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Antigenic Characterization of H3 Subtypes of Avian Influenza A Viruses from North America

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SUMMARY

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 2007 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 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 IAVs cross-reacted with ferret sera derived from contemporary swine and human IAVs. Our results showed that the H3 avian IAVs we tested lacked significant antigenic diversity, and these viruses were antigenically different from those circulating in swine and human populations. This suggests that H3 avian IAVs in North American waterfowl are antigenically relatively stable.

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Keywords

Influenza A virus; avian influenza virus; migratory waterfowl; H3; low pathogenic avian influenza virus; antigenic cartography; migratory flyway

INTRODUCTION

Specific strains of influenza A virus (IAV) can cause a pandemic disaster impacting human health on multiple continents and then persist causing seasonal influenza epidemics (1, 23). 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 (21, 31). 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 US (8, 46, 47).

IAVs have been recovered from at least 105 wild bird species from 26 different families (2, 29). 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 major natural IAV reservoirs (25, 50). Infected wild birds, especially migratory waterfowl, are believed to serve as a vector facilitating the spread of avian IAVs among wild bird species and different geographic locations, and are one source for viral transmission into domestic poultry (34, 50), swine (19), and humans (16).

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 (H1-H18) and 11 NA subtypes (N1-N11) have been reported in IAVs (48) although no isolates have been recovered for H17 and H18 subtypes. HA and NA are commonly used in combination to identify viral subtypes (i.e. H1N1). Antigenic changes in the HA and NA, the two surface glycoproteins, are mainly due to accumulating point mutations resulting in antigenic drift or genomic reassortment. These changes in the HA are especially important as they allow an IAV to evade the population immunity established from previous influenza infections or vaccination (26).

The H3 IAVs have very diverse host ranges in addition to infecting many species of birds. After the emergence of the pandemic virus A/Hong Kong/1968 (H3N2), this strain of IAV became endemic after the first year and has since been causing yearly seasonal epidemics in humans. The H3 IAVs circulate in swine, equine, canine, and avian species, and cause sporadic outbreaks in sea mammals. H3N2 is one of the predominant IAVs infecting both domestic and feral swine (14, 45, 49); H3N8 was shown to infect seals (22); H3N8 is endemic in domestic dogs in North America and equine worldwide (9, 17); and the avian origin H3N2 infected domestic dogs in Asia (24, 40).

Previous studies documented substantial antigenic diversity in the H3 IAV in mammals (14). Since 1968, antigenic drift has led to more than 28 updates of the H3N2 components in the seasonal influenza vaccines (37, 43). In swine, the H3N2 IAVs are genetically and antigenically diverse. Four genetic clusters of H3N2 IAVs (clusters I–IV) have been identified in US swine populations (19, 30, 33, 43), and the viruses in these four H3N2 clusters are antigenically different from each other. In addition, Cluster IV, which has become predominant among the US swine population, has further evolved into two antigenic clusters: H3N2SIV- α and H3N2SIV- β (14). Additionally, both H3N2 and H3N8 canine IAVs are antigenically distinct from contemporary human influenza viruses (54).

In wild migratory birds, H3 is one of the more frequent HA subtypes recovered in IAV surveillance projects (27). 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 36 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/179629/2011 (H3N8), was also included in this study. These viruses were recovered and propagated in 9–11 day-old SPF embryonated chicken eggs before serological characterization (28).

Sera

Two-week old broiler chicks, hatched from SPF eggs, were group housed in HEPA filtered, ventilated caging in ABSL-2 approved facilities. Six birds were housed per isolator and used for each challenge virus. Chicks were provided commercial poultry ration and water *ad libitum*, and were maintained on a 12-hour light cycle at a room temperature of 20–22 °C. Each bird was inoculated nasally with 10⁶, 50% tissue culture infective dose (TCID₅₀) in 100 μ L volume of a selected H3 isolate and boosted 14 days later with the same amount of virus. Six birds were used for each isolate, and the sera with at least a HI titer 1:40 were pooled. The sera were stored at –20 °C until the characterization. Reference sera were raised using isolates selected to cover two genetic clusters of North America origin H3 avian IAVs

