp). Avian Pathol. 35:250-253. 2006.

4. Archetti, I., and Horsfall, F. L. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum. J. Exp. Med. 92:441-462. 1950.

5. Barbeito, M. S., G. Abraham, M. Best, P. Cairns, P. Langevin, W. G. Sterritt, D. Barr, W. Meulepas, J. M. Sanchez-Vizcaino, and M. Saraza. Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. First International Veterinary Biosafety Workshop 14:873-887. 1995.

6. Belshe, R. B., M. H. Smith, C. B. Hall, R. Betts, and A. J. Hay. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J Virol 62:1508-1512. 1988.

7. Bender, C., H. Hall, J. Huang, A. Klimov, N. Cox, A. Hay, V. Gregory, K. Cameron, W. Lim, and K. Subbarao. Characterization of the Surface Proteins of Influenza A (H5N1) Viruses Isolated from Humans in 1997-1998. Virology 254:115-123. 1999.

8. Bright, R. A., D. K. Shay, B. Shu, N. J. Cox, and A. I. Klimov. Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the united states. JAMA 295:891-894. 2006.

9. Capua, I., F. Mutinelli, C. Terregino, G. Cattoli, R. J. Manvell, and F. Burlini. Highly pathogenic avian influenza (H7N1) in ostriches farmed in Italy. Vet. Rec. 146:356. 2000.

10. Capua, I., S. Marangon, L. Selli, D. J. Alexander, D. E. Swayne, M. D. Pozza, E. Parenti, and F. M. Cancellotti. Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997-January 1998. Avian Pathol. 28:455-460. 1999.

11. Caton, A. J. a. B., G. G. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417-427. 1982.

12. Cauthen, A. N., D. E. Swayne, S. Schultz-Cherry, M. L. Perdue, and D. L. Suarez. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J. Virol. 74:6592-6599. 2000.

13. 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.

14. Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351:472-477. 1998.

15. Cooley, A. J. H. V. C., M. S. Philpott, V. C. Easterday, and V. S. Hinshaw. Pathological lesions in the lungs of ducks with influenza A viruses. Vet. Path. 26:1-5. 1989.

16. Craig, J. V., W. F. Dean, G. B. Havenstein, K. K. Kruger, K. E. Nestor, G. H. Purchase, P. B. Siegel, and G. L. van Wicklen Guidelines for poultry husbandry. In: Guide for the care and use of agricultural animals in agricultural research and teaching. Federation of American Societies of Food Animal Sciences, Savoy, IL. 1999.

17. Edan, M., and N. Bourgeois. Review of free-range duck farming systems in Northern Vietnam and assessment of their implication in the spreading of the highly pathogenic (H5N1) strain of avian influenza (HPAI). A report from Agronomes et Veterinaires sans Frontieres for the Food and Agriculture Organization of the United Nations:1-101. 2006.

18. Ellis, T. M., R. B. Bousfield, L. A. Bissett, K. C. Dyrting, G. S. Luk, S. T. Tsim, K. Sturm-Ramirez, R. G. Webster, Y. Guan, and J. S. Malik Peiris. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Path. 33:492-505. 2004.

19. Fouchier, R. A., P. M. Schneeberger, F. W. Rozendaal, J. M. Broekman, S. A. Kemink, V. Munster, T. Kuiken, G. F. Rimmelzwaan, M. Schutten, G. J. Van Doornum, G. Koch, A. Bosman, M. Koopmans, and A. D. Osterhaus. Avian influenza A virus

(H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc. Natl. Acad. Sci. U S A 101:1356-1361. 2004.

20. Garcia, M., J. M. Crawford, J. W. Latimer, E. Rivera-Cruz, and M. L. Perdue. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J. Virol. 77:1493-1504. 1996.

21. Govorkova, E. A., J. E. Rehg, S. Krauss, H.-L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffmann. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. Journal of Virology 79:2191-2198. 2005.

22. Ha, Y., D. J. Stevens, J. J. Skehel, and D. C. Wiley. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proceedings of the National Academy of Sciences of the United States of America 98:11181-11186. 2001.

23. Happold, J. R., I. Brunhart, H. Schwermer, and K. D. C. Stark. Surveillance of H5 Avian Influenza Virus in Wild Birds Found Dead. Avian Dis. 52:100-105. 2008.

24. Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith. The molecular basis of the specific anti-influenza action of amantadine. EMBO 4:3021-3024. 1985.

25. Hooper, P. T., G. W. Russell, P. W. Selleck, and W. L. Stanislawek. Observations on the relationship in chickens between the virulence of some avian influenza viruses and their pathogenicity for various organs. Avian Dis. 39:458-464. 1995.

26. Horimoto, T., and Y. Kawaoka. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68:3120-3128. 1994.

27. 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.

28. Katz, J. M., Lu, X., Tumpey, T.M., Smith, C.B., Shaw, M.W., & Subbarao, K. Molecular correlates of inlfuenza A H5N1 virus pathogenesis in mice. J. Virol. 74:10807-10810. 2000.

29. 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.

30. Keawcharoen, J., K. Oraveerakul, T. Kuiken, R. A. M. Fouchier, A. Amonsin, S. Payungporn, S. Noppornpanth, S. Wattanodorn, A. Theamboonlers, R. Tantilertcharoen, R. Pattanarangsan, N. Arya, P. Ratanakorn, A. D. M. E. Osterhaus, and P. Y. Avian influenza H5N1 in tigers and leopards. Emerg. Infect. Dis. 10:2189-2191. 2004.

31. Kishida, N., Y. Sakoda, N. Isoda, K. Matsuda, M. Eto, Y. Sunaga, T. Umemura, and H. Kida. Pathogenicity of H5 influenza viruses for ducks. Arch. Virol. 150:1383-1392. 2005.

32. Kobayashi, Y., T. Horimoto, Y. Kawaoka, D. J. Alexander, and C. Itakura. Neuropathological studies of chickens infected with highly pathogenic avian influenza viruses. J. Comp. Pathol. 114:131-147. 1996.

33. Kobayashi, Y., T. Horimoto, Y. Kawaoka, D. J. Alexander, and C. Itakura. Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. Avian Pathol. 25:285-304. 1996.

34. Kwon, Y. K., S. J. Joh, M. C. Kim, H. W. Sung, Y. J. Lee, J. G. Choi, E. K. Lee, and J. H. Kim. Highly pathogenic avian influenza (H5N1) in the commercial domestic ducks of South Korea. Avian Pathol. 34:367-370. 2005.

35. Lee, C. W., D. L. Suarez, T. M. Tumpey, H. W. Sung, Y. K. Kwon, Y. J. Lee, J. G. Choi, S. J. Joh, M. C. Kim, E. K. Lee, J. M. Park, X. Lu, J. M. Katz, E. Spackman, D. E. Swayne, and J. H. Kim. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J. Virol. 79:3692-3702. 2005b.

36. Lee, C. W., and D. L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Animal Health Research Reviews 6:1-15. 2005a.

37. Lee, C. W., Senne, D. A., and Suarez, D. L. Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clinical and Vaccine Immunology 13:395-402. 2006.

38. 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.

39. Li, Y., Z. Lin, J. Shi, Q. Qi, G. Deng, Z. Li, X. Wang, G. Tian, and H. Chen. Detection of Hong Kong 97-like H5N1 influenza viruses from eggs of Vietnamese waterfowl. Archives of virology 151:1615-1624. 2006.

40. Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. W. Zhang, X. L. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. F. Gao. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309:1206. 2005.

41. Ludwig, S., U. Schultz, J. Mandler, W. M. Fitch, and C. Scholtissek. Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology 183:566-577. 1991.

42. Mase, M., N. Tanimura, T. Imada, M. Okamatsu, K. Tsukamoto, and S. Yamaguchi. Recent H5N1 avian influenza A virus increases rapidly in virulence to mice after a single passage in mice. Journal of General Virology 87:3655-3659. 2006.

43. Matrosovich, M., N. Zhou, Y. Kawaoka, and R. Webster. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J. Virol. 73:1146-1155. 1999.

44. Mo, I. P., M. Brugh, O. J. Fletcher, G. N. Rowland, and D. E. Swayne. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. Avian Dis. 41:125-136. 1997.

45. Nguyen, D. C., T. M. Uyeki, S. Jadhao, T. Maines, M. Shaw, Y. Matsuoka, C. Smith, T. Rowe, X. Lu, H. Hall, X. Xu, A. Balish, A. Klimov, T. M. Tumpey, D. E. Swayne, L. P. Huynh, H. K. Nghiem, H. H. Nguyen, L. T. Hoang, N. J. Cox, and J. M.

Katz. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J. Virol. 79:4201-4212. 2005.

46. Nguyen, T. D., T. V. Nguyen, D. Vijaykrishna, R. G. Webster, Y. Guan, J. S. M. Peiris, and G. J. D. Smith. Multiple sublineages of influenza A virus (H5N1), Vietnam, 2005-2007. Emerg. Infect. Dis. 14:632-636. 2008.

47. Normile, D. Vietnam battles bird flu...and critics. Science 309:368-373. 2005.

48. OIE Highly pathogenic avian influenza, Vietnam. In. OIE World Health Animal Health Information Database. 2008.

49. Pantin-Jackwood, M., 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. 2007b.

50. 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. 2007a.

51. 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.

52. Perkins, L. E., and D. E. Swayne. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet. Pathol. 38:149-164. 2001.

53. Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupa, R. Kijphati, and P. Sawanpanyalert.

Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. 86:423-433. 2005.

54. Reed, L. J., and H. Muench. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27:493-497. 1938.

55. Salzberg, S. L., C. Kingsford, G. Cattoli, D. J. Spiro, D. A. Janies, M. M. Aly, I. H. Brown, E. Couacy-Hymann, G. M. De Mia, H. Dung do, A. Guercio, T. Joannis, A. S. Maken Ali, A. Osmani, I. Padalino, M. D. Saad, V. Savic, N. A. Sengamalay, S. Yingst, J. Zaborsky, O. Zorman-Rojs, E. Ghedin, and I. Capua. Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg. Infect. Dis. 13:713-718. 2007.

56. Schmidtke, M., R. Zell, K. Bauer, A. Krumbholz, C. Schrader, J. Suess, and P. Wutzler. Amantadine resistance among porcine H1N1, H1N2, and H3N2 influenza A viruses isolated in Germany between 1981 and 2001. Intervirology 49:286-293. 2006.

57. Seo, S. H., E. Hoffmann, and R. G. Webster. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8:950-954. 2002.

58. Shinya, K., M. Hatta, S. Yamada, A. Takada, S. Watanabe, P. Halfmann, T. Horimoto, G. Neumann, J. H. Kim, W. Lim, Y. Guan, M. Peiris, M. Kiso, T. Suzuki, Y. Suzuki, and Y. Kawaoka. Characterization of a human H5N1 influenza A virus isolated in 2003. J. Virol. 79:9926-9932. 2005.

59. 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.

60. Sims, L. D. Lessons learned from Asian H5N1 outbreak control. Avian Dis. 50:174-181. 2007.

61. Smith, G. J., X. H. Fan, J. Wang, K. S. Li, K. Qin, J. X. Zhang, D. Vijaykrishna, C. L. Cheung, K. Huang, J. M. Rayner, J. S. Peiris, H. Chen, R. G. Webster, and Y. Guan. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U S A 103:16936-16941. 2006.

62. 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.

63. Stallknecht, D. E. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorevirds, pelicans, cormorants, etx. Proceedings of the Fourth International Symposium on Avian Influenza:61-69. 1998.

64. Stevens, J., O. Blixt, T. M. Tumpey, J. K. Taubenberger, J. C. Paulson, and I. A. Wilson. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 312:404-410. 2006.

65. Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J. Virol. 79:11269-11279. 2005.

66. Sturm-Ramirez, K. M., T. Ellis, B. Bousfield, L. Bissett, K. Dyrting, J. E. Rehg, L. Poon, Y. Guan, M. Peiris, and R. G. Webster. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J. Virol. 78:4892-4901. 2004.

67. Suarez, D. L., and S. Schultz-Cherry. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis. 44:861-868. 2000.

68. 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.

69. Subbarao, E. K., W. London, and B. R. Murphy. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. 67:1761-1764. 1993.

70. Swayne, D. E. Understanding the Complex Pathobiology of High Pathogenicity Avian Influenza Viruses in Birds. Avian Dis. 50:242-249. 2007.

71. Swayne, D. E., D. A. Senne, and C. W. Beard Avian Influenza. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. J. R. Glisson, Swayne, D. E., Jackwood, M. W., Pearson, J. E., and Reed, W. M., ed. American Association of Avian Pathologists, Kennet Square, PA. pp 150-155. 1998.

72. Swayne, D. E. Pathobiology of H5N2 Mexican avian influenza virus infections of chickens. Vet Pathol 34:557-567. 1997.

73. Swayne, D. E., and D. L. Suarez. Highly pathogenic avian influenza. Rev. Sci. Tech. 19:463-482. 2000.

74. Swayne, D. E., and Halvorson, D. A. Influenza. In: Disease of Poultry. Y. M. Said, Barnes, H. J., Glisson, J. R., Fadly A. M., McDougald, L. R., and Swayne, D. E., ed. Iowa State University Press, Ames. pp 135-160. 2003.

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

76. Treanor, J. J., M. H. Snyder, W. T. London, and B. R. Murphy. The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology 171:1-9. 1989.

77. Tumpey, T. M., D. L. Suarez, L. E. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. J. Virol. 76:6344-6355. 2002.

78. Tumpey, T. M., D. L. Suarez, L. E. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne. Evaluation of a high-pathogenicity H5N1 avian influenza A virus isolated from duck meat. Avian Dis. 47:951-955. 2003.

79. Wan, X. F., Nguyen, T., Davis, C. T., Smith, C. B., Zhao, Z. M., Carrel, M., Inui, K., Hoa, T. D., Mai, D. T., Jadhao, S., Balish, A., Shu, B., Luo, F., Emch, M., Matsuoka, Y., Sindstrom, S. E., Cox, N. J., Nguyen, C. V., Klimov, A., and Donis, R. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. PLoS ONE 3:e3462. 2008.

80. Wang, J., D. Vijaykrishna, L. Duan, J. Bahl, J. X. Zhang, R. G. Webster, J. S. M. Peiris, H. Chen, G. J. D. Smith, and Y. Guan. Identification of the Progenitors of Indonesian and Vietnamese Avian Influenza A (H5N1) Viruses from Southern China. Journal of Virology 82:3405-3414. 2008.

81. Webster, R. G., Y. Guan, M. Peiris, D. Walker, S. Krauss, N. N. Zhou, E. A. Govorkova, T. M. Ellis, K. C. Dyrting, T. Sit, D. R. Perez, and K. F. Shortridge. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J. Virol. 76:118-126. 2002.

82. Webster, R. G., and E. A. Govorkova. H5N1 influenza--continuing evolution and spread. N. Engl. J. Med. 355:2174-2177. 2006.

83. WHO. H5N1 avian influenza: Timeline of major events. 2008.

84. WHO. H5N1 avian influenza: timeline of major events. 2009.

85. WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines. 2007.

86. WHO H5N1 nomenclature. In. World Health Organization H5N1 Evolution Working Group. Geneva, Switzerland. http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html. 2007.

87. WHO Evolution of H5N1 avian influenza viruses in Asia. Emerg. Infect. Dis. . In. Global Influenza Program Surveillance Network. pp 1515-1521. . 2005.

88. Wright, P. F., G. Neumann, and Y. Kawaoka Orthomyxoviruses. In: Fields Virology. D. M. Knipe, and P. M. Howley, ed. Lippincott Williams & Wilkins. pp 1691-1740. 2006.

89. Xu, X., K. Subbarao, N. J. Cox, and Y. Guo. 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 261:15-19. 1999.

90. Zhou, J. Y., H. G. Shen, H. X. Chen, G. Z. Tong, M. Liao, H. C. Yang, and J. X. Liu. Characteriation of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China. J. Gen. Virol. 87:1823-1833. 2006.

CHAPTER 4

EFFICACY OF COMMERCIAL VACCINES IN PROTECTING CHICKENS AND DUCKS AGAINST H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES FROM VIETNAM[1]

[1]J. Pfeiffer, D. L. Suarez, L. Sarmento, T. L. To, T. Nguyen, and M. J. Pantin-Jackwood. To be submitted to Avian Diseases.

SUMMARY.

