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Antigenic characterization of H3 and H7 avian influenza A virus from migratory waterfowl in North America

By

Laura Elizabeth Bailey

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2015

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Antigenic characterization of H3 and H7 avian influenza A virus from migratory waterfowl in North America

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Avian influenza A viruses pose threats to public health and agriculture stability. Historically, each of the four influenza A viruses responsible for pandemics in the last century contained at least one gene segment of avian origin. Migratory waterfowl are natural reservoirs of influenza A viruses and are capable of widespread dissemination. In this study, we aim to characterize the antigenic profiles of H3 and H7 avian influenza A viruses currently circulating in migratory waterfowl within North America. By understanding the antigenic diversity of these subtypes, we can understand the natural history of influenza evolution and develop potential disease preventive strategies.

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DEDICATION

I would like to dedicate this thesis to my family: My mother, Laura Bailey; my father, Dr. Ennis E. "Chip" Bailey; my sister, Molley Bailey, and Brant McNeece. Thank you for all of your support, encouragement and steadfast love throughout my graduate education. I cannot begin to express the level of gratitude I have for all of you and your patience with me as I began and completed this chapter in my life. I would also like to thank Maggie, Jack and Charlie for their company during late nights studying and writing. I love you all.

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CHAPTER I

REVIEW OF CURRENT LITERATURE

Influenza A virus

The influenza A virus (IAV) is a member of the *Orthomyxoviridae* family of viruses and causes infections in a wide-range of hosts including humans, canine, avian, equine, swine and sea mammal species. IAV is a negative sense, single stranded RNA virus. The genome consists of 8 segments, which encode at least 11 proteins, including hemagglutinin (HA), neuraminidase (NA), RNA polymerase subunit 1 (PB1), RNA polymerase subunit 2 (PB2), RNA polymerase unit (PA), nucleoprotein (NP), two non-structural proteins (NS1 and NS2), and two matrix proteins (M1 and M2). Strains of influenza are designated by the surface glycoproteins, the hemagglutinin (H) and the neuraminidase (N). To date, a total of 18 HA and 11 NA subtypes have been identified. IAV can evade the host immune system by changes of H and/or N proteins, including mutations, which lead to antigenic drift and reassortments, which cause antigenic shift. Antigenic drift poses seasonal threats, and antigenic shift poses occasional pandemic threats. Four documented influenza epidemics include 1918 Spanish Influenza (H1N1), 1957 Asian Influenza (H2N2), 1968 Hong Kong Influenza (H3N2), and more recently the 2009 Pandemic Influenza (H1N1) [1].

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Influenza pandemics in human

The 1918 H1N1 influenza pandemic was responsible for over 40 million deaths including the deaths of troops from the U.S. between the months of September and December [2]. This virus claimed the lives of more people than any other disease outbreaks documented in history [3]. Medical practitioners and nurses also contracted this rampant virus from exposure [4]. Debate arose as to the initial source of the virus, however conclusively it was determined that the root of the Spanish (H1N1) influenza virus begun in the United States [5]. Evolutionary analyses by Dr. Jeffrey Taubenberger's group suggested that the 1918 viruses are genetically similar to the swine influenza viruses reported in the 1930s [5, 6]. What we can learn from the 1918 pandemic is that the influenza virus has ample capability to disseminate quickly and its mortality should not be ignored.

The 1957 Asian Influenza (H2N2) was comprised of novel avian-like hemagglutinin, PB1 and neuraminidase genes [7]. This reassortant virus came after the first influenza virus was isolated from a human infection in 1933 [8]. More recent studies suggest that certain strains of H2 not only carry avian-like properties but these properties were mitigated by a swine-origin. These studies also suggest that these swine-avian reassortant H2 viruses show adaptation to mammals before infecting humans [7]. In 1968, the last case of A/H2N2 was reported in Australia [9] [10], and no human case of H2N2 virus has been reported since. This raises the question of whether or not the current naïve population is at risk should this subtype re-emerge [11].

The 1968 Hong Kong Influenza (H3N2) virus contained genomic segments similar to those of the 1957 H2N2 virus except the hemagglutinin. Between 1968 and

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1969 the United States experienced increased mortality [12] and was the only reported nation affected until an outbreak in England between 1969 and 1970 in which the virus was more severe [13, 14]. Epidemiological data in the United States indicated a large outbreak in 1968 followed by a milder seasonal epidemic in 1969-1970. The remaining four countries; Australia, France, England and Wales, and Japan showed completely different profiles suggesting the initial epidemic was mild followed by a larger epidemic, just opposite of that in the U.S. and Canada [15]. Pandemic patterns associated with the Hong Kong A/H3N2 pandemic represent two geographically distinct mortality patterns: The North American pattern (U.S. and Canada) and the "smoldering" pattern [15].

The 2009 Pandemic Influenza (H1N1) is the first influenza pandemic in this century. Since this novel 2009 H1N1 influenza virus was first isolated from humans in the United States in early April, laboratory confirmed cases were reported in at least four countries until the end of May of 2009 [16, 17] In the United States, until August 2009, 477 deaths associated with the H1N1 virus had been reported to the CDC, 36 of which were children under the age of 18 [17]. The genome of the 2009 H1N1 pandemic virus consisted of NA and M gene segments from the Eurasian lineages of H1N1 swine influenza virus, HA, NP, and NS gene from classical lineages of H1N1 swine influenza virus, PB2, and PA gene segments from a swine triple reassortant, and PB1 from human influenza viruses [18] [19].

Avian influenza virus

Avian influenza A virus (AIV) infects both domestic and wild birds. AIV requires terminal α-2,3 linked sialic acid receptors for binding and proliferation which is found in the intestinal tract of avian species. These α-2,3 linked sialic acid receptors are

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also found in the lower respiratory tract of humans [20, 21]. However, in order for the virus to attach, it has a great distance to travel due to the location within the lower lung and therefore it is not only difficult to contract but consequently difficult to transmit human to human.[22, 23] Avian influenza is separated into two categories based on their ability to cause disease in chickens: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). Low pathogenic avian influenza is generally noted as asymptomatic in birds and do not pose a significant threat to human health [24] . Highly pathogenic avian influenza however disseminates quickly with high fatality in commercial poultry such as chickens and turkeys which can result in a large economic loss. LPAI and HPAI have been detected in the United States and Canada. Factors such as illegal bird trade, exotic bird importation, and migration are all key in the potential introduction of a HPAI in North America. Waterfowl have been named as natural influenza reservoirs [25, 26] as they are constantly hosting and shedding the virus. Considering their role in dissemination of virus, it is important to monitor their migrations as well as any antigenic changes which may arise from isolated avian influenza viruses.

Effect on industry

As of January, the USDA reported that broiler export forecasts have dropped 40 million pounds since their December forecast due to the temporary ban of U.S. poultry imports to South Korea and China, due to a reported avian influenza outbreak in the northwestern United States [27].

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Low pathogenicity avian influenza in the United States

In 1975, a LPAI (H5N1) virus was detected in two waterfowl in Wisconsin during routine sampling and was isolated as A/mallard/Wisconsin/428/1975 (H5N1). According to the WHO, the 1983 LPAI (H5N1) Pennsylvania outbreak took two years to control after it invaded the Pennsylvania poultry industry. The virus was isolated from ring-billed gulls, a species of waterfowl. One year later, H5N2 (HPAI) was reported in turkeys and chickens in the same area. The WHO reported a direct cost of $62 million to euthanize 17 million birds and further estimates the final indirect cost to be greater than $250 million. In addition, there was a reported 30% increase in the cost of eggs as a direct result.

In addition, four smaller outbreaks occurred to include: 1981 and 1985 LPAI (H5N1) in Minnesota; 1986 LPAI (H5N1) was confirmed in a wild mallard in Ohio during routine sampling; and in 2005 LPAI (H5N1) was confirmed in Manitoba, Canada.

In 2006, LPAI (H5N1) was detected in two mute swans in Michigan and mallard ducks in Maryland and Pennsylvania. According to the USDA Fact Sheet, Delaware reported LPAI (H5N1) in green-winged teals in conjunction with USDA avian influenza surveillance sampling.

High pathogenicity avian influenza in the United States

A 2004 HPAI (H5N2) outbreak in Texas chickens was contained and eradicated and resulted in minimal economic loss. [28].This outbreak reported no transmission of HPAI H5N2 to humans. [29] [28] No HPAI (H5N1) virus has been detected in the U.S. as of 2014. However, the HPAI H5N2 and HPAI H5N8 have been reported in Texas and California in 2014, respectively.

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Most recently, a California commercial turkey flock was reported having HPAI H5N8 in January 2015. Amid this outbreak, no human cases have been detected and the facility in which the outbreak occurred has been quarantined.

Ecology of avian influenza virus

Surveillance studies indicate dabbling ducks host the most wide-range of HA subtypes [30]. Dabbling ducks are defined as ducks which feed on vegetation beneath the water surface. Mallards are the most common of the dabbling ducks associated with transmission of AIV. A Positive correlation between AIV ecology and seasonality has been identified and is consistent with the migration patterns exhibited in migratory waterfowl [31] [32] [33]. As previously mentioned, AIV differs from influenza A virus in humans in the preferential binding and transmission route. In AIV, the virus preferentially binds to terminal α-(2,3) sialic acid receptors which are commonly found in the intestinal tract of waterfowl which is why cloacal swabbing is the preferred route of sampling. [34, 35]

Life Cycle

The surface protein hemagglutinin is responsible for initial binding to host sialic acid residues. Once bound, the virus is endocytosed by the host cell plasma membrane. Fusion with intracellular endosomes occurs immediately after endocytosis. A drop in pH triggers the viral envelope to fuse with the endosome membrane [36] [37]. The low pH induces a conformational change in the hemagglutinin resulting in movement of the HA2 subunit to better attach to the host membrane [38] [39]. The virus gains access to the

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cytosol via formation of a fusion pore in which can translocate the viral RNA [40]. It is in the cytoplasm that the viral RNPs are released from the endosome. [41]

Persistence in the environment

Direct contact with AIV infected waterfowl or poultry is the most common route of exposure, however environmental contamination of AIV is also responsible for indirect transmission of AIV to other avian species.[42] [43]. Avian influenza viruses have been isolated in duck meat [44] [45] bodies of water [46] [47] [48] [49], lake sediment [50], duck feathers [51], and duck feces[52, 53]. The low temperature profile assists with thermostability of the virus and promotes longevity in the lifespan of the virus in the environment. It has been noted that AIV in lake water has a shorter life span than that of AIV in sediments [54]

Migration influence

Migratory waterfowl travel trans-continentally on an annual basis. The ability for migratory waterfowl to serve as a vector for trans-continental dissemination of AIV raises concern about their role in pandemic potential. The major flyways of importance are East Asia/Australian flyway, Central Asia flyway, East Africa West/Asia flyway, Black Sea/Mediterranean flyway, East Atlantic flyway, Atlantic Americas flyway, Pacific Americas flyway, Mississippi flyway, and the Central flyway. There is overlap between the Pacific Americas flyway and the East Asia/Australian flyway in which the migration occurs across the Pacific Ocean initiating in east Asia and moving into Alaska [55] [56] and northwest Canada.[57] The Central Asia flyway does not span trans-continentally however it does overlap with the Black Sea/Mediterranean flyway, East Africa/West Asia

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flyway, the East Asia/Australian flyway, and the East Atlantic flyway rendering it an influential flyway regarding dissemination of AIV. [58] The East Atlantic flyway covers Northeast Canada, West Africa, and North Asia as well as Iceland. Due to the overlap in flyways, the movement of non-native AIVs becomes a serious concern especially from China to the pacific coast of North America.[59] This inter-continental movement explains the presence of various "type" lineages within subtypes, particularly H6. H6 in North America has been liked to both North American-like lineages as well as Eurasian-like lineages. [60]

Influenza Surveillance in the United States

Current surveillance efforts in North America cover wintering grounds of migratory waterfowl [61] as well as areas with heavy populations of commercial poultry, paying particular attention to where these two overlap. [62] In order to generate up-to-date vaccines, surveillance efforts must be continuous to help identify antigenic similarities as well as the inclusion of some variant strains. Long-term use of a vaccine can lead to antigenic drift [63] which renders the vaccines ineffective and the host unprotected. Using insufficient vaccine strategies can lead to a widespread infection of AIV resulting in large economic losses. It is suggested that vaccine strains should be tested against new circulating viruses every 2-3 years [64].