in the phylogenetic trees (Figure 1). In addition, different years, locations, and host species were also considered to maximize the potential coverage. Consequently, 11 viruses were selected, including A/snow goose/Nunavet/03438/2010 (H3N8), A/hooded merganser/New Brunswick/03749/2009 (H3N8), A/blue-winged teal/Nova Scotia/00069/2010 (H3N8), A/American black duck/Nova Scotia/02213/2007 (H3N8), A/American black duck/New Brunswick/03451/2009 (H3N9), A/American black duck/New Brunswick/02650/2007 (H3N2), A/long tailed duck/Maine/295/2011 (H3N8), A/harbor seal/New Hampshire/179629/2011 (H3N8), A/Ring-Necked Duck/Nova Scotia/03378/2009 (H3N8), A/Mallard/New Brunswick/00593/2010 (H3N7), A/American Black Duck/New Brunswick/00618/2010 (H3N6), and A/Blue Wing Teal/Prince Edward Island/00803/2010 (H3N8) (Table 1).

To assess the cross-reactivity with the contemporary human and swine H3 IAVs, we also included a set of ferret sera (14) specifically against 20 H3 IAVs, including eight swine and 12 human IAVs [A/swine/Ohio/09SW0964/2009 (H3N2), A/swine/Ohio/09SW96/2009 (H3N2), A/swine/Ohio/10SW130/2010 (H3N2), A/swine/Ohio/10SW156/2010 (H3N2), A/swine/Ohio/10SW215/2010 (H3N2), A/swine/Ohio/11SW111/2011 (H3N2), A/swine/Ohio/11SW208/2011 (H3N2), A/swine/Ohio/11SW347/2011 (H3N2) A/Bangkok/1/1979 (H3N2), A/Philippines/2/82 (H3N2), A/Caen/1/1984 (H3N2), A/Mississippi/1/1985 (H3N2), A/Leningrad/360/1986 (H3N2), A/Sichuan/02/1987 (H3N2), A/Sichuan/60/1989 (H3N2), A/Ann Arbor/03/1993 (H3N2), A/Johannesburg/33/1994 (H3N2); A/Nanchang/933/1995 (H3N2), A/Sydney/05/1997 (H3N2), and A/Wisconsin/67/2005 (H3N2)].

Hemagglutination and hemagglutination inhibition assays

The HA and HI assays were performed according to OIE guidelines (13) using 1% chicken red blood cells (Lampire Labs, Pipersville, PA).

Antigenic cartography

Antigenic cartography projects influenza viruses into a two or three-dimensional map based on antigenic relationships embedded in the serological data. Antigenic cartography has been used as an effective method in characterizing antigenic diversity for IAVs (7, 38). In this study, AntigenMap (4, 7) was used to assess antigenic variability among the H3 IAVs we selected. A threshold of 1:10 was used to define the low reactor.

Phylogenetic analysis and molecular characterization

Multiple sequence alignments of the HA genes of H3 subtypes were created by using the MUSCLE software package (12). Phylogenetic analyses were performed using maximum likelihood by the GARLI version (5), and bootstrap resampling analyses were conducted using PAUP* 4.0 Beta (52) with a neighborhood joining method, as previously described (6).

Analysis of antibody binding sites

To analyze the sequence diversity among the antibody binding sites of hemagglutinin protein, sequence diversity profiles were generated for antibody binding sites in the viruses we studied using WebLogo 3 webserver (10). Antibody binding sites were annotated based on

previous studies (53), and they were mapped in a three dimensional structure of hemagglutinin protein using the PDB entry 1MQL (18) as the template. In addition, a total of 1,265 hemagglutinin protein sequences for North America avian origin H3 IAVs were retrieved from the NCBI Influenza Virus Database (3), and their sequence logos for antibody binding sites were also analyzed.

RESULTS

There is a large genetic diversity in H3 avian IAVs in North America

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 (Figure 1). 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 representative strains for contemporary H3 avian IAVs in North America. The nucleotide sequence identities among these H3 avian influenza viruses from 2007 to 2010 in North America varied from 81.94% to 100%. These viruses were genetically distinct from the human, equine, and swine H3 IAVs (Figure 1). The isolate from the harbor seal was genetically close to lineage I of North American H3 avian IAVs.