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

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

INTRODUCTION

The first case of Asian lineage highly pathogenic (HP) H5N1 AI virus was isolated in 1996 from a goose in the Guangdong province of China (55, 60). A similar virus proceeded to cause an outbreak among poultry in Hong Kong in 1997 (54). Massive culling of all poultry led to the eradication of these viruses, but the Goose/Guangdong-like viruses continued to circulate among ducks in China (41, 55). Cauthen *et al.* also demonstrated that H5N1 viruses obtained from cages where geese were housed, in 1999, were nearly identical to the Goose/Guangdong/1/96 virus (4). Evidence of continued circulation of virus in the region included H5N1 from exported Chinese duck meat in 2001, and H5N1 viruses being isolated in live bird markets (LBM) in Vietnam in 2001 and Hong Kong in 2002 (13, 22, 48). However, starting late in 2003, an unprecedented spread of the virus occurred in Southeast Asia that eventually moved to Europe, Africa, and the Indian subcontinent (55). These H5N1 viruses have become endemic in several countries in Asia and Africa and variant strains continue to emerge (5, 19, 33, 52, 58). Vaccination has been implemented and is still encouraged as part of a control program in poultry in parts of Asia including Vietnam, Indonesia, China, and Egypt (Thanhnien news; 7/15/05) (7).

In addition to preventing clinical disease, a major goal of vaccination against AI (particularly H5 and H7 subtypes), when used as part of a control program, is to reduce levels of virus shed into the environment (42, 49). Influenza viruses tend to accumulate point mutations in their hemagglutinin (HA) and neuraminidase (NA) surface proteins over time, resulting in antigenic drift, which enhances the ability of the virus to evade the host immune response induced by vaccination or natural infection, allowing higher levels

of replication (10, 37, 59). With the increased amount of virus replication, higher levels of virus shedding by infected birds increase the likelihood of transmission to other susceptible birds or flocks**.** Earlier studies have shown that the closer the HA amino acid sequence of the vaccine virus is to that of the challenge virus, the lower the levels of virus that are shed from the oropharynx (46). Therefore, it is important when selecting a vaccine virus to take into consideration the amino acid sequence similarity between the circulating viruses and the vaccine virus candidates.

For human influenza viruses, the importance of antigenic drift in vaccine seed strains has resulted in the development of the World Health Organization Global Influenza Surveillance Network whose principal function is to recommend what vaccine seed strains should be included in commercial vaccines. The factors that they evaluate are genetic differences in circulating strains, the prevalence of important variants, and the antigenic differences these viruses have from the current vaccine strains. A four-fold difference in hemagglutination inhibition (HI) titers between antibody against the current vaccine viruses and antigen of reference strains is an indication that the vaccine seed strain needs to be changed to maintain optimal protection from the vaccine (http://www.who.int/csr/disease/influenza/surveillance/en/index.html). Avian influenza viruses at one time were thought to be less susceptible to antigenic drift as it related to vaccine efficacy, at least as measured by morbidity or mortality in vaccinated birds (53). Because cross-protection has been provided by vaccines produced from heterologous viruses (45, 50), frequent changing of AI virus vaccine strains was not considered to be necessary (44, 46). This difference in human and AI viruses was believed to be a combination of less selection pressure in the birds due to the infrequent vaccination and

short production lives of the birds and the use of strong adjuvants that were commonly used with poultry vaccines (49). However, long-term vaccination for AI in poultry has recently become more common, and in countries like Mexico where long-term vaccination without eradication of the low pathogenic H5N2 avian influenza circulating there, similar degrees of antigenic drift in human and AI viruses seems to occur at the antigenic level (17). In the study by Lee *et al.* (2004) comparing different Mexican lineage viruses isolated before and during the vaccination campaign, up to a 16-fold difference was seen in HI activity between the vaccine seed strain and the more recent AI isolates. Additionally, amounts of virus shed by vaccinated birds that were challenged with distantly related viruses were comparable to birds that had not been vaccinated (18). The findings from this study underscore the need for frequent evaluation of AI vaccines in their abilities to control viral shedding. Furthermore, it emphasizes the importance of carefully matching vaccine strains with circulating viruses.

In Vietnam, customary duck management practices often allow exposure of domestic ducks to the wild waterfowl population, which enables ample opportunity for AI viruses to infect domestic ducks and to potentially proceed to infect chickens and other poultry (3, 9). Because ducks can serve as silent carriers of AI and also due to the fact that ducks make up a large part of the poultry population in Vietnam, vaccination of ducks has been included in the vaccine regimen (34). Some concern that this increased vaccination pressure may result in faster antigenic drift away from the vaccine strains has been raised. It has been suggested to evaluate vaccine efficacy at least biennially (42) to ensure that optimal levels of protection against clinical disease and viral shedding are met. While various vaccines' protective abilities against AI challenge in chickens is

relatively well understood, less is known about how well AI vaccines perform in ducks. Here, we evaluated the abilities of three currently used commercial vaccines and two experimental vaccines homologous to the challenge strain to protect both chickens and ducks against lethal exposure of highly pathogenic H5N1 viruses that were obtained from chickens and ducks in Vietnam. The challenge viruses selected were classified as clade 2.3.2 or 2.3.4 viruses and were unusually virulent for ducks as well as being characteristically virulent for chickens (23, 28, 58).

MATERIALS AND METHODS

Viruses

The H5N1 viruses A/Duck/Vietnam/203/05 (DK/VN/203), A/Chicken/Vietnam/209/05 (CK/VN/209), and A/Duck/Vietnam/218/05 (DK/VN/218) were isolated from either ducks or chickens in Vietnam and were obtained from the National Center for Veterinary Diagnosis**,** Hanoi, Vietnam. The DK/VN/203 virus is a clade 2.3.2 virus and the two latter viruses belong to clade 2.3.4 (23, 28, 58), and were isolated in northern Vietnam in December of 2005 (28). Isolates were inoculated into the allantoic cavity of embryonating chicken eggs and grown for 24-30 hours at 37°C. Allantoic fluid was harvested, titered as previously described (28), and frozen at -70°C until further use.

Vaccines

Three commercially available vaccines used in this study were generated from the following whole, killed viruses, and contained an oil adjuvant: 1) A/TK/England/N-

28/73, subtype H5N2 (referred to as N28), 2) a genetically modified reassortant H5N1 low pathogenic virus, A/Harbin/Re-1/2003 (referred to as Re-1) (29), and 3) A/CK/Mexico/232/94 (H5N2) (referred to as Mexican vaccine) (Table 4.1). The first two vaccines were obtained from Vietnam and were produced by Weike Biological Company, of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China), and the third vaccine used was from Intervet Inernational/ Investigacion Aplicada S.S. (IASA), Tehuacan, Puebla, Mexico. The Re-1 vaccine was produced through reverse genetics and derived its HA and NA genes from A/Goose/Guangdong/96 (29). This virus was attenuated by removing the multiple basic amino acids at the HA cleavage site (47). The six internal genes of this recombinant virus were derived from the high-growth A/Puerto Rico/8/34 (PR8) virus.

Two experimental vaccines containing either DK/VN/203 or CK/VN/209 antigen (Ag) were prepared on site, as previously described (39). Briefly, viruses were grown in 10-day old embryonating chicken eggs for one day. Allantoic fluid from eggs infected with one of each particular virus was harvested and pooled. Following inactivation of each virus with 0.1% β-propiolactone (Sigma, St. Louis, MO), HA titers were determined by the HA test to be the following: DK/VN/203: 256 HA units and CK/VN/209: 256 HA units. One part aqueous Ag (10 ml) was emulsified in four parts (40 ml) oil phase. The oil phase consisted of 36 ml Drakeol 6 VR pharmaceutical grade mineral oil (Penreco, Burler, PA), 3 ml 7.5% sorbitan monooleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 1 ml 2.5% polysorbate (Tween 80, ICI United States, Inc.). Vaccines produced on site were prepared five or six days prior to administration through

homogenization using a Waring blender (Fisher Scientific International Inc., Hampton, NH) (40), and were stored at 4°C.

Evaluation of sequence similarity

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

Animal experiments

Two-week old specific pathogen-free (SPF) white leghorn chickens from our flock at SEPRL, either eight or 10 per group, were vaccinated once, subcutaneously in the nape of the neck with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine), or with 0.5 ml of experimental vaccine (39). Normal allantoic fluid in the form of an oil emulsion vaccine was used as negative control. Two weeks post-vaccination, all birds were bled via the wing vein. Three weeks post-vaccination, all birds were challenged with 10^6 EID_{50} (50% embryo infectious dose) of either DK/VN/203 or CK/VN/209 viruses in a total volume of 0.2 ml brain heart infusion (BHI) broth per bird, via the choanal slit. Birds were evaluated for clinical signs for 10 days following challenge. Oropharyngeal swabs were taken at days two and four post challenge (DPC) for determining viral shedding. At 10 DPC, all survivors were bled via the wing vein, and then euthanized with 0.2 ml sodium pentobarbital (5 gr/ml) per bird.

One-week-old white Pekin ducks (*A. platyrhynchos*) obtained from a commercial farm were divided into six groups of 10 birds. Blood samples for serology were collected from the saphenous vein of a representative number of ducks to ensure that the birds were serologically negative for AI. Ducks were vaccinated once, subcutaneously in the nape of the neck with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine). Two groups served as nonvaccinated controls receiving allantoic fluid in the form of an oil emulsion vaccine. Two weeks post-vaccination, blood samples were collected from all ducks for serology. At this same time, the ducks were challenged via the choanal slit with $10^{5.0}$ EID_{50} of DK/VN/203 or DK/VN/218 influenza virus in 0.1 ml. Ducks were observed daily for clinical signs of disease. Oropharyngeal and cloacal swabs were collected at 2, 3, 5, 7, and 11 DPC to determine viral shedding. One duck per group was euthanized with 0.2 ml sodium pentobarbital (5 gr/ml) per bird at 3 DPC, and tissues collected for virus detection by quantitative real-time RT-PCR (36). Blood samples were collected at 11 DPC from all surviving ducks. Ducks remaining at the end of the experiment were euthanized as previously mentioned.

Hemagglutination inhibition (HI) test

Hemagglutination inhibition antibody titers against AI were determined by using the HI test (43). Either homologous or heterologous β-propiolactone-inactivated Ag was diluted in PBS to make a concentration of four HA units. Homologous Ag refers to the same strain of virus used to produce the vaccine. Heterologous Ag refers to any of the three viruses used to produce the vaccines tested in this study, which were not identical to

the vaccine virus administered. Fifty microliters of Ag were added per well of a 96-well plate, where test serum was two-fold, serially diluted. Plates were incubated 15 min. at room temperature before 0.5% chicken red blood cells were added to each well. Plates were shaken for 15 s, and incubated for 45 min. at room temperature. Results were interpreted as the reciprocal of the last well that had complete inhibition of hemagglutinating activity.

Determination of viral shedding

Oropharyngeal swab samples from chickens and ducks, and cloacal swab samples from ducks were suspended in 2 ml sterile BHI broth (Sigma-Aldrich, St. Louis, MO) containing 1X antibiotic/antimycotic (Mediatech, Herndon, VA), and frozen at -70°C until RNA extraction. Total viral RNA was extracted using Trizol or MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol (36). The procedure for RNA isolation was carried out using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA).

Quantitative real-time RT-PCR (RRT-PCR) was performed using primers and probe specific for type A avian influenza matrix gene (35), but with modifications. Two and three nucleotide changes were detected between the DK/VN/203/05 and CK/VN/209/05 matrix genes and the reverse primer created by Spackman *et al.* (2002) so new primers were designed specific for these changes. The primer sequences are as follows: DK/VN/203/05 MA -124: 5' TGCAAAGACATCTTCAAGTTTCTG 3' and CK/VN/209/05 MA -124: 5' TGCAAAGACATCCTCAAGTTTCTG 3'. Qiagen (Valencia, CA) OneStep RT-PCR Kit was used under the following conditions: 1X

buffer, 3.75 mM $MgCl_2$, 10 pmol each primer, 320 µM each dNTP, 0.12 µM probe, and 13 units Rnase Inhibitor (Promega, Madison, WI). Eight µl of the RNA sample (mentioned above) and nuclease-free water were added to make a final volume of 25 µl. The reverse transcription reaction consisted of one cycle of 30 min. at 50°C, followed by 15 min. at 95°C. Forty-five cycles of 1s denaturation at 94°C, followed by annealing for 20s at 60°C were carried out in the PCR reaction. Both reactions were carried out in a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine. The EID_{50}s of virus from the swab samples were extrapolated from the cycle thresholds by using standard curves generated from the known amounts of RNA of the challenge viruses used (16).

Statistical Analysis

Hemagglutination inhibition and swab sample data were analyzed using Prism v5 Software package (GraphPad Software Inc., San Diego, CA). One-way ANOVA with Tukey's post hoc test was used to analyze log2 HI titers and viral shedding data. Results with *P* values < 0.05 were considered to be statistically significant. Detection limits of individual RRT-PCR reactions were calculated from the standard curve, setting the cycle threshold (C_t) value equal to the number of cycles run (36). Samples that were RRT-PCR-negative in this study were assigned titer values equal to the detection limit of the RRT-PCR run minus $10^{0.1}$ EID_{50}/ml, as previously described (36).

RESULTS

Vaccine efficacy experiments

Three commercially available vaccines and two experimental vaccines containing viruses homologous to the challenge strains were used. Two of the commercial vaccines (N28 and Re-1) are of the Asian lineage of influenza and have sequence similarities to the challenge viruses ranging from 87.1% to 92.5% (Table 4.1). The third virus is of the North American Lineage and is approximately 84% similar to the challenge strains (Table 4.1).

DK/VN/203/05 challenge in chickens

Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or the homologous vaccine and challenged three weeks later with DK/VN/203, a clade 2.3.2 virus. Results are shown in Table 4.2A. All negative control birds died by day two post-challenge. Five of eight birds vaccinated with N28 vaccine displayed signs of mild sinusitis, conjunctivitis, and were less active by 2 DPC, but all birds recovered. No clinical signs were noted in any vaccinates in the Re-1, Mexican or homologous vaccine groups.

Total viral RNA was isolated from oropharyngeal swab samples and quantitative RRT-PCR was performed to compare levels of virus shed by birds from the different vaccine groups both two and four days following challenge. At 2 DPC, virus shedding from negative control birds was significantly higher than that from all vaccinated birds.

At 4 DPC, no significantly different levels of virus were shed between any of the vaccine groups.

Hemagglutination inhibition titers were evaluated using both homologous and heterologous Ag. At two weeks post vaccination, all vaccinated birds had HI titers (Table 4.3A). The Mexican vaccine induced the highest HI titers, which averaged to be 70 at two weeks post-vaccination, when homologous Ag was used (Table 4.3A). Correspondingly, 100% of the birds in these groups did not show any clinical signs (Table 4.2A). The Re-1 vaccine group had the second-highest titers, with a geometric mean titer of 58 two weeks post vaccination with homologous Ag (Table 4.3A). The DK/VN/203 and N28 vaccine groups' HI titers were close to 30 at two weeks post vaccination when homologous Ag was used (Table 4.3A). An HI titer of 40 is considered to provide consistent and reproducible protection after virulent challenge, but lower titers may also be protective. This was demonstrated in DK/VN/203 and the N28 vaccine groups, where most birds had no or only mild clinical disease (Table 4.2A). At 10 D PC, HI titers in all surviving birds were at least 40, regardless if the Ag was homologous or not (Table 4.3A). As seen with the two-week post vaccination sera, HI titers were highest with homologous Ag. Even though the group vaccinated with the Ag that was homologous to the challenge strain did not have the highest HI titers, all of these birds were protected from disease and death (Table 4.2A).

CK/VN/209/05 challenge in chickens

Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or with the homologous vaccine and challenged three weeks later

with CK/VN/209, a clade 2.3.4 virus. Results are shown in Table 4.2B. Similar to the DK/VN/203 challenge group, all control birds died by 2 DPC. Five out of 10 birds in the N28 vaccine group displayed signs of depression and conjunctivitis. At 5 DPC, one bird had died and one, which had displayed severe respiratory signs, was euthanized. In the Re-1 vaccine group, two birds died at 7 DPC. Two birds in the homologous vaccine group also died 2 and 3 DPC. None of the deaths from the latter three groups was statistically significant and all but one of the birds had HI titers below the protective level of 40, two weeks following vaccination. All birds in the Mexican vaccine group were active and eating normally.