Currently, there are three applied methods for poultry vaccination: inactivated AIV, live wild-type LPAIV and live attenuated LPAIV. The inactivated AIV vaccinates against H5 and H7 subtypes and covers a broad range of species to include chickens, turkeys, duck, geese, and zoo birds [65]. The inactivated AIV vaccine is administered sub-cutaneous, intra-muscular, or in ovo. The two classes of live AIV vaccines are only

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approved for chickens and both cover H5 and H7 subtypes. The live wild-type LPAIV vaccine can be administered intra-muscular or intra-nasal via a spray. The live attenuated LPAIV vaccine can only be administered via intra-nasal spray. [66]

Techniques for influenza diagnosis and surveillance

Viral propagation

Avian influenza viruses are propagated in 9-11 day old embryonated chicken eggs as devised by Frank Macfarlane Burnet in the 1930s. (Burnet and Bull, 1943) This method allows viral replication in the allantoic cavity of an embryonated chicken egg. While some avian influenza viruses may replicate in mammalian cell lines, the most effective measure is the use of embryonated chicken eggs due to the presence of sialic-acid α-(2,3) receptors in the allantoic cells. [67] The limitations to allantoic fluid as a means for viral isolation is the generation of egg-adapted mutations. Egg-adapted mutations have specific signatures and acquire specific substitutions during egg-adaptation. [68, 69] Currently, the major representative sites of egg-adapted mutations in the hemagglutinin surface protein occur at amino acid positions 145, 156, 163, 186, 189, 190, 225, and 246. [68]

Sera generation

For this study, serum was generated in-house by intranasal inoculation volume of 100uL containing 10^6 TCID50 avian influenza virus into specific pathogen free (spf) chickens at three weeks post-hatch. At five weeks post-hatch, the chickens were boosted with an additional intranasal inoculation volume of 100uL which contained 10^6 TCID50. Two weeks post-boost, chickens were euthanized in accordance with MSU-CVM IACUC

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regulations and 12-15mL blood was removed directly from the heart. The blood was allowed to clot and then centrifuged at 2,000rpm for 10 minutes in order to separate the serum from the red blood cells. After centrifugation, serum was removed and aliquots stored at -20°C.

Hemagglutination assay

Hemagglutination assays are a widely-used tool in influenza research in which viral isolates are measured by their ability to agglutinate red blood cells. The antigen-red blood cell interaction forms a lattice which is termed hemagglutination. Influenza virus actively agglutinates red blood cells, therefore a positive identification of a viral isolate can be confirmed by its ability to agglutinate red blood cells. Various red blood cell types used include chicken, turkey, guinea pig, horse, and beagle. Between 1990 and 2003 several H3N2 IAVs were found to have lost the ability to bind chicken red blood cells due to a mutation in the HA [70].

Hemagglutination inhibition assay

Hemagglutnation inhibition assay has often been referred to as the gold-standard of influenza serology techniques. Antisera are serially diluted and 4 HA units of virus are added to each well of the diluted sera. Because the sera is diluted and the virus is not, it provides a visualization of antigen:antibody reactivity by showing hemagglutination inhibition. Hemagglutination inbition is defined as the inability of the virus to agglutinate the red blood cells being used. Therefore, the formation of a lattice indicates the virus was able to agglutinate the red blood cells while the presence of a tear dropping pattern indicates the inhibition of agglutination.

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The main objectives of this study

The main objective of this study is to identify the presence of antigenic similarities among H3 and H7 AIV from migratory waterfowl in North America while also differentiating between varying antigenic lineages. This study aims to define the antigenic properties of these virus subtypes in an effort to provide a risk-assessment for current AIVs circulating in North America which may come into contact with commercial poultry as well as propose the shift to new vaccines more representative of the circulating AIVs which pose considerable threat.

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CHAPTER II

ANTIGENIC CHARACTERIZATION OF H3 AVIAN INFLUENZA A VIRUS FROM MIGRATORY WATERFOWL IN NORTH AMERICA.

Abstract

Besides humans, H3 subtypes of influenza A viruses (IAVs) can infect various animal hosts including avian, swine, equine, canine, and sea mammals. These H3 viruses are both antigenically and genetically diverse. Here we characterized the antigenic diversity of contemporary H3 avian IAVs recovered from migratory birds in North America. Hemagglutination inhibition (HI) assays were performed on 37 H3 isolates of avian IAVs recovered from 1999 to 2011 using generated reference chicken sera. These isolates were recovered from samples taken in the Atlantic, Mississippi, Central, and Pacific waterfowl migration flyways. Antisera to all the tested H3 isolates cross-reacted with each other, and, to a lesser extent with those to H3 canine IAVs and H3 equine IAVs. Antigenic cartography showed that the largest antigenic distance among the 37 avian IAVs is about 4 units, and each unit corresponds to a 2log2 difference in the HI titer. However, none of the tested H3 AIVs cross-reacted with ferret sera derived from contemporary swine and human IAVs. Our results showed that these H3 avian IAVs we tested lack significant antigenic diversity, and these viruses are antigenically different from those viruses circulating in swine and human populations. This suggests that H3 avian IAVs in North American waterfowl are antigenically relatively stable.

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Introduction

The influenza A virus (IAV) causes a pandemic disaster impacting human health on multiple continents and then persist causing seasonal influenza epidemics [71, 72]. Four documented influenza A pandemics occurred in the last hundred years, 1918, 1957, 1968, and 2009. About 50 million people died of the 1918 H1N1 influenza A pandemic [73, 74]. The annual inter-pandemic influenza season in the northern hemisphere, from October to April, results in over 200,000 hospitalizations and up to 49,000 deaths annually in the United States [75-77].

IAVs have been recovered from at least 105 wild bird species from 26 different families [78, 79]. Among these birds, those living in wetland and aquatic environments (e.g., *Anseriformes* spp., particularly ducks, geese, and swans, and *Charadriiformes* spp., particularly gulls, terns, and waders) are by far the major source of these isolates and are generally accepted as a major natural IAV reservoir [24, 80]. Infected wild birds, especially migratory waterfowl, are believed to serve as a bridge facilitating the spread of IAVs between different wild avian species and different geographic locations, and are one source for viral transmission into domestic poultry [80, 81], swine [82], and humans [83].

The IAV has 8 genomic segments (segment 1-8) encoding for at least 11 proteins: PB2 by segment 1, PB1 and PB1-F2 by segment 2, PA by segment 3, hemagglutinin (HA) by segment 4, nucleoprotein (NP) by segment 5, neuraminidase (NA) by segment 6, matrix proteins M1 and M2 by segment 7, and nonstructural protein NS1 and NS2 by segment 8. To date, 18 HA and 11 NA subtypes have been reported in IAVs [84] although no isolates have been recovered for H17 and H18 subtypes. Antigenic changes

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in the HA and NA, the two surface glycoproteins, are mainly due to accumulating point mutations resulting in antigenic drift or genomic reassortment resulting in antigenic shift. These changes in the HA are especially important as they allow an IAV to evade the herd immunity established from previous influenza infections or vaccination [21].

The H3 IAVs have very diverse host ranges in addition to infecting many species of birds they are widely found in mammals. After the emergence of the A/Hong Kong/1968 (H3N2) and 1968 pandemic this strain of IAV became endemic after the first year and has since been causing yearly seasonal epidemics in humans. The H3 IAVs maintained in infect swine, equine, canine, and avian species and cause sporadic outbreaks in sea mammals. H3N2 is one of the predominant IAVs infecting both domestic swine and feral swine [85-87]; H3N8 was shown to infect seals [88]; H3N8 is endemic in domestic canine in North America and equine worldwide [89, 90]; and the avian origin H3N2 infected domestic dogs in Asia [91, 92].

Previous studies documented substantial antigenic diversity in the H3 IAV in mammals [87]. Since 1968, the extent of antigenic drift has led to more than 28 updates of the H3N2 components in the seasonal influenza vaccines [93, 94]. In swine, the H3N2 IAVs are genetically and antigenically diverse. Four genetic clusters of H3N2 IAVs (clusters I–IV) have been identified in United States swine populations [82, 94-96], and the viruses in these four H3N2 clusters are antigenically different from each other. In addition, Cluster IV, which has become predominant among the United States swine population, has further evolved into two antigenic clusters: H3N2SIV-α and H3N2SIV-β [87]. Additionally, both H3N2 and H3N8 canine IAVs are antigenically distinct from contemporary human influenza viruses [97].

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In wild migratory birds, H3 is one of the more frequent hemagglutinin subtypes recovered in IAV surveillance projects [33]. However, the antigenic diversity of these H3 viruses is not well characterized. This study aims to characterize the antigenic diversity of contemporary H3 IAVs recovered from migratory birds in North America. This information helps us understand the natural history of influenza evolution and may also help improve knowledge on influenza prevention and control.

Materials and Methods

Viruses

A total of 37 H3 isolates (H3N1, H3N2, H3N6, H3N7, H3N8, or H3N9) recovered from migratory birds in North America from 2007 to 2011 were included in this study (Table A.2). These viruses were recovered from American black duck (*Anas rubripes*), mallard (*Anas platyrhynchos*), ring-necked duck (*Aythya collaris*), hooded merganser (*Lophodytes cucullatus*), northern pintail (*Anas acuta*), snow goose (*Chen caerulescens*), blue-winged teal (*Anas discors*), and long-tailed duck (*Clangula hyemalis*). These H3 isolates were recovered from samples collected in Canadian locations in Nova Scotia, New Brunswick, Nunavut, and Prince Edward Island, as well as the US states of Maine, New Hampshire, North Dakota, Colorado, Oregon, Washington, Wisconsin, Iowa, Maryland, and New York, encompassing regions across the Atlantic, Mississippi, Central, and Pacific Bird Migratory Flyways in North America. In addition, one H3N8 isolate from a harbor seal (*Phoca vitulina*), A/harbor seal/New Hampshire/2011 (H3N8), was also included in this study. These viruses were recovered and propagated in 9-11 day-old specific pathogen free (SPF) embryonated chicken eggs before serological characterization.