There is lack of antigenic diversity in H3 avian IAVs in North America

Our results demonstrated that all sera raised to the avian H3 IAVs tested were highly cross-reactive with each other (Table S1). Antigenic cartography showed that all 36 viruses were antigenically similar to each other (Figure 2). 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) (Figure 2). The antigenic distances for all 35 isolates except A/mallard/Washington/A00461816/2008(H3N?) were approximately two units.

H3 avian IAVs are antigenically distinct from human, swine, and canine H3 subtypes

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. None of the H3 avian IAVs cross-reacted with ferret sera against H3N2 human viruses (data not shown) or the ferret sera against H3N2 swine viruses (Table S3). The ferret sera against H3N8 canine influenza virus had a HI titer of 1:40 against 3 of the 22 tested isolates, whereas all these viruses were negative against the ferret sera against H3N2 avian origin canine influenza viruses (Table S2). The ferret sera against two H3N2 avian influenza viruses showed that the one against the virus from 1999 had a HI titer of 1:40 against 15 of the tested 22 isolates where the one against the virus from 2011 had a HI titer of 1:40 against 14 of the tested 22 isolates.

HI analyses showed that the sera to the seal isolate cross-reacted well with 4 of the 35 avian H3 IAVs tested, with a titer 1:40 and a maximum titer of 1:160 (Table S1).

There is lack of divergence in the antibody binding sites of H3 avian 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 (53), for all H3 avian IAVs from North America in the public database (Figure 3). There was a lack of divergence of these residues in the five sites (Figure 3A and 3C). 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. The antibody binding sites of the viruses we tested are similar to those from the public database (Figure 3B and 3C).

DISCUSSION

The evolution of IAVs in waterfowl was proposed to be static (41, 42). However, other studies have shown that the H5N1 highly pathogenic IAVs circulating in ducks have rapidly evolved (20, 41). A recent serological surveillance of wild birds suggested that wild birds may have been exposed to multiple, antigenically diverse H5 IAVs (15). 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 little 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 of the static evolution of H3 viruses in migratory waterfowl (41).

Waterfowl serve as reservoirs for IAVs and, consequently, IAVs emerging from these hosts could 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 (35, 36, 51). 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, 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 may be lack of immunological protection in the human populations against these H3 avian IAVs. These H3 avian IAVs could potentially lead to future 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 (11), a more recent study demonstrated that an H3N8 avian IAV isolated from a harbor seal could be transmitted among ferrets through aerosol droplets (22) and infect pigs without previous adaptation (39). This same strain was shown to have antigenic properties similar to those from migratory

waterfowl but distinct from human viruses (Table 1). 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 North 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 (44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HA	hemagglutinin
HI	Hemagglutination Inhibition Assay
IAV	influenza A virus
NA	neuraminidase
NCBI	National Center for Biotechnology Information
NWRC	National Wildlife Research Center
US	United States
USDA	United States Department of Agriculture
USGS	United States Geological Survey
SPF	specific pathogen free
TCID₅₀	Tissue Culture Infectious Dose at 50%

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Figure 1.

Phylogenetic analysis of HA genes of H3 IAV isolates from migratory waterfowl in North America (2007–2011). The H3 IAV isolates included in antigenic characterization are marked with a star. The phylogenetic analyses were performed using maximum likelihood by the GARLI version (5), and bootstrap resampling analyses were conducted using PAUP* 4.0 Beta (52) with a neighborhood joining method, as previously described (6).

Figure 2.

Antigenic cartography for H3 avian IAVs in North America (2007–2011) based on HI data from chicken sera. Antigenic cartography was constructed using AntigenMap (<http://sysbio.cvm.msstate.edu/AntigenMap>) (4, 7).

Figure 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 (53). The conservation analysis were characterized using WebLogo 3 webserver (10). The locations of these antibody binding sites were visualized using Chimera (32) based on the template of PDB entry 1MQL (18).

TABLE 1

Summary of H3 influenza A viruses from migratory birds in North America

Virus ^d	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/Numavet/03438/2010 (H3N8)	Kivalliq	Numavet	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/179629/2011 (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
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

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Virus^a	County^b	State/Province	Date Collected	Flyway^c	Sex	Age Class
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:

^aViruses in bold are those selected to generate chicken antiserum;

^bNA, not available;

^cFlyways are as follows: PAC=Pacific, CENT=Central, MISS=Mississippi, ATLA=Atlantic