Two days following challenge, all vaccinated birds shed significantly less virus than negative control birds (Table 4.2B). At four dpch, birds that were vaccinated with the N28 or Re-1 vaccine shed significantly higher levels of virus than birds that had received the homologous vaccine (Table 4.2B). There was no correlation between oropharyngeal virus shedding and survival of the birds; not all birds that died were shedding detectable levels of virus, based on RRT-PCR. However, several birds that did shed detectable levels of virus, based on RRT-PCR, survived.

All vaccinated birds seroconverted to AIV at 2 weeks following vaccination (Table 4.3B). The Mexican vaccine induced the highest HI titers (geometric mean titer: 108) and the Re-1 vaccine induced the second-highest titers (geometric mean titer: 45) two weeks post-vaccination, when homologous Ag was used (Table 4.3B). Two weeks following vaccination, the group vaccinated with the homologous vaccine had HI titers near 30. However, only one of these birds displayed clinical signs (Table 4.2B). Birds that had been vaccinated with N28 had significantly lower HI titers than birds vaccinated

with the Mexican vaccine, when homologous antigen was used (Table 4.3B). Similar to the DK/VN/203 challenge study, all surviving birds had HI titers greater than 40 following challenge (Table 4.3B). At 4 DPC, birds in the Re-1 vaccine group had significantly higher HI titers than those in the homologous vaccine group (Table 4.3B). As mentioned, all but one of the six vaccinated birds that died following challenge had HI titers less than 40, when homologous Ag was used.

DK/VN/203/05 challenge in ducks

One-week-old Pekin ducks were vaccinated once with one of the three commercial vaccines and challenged two weeks later with DK/VN/203. Results are shown in Table 4A. All control birds died at four DPC. One duck vaccinated with Re-1 presented neurological signs such as head tilting and incoordination, but survived.

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

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

groups when homologous antigen was used on serum collected 11 DPC (Table 5A). However, ducks vaccinated with the Mexican vaccine had significantly higher HI titers than those vaccinated with N28 when heterologous Ag was used (Table 5A).

DK/VN/218/05 challenge in ducks

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

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

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

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

DISCUSSION

When used as part of an effective control strategy against AI, vaccination should not only prevent clinical signs and illness, but also significantly reduce the amount of viral shedding that could be a source of infection for other birds (15, 42). The Asian lineage of H5N1 AI first caused disease outbreaks in poultry in Vietnam at the start of 2004 (56), and it spread widely across the country. In 2005, a campaign to vaccinate 220 million domestic fowl was launched in an attempt to control the outbreak (http://www.globalsecurity.org/security/library/news/2009/02/sec-090211-irin01.htm) (24) and it appeared to be a success over the next year by greatly reducing the number of reported outbreaks and human infections in Vietnam (57). During the latter part of 2006, H5N1 viruses were reported in unvaccinated, asymptomatic ducks, upon routine surveillance (57). Even though vaccination is still being used in Vietnam, poultry outbreaks as well as human cases continue to be reported (57). The factor of low immunity rate in poultry, because of the difficulty to vaccinate and booster poultry populations, is thought to contribute to the resurgence of the disease. In addition, the viruses in Vietnam continue to change both by antigenic drift as well as new variants being introduced from other countries in the region. One of the primary goals of this study was to evaluate if the change of the lineage of virus circulating in northern Vietnam, from clade 1 to clade 2, contributed to the increase of poultry outbreaks in spite

of the continued efforts at vaccination. For this, we evaluated the level of protection obtained with three widely available commercial vaccines in chickens and ducks after challenge with viruses representative of strains circulating in Vietnam.

While H5N1 HPAI viruses display virulent phenotypes in chickens, ducks may become infected and shed virus without presenting any signs of illness (1, 6, 14, 25-27, 31). Therefore, ducks have been linked to transmitting AI to poultry (32) by 'silently' spreading virus, contributing to its circulation and further propagation among poultry. As a result, the HPAI H5N1 viruses continue to threaten both human and veterinary/poultry health. On the other hand, some HPAI viruses circulating in Vietnam have shown to produce high mortality in domestic ducks (28), directly affecting this important segment of this country's poultry industry. The duck raising practices in Vietnam include the production of free-range ducks, which because of the low biosecurity inherent with this production practice, poses a high risk of spread and maintenance of H5N1 in the country (20). Consequently, outbreaks continue to occur in unvaccinated ducks (8, 57). If efficacious vaccines could be given to ducks in this production system, it could significantly improve the control of AI.

Because of AI's tendency to antigenically drift, AI vaccines should be tested periodically to ensure sufficient protection from clinical disease and virus shedding (30, 42). The challenge viruses for this study were not only highly pathogenic in chickens, but unusually virulent for ducks, as well (28). Specifically for ducks, an increase in tissue tropism, lesion severity, viral replication, and one of the shortest mean death times reported in both two- and five-week-old Peking ducks was observed with these viruses, as compared to previous H5N1 viruses tested in ducks (28). The level of protection

rendered by the vaccines was influenced by the virus that served as the challenge strain. Though both of the groups of viruses were extremely virulent in chickens and ducks, the clade 2.3.4 viruses (CK/VN/209 and DK/VN/218) appeared more pathogenic than the clade 2.3.2 (DK/VN/203) virus, and caused higher morbidity and virus shedding in ducks.

Hemagglutination inhibition titers are commonly used to predict levels of protection against viral infection and disease in vaccinated birds. In chickens, the Mexican vaccine surpassed the others in its ability to induce high levels of HI antibodies (Tables 4.3A, 4.3B) and to protect the birds from clinical disease (Tables 4.2A, 4.2B). This is quite interesting because, compared to the other vaccine virus HA sequences, it has the lowest sequence similarity with the challenge viruses. Similar results were seen by Swayne *et al.* (2006) and Veits *et al.* (2008), when testing the efficacy of H5N2 vaccines to protect chickens against HPAI viruses that were less than ideally matched to the vaccine viruses (45, 51). Such remarkable immunogenicity could possibly be attributed to the proprietary adjuvant used in the formulation of the Mexican vaccine, the antigen mass used in the vaccine, or the inherent antigenicity of the hemagglutinin protein itself (44). With regards to shedding, chickens vaccinated with the CK/VN/209/05 vaccine (and challenged with the same virus) shed significantly lower amounts of virus from the respiratory tract, compared to the N28 vaccine, which was a low pathogenic turkey virus of Eurasian lineage isolated in 1973. In addition, several of the birds vaccinated with this older virus also displayed clinical signs. Another study tested the Re-1 vaccine in chickens and demonstrated, as did we, that all of the vaccinated birds were completely protected from disease and death, upon challenge with

either homologous virus or heterologous viruses from 2004 (47). There was also virus detected in oropharyngeal swab samples from some of the vaccinated birds (47), similar to our findings.

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

In a previous duck study, a two-dose vaccination program starting in ducks at one day of age, followed by a booster at four weeks of age, was used because of its compatibility with the duck husbandry practices in Asia, and was shown to be effective (2). It is important to vaccinate ducks at an early age to try to provide immunity as early as possible, but also because after the ducks are released into the fields, it becomes much more difficult to vaccinate them thereafter. We chose to use one-week-old ducks on a single-dose regimen to see if vaccinated at this age, they would obtain good protection and a reduction in virus shedding after challenge. We also chose to use one-week-old ducks because with increased vaccination of poultry in Vietnam, day-old birds may have maternal antibodies that could interfere with vaccine efficacy, and one-week vaccination

is potentially a suitable compromise with ease of vaccination and less interference by maternal antibody.

Other duck studies involving various vaccination regimens followed by challenge have also demonstrated clinical protection and reduced virus shedding (11, 21, 37, 38, 50). However, these results were obtained, as ours, in laboratory settings. It is important to keep this in mind when applying experimental vaccine data to the field. Experimental data can not be directly extrapolated to the field setting because of differences in circumstances between the two. Unlike poultry raised in the field, the experimental animals do not have pre-existing immunity to AI from maternal antibodies or prior AI infection and their immune systems are not compromised by other unrelated pathogens, which may be concomitantly circulating among flocks in a field setting. In any case, vaccination is not likely to prevent infection and provide sterilizing immunity. In a previous study, prevention of tracheal and cloacal shedding was achieved when a large dose (1 μg) of antigen was administered (12). Unfortunately, the large quantities of antigen or adjuvant required to induce such a potent immune response may be greater than could be realistically administered in the field. With an appropriate vaccination program, however, shedding of infectious virus into the environment could be reduced to a minimum and consequently prevent transmission.

Kim *et al.* (2008) tested vaccines that contained the HAs of either clade 1, clade 2.2, or clade 2.3.4 viruses, in their abilities to protect SPF white Pekin ducks from an extremely virulent H5N1 virus, Duck/Laos/25/06. Despite low or undetectable HI titers, all of the challenged, vaccinated birds were completely protected from morbidity and mortality after one vaccination (12). Regardless of the time point, the HI titers of

vaccinated ducks in our study were much lower than those of chickens (Tables 4.3A, 4.3B, 4.5A, and 4.5B). The results of their study support our findings that even if the humoral immune response to the vaccine viruses is not always detectable in ducks, the immune response may still be protective.

At 10 and 11 days post challenge, HI titers in all surviving birds, chickens and ducks alike, were at least 40, when homologous Ag was used (Table 4.3A, 4.3B, 4.5A, 4.5B). However, compared to titers produced when heterologous Ag was used, the homologous HI titers were between four- and 16-fold higher. This suggests that following challenge, the antibodies produced were not only against the challenge strain, but were the result of a memory response against the vaccine virus, as well.

Based on our results, the current vaccines provided both chickens and ducks protection from disease, and reduced viral shedding, upon challenge with either of two different isolated clade 2 H5N1 highly pathogenic AI viruses from Vietnam. Though most birds vaccinated with the N28 vaccine did have clinical protection from virulent challenge, as compared to the unvaccinated birds, the HI titers pre-challenge were the lowest of the vaccines tested and the reduction of viral shedding was marginal. This vaccine would seem to be a poor option for a vaccine program, although the reasons for a poor response from this antigen were not fully investigated. The adjuvant used was assumed to be similar to the Re-1 vaccine because it was made by the same manufacturer, but differences in antigen mass, antigenicity of the hemagglutinin, or antigenic differences based both on HI data and sequence similarity may have all contributed to the poorer results. This vaccine would not be recommended for further use. The Re-1 and the Mexican lineage vaccine still appear to provide good protection from challenge, but

antigenic variability based on HI data and sequence similarity, raises concern that vaccines made with these viruses will lose protectiveness as the field viruses continue to drift. The need to update vaccine seed strains is critical if optimal protection from vaccination is to be realized. However, good surveillance is needed to understand what viruses are circulating in a region or country. For northern Vietnam in December 2005, both clade 2.3.2 and 2.3.4 viruses were co-circulating in the region, and therefore a single vaccine is unlikely to provide optimal protection. Availability of vaccines currently is problematic, not only because of antigenic drift, but also due to differential immune responses to vaccines in chickens as well as in individual duck species (Pantin-Jackwood and Suarez, unpublished data). It will be useful to continue evaluating the current vaccines not only in chickens, but various species of ducks, also.

ACKNOWLEDGMENTS

We would like to thank Suzanne DeBlois, Aniko Zsak, and Diane Smith for excellent technical support and also Ronald Graham and Roger Brock for providing care to the animals. The authors also wish to thank Colleen Thomas for assistance with statistical analysis.

Table 4.1. Sequence similarity[A] between commercial vaccines and challenge virus HA1 proteins

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

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

Table 4.2A. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 weeks of age and intranasally challenged at 5 weeks of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus

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

[A]MDT, mean death time denoted in days

[B] Swab samples were taken from all birds remaining at each time point post challenge. O/P, oropharyngeal; NA= not applicable

[C]Log EID_{50} was determined using real-time RT-PCR specific for type A avian influenza matrix gene (35). Numbers in parentheses are averages of viral titers shed from birds in each group.

Different lowercase superscripts denote significance between treatment groups ($p<0.05$) as determined by one-way ANOVA.

Table 4.2B. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 weeks of age and intranasally challenged at 5 weeks of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus

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

[A]MDT, mean death time denoted in days

[B] Swab samples were taken from all birds remaining at each time point post challenge. O/P, oropharyngeal; NA= not applicable

[C]Log EID_{50} was determined using real-time RT-PCR specific for type A avian influenza matrix gene (35). Numbers in parentheses are averages of viral titers shed from birds in each group.

Different lowercase superscripts denote significance between treatment groups ($p<0.05$) as determined by one-way ANOVA.

Table 4.3A. Hemagglutination inhibition (HI) titers of chickens vaccinated at two weeks of age and challenged intranasally at five weeks of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus

	Range of pre-challenge HI titers[A]		Range of post challenge HI titers[B]	
Vaccine group	Homologous Ag[C]	DK/VN/203/05 Ag	Homologous Ag[C]	DK/VN/203/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	16-64 (30)[b]	4-16 (10)[bc]	32-1024 (304)[a]	32-512 (152)[a]
Re-1	32-128 (58)[bc]	4-32 (14)[bc]	128-2048 (832)[a]	32-1024 (165)[a]
Mexican	32-128 (70)[c]	0-16[1] (7)[b]	256-2048 (1323)[a]	64-1024 (278)[ab]
DK/VN/203/05	8-64 (27)[b]	8-64 (27)[c]	512-2048 (776)[a]	512-2048 (776)[b]

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

Table 4.3B. Hemagglutination inhibition (HI) titers of chickens vaccinated at two weeks of age and challenged intranasally at five weeks of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus

	Range of pre-challenge HI titers[A]		Range of post challenge HI titers[B]	
Vaccine group	Homologous Ag[C]	CK/VN/209/05 Ag	Homologous Ag[C]	CK/VN/209/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	8-128 (24)[b]	0-32[1] (13)[b]	128-2048 (861)[ab]	128-256 (194)[a]
Re-1	2-256 (45)[bc]	4-64 (18)[b]	512-4096 (1722)[a]	64-512 (181)[a]
Mexican	32-512 (108)[c]	4-32 (13)[b]	512-4096 (891)[ab]	64-1024 (194)[a]
CK/VN/209/05	4-128 (35)[bc]	4-128 (35)[b]	256-512 (362)[b]	256-512 (362)[a]

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

Table 4.4A. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 week of age and intranasally challenged at 3 weeks of age with 10^5 EID_{50} of DK/VN/203/05 H5N1 HPAI virus

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

[A]Log EID_{50} was determined using real-time RT-PCR specific for type A avian influenza matrix gene (35).
[B] MDT, mean death time denoted in days.

[C]O/P, oropharyngeal

[D] number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

Table 4.4B. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 week of age and intranasally challenged at 3 weeks of age with 10^5 EID_{50} of DK/VN/218/05 H5N1 HPAI virus

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

[A]Log EID_{50} was determined using real-time RT-PCR specific for type A avian influenza matrix gene (35).

[B] MDT, mean death time denoted in days.

[C]O/P, oropharyngeal

[D] number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

Table 4.5A. Hemagglutination inhibition (HI) titers of ducks vaccinated at one week of age and challenged intranasally at three weeks of age with 10^5 EID_{50} of DK/VN/203/05 H5N1 HPAI virus

	Range of pre-challenge HI titer[A]		Range of post challenge HI titer[B]	
Vaccine group	Homologous Ag[C]	DK/VN203/05 Ag	Homologous Ag[C]	DK/VN/203/05 Ag
Negative control	0[a]	0	N/A	N/A
N28	16-32 (17)[b]	0	64-256 (166)[a]	0-16[1] (3)[a]
Re-1	32-128 (42)[c]	0	512-1024 (446)[a]	0-32[2] (7)[ab]
Mexican	16-128 (23)[b]	0	128-1024 (276)[a]	8-64 (16)[b]

[A]Serum samples were taken two weeks post vaccination. In parentheses: geometric mean of HI titers.
[B]Serum samples were collected 11 days post infection.
[C]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
Different lower case superscript letters denote significance between groups ($p<0.05$) as determined by one-way ANOVA.
[1]Four out of nine birds did not have detectable levels of HI antibodies against this antigen.
[2]Two out of eight birds did not have detectable levels of HI antibodies against this antigen.
NA= not applicable

Table 4.5B. Hemagglutination inhibition (HI) titers of ducks vaccinated at one week of age and challenged intranasally at three weeks of age with 10^5 EID_{50} of DK/VN/218/05 H5N1 HPAI virus

	Range of pre-challenge HI titer[A]		Range of post challenge HI titer[B]	
Vaccine group	Homologous Ag[C]	DK/VN/218/05 Ag	Homologous Ag[C]	DK/VN/218/05 Ag
Negative control	0[a]	0	N/A	N/A
N28	16-32 (20)[b]	0	256-1024 (380)[a]	8-32 (17)[ab]
Re-1	16-128 (34)[b]	0	128-1024 (474)[a]	0-32[1] (7)[a]
Mexican	16-256 (26)[b]	0	128-1024 (406)[a]	16-128 (64)[b]

[A]Serum samples were taken two weeks post vaccination. Titers are expressed as geometric mean titers
[B]Serum samples were collected 11 days post infection.
[C]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
Different lower case superscript letters denote significance between groups ($p<0.05$) as determined by one-way ANOVA.
[1]Three out of nine birds did not have detectable levels of HI antibodies against this antigen.