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Sera

A total of 37 H3 isolates (H3N1, H3N2, H3N6, H3N7, H3N8, or H3N9) recovered from migratory birds in North America from 2007 to 2011 were included in this study (Table 1). These viruses were recovered from American black duck (*Anas rubripes*), mallard (*Anas platyrhynchos*), ring-necked duck (*Aythya collaris*), hooded merganser (*Lophodytes cucullatus*), northern pintail (*Anas acuta*), snow goose (*Chen caerulescens*), blue-winged teal (*Anas discors*), and long-tailed duck (*Clangula hyemalis*). These H3 isolates were recovered from samples collected in Canadian locations in Nova Scotia, New Brunswick, Nunavut, and Prince Edward Island, as well as the US states of Maine, New Hampshire, North Dakota, Colorado, Oregon, Washington, Wisconsin, Iowa, Maryland, and New York, encompassing regions across the Atlantic, Mississippi, Central, and Pacific Bird Migratory Flyways in North America. In addition, one H3N8 isolate from a harbor seal (*Phoca vitulina*), A/harbor seal/New Hampshire/2011 (H3N8), was also included in this study. These viruses were recovered and propagated in 9-11 day-old specific pathogen free (SPF) embryonated chicken eggs before serological characterization.

Hemagglutination assay (HA) and hemagglutination inhibition assay (HI)

The HA and HI assays were performed according to OIE guidelines [98] using 1% chicken red blood cells (Lampire Labs, Pipersville, PA).

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Phylogenetic analysis and molecular characterization.

Multiple sequence alignments of H3 subtype of hemaglutinin genes were created by using the MUSCLE software package [99]. Phylogenetic analyses were performed using maximum likelihood by the GARLI version [100], and bootstrap resampling analyses were conducted using PAUP* 4.0 Beta [101] with a neighborhood joining method, as previously described [102].

Analysis of antibody binding sites

To analyze the sequence diversity among the antibody binding sites of hemaglutinin protein, sequence diversity profiles were generated for antibody binding sites in the viruses we studied using WebLogo 3 webserver [103]. Antibody binding sites were annotated based on previous studies [104], and they were mapped in a three dimensional structure of hemaglutinin protein using the PDB entry 1MQL [105] as the template. In addition, a total of 1,265 hemaglutinin protein sequences for North America avian origin H3 IAVs were retrieved from the NCBI Influenza Virus Database [106], and their sequence logos for antibody binding sites were also analyzed.

Results

Phylogenetic analysis

Phylogenetic analysis showed that the H3 genes of avian IAVs in North America formed two genetic clusters, and both were genetically distinct from Eurasian H3 avian IAVs from wild birds (0). The HA genes of H3 avian IAVs from North America we tested were distributed throughout the branch containing other IAVs from public databases, indicating the viruses we selected were genetically representative strains for

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contemporary H3 avian IAVs in North America. These viruses were genetically distinct from the H3 IAVs from human, equine, and swine (0). The isolate from harbor seal was genetically close to lineage I of North American H3 avian IAVs.

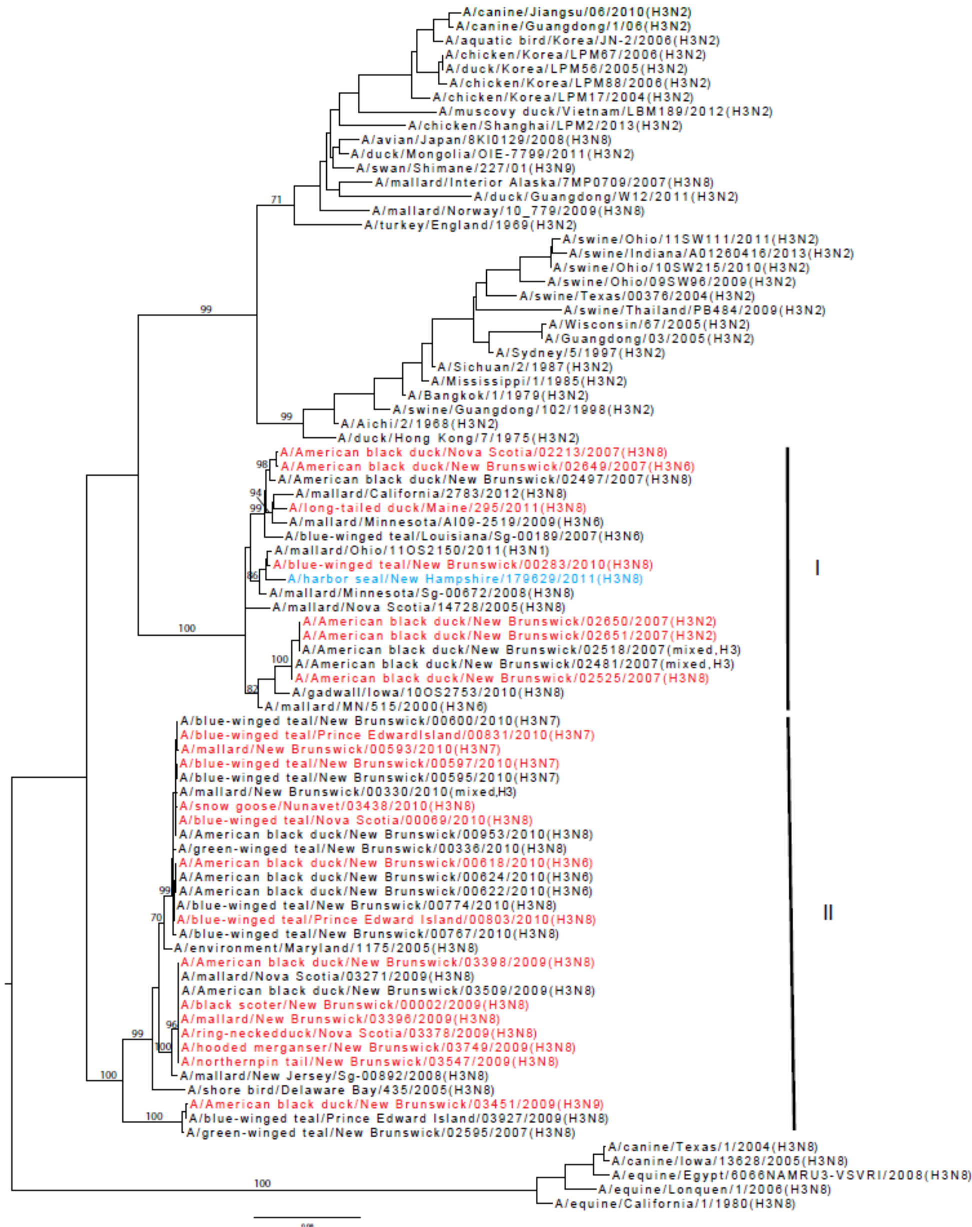

Figure 2.1 Phylogenetic analysis of H3 AIV in North America

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There is lack of antigenic diversity among H3 AIVs in North America

Our results demonstrated that all sera raised to the avian H3 IAVs tested were highly cross-reactive with each other (Table A.1). Antigenic cartography showed that all the viruses were antigenically similar to each other (0). The average antigenic distance in the cartography was estimated to be 0.99 units (standard deviation 0.62 units). The maximum antigenic distance observed among these viruses was 3.62 units, and occurred between virus A/mallard/Washington/A00461816/2008(H3N?) and A/mallard/Oregon/A00282275/2007 (H3N8) (0).

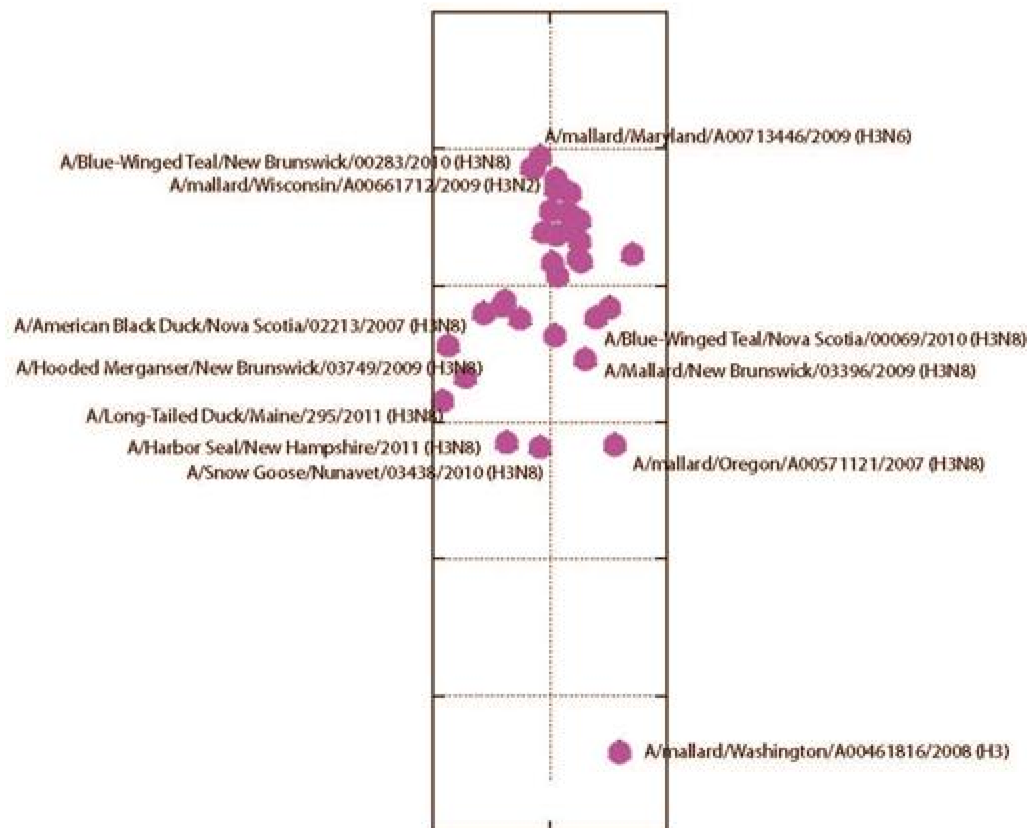

Figure 2.2 Antigenic cartography for H3 AIVs from migratory waterfowl in North America (2007-2011) based on HI data.

Antigenic cartography was constructed using AntigenMap
http://sysbio.cvm.msstate.edu/AntigenMap

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H3 AIVs are antigenically distinct from H3 subtypes of IAVs from human, swine, and canine species

To test the antigenic divergence of H3 avian IAVs from North American migratory birds and from other hosts, we performed HI experiments on the ferret sera raised to H3N2 viruses, representing antigenic clusters from 1979 to 2002 human IAVs, eight H3N2 influenza viruses representing both H3N2-*alpha* and -*beta* circulating in swine populations, and H3N2 and H3N8 canine influenza viruses. Results showed that none of the H3 avian IAVs cross-reacted with the ferret sera against H3N2 human viruses (data not shown) or the ferret sera against H3N2 swine viruses (Table A.5). The ferret sera against H3N8 canine influenza virus had a HI titer of 1:40 against 3 of the 37 tested isolates whereas all these viruses were negative against the ferret sera against H3N2 avian origin canine influenza viruses (Table A.4)

Two-way HI analyses showed that the sera to the seal isolate cross-reacted well with all the 37 avian H3 IAVs tested, with a minimum titer of 1:8 and a maximum titer of 1:128.

