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ORIGINAL ARTICLE

Possible basis for the emergence of H1N1 viruses with pandemic potential from avian hosts

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Influenza A viruses of the H1N1 subtype have emerged from the avian influenza gene pool in aquatic birds and caused human pandemics at least twice during the past century. Despite this fact, surprisingly little is known about the H1N1 gene pool in the aquatic bird reservoir. A preliminary study showed that an H1N1 virus from a shorebird of the Charadriiformes order was transmitted between animals through the airborne route of infection, whereas an H1N1 virus from a bird of the Anseriformes order was not. Here we show that two of the three H1N1 viruses isolated from Charadriiformes species in 2009 were transmitted between animals through the airborne route of infection, and five H1N1 isolates from Anseriformes species were not. The one H1N1 virus from a Charadriiformes species that failed to transmit through the airborne route was a reassortant possessing multiple internal gene segments from Anseriformes species. The molecular differences between the airborne-transmissible and non-airborne-transmissible H1N1 viruses were multigenic, involving the selection of virus with human-like receptor-binding specificity (α2-6 sialic acid) and multiple differences in the polymerase complex, mainly in the PB2, PB1-F2, and nonstructural genes.
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INTRODUCTION

The ''one health–one world'' concept is well accepted as being relevant to understanding the genesis of pandemic H1N1 influenza viruses that originate in wild aquatic birds.[1] Each of the eight gene segments of the 2009 H1N1 pandemic strain can be traced to the aquatic bird reservoir.[2] Surveillance of influenza viruses in their natural reservoirs has established that there are 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes of influenza A viruses maintained in the aquatic birds of the world,[3,4] and two influenza subtypes are maintained in bats (H17N10 and H18N11).[5] The H1N1, H2N2, and H3N2 influenza A subtypes emerged from the wild aquatic bird reservoir during the past century to cause pandemics in humans.[6] It is noteworthy that H1N1 caused the Spanish pandemic of 1918 and later re-emerged and caused the H1N1 pandemic of 2009; H1N1 was also responsible for the Russian pseudo-pandemic in 1977.[7]

Although H1N1 influenza viruses have frequently caused pandemics in humans, little attention has been given to the incidence or characteristics of H1N1 influenza in the aquatic bird reservoir. Here we determined the frequency of isolation and the pandemic potential of H1N1 influenza viruses from Anseriformes (primarily from migratory ducks) and Charadriiformes (primarily from shorebirds) species, at two long-term surveillance sites in North America: Alberta, Canada and Delaware Bay, New Jersey, USA.

MATERIALS AND METHODS

Ethics statement

All animal experiments were conducted in an Animal Biosafety Level 2+ (i.e., level 2 with enhanced biocontainment for pandemic H1N1 influenza A virus) facility at St. Jude Children's Research Hospital. All experiments were done in compliance with the policies of the National Institutes of Health and the Animal Welfare Act and with the approval of the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee (Protocol NO: 081, approval date: July 31, 2014).

Surveillance in aquatic birds

Long-term surveillance of influenza viruses in ducks was conducted in Alberta, Canada, from 1976 through 2014, and in shorebirds from 1985 through 2014 at Delaware Bay, New Jersey, USA. The details of surveillance, virus isolation, subtype characterization, and sequence analyses are as described.[8] Briefly, influenza viruses were isolated in chicken eggs, characterized antigenically by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays, and passaged no more than once before being entered into our repository.

Viruses

We tested the disease potential and transmission of eight North American avian H1N1 influenza viruses in ferrets. The viruses were selected based on their previously described pathogenicity, which was