NA= not applicable

REFERENCES

1. 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.

2. Beato, M. S., A. Toffan, R. DeNardi, 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 doi:10.1016/j.vaccine.2007.02.042. 2007.

3. Bragstad, K., P. H. Jorgensen, K. J. Handberg, S. Mellergaard, S. Corbet, and A. Fomsgaard. New avian influenza A virus subtype combination H5N7 identified in Danish mallard ducks. Virus Res. 109:181-190. 2005.

4. Cauthen, A. N., D. E. Swayne, S. Schultz-Cherry, M. L. Perdue, and D. L. Suarez. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J. Virol. 74:6592-6599. 2000.

5. Chutinimitkul, S., T. Songserm, A. Amonsin, S. Payungporn, K. Suwannakam, S. Damrongwatanapokin, A. Chaisingh, B. Nuansrichay, T. Chieochansin, A. Theamboonlers, and Y. Poovorawan. New strain of influenza A virus (H5N1), Thailand. Emerg. Infect. Dis. 13:506-507. 2007.

6. Cooley, A. J. H. V. C., M. S. Philpott, V. C. Easterday, and V. S. Hinshaw. Pathological lesions in the lungs of ducks with influenza A viruses. Vet. Path. 26:1-5. 1989.

7. Cristalli, A., and I. Capua. Practical problems in controlling H5N1 high pathogenicity avian influenza at village level in Vietnam and introduction of biosecurity measures. Avian Dis. 51:461-462. 2007.

8. FAO. Summary of highly pathogenic avian influenza (HPAI) situation in Viet Nam.1-6. 2008.

9. Fear, C. J. Fish farming and the risk of spread of avian influenza. Bird Life International. 2006.

10. Flint, S. J., Enquist, L. W., Krug, R. M., Racaniello, V. R., and Skalka, A. M. Principles of virology, molecular biology, pathogenesis, and control. ASM Press, Washington D.C. 2000.

11. Isoda, N., Sakoda, Y., Kishida, N., Soda, K., Sakabe, S., Sakamoto, R., Imamura, T., Sakaguchi, M., Sasaki, T., Kokumai, N., Ohgitani, T., Saijo, K., Sawata, A., Hagiwara, H., Lin, Z., and Kida, H. Potency of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 reassortment virus generated between isolates from migratory ducks in Asia. Archives of virology 153:1685-1692. 2008.

12. Kim, J. K., Seiler, P., Forrest, H. L., Khalenkov, A. M., Franks, J., Kumar, M., Karesh, W. B., Gilbert, M., Sodnomdarjaa, R., Douangngeun, B., Govorkova, E. A., and Webster, R. G. Pathogenicity and vaccine efficacy of dfferent clades of Asian H5N1 avian influenza A viruses in domestic ducks. J Virol doi:10.1128/JVI.01176-08. 2008.

13. Kung, N. Y., R. S. Morris, N. R. Perkins, L. D. Sims, T. M. Ellis, L. Gissett, M. Chow, K. F. Shortridge, Y. Guan, and M. J. S. Peiris. Risk for infection with highly pathogenic influenza A virus (H5N1) in Chickens, Hong Kong, 2002. Emerg. Infect. Dis. 13:412-418. 2007.

14. Laudert, E. A., V. Sivanandan, and D. A. Halvorson. Effect of intravenous inoculation of avian influenza virus on reproduction and growth in mallard ducks. J. Wildl. Dis. 29:523-526. 1993.

15. Lee, C. W., and D. L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Animal Health Research Reviews 6:1-15. 2005a.

16. Lee, C. W., and D. L. Suarez. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype of avian influenza virus. J. Virol. Methods 119:151-158. 2004.

17. Lee, C. W., D. A. Senne, J. A. Linares, P. R. Woolcock, D. E. Stallknecht, E. Spackman, D. E. Swayne, and D. L. Suarez. Characterization of recent H5 subtype avian influenza viruses from US poultry. Avian Pathol. 33:288-297. 2004.

18. Lee, C. W., Senne, D., and Suarez, D. L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78:8372-8381. 2004.

19. 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.

20. Mabbett, T. Vietnam under H5N1 attack on all sides. In. 2008.

21. Middleton, D., J. Bingham, P. W. 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.

22. Nguyen, D. C., T. M. Uyeki, S. Jadhao, T. Maines, M. Shaw, Y. Matsuoka, C. Smith, T. Rowe, X. Lu, H. Hall, X. Xu, A. Balish, A. Klimov, T. M. Tumpey, D. E. Swayne, L. P. Huynh, H. K. Nghiem, H. H. Nguyen, L. T. Hoang, N. J. Cox, and J. M. Katz. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J. Virol. 79:4201-4212. 2005.

23. Nguyen, T. D., T. V. Nguyen, D. Vijaykrishna, R. G. Webster, Y. Guan, J. S. M. Peiris, and G. J. D. Smith. Multiple sublineages of influenza A virus (H5N1), Vietnam, 2005-2007. Emerg. Infect. Dis. 14:632-636. 2008.

24. Normile, D. Vietnam battles bird flu...and critics. Science 309:368-373. 2005.

25. Pantin-Jackwood, M., 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. 2007b.

26. 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. 2007a.

27. 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.

28. Pfeiffer, J., Pantin-Jackwood, M., To, T. L., Nguyen, T., and Suarez, D. L. Phylogenetic and Biological Characterization of Highly Pathogenic H5N1 Avian Influenza Viruses (Vietnam 2005) in Chickens and Ducks. Virus Research in press. 2009.

29. Qiao, C., Tian, G., Jinag, Y., Li, Y., Shi, J., Yu, K., and Chen, H. Vaccines developed for H5 highly pathogenic avian influenza in China. Ann. N. Y. Acad. Sci. 1081:182-192. 2006.

30. Romer-Oberdorfer, A., J. Veits, D. Helferich, and T. C. Mettenleiter. Level of protection of chickens against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live attenuated vector vaccine depends on homology of H5 sequence between vaccine and challenge virus. Vaccine doi.:10.1016/j.vaccine.2008.02.061. 2008.

31. 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.

32. Sims, L. D. Lessons learned from Asian H5N1 outbreak control. Avian Dis. 50:174-181. 2007.

33. Smith, G. J., X. H. Fan, J. Wang, K. S. Li, K. Qin, J. X. Zhang, D. Vijaykrishna, C. L. Cheung, K. Huang, J. M. Rayner, J. S. Peiris, H. Chen, R. G. Webster, and Y. Guan. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U S A 103:16936-16941. 2006.

34. Socialist Republic of Vietnam, and Vietnam integrated national plan for avian influenza control and human pandemic influenza preparedness and response. In. pp 1-19. 2006.

35. 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. Journal of clinical microbiology 40:3256-3260. 2002.

36. Steel, J., S. V. Burmakina, C. Thomas, E. Spackman, A. Garcia-Sastre, D. E. Swayne, and P. Palese. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26:522-531. 2008.

37. 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.

38. Steensels, M., Bublot, M., Van Borm, S., De Vriese, J., Lambrecht, B., Richard-Mazet, A., Chanavat-Bizzini, S., Duboeuf, M., Le Gros, F.-X., and van den Berg, T. 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.

39. Stone, H., B. Mitchell, and M. Brugh. *In ovo* vaccination of chicken embryos with experimental Newcastle disease and avian influenza oil-emulsion vaccines. Avian Dis. 41:856-863. 1997.

40. Stone, H. D., Brugh, M., Hopkins, S. R., Yoder, H. W., and Beard, C. W. Preparation of inactivated oil-emulsion vaccines with avian viral or Mycoplasma antigens. Avian Dis. 22:666-674. 1978.

41. Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne. Comparison of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J. Virol. 72:6678-6688. 1998.

42. 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.

43. Swayne, D. E., D. A. Senne, and C. W. Beard Avian Influenza. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. J. R. Glisson, Swayne, D. E., Jackwood, M. W., Pearson, J. E., and Reed, W. M., ed. American Association of Avian Pathologists, Kennet Square, PA. pp 150-155. 1998.

44. Swayne, D. E., J. R. Beck, M. Garcia, and H. D. Stone. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Path. 28:245-255. 1999.

45. Swayne, D. E., C. W. Lee, and E. Spackman. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Path. 35:141-146. 2006.

46. Swayne, D. E., M. L. Perdue, J. R. Beck, M. Garcia, and D. L. Suarez. Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years. Veterinary Microbiology 74:165-172. 2000.

47. Tian, G. B., S. H. Zhang, and Y. B. Li. Protective efficacy in chickens, geese, and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology 341:153-162. 2005.

48. Tumpey, T. M., D. L. Suarez, L. E. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. J. Virol. 76:6344-6355. 2002.

49. van den Berg, T., B. Lambrecht, S. Marche, M. Steensels, S. Van Borm, and M. Bublot. Influenza vaccines and vaccination strategies in birds. Comp. Immunol. Microbiol. Infect. Dis. 2007.

50. van der Goot, J. A., van Boven, M., Stegeman, A., van de Water, S. G. P, de Jong, M. C. M., and Koch, G. Transmission of highly pathogenic avian influenza H5N virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. Virol doi: 10.1016/j.virol.2008.08.037. 2008.

51. Veits, J., A. Romer-Oberdorfer, D. Helferich, M. Durban, Y. Suezer, G. Sutter, and T. C. Mettenleiter. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine 26:1688-1696. 2008.

52. Wan, X. F., Nguyen, T., Davis, C. T., Smith, C. B., Zhao, Z. M., Carrel, M., Inui, K., Hoa, T. D., Mai, D. T., Jadhao, S., Balish, A., Shu, B., Luo, F., Emch, M., Matsuoka, Y., Sindstrom, S. E., Cox, N. J., Nguyen, C. V., Klimov, A., and Donis, R. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. PLoS ONE 3:e3462. 2008.

53. 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.

54. Webster, R. G., Y. Guan, M. Peiris, D. Walker, S. Krauss, N. N. Zhou, E. A. Govorkova, T. M. Ellis, K. C. Dyrting, T. Sit, D. R. Perez, and K. F. Shortridge. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J. Virol. 76:118-126. 2002.

55. Webster, R. G., and E. A. Govorkova. H5N1 influenza--continuing evolution and spread. N. Engl. J. Med. 355:2174-2177. 2006.

56. WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines. 2007.

57. WHO. H5N1 avian influenza: Timeline of major events. 2008.

58. WHO Evolution of H5N1 avian influenza viruses in Asia. Emerg. Infect. Dis. . In. Global Influenza Program Surveillance Network. pp 1515-1521. . 2005.

59. Wright, P. F., G. Neumann, and Y. Kawaoka Orthomyxoviruses. In: Fields Virology. D. M. Knipe, and P. M. Howley, ed. Lippincott Williams & Wilkins. pp 1691-1740. 2006.

60. Xu, X., K. Subbarao, N. J. Cox, and Y. Guo. 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 261:15-19. 1999.

CHAPTER 5

ANTIGENIC ANALYSIS OF AMINO ACID POINT MUTATIONS IN HEMAGGLUTININ PROTEINS OF AVIAN INFLUENZA ISOLATES FROM MEXICO[1]

[1]J. Pfeiffer, C. W. Lee, S. J. Jadhao, and D. L. Suarez. To be submitted to Virology.

ABSTRACT

Avian influenza (AI) viruses in poultry undergo rapid antigenic drift that is important in virus shedding in vaccinated birds, but the antigenic drift does not play as critical a role in clinical protection in poultry as is seen with human influenza. This difference is likely from the differences in pathogenesis of the virus in the different species. In Mexico, where vaccination of poultry has been employed since 1995, the circulating low pathogenic (LP) H5N2 viruses continue to drift, with a higher proportion of the amino acid changes occurring at the putative antigenic sites of the hemagglutinin (HA) protein. To determine which of these amino acids influenced antigenicity, we used site-directed mutagenesis to introduce mutations found in the HAs of drift variants into the HA of an early Mexican strain that is used as a vaccine seed strain to make eukaryotic expression plasmids and mutant viruses by reverse genetics. We used the hemagglutination inhibition (HI) test, virus neutralization in embryonating chicken eggs, and performed a challenge study on chickens, to evaluate the influence that these particular residues have on the ability of these viruses to evade the host's antibody response. Smaller than expected differences in antigenic diversity were seen with the targeted amino acids in HI and virus neutralization assays. In general, vaccinated birds shed significantly less virus from the oropharynx than unvaccinated birds, but little difference was seen between the different seed strains used in the vaccinated birds. The specific amino acids important for antigenic differences were partially determined, but additional changes appear to also influence antigenicity.

INTRODUCTION

Over the past decade, vaccination of poultry against avian influenza (AI) has become commonly used as part of a control strategy in multiple countries around the world (30). Despite

the presumption made nearly two decades ago, that vaccines may contribute to the maintenance of viruses and continuation of the disease problem in poultry by producing antigenic variants (8), this conjecture has only recently been scientifically documented in poultry (16). There is now evidence that these viruses, when faced with vaccine pressure, undergo antigenic drift away from the vaccine virus with loss of protection, in order to evade the immune responses of the vaccinated hosts (16).

In 1994, an outbreak of a low pathogenic (LP) H5N2 avian influenza began in Mexico (9, 16). The low pathogenic virus mutated to the highly pathogenic form of the virus between 1994 and 1995(32), and the highly pathogenic viruses were eradicated, in part because of a vaccine strategy that was employed. However, the low pathogenic viruses were not eradicated and continue to circulate among chickens in Mexico and eventually spread to El Salvador and Guatemala (24). A vaccination program that was implemented in 1995 continues to be used (24).

Unlike flu viruses that circulate among humans, avian influenza viruses were thought to remain antigenically stable (34). However, Lee *et al.*(2004) analyzed viral strains isolated between 1994 and 2002 in Mexico and other neighboring regions, including Guatemala, and found that this is not necessarily true. It was shown that over a long period of time, in a region where the outbreak had persisted and vaccination was widely used, antigenic drift had occurred (16). The antigenic drift cannot be completely tied to vaccination pressure, because host antibody to infection and the likelihood of reinfection also likely shaped the viral response. The result of the antigenic drift is large differences in hemagglutination inhibition (HI) titers between vaccine viruses and antibodies against more recent isolates such as CK/Guatemala/194573/02 (CK/Guat). Multiple amino acid changes were detected in the HA1 protein regions of these

isolates, some of which occurred at the proposed antigenic sites. Antigenic analysis through cross-HI further supported this finding when over 16-fold differences in HI titers were seen between the vaccine and recent field strains (16). This is worth noting because with human influenza viruses, if serum against the current vaccine has a four-fold drop in HI titer to an emerging influenza strain, the new strain may be considered for inclusion in the upcoming year's vaccine (13). In a vaccine study on chickens, those that were vaccinated with the initial seed strain from 1994 and then challenged with more recent virus strains, from either 1998 or 2002, shed amounts of virus comparable to that shed from unvaccinated birds, as compared to those challenged with homologous virus (16).