Molecular characterized of antibody binding sites in avian H3 IAVs.

To identify the potential variations of antibody binding sites of H3 avian IAVs, we compared the reported five antibody binding sites A, B, C, D, and E, which were annotated by [104], for all H3 avian IAVs from North America in the public database (0). Results showed that there is lack of divergence of these residues in these five reported sites (0). We further characterized the profiles of the antibody binding sites of the 37 IAVs evaluated here, and compared them with those from the public database. Results

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showed that the antibody binding sites of the viruses we tested are similar to those from the public database (0).

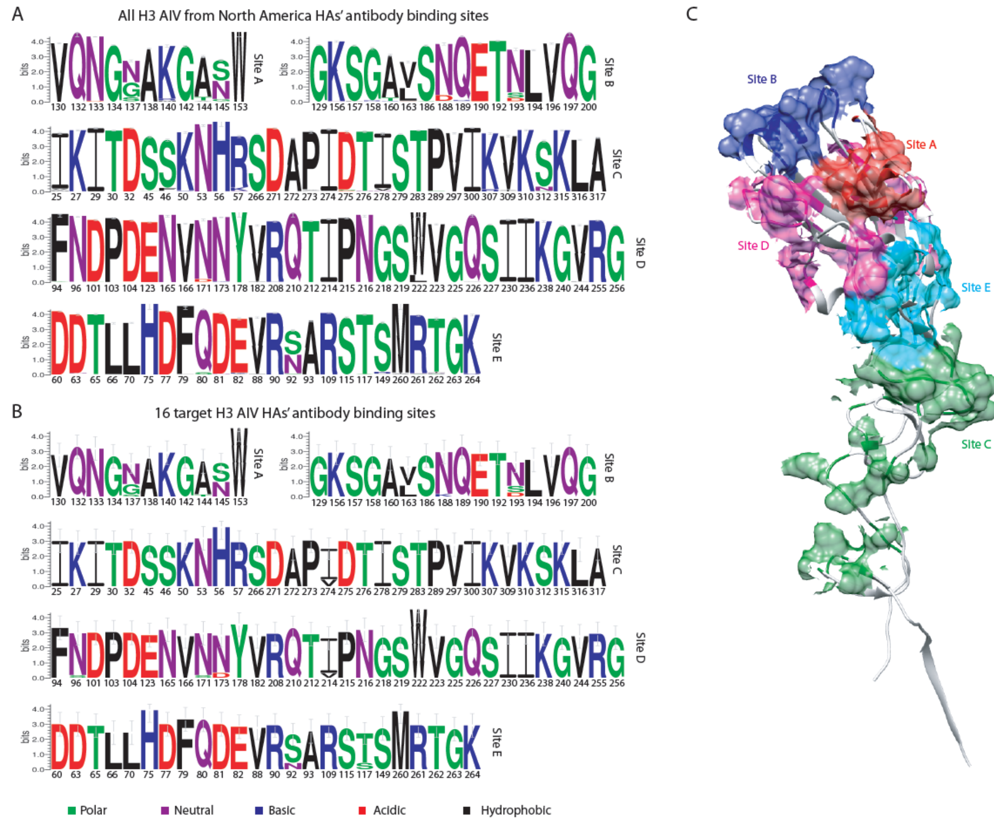

Figure 2.3 Conservation among the reported antibody binding sites among the HA protein of H3 avian influenza viruses from North America:

(A) Amino acid variations of antibody binding sites on all H3 AIVs from North America; (B) Amino acid variations of antibody binding sites on 16 of the 37 target H3 AIV HAs; and (C) A structural model of H3 HA with antibody binding sites mapped. The antibody binding sites were annotated by [104]. The conservation analysis were characterized using WebLogo 3 webserver [103]. The locations of these antibody binding sites were visualized using Chimera [107] based on the template of PDB entry 1MQL [105].

Discussion

Sturm-Ramirez et al. proposed that the evolution of IAVs in waterfowl is static [108]. However, other studies have shown that the H5N1 IAVs circulating in ducks have

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rapidly evolved [108, 109]. A recent serological surveillance of wild birds suggested that wild birds may have been exposed to multiple, antigenically diverse H5 IAVs [110]. Thus, understanding the antigenic diversity of avian IAVs in migratory waterfowl is critical for understanding natural history of these viruses.

This study evaluated H3 viruses from 2007 to 2011, and those from all four migratory flyways in North America. Thus, we considered these viruses to be representative of the antigenic diversity of H3 IAVs in migratory birds. Antigenic characterization of the 37 IAVs in this study demonstrated that there is lack of antigenic diversity for contemporary H3 IAVs in migratory birds. Such results suggest that H3 IAVs probably had been exposed to little selective pressure for antigenic evolution, supporting the previous concept on the static evolution of H3 viruses in migratory waterfowl [108].

Waterfowl serve as reservoirs for IAVs and, consequently, IAVs emerging from these species could potentially threaten human health. Genetic reassortments can lead to antigenic shift and the generation of epidemic and pandemic influenza strains. At least two of four documented pandemic viruses had HA genes of avian origin [111-113]. The 1957 H2N2 pandemic strain probably emerged from the avian H2N2 IAV and H1N1 human influenza IAV. The 1968 H3N2 pandemic strain was likely a reassortment of the avian H3 IAV and the human H2N2 IAV. Thus, the antigenically distinct HA genes of avian IAVs can contribute to generating a novel pandemic virus through antigenic shift. This study demonstrated that the contemporary H3 avian IAVs in migratory birds are antigenically distinct from those causing seasonal outbreaks in humans as well as swine, and thus, there is lack of immunological protection in human population against these H3

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avian IAVs. These H3 avian IAVs could potentially lead to future human outbreaks either through direct or indirect spill-over transmission to humans.

In addition, there are genetically diverse IAVs from wild birds, and characterization of the transmissibility of these viruses in mammals is very limited. Although some studies showed that avian IAVs have little transmissibility in mammals [114], a more recent study demonstrated that an H3N8 avian IAV isolated from a harbor seal could be transmitted among ferrets through aerosol droplets [88]. This same strain was shown to have antigenic properties similar to those from migratory waterfowl but distinct from human viruses. Further studies are needed to compare the antigenic divergence of H3 IAVs in North America to those from other regions of the world, especially Eurasia.

In summary, this study showed that H3 IAVs from migratory waterfowl in Northern America lack antigenic diversity and that these viruses are antigenically distinct from those contemporary H3 IAVs in humans, swine, and canine. Although we included H3 IAVs with diverse geographic and temporal coverage, it is likely some H3 antigenic variants in migratory birds could have been missed. Additional studies are needed to compare antigenic diversity of waterfowl origin IAVs to those from domestic poultry, especially those in the areas where H3 vaccination is used in turkeys [115].

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CHAPTER III

ANTIGENIC CHARACTERIZATION OF H7 AVIAN INFLUENZA A VIRUS FROM MIGRATORY WATERFOWL IN THE UNITED STATES

Abstract

H7 subtypes of avian influenza A viruses (AIV) have caused at least 500 human cases. Highly pathogenic H7 AIVs could cause devastating diseases in domestic poultry, and there were more than 10 sporadic outbreaks in poultry during the past two decades. Understanding antigenic diversity of H7 AIVs is critical for developing effective strategies for disease prevention and control. In this study, a total of 94 isolates were characterized antigenically, including 87 migratory waterfowl origin isolates (1976 to 2010) and 7 representative isolates from domestic poultry (1971 to 2012). Geographically, these isolates were recovered from Canada, Mexico, and 29 states in the United States. The HI assays were conducted against a panel of 21 representative chicken reference sera. Serological analyses showed that the 94 isolates cross-reacted with each other to different extents. Antigenic cartography analyses showed that the average antigenic distance was 1.06 (±0.68) units and that the maximum antigenic distance was 3.21 units, each unit corresponding to a 2-fold change in HI titers. The H7 isolates from domestic poultry were antigenically similar to those from migratory waterfowl; no clear correlation between antigenic diversity and temporal order was observed. Our results suggested that there is limited antigenic diversity among the H7 isolates we tested. These

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findings would be useful in pandemic preparedness against the emerging H7 outbreaks as well as selection of vaccine strains against potential H7 outbreaks in domestic poultry in North America.

Introduction

H7 subtypes of avian influenza A viruses (AIV) have caused at least 500 human cases. Highly pathogenic H7 AIVs could cause devastating diseases in domestic poultry, and there were more than 10 sporadic outbreaks in poultry during the past two decades. In North America, H7 low pathogenic avian influenza A viruses (LPAIVs) have been identified in the the live-bird markets from 1994-2006 [116, 117]. Frequent cases of H7 subtype influenza infections were documented in both domestic poultry and humans. In 2002, one confirmed H7N2 viral infection occurred in Virginia in a poultry worker with direct contact to infected chickens and turkeys. In 2003, another human infection of LPAI H7N2 was confirmed in New York, though the source was unknown [118] [119]. In 2004, two cases of human infection of H7N3 LPAI in Canada were reported to be caused by contact with infected poultry. [120, 121] However, there is still lack of evidence showing human-to-human transmission of H7 viruses. [118] [122]

Waterfowl are the natural reservoirs of influenza A viruses, and H7 is one of frequent subtypes identified in migratory waterfowl, having supplied the sources of emergence of H7 viruses in domestic poultry. Understanding antigenic diversity of H7 AIVs is critical for developing effective strategies for disease prevention and control. The objective of this study is to characterize the antigenic diversity of H7 viruses in migratory poultry and compared it with those H7 viruses in domestic poultries.

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Materials and Methods

Virus

A total of 94 isolates were characterized antigenically, including 87 migratory waterfowl origin isolates (1976 to 2010) and 7 representative isolates from domestic poultry (1971 to 2012). Geographically, these isolates were recovered from Canada, Mexico, and 29 states in the United States.

Viral propagation

The viruses were propagated using 9 day old spf embryonated chicken eggs (sunset farms). Eight to ten eggs were used per virus. After eggs were inoculated, they were incubated at 37°C for 72 hours. At 72 hours the eggs were transferred to 4°C for 24 hours to euthanize the embryos. After the 24 hour euthanization, the egg shells were removed at the air sac and the allantoic fluid was removed from each egg via 18G ½' needle and 10mL syringe and transferred to 15mL conical tubes. Conical tubes were then centrifuged at 2,000 x rpm for 10 minutes and supernatant was determined to be positive or negative for viral replication based on hemagglutination assay. Allantoic fluid of different eggs of the same virus were combined.