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determined per their pathogenicity index (PI) in DBA/2J mice.[9] A/mallard/Alberta/119/1998 (H1N1), A/mallard/Minnesota/AI07-3100/2007 (H1N1), A/pintail/Alberta/210/2002 (H1N1), and A/shorebird/Delaware/324/2009 (H1N1) were selected from the most pathogenic viruses (PI-4); A/red-headed duck/Minnesota/Sg-00123/2007 (H1N1) and A/gull/Delaware/428/2009 (H1N1) were selected from moderately pathogenic viruses (PI-3); A/shorebird/Delaware/274/2009 (H1N1) was selected from low pathogenic viruses (PI-2); and A/green-winged teal/Louisiana/Sg-00090/2007 (H1N1) was the least pathogenic virus (PI-1).[9] Viruses used to inoculate ferrets were minimally serially passaged in 10- to 11-day-old embryonated chicken eggs as previously described.[9] The H1N1 viruses used in glycan array analysis were grown in embryonated chicken eggs, inactivated using 0.05% paraformaldehyde at 4℃ overnight, and purified and characterized on glycan arrays as previously described.[10] HA titer did not change after paraformaldehyde inactivation under the mild conditions described.

Animals

Three- to four-month-old outbred male ferrets were purchased from Triple F Farms (Sayre, PA, USA). Upon arrival, all ferrets were quarantined in the Animal Resources Center for one week before virus inoculation and were given food and water *ad libitum*. Temperature transponder microchips were placed under the skin of ferrets during their quarantine period. The ferrets used in this study were confirmed H1 and H3 influenza-seronegative by HI assay using turkey red blood cells, i.e., their HI titers were less than 10 against A/Perth/16/2009 (H3N2) and A/California/04/2009 (H1N1) viruses.

Pathogenicity and transmission in ferrets

Two donor ferrets were anesthetized with 3% isoflurane (supplied with 2% oxygen) until the animals were initially sedated and inoculated intranasally with 10^6 egg infectious dose 50% (EID_{50}) of one of the eight avian H1N1 viruses listed above in 0.5 mL phosphate-buffered saline. On the first day post-infection (dpi), one direct-contact ferret was placed in the same cage with each donor ferret, while one airborne-contact ferret was placed in an adjacent cage but separated by a wire grill from the donor- and direct-contact ferrets.[9] Six ferrets were used to assess each virus: two donors, two direct contacts, and two airborne contacts. For 16 days, all ferrets were monitored daily for weight loss, body temperature, and clinical signs of influenza infection (e.g., lethargy, sneezing, nasal discharge, and coughing). Every two days, the animals were lightly anesthetized with 40 mg/kg ketamine and nasal wash specimens were collected in 1 mL sterile phosphate-buffered saline as described previously.[9] Seroconversion of the ferrets were determined by HI assay using horse red blood cells that express sialic acids with α2,3 linkages to which avian influenza viruses preferentially bind.

Virus genome sequences

The wild-type genome of the viruses used for genomic comparison were previously sequenced and uploaded into the GenBank database.[11] The GenBank accession NOs for the whole-genome sequences of those viruses are as follows: KF424175-KF424182 for A/mallard/Alberta/119/1998, KF424015-KF424022 for A/mallard/Minnesota/AI07-3100/2007, KF424127-KF424134 for A/shorebird/Delaware/300/2009, KF424111-KF424118 for A/pintail/Alberta/210/2002, KF424079-KF424086 for A/shorebird/Delaware/324/2009, KF424191-KF424198 for A/red-headed duck/Minnesota/Sg-00123/2007, KF424023-KF424030 for A/gull/Delaware/428/2009, KF424055-KF424062 for A/mallard/Minnesota/Sg-00627/2008, KF424063-KF424070 for A/shorebird/Delaware/274/2009, and KF424087-KF424094 for A/green-winged teal/Louisiana/Sg-00090/2007.

Phylogenetic analyses

Because we did not detect any H1N1 viruses in Charadriiformes since 2009 in our surveillance studies, only the nucleotide sequences of the avian viruses isolated in the United States, Canada, and Mexico as recently as 2009 and those of human viruses isolated in Mexico in 2009 were downloaded from the Influenza Research Database (www.fludb.org). The total numbers of taxa used to assess the phylogeny of each gene were as follows: 284 taxa for PB2, 335 taxa for PB1, 271 taxa for PA, 158 taxa for HA, 269 taxa for NP, 162 taxa for NA, 246 taxa for M, and 258 taxa for NS1. A/equine/Prague/1/1956 (H7N7) virus was used to root the trees. For HA and NA gene segments, only the H1 and N1 subtypes of viruses were used. A/California/04/2009 and A/California/07/2009 were included as reference 2009 pandemic viruses. Multiple sequence alignment was done using ClustalW in BioEdit version 7.2.5, and coding-region sequences were used to construct the phylogenetic trees. Neighbor-joining phylogeny was performed for each gene segment by using MEGA6.06 under Kimura-2-parameter model with the assumption of uniform rates among sites.[12] The robustness of the branch support was determined using 1000 bootstrap replicates.