In 2004, an H5N2 virus was isolated from a pet parrot in California. The HA of this virus clustered with the Mexican lineage of viruses, indicating that the parrot was most likely smuggled from Mexico (7). The HA gene of this isolate was even more genetically distant from the vaccine virus strain than was the HA gene of the CK/Guat isolate. Some of the differences between the CK/Guat and the parrot isolates were located at the proposed antigenic sites. These changes in the parrot isolate indicate that the virus had since 2002, continued to drift from the vaccine seed strain virus. Further support was found in a study by Escorcia *et al.* (2008), which phylogenetically and antigenically analyzed 18 viruses isolated from poultry in Mexico between 2002 and 2006. Findings similar to those mentioned above were detected; the more recent viruses demonstrated continued drift from both the vaccine seed virus and viruses isolated in 2002 (5). This antigenic drift is exhibited by the fact that vaccinated chickens in the field are infected and develop clinical signs of low pathogenic avian influenza (5).

As mentioned, mutations acquired by circulating viruses may allow for the viruses to evade antibodies induced in the host by vaccination. As a result, the circulating virus may be better able to replicate and more virus is likely to be shed by infected birds, ultimately spreading from infected flocks to uninfected, neighboring flocks. One of the criteria for validating a virus as a 'good' vaccine strain is its ability to prevent death upon virulent challenge (5). However, with routine use of vaccination, it is becoming important to ensure that a vaccine virus also reduces virus shed in order to fully achieve eradication (5). For a vaccine against AI to provide optimal protection against morbidity and mortality, it should be closely matched to the circulating viruses not only genetically, but also antigenically. This would ensure that optimal protection would be induced by the vaccine, and minimal levels of virus shedding would occur (13). It has been shown that even though viruses may have high HA sequence similarity, they may not be antigenically similar (37). If one were able to pinpoint particular amino acids of the HA protein that are involved in evasion of the antibody response, vaccine seed viruses could more appropriately and more easily be chosen, based primarily on sequence information. The vaccine seed strain candidate HA sequences, particularly the amino acids involved in antibody neutralization, could be compared to those sites within the HAs of circulating viruses. The most effective vaccine seed strain virus could be selected based on which or how many amino acids are similar at the proposed antigenic sites, in comparison to the circulating field strains.

To determine the influence that the above-mentioned amino acid changes at the potential antigenic sites had on the antigenicity of the HAs, we used reverse genetics to produce reassortant viruses (rg viruses) containing selected mutations. The viruses were identical, except for their individual HA genes, which were either of the wild type (WT) virus sequence or the parental Mexican vaccine HA containing various amino acid changes, based on the sequence

data. We used the cross-HI and virus neutralization tests to antigenically compare the immune response to the different viruses. We also conducted a study in chickens to compare the levels of oropharyngeal viral shedding between groups that had been vaccinated with one of the five live rg viruses and then challenged with one of two wild type Mexican viruses containing HAs that were most genetically distant from each other. We compared levels of the serum acute phase protein (APP) α_1-acid glycoprotein (AGP) between selected vaccine groups to determine if these proteins could serve as additional indicators of the degree of protection that particular vaccine viruses can provide.

MATERIALS & METHODS

Viruses

Avian influenza virus isolates CK/Hidalgo/28159-232/94 (CK/Hidalgo), CK/Guatemala/194573/02 (CK/Guat), Parrot/CA/6032/04 (Parrot/CA), and Yellow-Headed Amazon/CA/500658-8/07 (Amazon/CA), all subtype H5N2, were obtained from the SEPRL repository or the National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, Ames, IA. All viruses were propagated in the allantoic cavities of 10-day-old embryonating chicken eggs (ECE) for three days. Allantoic fluid was harvested and titered using 10-fold serial dilutions to determine the median embryo infectious dose (EID_{50}) (22). Stocks were stored at -70°C until use. Viruses produced through reverse genetics are described below.

Cloning of HA genes

Viral RNA was extracted from infectious allantoic fluid from embryonating chicken eggs, using Trizol LS reagent (Life Technologies, Rockville, MD). Isolated RNA from the wild

type (WT) CK/Guat, and Parrot/CA viruses were used as templates for amplification of the HA open reading frame or entire HA gene, respectively, by RT-PCR using sequence-specific primers containing the *Mlu* I and *Sal* I restriction sites in the 5' and 3' primers, respectively. The PCR products were digested with *Mlu* I and *Sal* I and directionally cloned into pCI eukaryotic expression vector (ProMega, Madison, WI), which had been cut with the same enzymes. Sequence analysis ABI Big Dye Terminator version 1.1 sequencing kit (Applied Biosystems, Foster City, CA) run on 3730 XL DNA Analyzer (Applied Biosystems) sequencer was used to confirm that the correct sequences were cloned into the vector in the proper orientation. The CK/Hidalgo HA gene was previously cloned (20).

Sequencing and phylogenetic analysis

The MegAlign program (DNASTAR, Madison, WI) using the Clustal V alignment algorithm was used to compare nucleotide sequences and create the multiple sequence alignment. The method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc, Sunderland, MA) was used for phylogenetic comparisons of the aligned sequences, using bootstrap resampling method with a heuristic search algorithm.

Generation of mutated HA genes

A series of seven point mutations corresponding to amino acid changes at positions 126, 136, 137, 154, 181, 188, and 275 of the HA1 portion (Fig. 1) of CK/Guatemala/194573/02 H5 were made de novo in the vaccine strain CK/Hidalgo/232/94 HA1 gene segment (Retrogen, Inc., San Diego, CA). The DNA with the altered section of approximately 1 kb in length was cut out of vector pCRBlunt (Invitrogen, Carlsbad, CA), using restriction enzymes *Xcm* I and *Stu* I. A

portion of the CK/Hidalgo/232/94 HA gene, already in pCI, was cut out using the same enzymes. The mutated CK/Hidalgo/232/94 HA1 segment was then directionally subcloned into the digested pCI vector. Proper mutations in the gene as well as proper ligation into the plasmid were confirmed by sequence analysis. This plasmid containing the mutated HA gene was designated as Mexican Mutant 7.

Additionally, the HA gene from an even more recent low pathogenic isolate, Parrot/CA, which has high sequence homology to the Mexican Central American phylogeny, was included in this study. Four additional mutations (as compared to the CK/Guat/02 HA) also found at proposed antigenic sites in the Parrot/CA HA1 sequence were de novo synthesized into the Mexican Mutant 7 HA sequence (DNA 2.0, Menlo Park, CA). The additional residues that were mutated, 133, 185, 273, and 276 (HA1 numbering) (Fig. 5.1), generated the HA1 protein of Mexican Mutant 11. The Mexican Mutant 11 HA gene was then subcloned into pCI.

Generation of reverse genetic reassortant viruses

Reverse genetics viruses were produced using DNA transfection, as previously described (15-18, 33). Briefly, 293T cells were transfected with 1 μg of each of eight transcription plasmids and four expression plasmids, using Lipofectamine 2000 transfection reagent (Invitrogen, San Diego, CA). After 48 h incubation time, supernatant was collected and inoculated into 10- or 11-day-old embryonating chicken eggs (ECE). Allantoic fluid containing reassortant virus was harvested 48 h later, and stored at -70°C. Partial sequencing was used to confirm the identity of each viral gene segment. The M, NS, PA, and PB2 genes originated from CK/Indonesia/7/03 (H5N1), and the NA, NP, and PB1 genes came from DK/Anyang/AVL-1/01 (H5N1) virus.

Hemagglutinin-specific antibody production

One hundred μg of pCI plasmid containing the HA genes of either the CK/Hidalgo, CK/Guat, Parrot/CA, Mexican Mutant 7, or Mexican Mutant 11 were administered as DNA vaccines into three- to 11- week old specific pathogen-free (SPF) chickens a total of three or four times, at approximately three-week intervals. At the end of the immunization period, the birds were sedated by ketamine/xylazine (66 mg/ml ketamine, 6.6 mg/ml xylazine) and the hyperimmune antisera was harvested from whole blood obtained by cardiac puncture and then the birds were euthanized.

Hemagglutination inhibition test

Hemagglutination inhibition titers were determined by using the HI test (27). All viral antigens (Ag) were β-propiolactone-inactivated and were diluted in PBS to make four HA units. Fifty microliters homologous or heterologous Ag were added per well of a 96-well plate, where test sera were two-fold, serially diluted, and the plates were incubated for 15 min. at room temperature before 50 microliters of 0.5% chicken red blood cells were added to each well. Plates were mixed for 15 seconds, and then incubated for 45 min. at room temperature. Results were interpreted as the reciprocal of the last well that had complete inhibition of hemagglutinating activity. All serum samples having an HI titer of two or greater were considered positive. Virus isolates and antisera used are listed in Table 5.1. Antisera against Environmental/DE/1346/01 and Avian/NY/315388/00 were kindly provided by Dr. Erica Spackman.

Virus neutralization

Virus neutralization was performed using the beta (diluted-serum, constant virus) procedure in 10- or 11-day-old SPF ECEs. The infectious allantoic fluid containing one of the above-mentioned rg viruses was standardized to an EID_{50} of $10^{3.5}$ /0.1 ml in sterile brain heart infusion (BHI) broth containing 1X antibiotics/antimycotics (Mediatech, Herndon, VA). Virus was incubated with dilutions of hyperimmune, hemagglutinin-specific antisera, produced by DNA vaccines as described above, at 37° C for 30 min. These mixtures were then inoculated into each of three ECEs (three eggs per serum dilution) in a total volume of 0.2 ml per egg. Three days post inoculation, (dpi), allantoic fluid was examined for hemagglutinating activity to detect presence of the virus. (1)

Effects of point mutations on antigenicity and oropharyngeal viral shedding from chickens

Ninety-six two-week-old white rock chickens were divided into 12 groups of eight birds per group. Sixteen birds were inoculated intramuscularly with 10^6 EID_{50} in a total volume of 0.2ml sterile BHI of one of the five live rg viruses that had been constructed or a sham vaccine containing sterile BHI alone. Thirteen days following vaccination, all birds were bled via the wing vein. Two weeks post vaccination, eight birds from each vaccine group were challenged intranasally with 10^6 EID_{50} of either CK/Hidalgo/232/94 (H5N2) or Parrot/CA/6032/04 (H5N2) wild type viruses. At one and two days post challenge (dpch), birds from the CK/Hid rg, Parr/CA rg, or sham vaccine groups were bled via the wing vein. Three and five dpch, oropharyngeal swab samples were obtained from all birds. At 10 dpch, all birds were bled by cardiac puncture, following sedation with 0.2 ml of ketamine/xylazine (66mg/ml/6.6 mg/ml, respectively), and were then euthanized.

Real-time RT-PCR

Determination of viral shedding

Oropharyngeal swab samples from chickens were suspended in two ml sterile BHI (Sigma-Aldrich, St. Louis, MO) containing 1X antibiotic/antimycotic (Mediatech, VA), and frozen at -70°C until RNA extraction. Total viral RNA was extracted using lysis buffer and MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol (26) using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA).

Quantitative real-time RT-PCR (RRT-PCR) was performed using primers and probe specific for type A avian influenza matrix gene (25). The RRT-PCR reaction used a lyophilized bead that contained primers, probe, $MgCl_2$ and buffer. Avian Influenza Real-time RT-PCR duplex Assay (Cepheid, Sunnyvale, CA) to detect the Matrix (M) gene of all type A influenza viruses with internal control was used under the following conditions: 0.03 μM each primer, 320 μM each dNTP, 0.12 μM probe, and 13 units Rnase Inhibitor (Promega, Madison, WI). Eight μl swab RNA sample and nuclease-free water were added to make a final volume of 25 μl per reaction. The reverse transcription reaction consisted of one cycle of 30 min. at 50°C, followed by 15 min. at 94°C. Forty-five cycles of 20s denaturation at 94°C, followed by annealing for 20s at 60°C were carried out in the PCR reaction. All reactions were carried out in a Smart Cycler II (Cepheid) real-time PCR machine. The EID_{50}s of virus from the swab samples were extrapolated from the cycle thresholds by using standard curves generated from the known amounts of RNA of the challenge viruses used (14).

Statistical analysis

Swab sample data were analyzed using Prism v5 Software package (GraphPad Software Inc., San Diego, CA). One-way ANOVA with Tukey's post hoc test was used to analyze viral shedding data. Results with *P* values < 0.05 were considered to be statistically significant. For statistical purposes, negative RRT-PCR samples were assigned titer values equal to the detection limit of the run, less $10^{0.1}$ EID_{50}/ml. Detection limits of individual RRT-PCR reactions were calculated from the standard curve, setting the cycle threshold (C_t) value equal to the number of cycles run.

Alpha one-acid glycoprotein (AGP) levels

To compare levels of the acute phase protein α_1- acid glycoprotein, single radial immunodiffusion Chicken α_1-Acid Glycoprotein Measurement Kits (Cardiotech Services, Inc., Louisville, KY) were used, according to manufacturer's instructions, but with modifications. Briefly, chicken AGP standards were diluted two-fold, serially, from 1000 to 62.5µg/ml. Five µl of standard or undiluted serum sample were loaded per well in duplicate, and incubated 48 hr at room temperature in a humidified chamber. The diameters (mm) of the precipitin rings were measured and mean serum AGP concentrations were interpolated from the standard curve using GraphPad Prism and four parameter logistic curve fit non-linear regression.

Molecular graphic illustration

Amino acid changes at antigenic sites of the HA1 molecule were determined by alignment of amino acid sequences using the MegAlign program (DNASTAR, Madison, WI). These changes were located on the HA monomer using the Rasmol software (version 2.6)

(Biomolecular Structures Group, Hertfordshire, UK) on the file 1JSM, of the DK/Singapore/3/97 H5 monomer, downloaded from the Protein Data Bank website (http://www.ncbi.nlm.nih.gov/sites/entrez).

RESULTS

Cross-HI test

Hemagglutinin-specific reference antisera against the viruses CK/Hidalgo, CK/Guat, Parrot/CA, Mexican Mutant 7, and Mexican Mutant 11, were produced in chickens vaccinated with plasmid DNA. The antisera were used in the cross HI test to evaluate antigenic differences between the viruses and to determine the effects that the mutations introduced into the vaccine seed H5 had on the antigenicity. As expected, most HI titers were highest when antiserum was used against the homologous virus (Table 5.1). A minimum of a four-fold difference but up to a 32-fold difference in HI titers was seen when serum specific for the vaccine seed virus was tested against more recent viral isolates CK/Guat, Parrot/CA, or Amazon/CA. However, the HI titer of the vaccine seed-specific serum was equally as high when tested against either of the mutant viral Ags, Mexican Mutant 7 and Mexican Mutant 11, as when tested against its homologous Ag (Table 5.1). When antisera of the Mexican mutant 7 virus was compared, it had a similar pattern as the CK/Hidalgo with a marked antigenic difference with the CK/Guat and the two parrot isolates. The Mexican Mutant 11 appeared to fall in between with at most a four-fold difference from all the antigens used in the table. Interestingly, no more than a two-fold difference was seen when CK/Hidalgo serum was used against either antigen produced from United States isolates, but there was a four- to sixteen-fold drop in HI titers when serum specific for either

mutant was assessed with the Avian/NY antigen (Table 5.1). The same degree of difference in HI titers was also seen when Parrot/CA serum was used against the Avian/NY antigen.

Virus neutralization

Similar to the HI results, distinct trends were detected whereby the overall sequence similarity but not necessarily the particular amino acid changes at the antigenic sites influenced the antigenic relatedness. CK/Hidalgo, Mexican Mutant 7, and Mexican Mutant 11 sera all poorly neutralized the CK/Guat rg and Amazon/CA viruses (Table 5.2, Fig. 5.2). This is supported by the fact that CK/Hidalgo, Mexican Mutant 7, and Mexican Mutant 11 HA1s all are genetically similar, with only 7 to 11 aa changes between them, (less than a 4% difference), while the CK/Guat and Amazon/CA viruses have between nine and 12% differences from the CK/Hidalgo and mutant viruses (Table 5.3). In contrast, CK/Guat serum maintained neutralizing abilities against itself and Amazon/CA viruses, when diluted four to 16 times more than the concentrations needed to neutralize the vaccine seed virus (Table 5.2). Overall, the Parrot/CA serum also neutralized in a manner that was dependent on HA sequence similarity of the virus being neutralized.

Antigenic relatedness (R) between isolates was determined, using the method of Archetti and Horsfall (1). There was a noticeable difference in antigenic relatedness when comparing the vaccine seed or mutant H5s, with either CK/Guat or Parrot/CA H5s. While the mutants were at least 35% antigenically related to the vaccine strain, neither of the more recent isolates from 2002 and 2004 was more than 3% similar to the vaccine strain (Table 5.2). No greater than 6% similarity was seen between the Parrot/CA virus and the vaccine seed virus or either mutant.