Generation of reference sera

Twenty-one of the 78 isolates were selected to generate chicken-sera which were used in the HI assay based on subtype diversity, species diversity, location and temporal range, including A/bufflehead/Virginia/A00120022/2008 (H7N2), A/mallard/New Jersey/A00122457/2008 (H7N8), A/mallard/Wisconsin/A00465618/2008 (H7N3), A/American green-winged teal/Colorado/A00551331/2007 (H7N3), A/American black duck/Deleware/A00870108/2010 (H7N3), A/mallard/Montana/A00750842/2009 (H7N3),

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A/mallard/Nebraska/A00709567/2009 (H7N3), A/American green-winged teal/Arizona/A00115995/2009 (H7N7), A/mallard/Iowa/A00558620/2008 (H7N3), A/mallard/Indiana/A00412205/2008 (H7N3), A/northern shoveler/Oklahoma/A00744384/2009 (H7N3), A/American green-winged teal/Colorado/A00660616/2008 (H7N3), A/American green-winged teal/Utah/A00461136/2009 (H7N1), A/blue-winged teal/Missouri/A00624484/2008 (H7N3), A/domestic duck/West Virginia/A00140913/2008 (H7N3), A/northern shoveler/Mississippi/A00630207/2009 (H7N6), A/northern shoveler/Utah/A00461133/2009 (H7N4), A/ring-necked duck/Texas/A00766403/2009 (H7N1), A/blue-winged teal/South Dakota/A00772794/2009 (H7N7), A/mallard/New York/A00723400/2009 (H7N4), and A/northern shoveler/Mississippi/A00769951/2010 (H7N3). The chicken specific sera were generated against these sera, six birds per virus.

Hemagglutination assay, Hemagglutination Inbition assay, Antigenic cartography

See Chapter II.

Results

The hemagglutination inhibition (HI) assays were conducted for all 94 H7isolates against a panel of 21 representative chicken reference sera. Serological analyses showed that the 94 isolates cross-reacted with each other to different extents. Antigenic cartography analyses showed that the average antigenic distance was 1.06 (±0.68) units and that the maximum antigenic distance was 3.21 units, each unit corresponding to a 2-fold change in HI titers. The H7 isolates from domestic poultry were antigenically similar to those from migratory waterfowl; no clear correlation between antigenic diversity and

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temporal order was observed. Our results suggested that there is limited antigenic diversity among the H7 isolates we tested. These findings would be useful in pandemic preparedness against the emerging H7 outbreaks as well as selection of vaccine strains against potential H7 outbreaks in domestic poultry in North America.

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Table 3.1 Hemagglutination inhibition data

Virus	Chicken serum																
	BUFF 120022	MAL L122457	MAL L465618	AGW T551331	ABD U870108	MAL L750842	MAL L709657	AGW T115995	MAL L558620	MAL L142205	AGW T660616	AGW T461136	BWT E624484	DUC K140913	RND U766403	BWT E772794	NSHO 769951
A/bufflehead/Virginia/A00120022/2008 (H7N2)	**80**	80	80	160	320	80	80	80	80	640	160	320	80	80	160	320	320
A/mallard/New Jersey/A00122457/2008 (H7N8)	80	**80**	80	80	160	80	40	20	80	160	80	80	40	20	160	320	160
A/mallard/Wisconsin/A00465618/2008 (H7N3)	80	160	**160**	160	160	160	40	40	80	160	80	320	80	40	320	320	160
A/American green winged teal/Colorado/A00551331/2007 (H7N3)	80	160	80	**80**	160	80	40	20	40	160	80	160	40	20	160	320	80
A/American black duck/Deleware/A00870108/2010 (H7N3)	80	80	80	160	**160**	80	80	80	160	160	80	160	40	40	320	640	160
A/mallard/Montana/A00750842/2009 (H7N3)	160	160	320	320	320	**320**	160	160	160	320	80	640	40	40	320	640	320
A/mallard/Nebraska/A00709657/2009 (H7N3)	80	320	80	80	160	80	**160**	160	80	160	160	320	80	80	320	640	160
A/American green winged teal/Arizona/A00115995/2009 (H7N7)	20	80	40	40	80	40	40	40	80	80	40	80	20	20	80	160	80
A/mallard/Iowa/A00558620/2008 (H7N3)	20	80	40	40	40	40	40	20	40	80	40	80	20	20	80	160	80
A/mallard/Indiana/A00412205/2008 (H7N3)	160	160	160	160	160	160	160	160	40	**80**	40	160	40	20	160	320	160
A/northern shoveler/Oklahoma/A00744384/2009 (H7N3)	80	320	80	80	160	80	80	80	320	160	80	160	40	40	160	320	80
A/American green winged teal/Colorado/A00660616/2008 (H7N3)	40	160	40	80	160	40	40	40	40	160	40	320	40	20	320	320	160
A/American green winged teal/Utah/A00461136/2009 (H7N1)	20	160	160	160	160	40	40	40	40	160	40	**160**	40	40	80	160	160
A/blue winged teal/Missouri/A00624484/2008 (H7N3)	160	160	160	160	160	160	160	160	80	320	160	640	**160**	80	640	1280	160
A/domestic duck/West Virginia/A00140913/2008 (H7N3)	80	80	80	80	80	80	40	40	40	80	80	80	40	40	80	80	80
A/northern shoveler/Utah/A00461133/2009 (H7N4)	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80
A/ring-necked duck/Texas/A00766403/2009 (H7N1)	40	160	80	80	160	80	40	40	40	160	80	160	40	40	**160**	320	80
A/blue winged teal/South Dakota/A00772794/2009 (H7N7)	160	640	320	320	320	320	320	320	640	640	320	640	160	160	640	**640**	320
A/mallard/New York/A00723400/2009 (H7N4)	40	80	80	160	160	80	40	40	64	160	80	80	40	40	160	160	80
A/northern shoveler/Mississippi/A00769951/2010 (H7N3)	160	320	160	160	160	160	160	40	160	320	40	320	40	20	320	640	**160**
A/northern shoveler/Utah/A00374996/2007 (H7N3)	80	160	160	160	160	160	80	40	80	160	80	320	40	40	320	320	160
A/American green winged teal/Arizona/A00115994/2009 (H7N3)	40	80	80	160	80	80	80	40	80	160	40	160	40	40	160	160	80
A/American green winged teal/Mississippi/A00468514/2009 (H7N7)	160	160	320	320	320	160	320	160	160	320	160	320	80	80	320	640	320
A/American green winged teal/Mississippi/A00630203/2009 (H7N6)	20	160	160	160	160	160	40	20	160	320	80	160	40	20	160	320	160

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Table 3.1 (Continued)

A/American green winged teal/Texas/A00604024/2009 (H7N3)	80160160160160 80 80 40 40160 8016040 40 160 320 80
A/American green winged teal/Texas/A00604029/2009 (H7N3)	40 80 80 80 80 40 40 40 40 80 40 8040 20 80 80 80
A/American green winged teal/Texas/A00604032/2009 (H7N3)	320160320320320160160 80320320 8064080 40 640 640160
A/American green winged teal/Texas/A00604814/2009 (H7N3)	160320160320160160 80 80320320 8032080 40 160 160160
A/American green winged teal/Texas/A00586649/2009 (H7N3)	40 80 80 80 80 40 40 40 20 80 40 8020 20 160 160 80
A/American green winged teal/Utah/A00833077/2009 (H7N3)	40160160 40160160 40 40 20160 2016040 2012801280640
A/American green winged teal/Utah/A00831743/2009 (H7N3)	160160160160160160 40 40 40320 8032080 40 320 640160
A/American green winged teal/Utah/A00654391/2009 (H7N3)	80 80 80 80 80 80 40 40 40 80 80 8040 40 80 80 80
A/American green winged teal/Utah/A00461135/2009 (H7N1)	80 80 80 80 80 80 80 80 80 80 80 8080 80 80 80 80
A/American green winged teal/Utah/A00614935/2009 (H7N3)	160320160160320160160160320320 8064080 80 320 320 80
A/American green winged teal/Utah/A00468772/2009 (H7N7)	40 80 80 80 80 40 40 40160160 8016040 40 320 320320
A/American green winged teal/Wyoming/A00230796/2008 (H7N3)	40 80 80 80 80 80 40 40 80160 8016040 20 160 320160
A/blue winged teal/Louisiana/A00637297/2009 (H7N3)	40 80 40 40 40 20 20 20 40 8016016020 20 160 320 80
A/blue winged teal/Louisiana/A00557206/2009 (H7N7)	160160160160160160 40 80160160 40 8040 20 160 160160
A/blue winged teal/Missouri/A00624483/2008 (H7N3)	160 80 40 40160 40 20 20 20160 2016040 20 160 320 40
A/blue winged teal/Texas/A00605473/2009 (H7N3)	20 80 40 40 40 20 20 20 20 40 20 8020 20 80 80 80
A/blue winged teal/Texas/A00676566/2009 (H7N3)	40320 80 80 80 80 80 80 8016016032040 40 320 320320
A/domestic duck/West Virginia/A00140912/2008 (H7N3)	40 80 80 80 80 40160 20 40 80 4016040 40 160 320 80
A/domestic duck/West Virginia/A00140915/2008 (H7N3)	40 80 80 80 80 80 40 20 40160 8016040 40 160 320 80
A/gadwall/Arizona/A00663934/2009 (H7N3)	80 80 80160160 80 40 20 40160 80 8040 40 80 160 80
A/mallard/Deleware/A00456271/2009 (H7N3)	80320320320320160 80 80160320 8032080 40 160 320320
A/mallard/Kansas/A00523306/2008 (H7N3)	80160 80160160 80 40 40 40160 8016040 40 320 320160
A/mallard/Oklahoma/A00449368/2009 (H7N3)	80160 80 80 80 40 40 40 80160 4016020 20 160 160 80
A/mallard/Oklahoma/A00449455/2009 (H7N3)	16 20 20 20 20 20 20 20 20 20 20 2020 20 40 80 40
A/mallard/South Dakota/A00649542/2008 (H7N3)	80160 80 80160 80 40 20 40160 8016040 20 160 320 80
A/mute swan/Rhode Island/A00325105/2008 (H7N3)	160160160 80160 40 40 40 40160 4016020 20 160 320160
A/mute swan/Rhode Island/A00325108/2008 (H7N3)	40 80 40 40 40 20 20 20 20 40 40 8020 20 40 40 40
A/mute swan/Rhode Island/A00325112/2008 (H7N3)	20 40 40 40 40 20 20 20 80 80 40 8020 20 40 160 40
A/mute swan/Rhode Island/A00325114/2008 (H7N3)	20 40 40 40 40 20 40 40 80 80 40 4020 20 80 160 40
A/mute swan/Rhode Island/A00325115/2008 (H7N3)	160160 40 80160 40 40 40 40160 40 8020 20 80 160 80
A/mute swan/Rhode Island/A00325117/2008 (H7N3)	40 80 40 40 40 20 20 20 20 40 20 8020 20 40 80 40
A/mute swan/Rhode Island/A00325125/2008 (H7N3)	40 80 40 40 80 40 40 20 40160 4016020 20 160 160 80
A/mute swan/Rhode Island/A00325129/2008 (H7N3)	20 40 40 40 40 20 20 20 40 40 20 8020 20 80 40 80
A/mute swan/Rhode Island/A00325136/2008 (H7N3)	40 80 80 80 80 40 40 20 40 80 40 8020 20 80 80 40
A/northern pintail/Texas/A00466052/2009 (H7N3)	40 80 40 80160 40 40 20 40 80 40 8020 20 160 160160
A/northern shoveler/Louisiana/A00557321/2009 (H7N3)	40 80 40 40 40 40 40 2032032016032040 40 160 320 80
A/northern shoveler/Mississippi/A00682947/2008 (H7N7)	160 8032032016032016016064032016032080 80 640 640320
A/northern shoveler/Mississippi/A00602284/2009 (H7N2)	16064032064064016032016016032032032080160 320 320160
A/northern shoveler/Mississippi/A00630207/2009 (H7N6)	160320320320320160 80 80 8032016032080 80 320 640320