Glycan array screening

Purified viruses were lightly labeled with Alexa-488 and run as previously described[10] on the Consortium for Functional Glycomics Glycan Array v5.0. Data analyses were performed as described in Gulati *et al.* (2012).[10]

RESULTS

Influenza surveillance in migratory aquatic birds

Previous studies have established that 16 subtypes of influenza A viruses cocirculate in aquatic birds, with cyclic dominance of a subtype for one or more years followed by its absence at that site for a number of years.[8,13] Surveillance of H1N1 influenza viruses in migratory ducks has shown similar cycles of dominance over the years (Figure 1). Since 1976, H1N1 has been isolated from wild ducks in Alberta during 23 of the 38 years in which annual surveillance was conducted. For that period, H1N1 virus was isolated from 0.7% of the migratory duck samples (126 isolates from 17 866 samples), accounting for 3.4% of all avian influenza virus isolates. H1N1 was the sixth most frequently identified HA-NA subtype of the 72 subtypes isolated.

In contrast, annual surveillance of avian influenza viruses in shorebirds and gulls at Delaware Bay, which began in 1985, failed to detect H1N1 in shorebirds until 2009.[8] Initially the NA subtype was determined based on serological characterization using the NI assay. However, subsequent characterization of these viruses by PCR and genomic sequencing revealed that our reference antisera to N4 cross-reacted to N1, indicating the presence of a cross-reactive antigenic determinant in these NAs. Therefore, many of the viruses isolated from shorebirds and gulls had been incorrectly characterized as H1N4 and were actually H1N1 viruses. Due to this, additional H1N1 influenza viruses are now reported from both migratory ducks and shorebirds, though isolations are less frequent from shorebirds than from ducks. Furthermore, we have failed to isolate H1N1 viruses from shorebirds since 2009 but have continued to isolate them from ducks.

The revised analysis of viral subtypes showed that H1N1 viruses were detected in shorebirds and gulls during four of the 29 years that

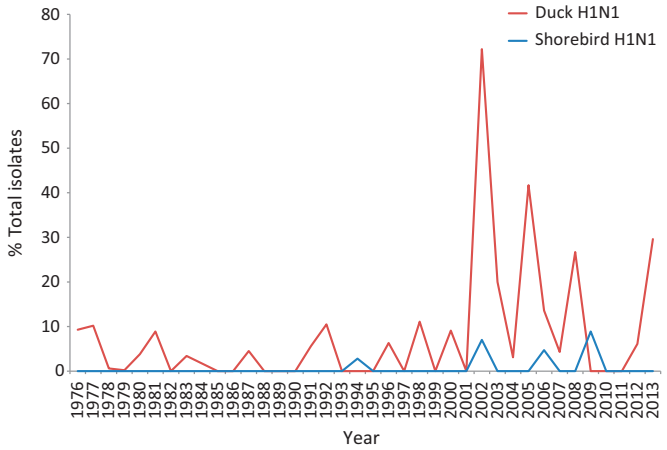


Figure 1 H1N1 isolates obtained from ducks in Alberta, Canada (red line) during 1976–2013, and from shorebirds at Delaware Bay, New Jersey, USA (blue line) during 1985–2013. H1N1 virus isolates are presented as a percentage of the total number of annual influenza virus isolates of all subtypes obtained from each wild bird taxonomic order.

surveillance was conducted: 1994, 2002, 2006, and 2009. The cyclical dominance of H1N1 virus isolation from shorebirds and gulls appears to be more irregular than that from migratory ducks (Figure 1), with an overall isolation rate of 0.15% (16 isolates per 11 030 samples). H1N1 viruses were isolated during fewer years from shorebirds and gulls and accounted for 1.4% of the avian influenza viruses found in those birds (16 H1N1 isolates per 1157 total isolates). H1N1 was the 18th most frequently identified HA-NA subtype of the 91 subtype combinations isolated. The interval 2002–2009 was the most active period for H1N1 in shorebirds and gulls during our surveillance study.