Protection against viral shedding

To evaluate the influence of the amino acid differences on the abilities of the different viruses to evade the host immune response and replicate, a vaccine-challenge study on chickens was performed. Two-week-old white rock chickens were vaccinated with one of the five live rg viruses or with BHI alone. All of the birds were then challenged with one of two wild type viruses having the most distantly related HA proteins of the viruses used in this study. Serum samples were taken 13 days post vaccination (dpv) to confirm that the vaccine viruses did indeed induce an immune response in their hosts. All vaccinated birds had positive HI titers (Tables 5.4A and 5.4B). Interestingly, when CK/Hidalgo antigen was used with pre-challenge serum specific for CK/Guat or Mexican Mutant 7 (which contained seven amino acid changes found in the CK/Guat HA), the HI titers were nearly identical (Table 5.4A, 5.4B). However, this phenomenon did not occur consistently when other viral antigens were used.

Real-time RT-PCR was used with RNA extracted from oropharyngeal (O/P) swab samples taken at both three and five dpch. Virus shedding was compared between birds in each challenge group. In the CK/Hidalgo challenge group, at three dpch, the birds that were vaccinated with the Mexican Vaccine rg virus shed significantly lower amounts of virus than the birds vaccinated with the CK/Guat or Parrot/CA rg viruses or the birds that received the sham vaccine (Table 5.5). There were no significant differences seen between the Mexican Vaccine rg-vaccinated birds and any of the mutant rg virus-vaccinated groups, which contained HAs identical to the Mexican Vaccine rg virus, except for the seven or 11 amino acid changes at the potential antigenic sites. In this same challenge group, at five dpch, all vaccinated birds shed significantly less virus than unvaccinated birds (Table 5.5). However, there were no significant

differences seen between any of the vaccinated birds (Table 5.5). No significant difference was seen at either time point from birds that were in the Parrot/CA challenge group (Table 5.5).

Serum samples were also taken from all birds 10 dpch and an increase in HI titers was seen in all groups (Tables 5.6A, 5.6B).

Alpha one-acid glycoprotein levels

At 24 hours prior to, and 24 and 48 hours following challenge, serum samples were obtains from chickens, in both challenge groups, that had been vaccinated with the Mexican Vacc rg virus, Parrot/CA rg virus, or sham vaccine. No significant difference was seen in levels of AGP between any of the vaccine groups.

DISCUSSION

Antigenic drift was at one time not thought to be an important consideration for vaccination of poultry. The humoral antibody produced by killed adjuvanted vaccines appears to be broadly protective within a subtype in blocking the viremia and the subsequent systemic replication, which contributes to the high mortality of HPAI. Although systemic replication may be blocked, mucosal replication and shedding of the virus from the oropharynx is influenced by antigenic variation or antigenic drift (31). It is important that an AI vaccine used on poultry protects not only from morbidity and mortality, but also prevents or reduces viral shedding. The smaller the amount of virus that is excreted into the environment means there is a reduced chance that the virus will spread within flock members or between flocks. Additionally, long-term vaccination of poultry for the control of HPAI was first attempted in the 1990's and little practical information was available on how the long-term vaccination of poultry would affect

antigenic drift. Lee *et al*. examined the Mexican H5N2 outbreak, where vaccination was widely used, and demonstrated that AI viruses undergo antigenic drift away from the vaccine virus (16). The antigenic drift in this case greatly affected the levels of virus replication and shedding, but the birds were still protected from morbidity and mortality with a HPAI challenge, demonstrating that both need to be considered in the vaccination policy if eradication is the goal.

Previous studies with fowlpox vectored H5 vaccine showed a clear correlation with amino acid sequence similarity of the HA1 protein and viral shedding, with closer sequence similarity resulting in less viral shedding (31). However, the viruses in this study were unrelated H5 viruses from different outbreaks, and this was more a study of viral variation within a subtype and not antigenic drift within a specific lineage of virus. With antigenic drift, because of positive selection, a higher percentage of amino acid changes will be at sites, which are important for antibody neutralization. In the study of Mexican viruses, the amino acid changes in the viruses examined were not randomly spread throughout the HA1 protein, but they were concentrated in the predicted antigenic sites known to be important for virus neutralization in human H1 and H3 influenza viruses (2, 35). With human influenza viruses the overall sequence similarity is correlated with antigenic variation; the better correlate of protection is the understanding of what specific amino acids are correlated to protection from neutralizing antibody. The goal of this study was to evaluate H5 influenza viruses to determine what specific amino acids were involved in the escape of circulating viruses from antibodies against the vaccine virus. With this knowledge, it would be possible to accurately select based on sequence information alone a vaccine virus that would be best able to protect against viral infection and shedding. We sought to determine which of the amino acids located at potential antigenic sites

of Mexican H5 isolates and to what extent they are actually involved in virus neutralization and in reducing viral shedding from infected chickens.

As was previously observed, the vaccine strain used for killed vaccines in Mexico, CK/Hidalgo/94, is antigenically quite different from the more recent field strains from Guatemala and the U.S. and differ by cross HI by four- to 32-fold (16). It was predicted that these antigenic differences could be traced to specific amino acids based on comparison with human H3 influenza viruses, where considerable work has been done to map the key antigenic sites. Based on this information, we selected 11 amino acids in the HA1 protein and created 2 variants of the CK/Hidalgo/94 virus that contained either 7 aa differences or all 11 aa differences in a DNA vaccine and a reverse genetics-produced virus. Using these viruses and the WT viruses for comparison, some antigenic differences were seen between CK/Hidalgo/94 and the mutant viruses, but the antigenic differences were lower than those seen with the wild type viruses. Clearly additional amino acids are necessary to explain the antigenic differences between the different viruses.

Even though the total antigenic differences could not be traced to the specific amino acid mutations examined when detected in the HI and virus neutralization titers, similar trends were seen between the HI and virus neutralization test results. Despite the fact that the receptor binding site is a separate entity from the Ab-binding site on the HA, they are both located near each other on the globular head of this protein. This may help to explain the similarities that we saw. Similar results to ours, where there was correlation between HI and virus neutralization tests have also been found in others' past experiments (21, 23, 36).

In this study for the cross HI tests, we used sera produced by a DNA vaccine that was administered multiple times. The advantage of the DNA vaccine is that it produces polyclonal

sera specific to the HA, and avoids issues of non-specific reactivity or steric hindrance associated with antibody to the neuraminidase protein. However, to produce high levels of antibody, 3 or 4 vaccinations were needed which may have effected the affinity and avidity of the immune response. Previous studies with human influenza viruses have shown that hyperimmune polyclonal antisera were unable to provide clear-cut evidence for antigenic differences between variants and the parental virus (6). To produce the antibody for cross HI tests to determine the strain selection for human influenza vaccines, a single dose of live virus is given to ferrets to produce an antibody response as selective as possible. The possibility exists that the antisera we used in the HI and virus neutralization tests did not have the specificity needed to discreetly differentiate antigenic changes because of the method used for antibody production.

For the animal challenge studies, we chose to use a single live virus vaccination given intramuscularly to obtain an immune response with a higher level of specificity. By keeping the immune response as specific as possible, we hoped to detect any differences in either HI titers or virus neutralization titers that would have occurred as a result of the amino acid changes at the proposed antigenic sites. First, viruses produced through rg are not always able to replicate in chickens to the same extent that the wild type viruses from which they were derived are (4), (unpublished data). To ensure that the birds would become infected and mount a measurable immune response against the vaccine viruses, we inoculated them intramuscularly. Upon challenge, an anamnestic response would be triggered. However, it was expected that only a poor mucosal response would develop, allowing for detectable virus replication on the mucosa. In the challenge studies in chickens using the CK/Hidalgo/94 virus as challenge, similar results to Lee *et al.* were seen where the birds vaccinated with the Guatemala and Parrot viruses shed much more virus than birds vaccinated with the homologous antigen. Although somewhat

paradoxically the opposite was not seen with the birds challenged with the parrot virus since no statistical differences in virus shedding were seen in any of the groups. This difference is likely to at least be partly the result of lower antibody levels seen with rg Parrot vaccine. This supports the idea that vaccine efficacy is the result of antigenic differences as well as the immune response to the vaccine (28, 29) A strong antibody response may compensate for a poor antigenic match. The CK/Hidalgo HA gene appeared to be the most immunogenic in this study, giving the highest antibody titers in all the vaccinated groups. This may help to explain why the Mexican origin vaccines, using the CK/Hidalgo virus as a seed strain, has consistently provided good protection to the antigenically distant Asian H5N1 viruses in part because it produces high antibody levels (29).

Both sequence evaluation and HI cross-neutralization studies are among the criteria used when selecting vaccine seed strains (13). While the amino acid sequence similarities between the HA1 proteins of the Mexican viruses used in this study ranged from 87.7% to 98.8% (Table 5.3), the R values representing antigenic relatedness were not nearly as high (Table 5.2). A similar scenario was seen in viruses isolated from turkey breeder hens in Ohio and Illinois in 2004 (37). The antigenic relatedness and sequence similarities between the circulating and vaccine viruses were assessed (37). While the circulating and vaccine virus (which was of swine origin) sequences were at least 95% similar at the amino acid level based on the HA1 molecule, they were shown to be antigenically distant based on the HI and virus neutralization test (37). The authors suggested that one of the reasons for the failure of protection by vaccination in the Illinois turkey flocks may be due to these antigenic differences (37). Eight amino acid changes at antigenic sites were found between the circulating turkey viruses and swine vaccine virus (37). Two of these changes were at amino acids 136 and 154 (H5 HA1 numbering), which were

examined in our study. However, changes at these and other additional sites did not show the complete difference in antigenicity of the Mexican H5 isolates. Perhaps the structural differences between the H5 and H3 molecules would explain the discrepancies. The findings of our and Yassine *et al.*'s studies emphasize the importance of taking into account not only sequence similarity but also antigenic relatedness to field strains when evaluating potential vaccine candidates.

A study was conducted to map the H9 of Swine/Hong Kong/9/98, variant viruses that had gone through either one or two rounds of selection by one or two monoclonal antibodies (11). Though two different viruses contained amino acid mutations at identical locations, one of the variants reacted with a particular monoclonal antibody while the other did not (11). It is worth noting that one of these viruses contained additional mutations in the HA molecule and the amino acid changes that occurred did not result in the same amino acid at the common position (11). However, their findings help to demonstrate that the occurrence of an amino acid change at a particular location in individual viruses does not necessarily render identical reactivity patterns to antibodies against the viruses (11). This may also help to explain why there were no distinct differences detected between the virus strains used in this study that were based solely on mutations at potential antigenic sites. There exists the possibility that the changes we introduced into the parental HA may have been sufficient for the variant viruses to escape antibodies, but clearly, they did not induce the same effect when introduced into the parental HA; these changes did not impart any additional ability of the mutants to evade the humoral response targeted at the parental HA.

In a previous study, a monoclonal antibody (mAb) was found to recognize a specific epitope on the HAs of both H1 and H2 subtype isolates (19). The mAb recognition site was

conformationally dependent, and the places of Ab binding were located in both the HA1 and HA2 subunits of the protein (19). When one amino acid change in the epitope occurred in the HA1, the mAb no longer was able to neutralize the virus (19). Interestingly, because part of the epitope was located in the HA2 stalk region, HI titers did not correlate with virus neutralization (19). All amino acid changes we incorporated into the HA proteins were located in the HA1 subunit. One explanation for the absence of detectable differences between HI and virus neutralization titers based on the point mutations is the possibility that an amino acid located in the HA2 subunit, which is part of a conformational epitope, may also play a part in affecting the antigenicity of these H5 viruses. It is possible that one of the amino acid changes we introduced caused a slight alteration in the epitope, but not enough to completely inhibit antibody binding.

With regard to human isolates, it was found that the HA1s of different pandemic strains had diverged to such a degree that is was impossible to map their sites of antigenic differences merely by comparing amino acid sequences (3). Since the time elapsed between initiation of vaccination in Mexican poultry and isolation of the most recent virus in this study was at least seven years, it is possible that such a numerous amount of changes occurred in the drift variant viruses that antigenically mapping these viruses is more complex than presumed at the onset of this study. It is possible that so many amino acid changes occurred in the viruses circulating in Mexican poultry over the duration of seven to 10 years that the maximum number of changes beyond which distinction of those amino acids directly involved in the antigenicity of these molecules has been reached.

It was found that in 10 variants selected with monoclonal antibodies, a proline in a particular location of the HA1 changed to serine, theonine, leucine, or histidine (3). Similar to the above, the interpretation of this result was that the region around this position was at or near

the antigenic site. In the early '80s, Jackson *et al.* stated that although there is no doubt that amino acid substitutions in the proposed antigenic regions induce antigenic alterations in the HA molecule, this does not necessarily prove that they represent the antigenic sites to which antibodies actually bind (10). The possibility also exists that substitutions in one region could affect an antibody-binding site some distance away by changing the conformation of the molecule (10). Laver *et al.* stated that the interface of antigen-antibody binding is absolutely dependent on the conformation of the native protein (12). Also similar to that stated above, it is possible that other changes that occurred in the HA that we did not look at in this study, though they may not have directly been involved in antibody binding, affected the folding of the HA. This may help to explain the decrease in virus neutralization and HI titers seen when the circulating viruses were tested against the vaccine seed virus (16), yet the lack of differences seen between the mutant HA rg viruses and the vaccine seed virus, in this study.

In this study, we set out to assess the influence that particular amino acid changes located in the previously identified antigenic sites had on the antigenicity of the viruses of the Mexican H5N2 lineage. The collection of amino acid mutations introduced into the parental HA protein did not appear to alter the abilities of the mutant rg viruses to escape antibodies against the parental HA. Apparently more is involved in the escape of viruses from the antibodies than only the 11 amino acids that we focused on here. The amino acids we studied were all located in the globular head of the HA1 molecule. Perhaps there are others also involved, which are located in the HA2 stalk region. These may interact with residues found in the globular head in a conformationally-dependent way. Alternatively, it is possible that there are epitopes in the HA2 that are recognized by neutralizing antibodies of which we are yet unaware.

Based on our findings, further investigation will be needed to identify the general location(s) of the amino acids that dictate the antigenic properties of the CK/Hidalgo/232/94 HA protein. The studies performed here only included mutant HAs that had changes in the HA1 subunit. The next step will be to determine if the antigenic regions of this protein are restricted to the HA1 subunit, as we had originally surmised, or if there are some amino acids in the HA2 subunit that contribute to the antigenicity. Until this is done, the HI and virus neutralization tests will most likely remain as the gold standards for assessing a vaccine candidate virus' ability to induce an effective immune response.

FIGURE LEGENDS

Figure 5.1. Diagram of the CK/Hidalgo/232/94 H5 monomer (based on protein structure of DK/Singapore/3/97 H5) containing amino acid changes at potential antigenic sites. Color scheme: purple: amino acid point mutations introduced into the HA molecule of the CK/Hidalgo/232/94 H5, yellow: residues located at the receptor binding site which determine host receptor binding specificity, cyan: HA1 subunit, orange: HA2 subunit.