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Table 3.1 (Continued)

A/northern shoveler/Nevada/A00505416/2008 (H7N6)	40 80 80 80 80 40 40 80 40 8040 804020 80 80 80
A/northern shoveler/Oregon/A00654616/2008 (H7N3)	80320 80 80160 80 801601601608032080406402048160
A/northern shoveler/Utah/A00831758/2009 (H7N3)	80160320320160160160160160640806408080320 640160
A/northern shoveler/Utah/A00468752/2009 (H7N3)	80 80 80 80 80 80 80 80 80160641604020160 160 80
A/northern shoveler/Utah/A00468715/2009 (H7N6)	20 20 20 20 40 20 20 20 40 4020 202020 20 40 40
A/northern shoveler/Utah/A00468766/2009 (H7N3)	80160 80 80160 80 40 40 20160403204020160 320 80
A/blue winged teal/Minnesota/A00137660/2009 (H7N3)	80160 80 80 80 80 80 40320320801604040320 320160
A/blue winged teal/Texas/A00463679/2010 (H7N3)	80320160160160 80 80 40160320803208040160 320 80
A/mallard/Illinois/A00325439/2009 (H7N3)	40 80 80 80 80 40 40 40 40 8040 804040 80 80 80
A/mallard/Illinois/A00755320/2009 (H7N3)	80320160160320160 80 8016032080 808080640 640160
A/mallard/Michigan/A00869519/2009 (H7N3)	80320 80 80 80 80 80 80320160803204040160 160 80
A/mallard/New Jersey/A00926089/2010 (H7N3)	80160 40 80 80 40 40 20 40 80641602020 80 80 40
A/mallard/New York/A00723392/2009 (H7N3)	20 80 40 40 40 20 20 20 40 8020 802020 80 160 40
A/mallard/Oklahoma/A00749161/2009 (H7N3)	40 80 40 40 80 40 40 40 40 8040 802020 80 80 80
A/mallard/Oklahoma/A00744383/2009 (H7N3)	80160 80160160 80160160320160803208040320 320160

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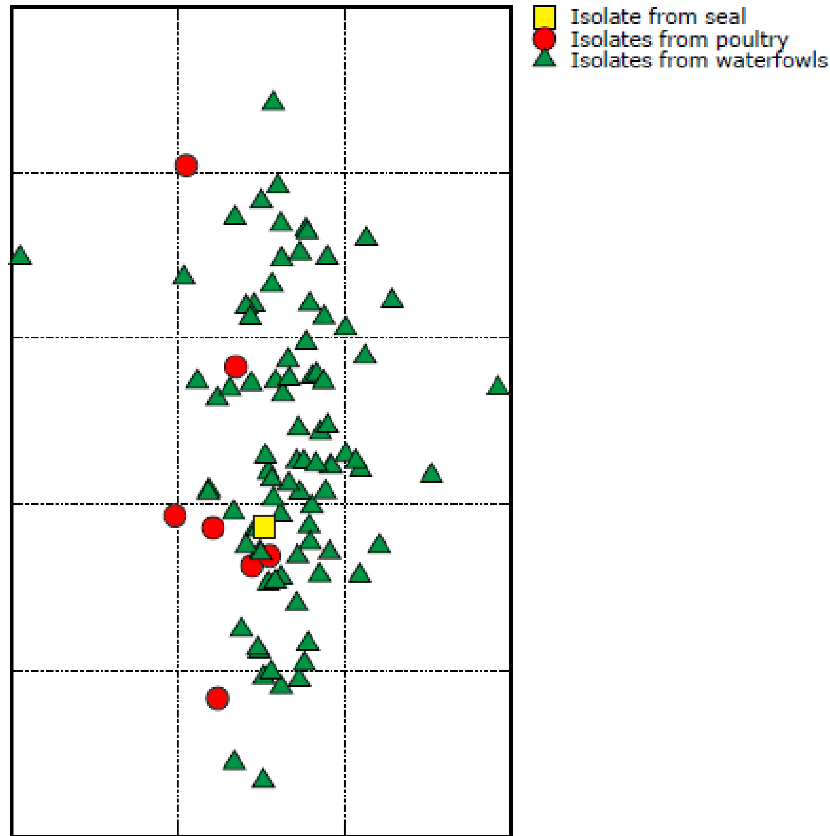

Figure 3.1 Antigenic cartography for H7 AIV

Discussion

The 2012 Mexico outbreak of HPAI (H7N3) in commercial poultry resulted in implementation of vaccination as a control measure. At the time of the outbreak, no commercial vaccine against H7 AIVs was available. An emergency vaccine seed strain A/cinnamon teal/Mexico/2817/2006 (H7N3) was selected and used in efforts to inhibition the dissemination of the AIVs to healthy commercial poultry farms [123]. As of 2013, Mexico implemented the vaccination of poultry against H7 AIVs and has up to date available vaccines. The H7 antigenic characterization suggest that H7 AIVs we tested, including from both domestic poultry and migratory waterfowl, have not undergone

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significant antigenic variations in the United States in the fast few years. Although the current H7 vaccine strains prepared from those isolates from domestic poultry could be effective in against future introduction, if vaccine strategy is necessary, it would important that continuous monitoring of antigenic variants of H7 viruses is needed.

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CHAPTER IV

CONCLUSIONS

Avian influenza vaccination is one important method for controlling and preventing avian influenza infection and outbreaks [124]. In the past 30 years, a number of inactivated avian influenza vaccines, such as H1N1, H1N2, H3N2, H6N2, and H7N3, have been licensed and used in chickens and turkeys [124, 125]. Conventional vaccination strategy is antigenic specific, and vaccine antigenic subtype is based on antigenic mapping on circulating influenza viruses in birds. Due to the rapid evolution of influenza viruses and the discrepancy and dynamics of antigenic subtypes, influenza vaccines have to be updated frequently. Even within the same HA subtype, multiple antigenic clusters are present and co-circulating [126-128]. It is difficult to anticipate what will cause an outbreak in poultry because of the rapid evolution of IAVs and frequent spillovers from other hosts, such as migratory birds [71, 129, 130], swine, [126, 131, 132] , and even from human carriers [133, 134].

In this study, the antigenicity of H3 and H7 avian influenza A viruses were characterized. The results demonstrating that there is lack of substantial antigenic variants in either H3 or H7 avian influenza viruses from North America we tested. The current study suggested that probably the currently licensed vaccine strains could provide protections again those viruses in migratory birds. However, although we included H3 and H7 IAVs with diverse geographic and temporal coverage, it is likely some H3 and

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H7 antigenic variants in migratory birds could have been missed. Continous studies are needed to monitor the antigenic evolution of H3 and H7 IAVs in migratory waterfowl.

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APPENDIX A

SUPPLEMENTARY HEMAGGLUTINATION INHIBITION TABLES AND GEOGRAPHIC LOCATIONS OF ISOLATES

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Table A.1 Hemagglutinaion inhibition data for H3 AIV against H3 chicken sera

Virus[1]	Titer for chicken antiserum[3]											
	MALL 00593	SNGO 03438	RNDU 03378	HDME 03749	BWTE 00803	BWTE 00069	ABDU 02213	ABDU 03451	ABDU 02650	ABDU 00618	LTDU 295	SEAL 2011
A/Mallard/New Brunswick/00593/2010 (H3N7)	**20**	20	20	20	<10	20	10	20	20	<10	<10	10
A/Snow Goose/Nunavet/03438/2010 (H3N8)	40	**80**	40	80	40	40	40	80	40	10	40	20
A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8)	40	20	**20**	40	20	20	40	20	<10	<10	20	10
A/Hooded Merganser/New Brunswick/03749/2009 (H3N8)	64	40	20	**40**	80	40	40	40	10	<10	20	10
A/Blue-Winged Teal/Nova Scotia/00069/2010 (H3N8)	20	40	10	40	20	**40**	20	40	20	20	20	20
A/American Black Duck/Nova Scotia/02213/2007 (H3N8)	20	40	40	20	80	40	**40**	10	10	<10	40	<10
A/American Black Duck/New Brunswick/03451/2009 (H3N9)	20	20	20	20	40	10	20	**64**	<10	10	40	<10
A/American Black Duck/New Brunswick/02650/2007 (H3N2)	20	10	20	10	<10	10	<10	10	**40**	<10	20	10
A/American Black Duck/New Brunswick/00618/2010 (H3N6)	<10	40	10	<10	10	<10	<10	10	20	**10**	<10	<10
A/Long-Tailed Duck/Maine/295/2011 (H3N8)	80	20	20	20	80	40	80	20	20	10	**80**	10
A/Harbor Seal/New Hampshire/2011 (H3N8)	40	40	40	80	80	40	40	20	20	10	40	**80**
A/American Black Duck/New Brunswick/03398/2009 (H3N8)	20	40	10	20	20	20	20	20	40	<10	<10	<10
A/Mallard/New Brunswick/03396/2009 (H3N8)	20	40	20	40	20	20	20	80	80	10	40	10
A/American Black Duck/New Brunswick/02525/2007 (H3N8)	40	20	20	20	80	10	10	20	40	<10	40	<10
A/American Black Duck/New Brunswick/02651/2007 (H3N2)	20	20	40	<10	<10	<10	<10	<10	20	<10	<10	<10
A/Northern Pintail/New Brunswick/03547/2009 (H3N8)	20	20	40	20	40	20	40	20	10	<10	20	10
A/Blue-Winged Teal/New Brunswick/00283/2010 (H3N8)	20	<10	<10	10	10	<10	<10	10	10	<10	<10	<10
A/Blue-Winged Teal/New Brunswick/00597/2010 (H3N7)	10	40	20	20	10	10	10	10	20	10	10	20
A/Blue-Winged Teal/Prince Edward Island/00831/2010 (H3N7)	10	<10	20	20	10	<10	10	<10	20	<10	<10	10
A/mallard/North Dakota/A00094822/2007 (H3N6)	10	<10	10	80	20	<10	20	40	20	10	<10	<10
A/mallard/Colorado/A00170366/2006 (H3N8)	20	20	20	40	30	10	40	20	80	20	10	10

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Table A.1 (Continued)