Replication and direct-contact transmission of avian H1N1 in ferrets

Our previous studies established that North American H1N1 influenza viruses from migrating waterfowl replicate to high titers in DBA/2J mice.[9] On the basis of weight loss and survival scores, we divided the viruses into four PI groups (Table 1).[9] PI-4 viruses caused 100% mortality, whilst PI-1 viruses were infectious but caused no mortality. Here we used ferrets to study the pathogenicity and transmissibility of these viruses because ferrets are the best animal model for studying influenza virus infection and transmission in humans.[14] Preliminary studies showed that two of the viruses, A/shorebird/Delaware/300/2009 (H1N1), a PI-4 virus, and A/mallard/Minnesota/Sg-00627/2008 (H1N1), a PI-2 virus, replicated and caused respiratory disease in ferrets. A/shorebird/Delaware/300/2009 (H1N1) was transmitted through the airborne route and caused disease in the contact animals, but the PI-2 virus did not. These results indicate that the pandemic potential of H1N1 viruses in shorebirds could be greater than is currently thought. To better understand this potential, we needed to confirm the preliminary findings and extend them to a larger number of avian H1N1 isolates in the ferret model.

To further assess the pathogenic potential of North American avian H1N1 viruses from aquatic birds, we tested eight H1N1 viruses, representing PI-4 through PI-1 viruses, in ferrets for their ability to replicate, cause disease signs, and transmit through the airborne route. All viruses replicated to high titers in donor ferrets (Figure 2). However,

Table 1 The clinical symptoms observed in donor, direct-contact, and airborne-contact ferrets upon infection with avian H1N1 influenza A viruses

H1N1 strain used for infection	Pathogenicity index[a]	Ferret	% Weight change loss (–)/gain (+)	Lethargy	Fever[b]	Sneezing	Coughing
A/mallard/Alberta/119/1998	4	Donor	–14/–13	–/+	+/+	+/–	+/–
		DC	+7/+10	–/–	–/–	–/+	–/–
		AC	+10/+8	–/–	–/–	–/–	–/–
A/mallard/Minnesota/AI07-3100/2007	4	Donor	+11/–5	–/–	–/–	–/+	–/–
		DC	–8/+37	–/–	–/–	+/–	–/–
		AC	+46/0	–/–	–/–	–/–	–/–
A/pintail/Alberta/210/2002	4	Donor	+12/+20	–/–	–/–	–/+	–/–
		DC	+17/+33	–/–	–/–	–/–	–/–
		AC	+19/+40	–/–	–/–	–/–	–/–
A/shorebird/Delaware/324/2009	4	Donor	+6/–7	–/–	+/+	+/+	+/–
		DC	+19/+27	–/+	+/–	+/+	–/+
		AC	+23/+6	–/+	–/+	–/+	–/–
A/red-headed duck/Minnesota/Sg-00123/2007	3	Donor	–14/–15	+/–	+/–	+/–	+/–
		DC	+9/+14	–/–	–/+	+/+	–/–
		AC	0/+9	–/–	–/–	–/–	–/–
A/gull/Delaware/428/2009	3	Donor	–6/–7	–/–	+/+	+/+	+/+
		DC	+13/+38	–/–	–/–	+/+	–/–
		AC	+19/+17	–/–	–/+	+/+	–/–
A/shorebird/Delaware/274/2009	2	Donor	+29/+25	–/–	–/–	+/+	–/–
		DC	+33/+36	–/–	–/–	+/–	–/–
		AC	+18/+17	–/–	–/–	–/–	–/–
A/green-winged teal/Louisiana/Sg-00090/2007	1	Donor	+24/+21	–/–	+/–	–/–	–/–
		DC	+17/+31	–/–	–/–	–/–	–/–
		AC	+19/+13	–/–	–/–	–/–	–/–

[a]Pathogenicity index values were previously calculated based on the weight loss and survival scores in the DBA/2J mouse model.[8]
[b]Fever was detected if the body temperature of ferrets was more than 1.5℃ higher than their baseline body temperatures. Clinical symptoms are shown for each ferret per group.
Abbreviations: DC, direct contact; AC, airborne contact.