Fig. 5.2. Phylogenetic tree of Mexican HA1s. The phylogenetic analysis using parsimony for the HA1 protein based on amino acid sequence. Tree was generated by general bootstrap analysis using 100 replicates and a heuristic search method, with PAUP 4.0b10 program. Branch lengths are indicated in the tree. The outgroup is CK/Scotland/59. Abbreviations used for identifying isolates: CK (chicken)

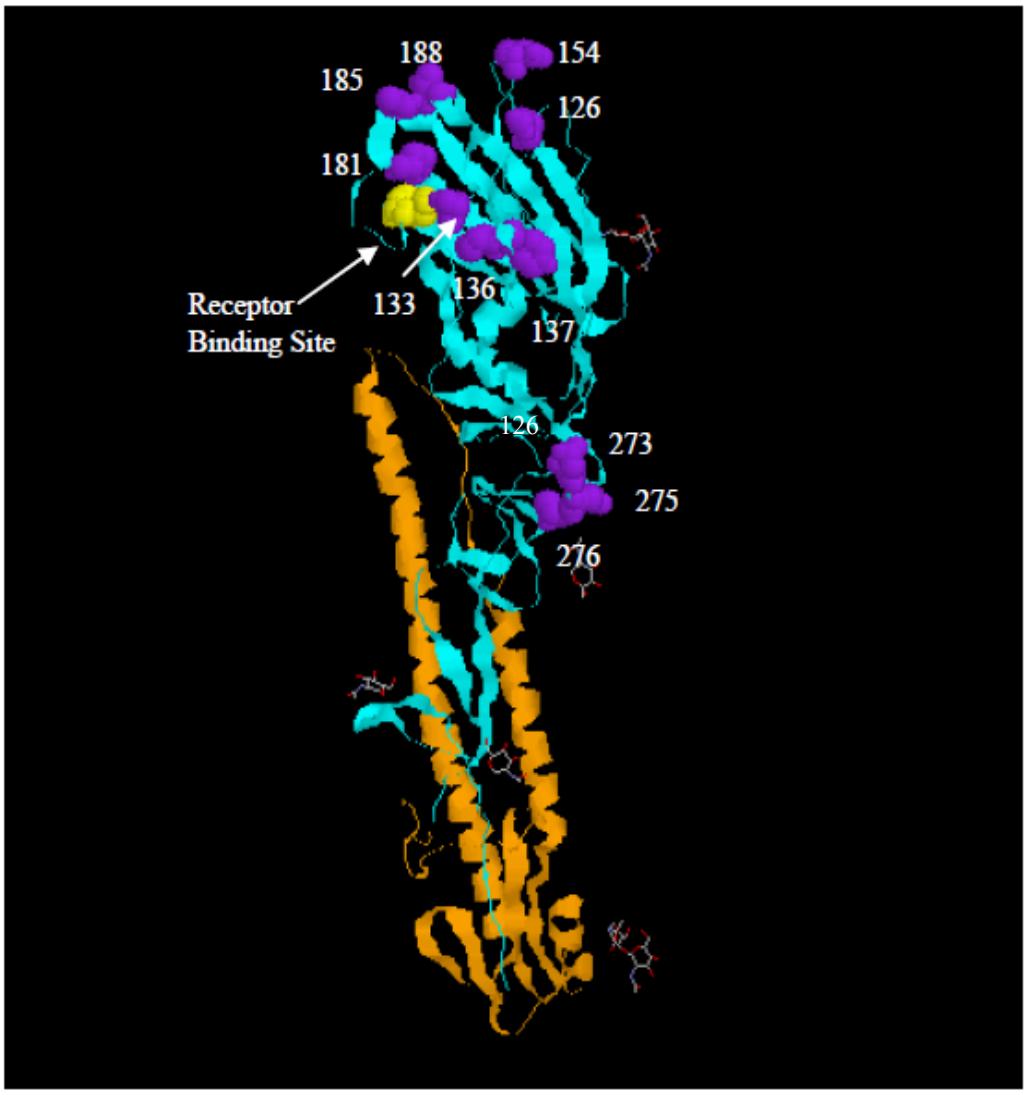
188
154
185
126
181
Receptor
Binding Site
133
136
137
126
273
275
276

Fig. 5.1

Table 5.1. Hemagglutination inhibition (HI) titers using the cross-HI test to evaluate antigenic differences between Mexican viruses

Antisera[A]	Viral Antigen[B]							
	CK/Hidalgo	Mex Mut 7	Mex Mut 11	CK/Guat	Parrot/CA	Env/DE	Avian/NY	YHA
CK/Hidalgo	**256**	256	256	8	64	256	128	16
Mexican Mutant 7	128	**512**	512	8	32	128	32	16
Mexican Mutant 11	128	256	**256**	64	128	256	64	64
CK/Guat	32	128	64	**256**	512	512	128	512
Parrot/CA/6032/04	256	128	512	512	**2048**	512	256	1024
Env/DE/1346/01	128	8	32	2	32	**2048**	128	4
Avian/NY/315388/00	128	64	64	64	128	1024	**512**	64

[A]Sera against all Mexican isolates were produced by using DNA vaccines in chickens, with plasmids encoding for the HA gene of the corresponding virus. Sera against United States virus isolates was produced using whole-killed oil emulsion vaccines
[B]Four HA units of β-propiolactone-inactivated virus was used as antigen
Isolate abbreviations: Avian/NY, Avian/New York/315388/00; CK/Hidalgo, CK/Hidalgo/28159-232/94; CK/Guat, CK/Guatemala/194573/02; Env/DE, Environment/DE/1346/01; Parrot/CA, Parrot/CA/6032/04; YHA, Yellow-Headed Amazon/CA/500658-8/07

Table 5.2. Cross-virus neutralization of homologous and heterologous serum dilutions/virus[A]

	Virus					
	CK/Hidalgo.	Mex Mut 7	Mex Mutant 11	CK/Guat	Parrot/CA	Amazon/CA
Antisera	Endpoint dilution[B]					
CK/Hidalgo	320	80	160	<2.5 [D]	<2.5	<2.5
Mex Mut 7	320	320	80	<2.5	2.5	<2.5
Mex Mut 11	20	160	80	2.5	5	<2.5
CK/Guat	2.5	160	5	40	5	10
Parrot/CA	10	20	5	40	80	80
	Percent antigenic relatedness[C]					
CK/Hidalgo	100%	50%	35%	2%	3%	ND
Mex Mut 7		100%	70%	17%	4%	ND
Mex Mut 11			100%	6%	6%	ND
CK/Guat				100%	25%	ND
Parrot/CA					100%	ND

[A]Beta-tests (2-fold serum dilutions beginning at 1:2.5 dilutions of serum with $10^{3.5}EID_{50}$ /0.1 ml virus) were performed in nine- to 11-day old ECEs. Three days post inoculation, the HA test was used to detect presence of virus in allantoic fluid. All viruses used, with the exception of Amazon/CA, were produced by reverse genetics.
[B]Highest dilution of serum that was able to completely neutralize virus (*ie* allantoic fluid from all eggs in serum dilution group demonstrated no hemagglutinating activity).
[C]Based on R value (see Materials and Methods for detailed description).
[D]Used when endpoints were not detected when serum was diluted at the initial dilution of 1:2.5.

Table 5.3. Amino acid sequence similarity[A] (%) between the HA1 proteins of Mexican reverse genetics viruses

	CK/Hidalgo	Mex Mutant 7	Mex Mutant 11	CK/Guat	Parrot/CA	Amazon/CA
CK/Hidalgo	100	97.9	96.7	91.0	87.7	87.7
Mex Mut 7		100	98.8	93.1	89.2	88.9
Mex Mut 11			100	91.9	90.4	88.3
CK/Guat				100	94.9	93.4
Parrot/CA					100	91.9

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

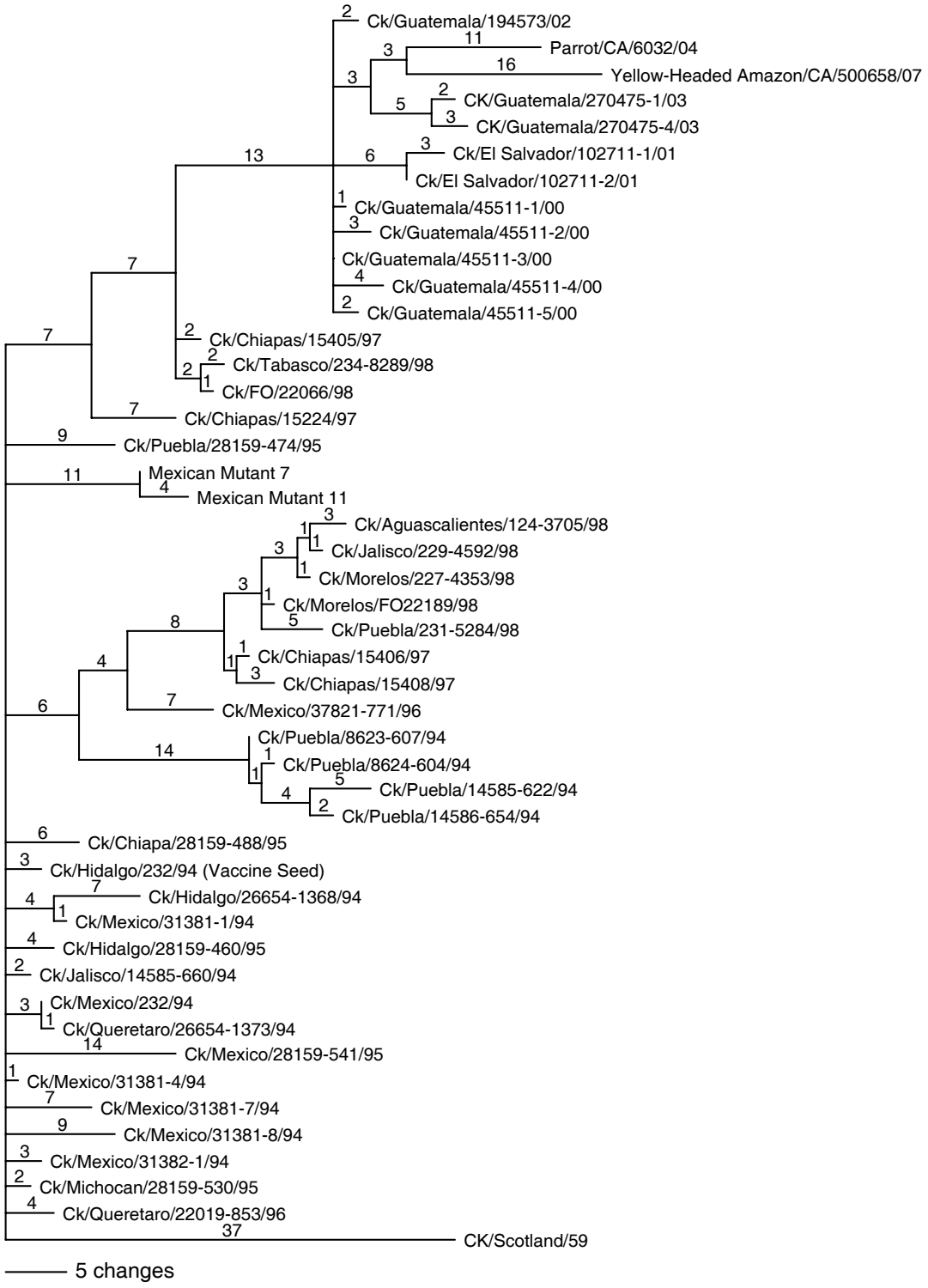
Ck/Guatemala/194573/02
Parrot/CA/6032/04
Yellow-Headed Amazon/CA/500658/07
CK/Guatemala/270475-1/03
CK/Guatemala/270475-4/03
Ck/El Salvador/102711-1/01
Ck/El Salvador/102711-2/01
Ck/Guatemala/45511-1/00
Ck/Guatemala/45511-2/00
Ck/Guatemala/45511-3/00
Ck/Guatemala/45511-4/00
Ck/Guatemala/45511-5/00
Ck/Chiapas/15405/97
Ck/Tabasco/234-8289/98
Ck/FO/22066/98
Ck/Chiapas/15224/97
Ck/Puebla/28159-474/95
Mexican Mutant 7
Mexican Mutant 11
Ck/Aguascalientes/124-3705/98
Ck/Jalisco/229-4592/98
Ck/Morelos/227-4353/98
Ck/Morelos/FO22189/98
Ck/Puebla/231-5284/98
Ck/Chiapas/15406/97
Ck/Chiapas/15408/97
Ck/Mexico/37821-771/96
Ck/Puebla/8623-607/94
Ck/Puebla/8624-604/94
Ck/Puebla/14585-622/94
Ck/Puebla/14586-654/94
Ck/Chiapa/28159-488/95
Ck/Hidalgo/232/94 (Vaccine Seed)
Ck/Hidalgo/26654-1368/94
Ck/Mexico/31381-1/94
Ck/Hidalgo/28159-460/95
Ck/Jalisco/14585-660/94
Ck/Mexico/232/94
Ck/Queretaro/26654-1373/94
Ck/Mexico/28159-541/95
Ck/Mexico/31381-4/94
Ck/Mexico/31381-7/94
Ck/Mexico/31381-8/94
Ck/Mexico/31382-1/94
Ck/Michocan/28159-530/95
Ck/Queretaro/22019-853/96
CK/Scotland/59
5 changes

Figure 5.2.

Table 5.4A. Prechallenge hemagglutination inhibition titers of chickens vaccinated at two weeks of age and challenged at four weeks of age with 10^6 EID_{50} CK/Hidalgo/232/94 WT virus.

Vaccine	Viral Antigen HA[A]				
	CK/Hidalgo	Mex Mut 7	Mex Mut 11	CK/Guat	Parrot/CA
CK/Hidalgo rg	**29.8**	9.8	6.1	7.4	3.5
Mex Mutant 7	7.5	**4.3**	3.7	2.8	1.7
Mex Mutant 11	12.1	9.8	**9.8**	4.9	3.5
CK/Guat rg	8.6	4.3	4	**16**	5.6
Parrot/CA rg	12.1	6.9	6.9	17.1	**19.7**

[A]All antigens were produced with viruses created through reverse genetics.
Numbers in bold face represent HI titers when homologous anigen and antiserum were used.

Table 5.4B. Prechallenge hemagglutination inhibition titers of chickens vaccinated at two weeks of age and challenged at four weeks of age with 10^6 EID_{50} Parrot/CA/6032/04 WT virus.

Vaccine	Viral Antigen HA[A]				
	CK/Hidalgo	Mex Mut 7	Mex Mut 11	CK/Guat	Parrot/CA
CK/Hidalgo rg	**29.8**	14.9	7.5	6	3.5
Mex Mutant 7	9.8	**8**	4.9	3.7	2.5
Mex Mutant 11	17	19.7	**13.9**	14.9	3
CK/Guat rg	9.8	5.6	5.3	**27.9**	8
Parrot/CA rg	6	2.8	4.9	8	**7.5**

[A]All antigens were produced with viruses created through reverse genetics.
Numbers in bold face represent HI titers when homologous anigen and antiserum were used.

Table 5.5. Virus isolation data from chickens vaccinated with live AI vaccine at 2 weeks of age and intranasally challenged at 4 weeks of age

	Viral RNA detection from oropharyngeal swab samples			
	CK/Hidalgo/28159-232/94 challenge groups		Parrot/CA/6032/04 challenge groups	
	3 days post ch	5 days post ch	3 days post ch	5 days post ch
Vaccine group	number positive/total[A], (Log EID_{50}/ml [B])		number positive/total, (Log EID_{50}/ml)	
CK/Hidalgo rg	4/8 (2.8)[a]	0/8[a]	5/8 (4.3)[a]	8/8 (5.6)[a]
Mexican Mut 7	5/8 (4.0)[ab]	3/8 (2.7)[a]	8/8 (5.5)[a]	8/8 (5.6)[a]
Mexican Mut 11	5/8 (4.2)[ab]	2/8 (2.5)[a]	8/8 (5.3)[a]	8/8 (6.0)[a]
CK/Guatemala rg	7/8 (5.0)[b]	2/8 (2.5)[a]	7/8 (5.3)[a]	8/8 (5.9)[a]
Parrot/CA rg	8/8 (5.5)[b]	3/8 (2.7)[a]	8/8 (5.5)[a]	8/8 (6.0)[a]
Negative control	8/8 (5.9)[b]	8/8 (5.2)[b]	8/8 (5.5)[a]	8/8 (6.1)[a]

[A]Swab samples were taken from all birds remaining at each time point.

[B] Log EID_{50} was determined using real-time RT-PCR specific type A avian influenza matrix gene (25)

Different lowercase superscripts denote significance between treatment groups (a vs b) ($p<0.05$)

Titers for negative samples were equal to the values of detection.

Table 5.6A. Hemagglutination inhibition titers[A] 10 days post challenge from chickens challenged intranasally with 10^6 EID_{50} CK/Hidalgo/232/94 WT virus.

Vaccine group	CK/Hidalgo WT Antigen[B]
CK/Hidalgo rg	789
Mex Mut 7	430
Mex Mut 11	394
CK/Guat rg	152
Parrot/CA rg	331
Neg. control	166

[A]Titers are expressed as geometric mean titers.
[B]Four HA units of antigen were used.

Table 5.6B. Hemagglutination inhibition titers[A] 10 days post challenge from chickens challenged intranasally with 10^6 EID_{50} Parrot/CA/6032/04 WT virus.