A/mallard/Oregon/A00282268/2007 (H3N8)	10	<10	20	20	20	<10	20	20	40	<10	10	<10
A/mallard/Oregon/A00282275/2007 (H3N8)	<10	<10	10	<10	10	<10	10	<10	10	<10	<10	<10
A/mallard/Washington/A00461816/2008 (H3)	160	160	160	320	160	160	160	160	320	320	160	160
A/mallard/Washington/A00466471/2008 (H3N8)	20	<10	20	20	10	10	10	40	80	20	10	<10
A/mallard/Oregon/A00571121/2007 (H3N8)	20	20	40	160	40	40	20	40	80	40	40	40
A/mallard/Wisconsin/A00661712/2009 (H3N2)	<10	<10	10	<10	10	<10	20	10	10	<10	10	<10
A/mallard/Iowa/A00683081/2008 (H3N8)	20	<10	20	20	20	10	20	20	20	20	20	10
A/mallard/Maryland/A00713446/2009 (H3N6)	10	<10	10	10	<10	<10	10	<10	<10	<10	<10	<10
A/mallard/Wisconsin/A00713769/2009 (H3N1)	20	10	20	<10	20	<10	20	80	40	10	10	10
A/mallard/Wisconsin/A00714818/2009 (H3N2)	10	<10	10	<10	10	<10	20	20	20	<10	10	<10
A/mallard/New York/A00723400/2009 (H3N6)	10	<10	<10	<10	<10	<10	20	10	20	<10	<10	<10
A/mallard/Wisconsin/A00751345/2009 (H3N8)	10	<10	10	40	<10	<10	20	20	20	<10	<10	<10
A/mallard/Wisconsin/A00751351/2009 (H3N6)	10	20	20	40	<10	<10	40	20	40	20	20	40
A/mallard/New York/A00755144/2009 (H3N6)	20	<10	20	10	10	<10	20	40	10	20	20	<10
A/mallard/Maryland/A00871428/2009 (H3N6)	10	<10	20	20	<10	<10	20	20	20	<10	<10	<10

[1] MALL00593, A/Mallard/New Brunswick/00593/2010 (H3N7); SNGO03438, A/Snow Goose/Nunavet/03438/2010 (H3N8); RNDU03378, A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8); HDME03749, A/Hooded Merganser/New Brunswick/03749/2009 (H3N8); BWTE00803, A/Blue Wing Teal/Prince Edward Island/00803/2010 (H3N8); BWTE00069, A/Blue Wing Teal/Nova Scotia/00069/2010 (H3N8); ABDU02213, A/American Black Duck/Nova Scotia/ 02213/2007 (H3N8), ABDU03451, A/American Black Duck/New Brunswick/03451/2009 (H3N9); ABDU02650, A/American Black Duck/New Brunswick/02650/2007 (H3N2); ABDU00618, A/American Black Duck/New Brunswick/00618/2010 (H3N6); LTDU295, A/Long Tailed Duck/Maine/295/2011 (H3N8); SEAL2011, A/Harbor Seal/New Hampshire/2011 (H3N8)
[2]Antigenic clusters were defined using the k-means clustering method.
[3]Values in bold are HI titres with homologous influenza virus isolates that were used to generate chicken antiserum.

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Table A.2 Summary of H3 influenza A viruses from migratory waterfowl in North America.

Virus[a]	County[b]	State/Province	Date Collected	Flyway[c]	Sex	Age Class
A/American Black Duck/Nova Scotia/02213/2007 (H3N8)	Cumberland	Nova Scotia	NA	ATLA	Female	Local
A/American Black Duck/New Brunswick/03451/2009 (H3N9)	Westmorland	New Brunswick	8/12/2009	ATLA	Male	Hatch Year
A/American Black Duck/New Brunswick/03398/2009 (H3N8)	Westmorland	New Brunswick	8/9/2009	ATLA	Male	Hatch Year
A/Mallard/New Brunswick/03396/2009 (H3N8)	Westmorland	New Brunswick	8/9/2009	ATLA	Female	Hatch Year
A/American Black Duck/New Brunswick/02650/2007 (H3N2)	Gloucester	New Brunswick	9/24/2007	ATLA	Female	Hatch Year
A/American Black Duck/New Brunswick/02525/2007 (H3N8)	Gloucester	New Brunswick	9/24/2007	ATLA	Male	Hatch Year
A/American Black Duck/New Brunswick/02651/2007 (H3N2)	Gloucester	New Brunswick	9/24/2007	ATLA	Male	Hatch Year
A/Hooded Merganser/New Brunswick/03749/2009 (H3N8)	Sunbury	New Brunswick	9/14/2009	ATLA	Male	Hatch Year
A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8)	Cumberland	Nova Scotia	8/8/2009	ATLA	Female	After Hatch Year
A/Northern Pintail/New Brunswick/03547/2009 (H3N8)	Gloucester	New Brunswick	9/11/2009	ATLA	Female	After Hatch Year
A/Snow Goose/Nunavet/03438/2010 (H3N8)	Kivalliq	Nunavet	7/20/2010	ATLA	Male	Adult
A/Blue-Winged Teal/Nova Scotia/00069/2010 (H3N8)	Cumberland	Nova Scotia	9/7/2010	ATLA	Male	Hatch Year
A/Blue-Winged Teal/New Brunswick/00283/2010 (H3N8)	Queens	New Brunswick	9/14/2010	ATLA	Female	Hatch Year
A/Blue-Winged Teal/New Brunswick/00597/2010 (H3N7)	Albert	New Brunswick	8/15/2010	ATLA	Male	Hatch Year
A/Blue-Winged Teal/Prince Edward Island/00831/2010 (H3N7)	Queens	Prince Edward Island	9/2/2010	ATLA	Male	Hatch Year
A/Mallard/New Brunswick/00593/2010 (H3N7)	Albert	New Brunswick	8/15/2010	ATLA	Male	Hatch Year
A/American Black Duck/New Brunswick/00618/2010 (H3N6)	Albert	New Brunswick	8/16/2010	ATLA	Male	Hatch Year
A/Long-Tailed Duck/Maine/295/2011 (H3N8)	Hancock	Maine	12/5/2011	ATLA	Female	After Hatch Year
A/Harbor Seal/New Hampshire/2011 (H3N8)	NA	New Hampshire	NA	ATLA	NA	NA
A/mallard/North Dakota/A00094822/2007 (H3N6)	Ramsey	North Dakota	10/10/2007	CENT	Female	After Hatch Year
A/mallard/Colorado/A00170366/2006 (H3N8)	Saguache	Colorado	9/2/2006	CENT	Female	Hatch Year
A/mallard/Oregon/A00282268/2007 (H3N8)	Klamath	Oregon	8/7/2007	PAC	Female	Hatch Year

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Table A.2 (Continued)

A/mallard/Oregon/A00282275/2007 (H3N8)	Klamath	Oregon	8/7/2007	PAC	Female	Hatch Year
A/mallard/Washington/A00461816/2008 (H3)	Whatcom	Washington	8/15/2008	PAC	Male	After Hatch Year
A/mallard/Washington/A00466471/2008 (H3N8)	Whatcom	Washington	10/9/2008	PAC	Female	Hatch Year
A/mallard/Oregon/A00571121/2007 (H3N8)	Lake	Oregon	9/11/2007	PAC	Male	Hatch Year
A/mallard/Wisconsin/A00661712/2009 (H3N2)	Dodge	Wisconsin	10/3/2009	MISS	Female	Hatch Year
A/mallard/Iowa/A00683081/2008 (H3N8)	Tama	Indiana	10/18/2008	MISS	Female	After Hatch Year
A/mallard/Maryland/A00713446/2009 (H3N6)	Dorchester	Maryland	11/27/2009	ATLA	Male	After Hatch Year
A/mallard/Wisconsin/A00713769/2009 (H3N1)	Marathon	Wisconsin	8/15/2009	MISS	Female	Hatch Year
A/mallard/Wisconsin/A00714818/2009 (H3N2)	Manitowoc	Wisconsin	8/13/2009	MISS	Female	After Hatch Year
A/mallard/New York/A00723440/2009 (H3N6)	Jefferson	New York	11/8/2009	ATLA	Male	Undetermined
A/mallard/Wisconsin/A00751345/2009 (H3N8)	Manitowoc	Wisconsin	8/13/2009	MISS	Male	Hatch Year
A/mallard/Wisconsin/A00751351/2009 (H3N6)	Manitowoc	Wisconsin	8/13/2009	MISS	Male	Hatch Year
A/mallard/New York/A00755144/2009 (H3N6)	Montgomery	New York	12/6/2009	ATLA	NA	NA
A/mallard/Maryland/A00871428/2009 (H3N6)	Dorchester	Maryland	11/27/2009	ATLA	Male	After Hatch Year

Note: [a]Viruses in bold are those selected to generate chicken antiserum; [b]NA, not available; [c]Flyways are as follows: PAC=Pacific, CENT=Central, MISS=Mississippi, ATLA=Atlantic

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Table A.3 Serological responses between the representative H3 AIVs from North America and the chicken sera generated against these viruses.

Virus[a]	Titer for chicken antiserum[b]										
	MALL 00593	SNGO 03438	RNDU 03378	HDME 03749	BWTE 00069	ABDU 02213	ABDU 03451	ABDU 02650	ABDU 00618	LTDU 295	SEAL 2011
A/Mallard/New Brunswick/00593/2010 (H3N7)	**16**	16	16	16	16	8	16	32	4	8	8
A/Snow Goose/Nunavet/03438/2010 (H3N8)	64	**128**	32	128	64	64	128	64	16	64	16
A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8)	32	16	**16**	32	16	32	32	8	4	16	8
A/Hooded Merganser/New Brunswick/03749/2009 (H3N8)	64	64	32	**64**	64	64	64	16	8	32	8
A/Blue-Winged Teal/Nova Scotia/00069/2010 (H3N8)	16	64	16	64	**32**	32	64	32	16	16	16
A/American Black Duck/Nova Scotia/02213/2007 (H3N8)	32	32	32	32	32	**64**	16	16	4	64	8
A/American Black Duck/New Brunswick/03451/2009 (H3N9)	32	16	16	32	16	16	**64**	8	8	16	8
A/American Black Duck/New Brunswick/02650/2007 (H3N2)	32	8	32	8	8	8	16	**32**	4	32	8
A/American Black Duck/New Brunswick/00618/2010 (H3N6)	8	32	8	8	4	4	16	16	**8**	4	2
A/Long-Tailed Duck/Maine/295/2011 (H3N8)	64	32	32	32	32	64	16	16	8	**128**	8
A/Harbor Seal/New Hampshire/2011 (H3N8)	64	64	64	128	64	64	32	16	8	32	**64**

Note: [a]MALL00593, A/Mallard/New Brunswick/00593/2010 (H3N7); SNGO03438, A/Snow Goose/Nunavet/03438/2010 (H3N8); RNDU03378, A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8); HDME03749, A/Hooded Merganser/New Brunswick/03749/2009 (H3N8); BWTE00803, A/Blue Wing Teal/Prince Edward Island/00803/2010 (H3N8); BWTE00069, A/Blue Wing Teal/Nova Scotia/00069/2010 (H3N8); ABDU02213, A/American Black Duck/Nova Scotia/ 02213/2007 (H3N8); ABDU03451, A/American Black Duck/New Brunswick/03451/2009 (H3N9); ABDU02650, A/American Black Duck/New Brunswick/02650/2007 (H3N2); ABDU00618, A/American Black Duck/New Brunswick/00618/2010 (H3N6); LTDU295, A/Long Tailed Duck/Maine/295/2011 (H3N8); SEAL2011, A/Harbor Seal/New Hampshire/2011 (H3N8)
[b]Values in bold are HI titres with homologous influenza virus isolates that were used to generate chicken antiserum.