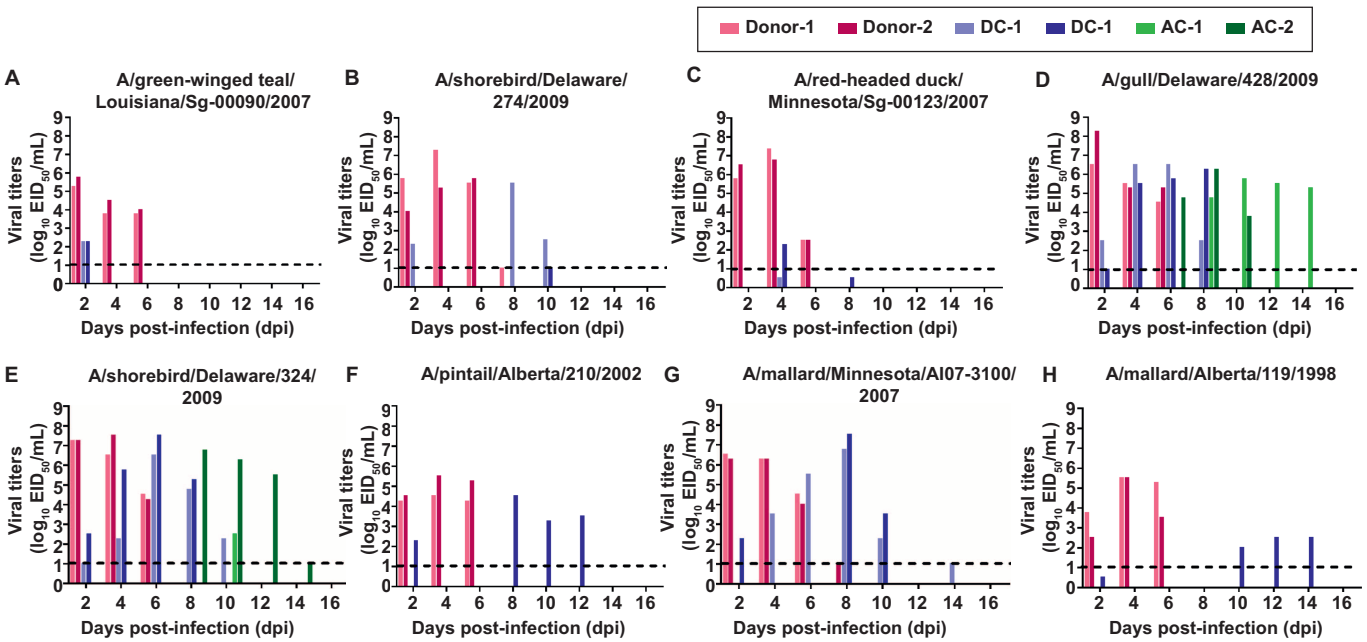


Figure 2 Viral titers in the nasal washes of ferrets after infection with one of the following avian H1N1 influenza A viruses: (**A**) A/green-winged teal/Louisiana/Sg-00090/2007, (**B**) A/shorebird/Delaware/274/2009, (**C**) A/red-headed duck/Minnesota/Sg-00123/2007, (**D**) A/gull/Delaware/428/2009, (**E**) A/shorebird/Delaware/324/2009, (**F**) A/pintail/Alberta/210/2002, (**G**) A/mallard/Minnesota/AI07-3100/2007, or (**H**) A/mallard/Alberta/119/1998. Light pink bars indicate donor-1 ferrets; dark pink bars, donor-2 ferrets; light blue bars, direct-contact (DC)-1 ferrets; dark blue bars, direct-contact (DC)-2 ferrets; and light green bars, airborne-contact (AC)-1 ferrets; dark green bars, airborne-contact (AC)-2 ferrets. Dashed lines represent the limit of detection.

the virus titers in donor ferrets infected with the PI-1 virus, A/green-winged teal/Louisiana/Sg-00090/2007 (H1N1), declined by 4 dpi, and there was marginal evidence of transmission even to direct-contact ferrets; virus titers were observed only at 2 dpi, and they were as low as 10^2 EID_{50}/mL (Figure 2A). All six ferrets in this group showed weight gain with absence of lethargy, sneezing, or coughing (Table 1).