Vaccine group	Parrot/CA WT Antigen[B]
CK/Hidalgo rg	394
Mex Mut 7	304
Mex Mut 11	724
CK/Guat rg	2233
Parrot/CA rg	4096
Neg. control	331

[A]Titers are expressed as geometric mean titers.
[B]Four HA units of antigen were used.

REFERENCES

1. Archetti, I., and Horsfall, F. L. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum. J. Exp. Med. 92:441-462. 1950.

2. Caton, A. J. a. B., G. G. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31:417-427. 1982.

3. Correspondent. Antigenic shift and drift. Nature 283:524-525. 1980.

4. Desheva, J. A., Lu, X. H., Rekstin, A. R., Rudenko, L. G., Swayne, D. E., Cox, N. J., Katz, J. M., and Klimov, A. I. Characterization of an influenza A H5N2 reassortant as a candidate for live-attenuated and inactivated vaccines against highly pathogenic H5N1 viruses with pandemic potential. Vaccine 24:6859-6866. 2006.

5. Escorcia, M., Vazquez, L., Mendez, S. T., Rodriguez-Ropon, A., Lucio, E., and Nava, G. M. Avian influenza: genetic evolution under vaccination pressure. Virol J 5:5-15. 2008.

6. Gerhard, W., and Webster, R. G. Antigenic drift in influenza A viruses I. Selection and characterization of antigenic variants of A/PR/8/34 [H0N1] influenza virus with monoclonal antibodies. J. Exp. Med. 148:383-392. 1978.

7. Hawkins, M. G., Crossley, B. M., Osofsky, A, Webby, R. J., Lee, C. W., Suarez, D. L., and Hietala, S. K. Avian influenza A virus subtype H5N2 in a red-lored amazon parrot. J Am Vet Med Asoc 228:236-241. 2006.

8. Hinshaw, V., Sheerar, M. G., and Larsen, D. Specific antibody responses and generation of antigenic variants in chickens immunized against a virulent avian influenza virus. Avian Dis. 34:80-86. 1990.

9. Horimoto, T., Rivera, E., Pearson, J., Senne, D., Krauss, S., Kawaoka, Y., and Webster, R. G. Origin and molecular changes associated with emergence of a higly pathogenic H5N2 influenza virus in Mexico. Virology 213:223-230. 1995.

10. Jackson, D. C., Murray, J. M., White, D. O, and Gerhard, W. U. Enumeration of antigenic sites of influenza virus hemagglutinin. Infection and Immunity 37:912-918. 1982.

11. Kaverin, N. V., Rudneva, I. A., Ilyushina, N. A., Lipatov, A. S., Krauss, S., and Webster, R. G. Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: analysis of H9 escape mutants. Journal of Virology 78:240-249. 2004.

12. Laver, W. G., Air, G. M., Webster, R. G., and Smith-Gill, S. J. . Epitopes on protein antigens: misconceptions and realities. Cell 61:553-556. 1990.

13. Lee, C. W., and D. L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Animal Health Research Reviews 6:1-15. 2005a.

14. Lee, C. W., and D. L. Suarez. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype of avian influenza virus. J. Virol. Methods 119:151-158. 2004.

15. Lee, C. W., Lee, Y. J., Senne, D. A., Suarez, D. L. Pathogenic potential of North American H7N2 avian influenza virus: a mutagenesis study using reverse genetics. Virol 353:388-395. 2006.

16. Lee, C. W., Senne, D., and Suarez, D. L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 78:8372-8381. 2004.

17. Lee, C. W., Senne, D., and Suarez, D. L. 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.

18. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Gogo, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E. Hobom, G., Kawaoka, Y. Generation of influenzA viruses entirely from clones cDNAs. Proceedings of the National Academy of Sciences of the United States of America 96:9345-9350. 1999.

19. Okuno, Y. I., Y., Sasao, F., and Ueda, S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J Virol 67:2552-2558. 1993.

20. Pfeiffer, J., Pantin-Jackwood, M., To, T. L., Nguyen, T., and Suarez, D. L. Phylogenetic and Biological Characterization of Highly Pathogenic H5N1 Avian Influenza Viruses (Vietnam 2005) in Chickens and Ducks. Virus Research in press. 2009.

21. Philpott, M., Easterday, B. C., and Hinshaw, V. S. Neutralizing epitopes of the H5 hemagglutinin from a virulent avian influenza virus and their relationship to the pathogenicity. J Virol 63:3453-3458. 1989.

22. Reed, L. J., and H. Muench. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27:493-497. 1938.

23. Richt, J. A., Lager, K. M., Janke, B. H., Woods, R. D., Webster, R. G., and Webby, R. J. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. Journal of clinical microbiology 41:3198-3205. 2003.

24. Senne, D. A. Avian influenza in North and South America, 2002-2005. Avian Dis. 50:167-173. 2007.

25. 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. Journal of clinical microbiology 40:3256-3260. 2002.

26. Steel, J., S. V. Burmakina, C. Thomas, E. Spackman, A. Garcia-Sastre, D. E. Swayne, and P. Palese. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26:522-531. 2008.

27. Swayne, D. E., D. A. Senne, and C. W. Beard Avian Influenza. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. J. R. Glisson, Swayne, D. E., Jackwood, M. W., Pearson, J. E., and Reed, W. M., ed. American Association of Avian Pathologists, Kennet Square, PA. pp 150-155. 1998.

28. Swayne, D. E., J. R. Beck, M. Garcia, and H. D. Stone. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Path. 28:245-255. 1999.

29. Swayne, D. E., C. W. Lee, and E. Spackman. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Path. 35:141-146. 2006.

30. Swayne, D. E. Avian influenza vaccines and therapies for poultry. Comp. Immunol. Microbiol. Infect. Dis.:doi: 10.1016/j.cimid.2008.1001.1006
2008.

31. Swayne, D. E., Garcia, M., Beck, J. R., Kinney, N., and Suarez, D. L. 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.

32. Villareal-Chavez, C. L., and Flores, A. O. The Mexican avian influenza (H5N2) outbreak. In: Proceedings of the Fourth International Symposium on Avian Influenza. D. E. Swayne, and Slemons, R. D., ed. United States Animal Health Association, Richmond, VA. pp 18-22. 1998.

33. Wasilenko, J. L., Lee, C. W., Sarmento, L., Spackman, E., Kapczynski, D. R., Suarez, D. L., and Pantin-Jackwood, M. J. NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens. J Virol 82:4544-4553. 2008.

34. 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.

35. Wiley, D. C., Wilson, I. A., and Skehel, J. J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289:373-378. 1981.

36. Yang, Z. Y., Wei, C. J., Kong, W. P., Lan, W., Xu, L., Smith, D. F., and Nabel, G. J. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science 317:825-828. 2007.

37. Yassine, H. M., Lee, C. W., Suarez, D. L., and Saif, Y. M. Genetic and antigenic relatedness of H3 subtype influenza A viruses isolated from avian and mammalian species. Vaccine 26:966-977. 2007.

CHAPTER 6

CONCLUSIONS

Many countries have included vaccination as part of a control strategy in poultry against avian influenza (AI). This virus has a tendency to antigenically drift, in part as a result of vaccination pressure. Therefore, it is important to continue characterizing new isolates in order to monitor them for any changes in their gene sequences and pathogenic phenotypes. In the event that changes requiring precautionary measures (such as culling of poultry or changes of vaccine seed strains) should occur, early detection would allow for appropriate decisions to be made in a timely manner. It is also important to ensure that the vaccines currently in use are effective at protecting infected poultry from disease and decreasing viral load that is shed into the environment. When selecting vaccine seed strains, it would be helpful to have a simple and easy yet reliable method, based on the amino acid sequences of their hemagglutinin (HA) surface glycoproteins. The scope of this dissertation encompasses studies on two separate lineages of subtype H5 AI viruses. In the first section, 19 H5N1 strains of the Asian lineage of highly pathogenic (HP) AI isolated from ducks and chickens in northern Vietnam in 2005 were characterized genetically, antigenically, and biologically, to determine how these viruses compared to other recently reported Asian H5N1s. Furthermore, commercial vaccines that are currently used in Vietnam in poultry against this Asian lineage of viruses were evaluated for their abilities to protect chickens and ducks from disease and viral shedding, upon challenge with the aforementioned HP H5N1 virus isolates. In the second portion, the influence that amino acid

changes that were found in proposed antigenic sites in HAs of drift variants of a Mexican H5N2 parental strain were evaluated for their effects on the antigenicity of this protein.

In the first part of this study, we found that the 19 Vietnamese AI viruses are closely related to other reported isolates from southern China. They cluster with clade 2 viruses, clade 2.3.2 and 2.3.4, and we referred to these 19 isolates as groups B and A, respectively. Since most other Vietnamese viruses isolated prior to these were clade 1, finding that these viruses are of clade 2 was unexpected.

Based on genetic analysis, these viruses contain some, but not all, amino acids that are associated with virulence in mammals. The antigenic surface proteins contained molecular markers of virulence. These viruses also appear to have a binding preference for avian receptors. Both of these traits were demonstrated by the highly pathogenic phenotype that they displayed in both chickens and ducks. The internal genes of these viruses do not contain known markers of virulence, and the likelihood of human infection by these viruses does not appear to be any greater than by other H5N1s that have recently circulated in poultry. Based on our findings, the prophylactic antivirals that are currently available should work efficiently against these viruses.

Differences in HI titers between the two subgroups, A and B, were detected. This antigenic drift further supported the genetic drift demonstrated upon phylogenetic analysis. Greater differences were seen when viruses of other clades or older isolates of the North American lineage were tested. Compilation of the antigenic analysis indicates that genetic drift has occurred not only between these Vietnamese viruses and other previous isolates, but also amongst themselves.

While HP H5N1 viruses are typically highly virulent in chickens, their virulence in ducks can vary from causing asymptomatic infection to being lethal. Pathogenicity in ducks is

dependent on the infecting virus strain as well as the duck breed and age. These Vietnamese viruses caused similar yet more severe lesions in the lungs, hearts, and spleens of ducks than chickens. These 19 isolates were also unique from other H5N1 viruses previously characterized because they were lethal in both two- and five- week- old ducks. Higher titers of these Vietnamese viruses were also shed from the oropharynx of ducks than other strains shown to be pathogenic in ducks. Overall, the viruses characterized in this study were more virulent in ducks than other previous H5N1 HPAIs, based on their increase in tissue tropism, lesion severity, and virus replication. This explains the increased and accelerated mortality observed in ducks that were infected with these viruses.

Based on the evidence of antigenic drift as well as enhanced virulence of these viruses, as particularly demonstrated in ducks, the importance of keeping a close watch on these viruses is underscored. After determining that antigenic drift has occurred in the H5N1 viruses that were circulating in northern Vietnam in 2005, we next tested the commercial vaccines that are currently used to keep these viruses in check.

A vaccination campaign in Vietnam was begun in 2005 in attempt to control the H5N1 AI viruses in poultry. It continues to be used, but outbreaks in poultry and human cases are still being reported. Because ducks account for such a large part of the poultry population in Vietnam and have been included in the vaccination regimen, we tested the vaccines in both chickens and ducks. As challenge virus, we used one representative virus from each Vietnamese subgroup, A or B.

In the chicken study, the N28 vaccine did not work as well as the other vaccines in the birds that were challenged with the DK/VN/203 virus. At two weeks post vaccination, the Mexican vaccine induced the highest HI titers of all the vaccines when homologous antigen was

used, and correspondingly, 100% of the birds that received this vaccine were protected from signs. The Re-1 vaccine induced the second-highest titers of HI antibodies when homologous antigen was used on serum collected two weeks post vaccination. Both the N28 and the homologous vaccines induced suboptimal HI titers in vaccinates. However, while many birds in the N28 vaccine groups had signs, only one bird vaccinated with homologous antigen displayed clinical signs. These results seen in chickens indicate that sequence similarity is not the only determining factor for predicting a vaccine's ability to provide protection against disease and viral shedding. If the HAs of vaccine viruses are not especially close in sequence similarity to the HAs of challenge viruses, but HI antibody titers are high enough, protection against disease can be achieved. On the other hand, even if the HI titers are not at the generally accepted protection level of HI titer of 40, if the sequence similarity of the vaccine and challenge virus HAs are close enough, protection may be rendered. All of the vaccines were able to significantly reduce the titers of virus that were shed compared to infected, negative control birds. However, the N28-vaccinated birds shed significantly higher titers of virus than birds vaccinated with the CK/VN/209 vaccine.

In general, all of the vaccines provided ducks protection from death, although the only vaccinated duck that did die had been vaccinated with the N28 vaccine. The Re-1 vaccine induced significantly higher prechallenge HI titers than either of the other two commercial vaccines, in the DK/VN/203 challenge group. Interestingly, when heterologous antigen was used, the HI test did not detect antibodies from any ducks, but following challenge, HI titers from homologous antigen were several-fold higher than HI titers when heterologous antigen was used. This indicated that the antibodies that provided protection from the challenge strain were not only those induced upon challenge, but were also those produced as the result of a memory

response against the vaccine virus, as well. This demonstrates the important role that vaccines play in protecting poultry from AI challenge. Viral shedding from both the oropharynx and cloaca were detected from all challenged ducks at two and three days post challenge. Although the different vaccines induced similar responses in ducks, the levels and duration of shedding following challenge differed, depending on the challenge virus.

Interestingly, duck HI titers were lower than those in chickens or were completely undetectable (depending on the antigen used in the test). However, the ducks were protected. Further studies on the immune response of vaccinated ducks that have been infected with flu may provide insight into the protective immune mechanism in ducks.

In an effort to understand the molecular basis for antigenic differences between viruses, drift variants isolated throughout a seven-year duration following implementation of a vaccination program in Mexico were analyzed. Amino acid changes located at potential antigenic sites of the variants' HA proteins were accompanied by large antigenic differences, as detected by the HI and virus neutralization tests. Furthermore, vaccines containing the parental virus were unable to prevent virus shedding when the most recently isolated variant served as challenge strain. The second portion of this dissertation addresses the impact that particular amino acid changes had on the antigenicity of the HA molecules of AI viruses of the Mexican lineage. The goal was to decipher which of the amino acid changes were causing the observed antigenic differences in order to lend insight for selecting vaccine viruses in the future.

The approaches taken to detect antigenic differences were the HI and virus neutralization tests, and a vaccine-challenge study on chickens. Collectively, the data from all three approaches inferred that the antigenicity was influenced more by the entire amino acid sequence than the particular amino acid changes that we introduced into the parental HA sequence. The

levels of the acute phase protein, α1-acid glycoprotein, were compared between vaccine groups to determine whether or not their concentrations in serum could be used as markers for predicting the level of protection that a vaccine virus would provide. No differences were seen between any of the groups.

No differences based solely on the amino acid point mutations that were introduced appeared to have an effect on the antigenicity of the HAs of the Mexican lineage of viruses that were used. The factors affecting HA antigenicity of AI viruses are very complex in nature; there are many possible explanations as to why such results were found. In the HI test, hyperimmune, polyclonal HA-specific antisera were used. If there was a more efficient means of producing HA-specific antisera after only one vaccination of the animals, clearer results may be seen. Additionally, monoclonal antisera against a known epitope may also help to distinguish specific amino acids involved in HA antigenicity. Perhaps there are epitopes located on the HA2 subunit, of which we are yet unaware that impacted the results. There also exists the possibility that the changes introduced altered the epitope, but not to a degree to completely abrogate antibody binding. Furthermore, the amino acids that were changed in this study may not be located at the actual antibody-binding sites, and may have an indirect role on influencing the antigenicity of the HA protein. Further studies will need to be performed to first determine if there are any amino acid changes in drift variants located at sites in the HA2 molecule that are antigenic in nature.

In conclusion, AI viruses, particularly those circulating in poultry in northern Vietnam as of 2005, are continuing to antigenically drift and have acquired the ability to be exceptionally virulent in chickens and Pekin ducks. It is very important to continue testing current AI vaccines in their abilities to prevent disease and reduce viral shedding of the current viruses in poultry and

consideration to discontinue using the N28 vaccine virus in Vietnam is encouraged. Finally, an attempt was made to develop an easier and more efficient method for better selecting new AI vaccine seed strains for use in poultry. However, the most reliable methods will most likely remain the more time consuming HI and in vivo studies.