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Table A.4 Hemagglutination inhibition data for H3 AIV from North America against ferret generated canine and avian sera.

	Canine		Ohio Avian	
Virus	CIVH3N2	CIVH3N8	99AIVH3N2	11AIVH3N2
A/American Black Duck/Nova Scotia/02213/2007 (H3N8)	<10	20	40	80
A/American Black Duck/New Brunswick/03451/2009 (H3N9)	<10	20	20	80
A/American Black Duck/New Brunswick/03398/2009 (H3N8)	<10	20	40	160
A/Mallard/New Brunswick/03396/2009 (H3N8)	<10	20	40	80
A/American Black Duck/New Brunswick/02650/2007 (H3N2)	<10	<10	40	10
A/American Black Duck/New Brunswick/02649/2007 (H3N6)	<10	40	40	160
A/American Black Duck/New Brunswick/0525/2007 (H3N8)	<10	20	20	80
A/American Black Duck/New Brunswick/02651/2007 (H3N2)	<10	20	80	20
A/Hooded Merganser/New Brunswick/03749/2009 (H3N8)	<10	10	20	80
A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8)	<10	20	20	80
A/Northern Pintail/New Brunswick/03547/2009 (H3N8)	<10	10	10	80
A/Black Scooter/New Brunswick/00002/2009 (H3N8)	<10	20	320	160
A/Snow Goose/Nunavet/03438/2010 (H3N8)	<10	<10	80	10
A/Blue-Winged Teal/Nova Scotia/00069/2010 (H3N8)	<10	<10	80	<10
A/Blue-Winged Teal/New Brunswick/00283/2010 (H3N8)	<10	20	20	160
A/Blue-Winged Teal/New Brunswick/00597/2010 (H3N7)	<10	40	80	40
A/Blue-Winged Teal/Prince Edward Island/00803/2010 (H3N8)	<10	20	80	20
A/Blue-Winged Teal/Prince Edward Island/00831/2010 (H3N7)	<10	<10	20	<10
A/Mallard/New Brunswick/00593/2010 (H3N7)	<10	20	80	40
A/American Black Duck/New Brunswick/00618/2010 (H3N6)	<10	<10	40	<10
A/Long-Tailed Duck/Maine/295/2011 (H3N8)	<10	<10	40	<10
A/Harbor Seal/New Hampshire/2011 (H3N8)	<10	40	40	320

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Table A.5 Hemagglutination inhibition data of H3 avian IAVs against ferret generated swine sera

	Swine Ferret Sera[a]							
Virus	9SW64	9SW96	0SW130	0SW156	0SW215	1SW111	1SW208	1SW347
A/American Black Duck/Nova Scotia/02213/2007 (H3N8)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/03451/2009 (H3N9)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/03398/2009 (H3N8)	10	10	10	10	10	10	10	10
A/Mallard/New Brunswick/03396/2009 (H3N8)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/02650/2007 (H3N2)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/02649/2007 (H3N6)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/0525/2007 (H3N8)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/02651/2007 (H3N2)	10	10	10	10	10	10	10	10
A/Hooded Merganser/New Brunswick/03749/2009 (H3N8)	10	10	10	10	10	10	10	10
A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8)	10	10	10	10	10	10	10	10
A/Northern Pintail/New Brunswick/03547/2009 (H3N8)	10	10	10	10	10	10	10	10
A/Black Scooter/New Brunswick/00002/2009 (H3N8)	10	10	10	10	10	10	10	10
A/Snow Goose/Nunavet/03438/2010 (H3N8)	10	10	10	10	10	10	10	10
A/Blue-Winged Teal/Nova Scotia/00069/2010 (H3N8)	10	10	10	10	10	10	10	10
A/Blue-Winged Teal/New Brunswick/00283/2010 (H3N8)	10	10	10	10	10	10	10	10
A/Blue-Winged Teal/New Brunswick/00597/2010 (H3N7)	10	10	10	10	10	10	10	10
A/Blue-Winged Teal/Prince Edward Island/00803/2010 (H3N8)	10	10	10	10	10	10	10	10

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Table A.5 (Continued)

A/Blue-Winged Teal/Prince Edward Island/00831/2010 (H3N7)	10	10	10	10	10	10	10	10
A/Mallard/New Brunswick/00593/2010 (H3N7)	10	10	10	10	10	10	10	10
A/American Black Duck/New Brunswick/00618/2010 (H3N6)	10	10	10	10	10	10	10	10
A/Long-Tailed Duck/Maine/295/2011 (H3N8)	10	10	10	10	10	10	10	10
A/Harbor Seal/New Hampshire/2011 (H3N8)	10	10	10	10	10	10	10	10

Note: [a]09SW64, A/swine/Ohio/09SW0964/2009 (H3N2); 09SW96, A/swine/Ohio/09SW96/2009 (H3N2); 10SW130, A/swine/Ohio/10SW130/2010 (H3N2); 10SW156, A/swine/Ohio/10SW156/2010 (H3N2); 10SW215, A/swine/Ohio/10SW215/2010 (H3N2); 11SW111, A/swine/Ohio/11SW111/2011 (H3N2); 11SW208, A/swine/Ohio/11SW208/2011 (H3N2); 11SW347, A/swine/Ohio/11SW347/2011 (H3N2)

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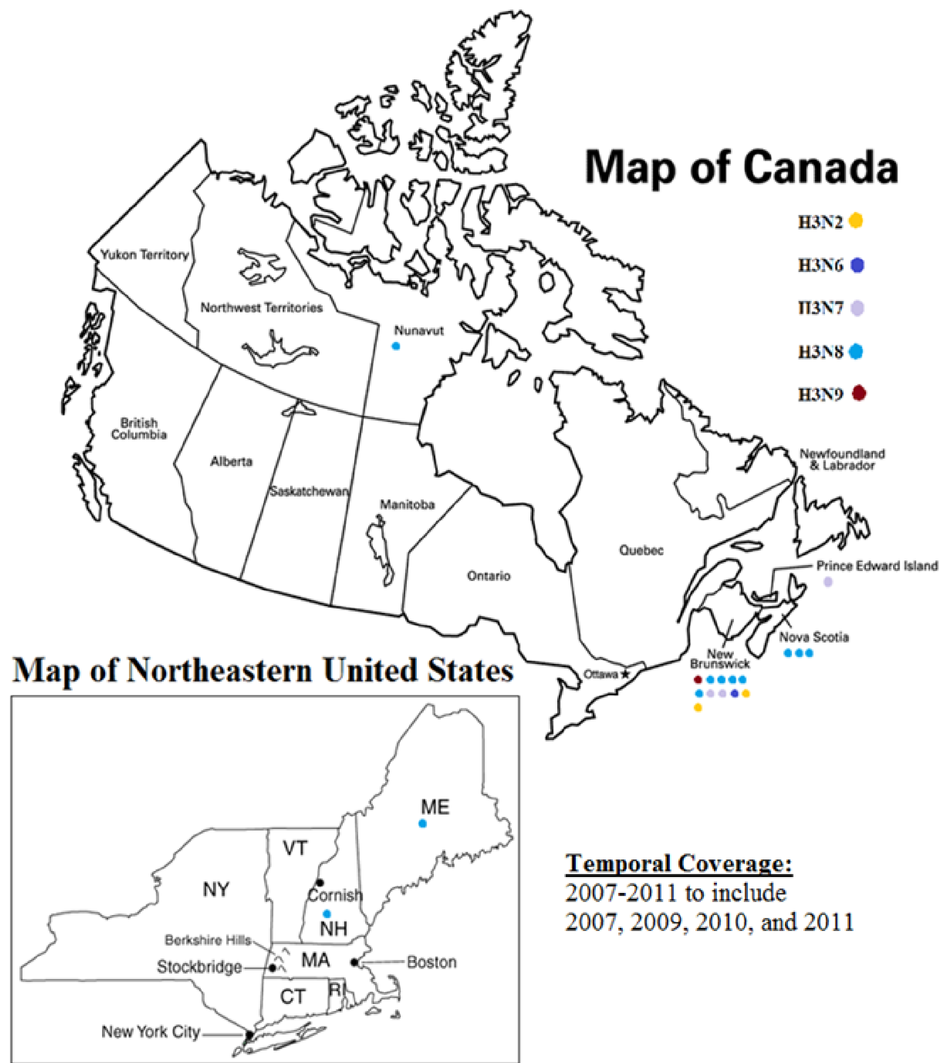

Figure A.1 Geographic location of all H3 isolates used in this study spanning across Canada and the northeastern U.S.

Temporal coverage is also included.

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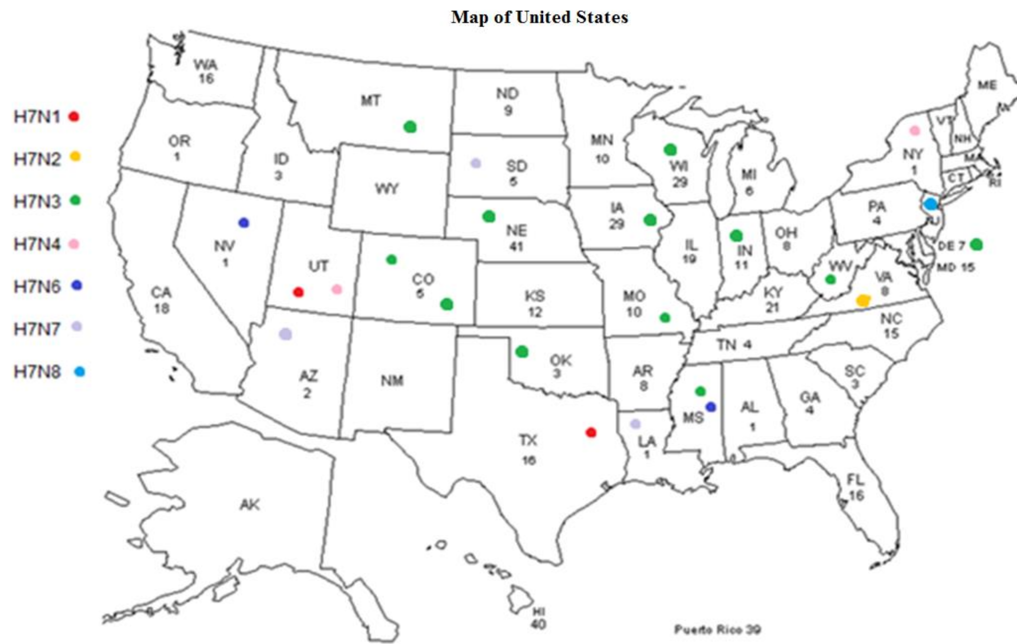

Figure A.2 Geographic location of all H7 isolates used in this study spanning cross the U.S.

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