The PI-2 virus, A/shorebird/Delaware/274/2009 (H1N1), replicated to high titers in both donor ferrets; sneezing was observed at 5–7dpi; and the virus was transmitted to the direct-contact animals. Virus was detected in one direct-contact animal at multiple time points (2, 8, and 10 dpi) while the other direct-contact ferret had detectable virus only on 10 dpi (limit of detection (10^1 EID_{50}/mL)) (Figure 2B). This ferret showed no significant weight loss or evidence of lethargy, fever, or coughing, but sneezing was observed at 9 dpi (Table 1).

The PI-3 viruses, A/red-headed duck/Minnesota/Sg-00123/2007 (H1N1) and A/gull/Delaware/428/2009 (H1N1), showed considerable variation. A/red-headed duck/Minnesota/Sg-00123/2007 (H1N1), which is of Anseriformes origin, infected donor ferrets, and high titers were detected on 2 and 4 dpi. This virus transmitted to one direct-contact ferret, causing very mild infection (10^2 EID_{50}/mL) (Figure 2C). Lethargy, fever, sneezing, and coughing were sporadically observed among donor and direct-contact ferrets (Table 1). In contrast, A/gull/Delaware/428/2009 (H1N1), of Charadriiformes origin, replicated to higher titers in donor ferrets on 2, 4, and 6 dpi (Figure 2D). Both direct-contact ferrets were infected, and viral titers as high as those of the donor ferrets were detected, which peaked on 4, 6, and 8 dpi. Slight weight loss, fever, sneezing, and coughing were observed in both donor ferrets. The direct-contact ferrets showed sneezing with absence of weight loss, lethargy, fever, and coughing (Table 1).

Ferrets exposed to the four PI-4 viruses showed a pattern similar to that of animals exposed to PI-3 viruses. A/shorebird/Delaware/324/2009 (H1N1) of Charadriiformes origin infected both donor ferrets and transmitted to both direct-contact ferrets, in which high virus titers, lethargy, fever, sneezing, and coughing but no substantial weight loss were observed on multiple days (Figure 2E and Table 1). High viral titers were detected in donor ferrets infected with the viruses of Anseriformes origin on 2, 4, and 6 dpi, and sporadic clinical signs were observed (lethargy, fever, and sneezing). The viruses were transmitted to one or both direct-contact ferrets with either no clinical signs or only sneezing at one time point (Figures 2F–H and Table 1).

Airborne transmission of avian H1N1 influenza virus in ferrets

Transmission of a virus among individuals of a host population is a key factor for determining viral fitness and assessing pandemic risk. The transmissibility of the eight avian H1N1 viruses through the airborne route was tested by separating animals by a wire mesh. The PI-3 virus A/gull/Delaware/428/2009 (H1N1) and the PI-4 virus A/shorebird/Delaware/324/2009 (H1N1) transmitted through airborne routes (Figures 2D and E and Table 2). Airborne-contact ferrets shed high titers of viruses and exhibited clinical symptoms, including fever, lethargy, sneezing, and coughing of similar severity to those of the donor ferrets (Figures 2D and E and Table 1). These contact ferrets recovered from infection and developed high anti-H1N1 antibody titers. The HI titer by horse red blood cells on 17 dpi was lower than 20 in one ferret and higher than 1280 in the other for the airborne contacts infected with A/shorebird/Delaware/324/2009 (H1N1); the HI titer was 160 for both ferrets infected with A/gull/Delaware/428/2009 (H1N1). Of special note, A/gull/Delaware/428/2009 (H1N1) transmitted very rapidly to one airborne contact, as viral titers were detected in that ferret as early as 6 dpi (Figure 2D). The viruses that transmitted through the airborne route were isolated from shorebirds or gulls in 2009, whilst most of those that did not were isolated from ducks. The one exception was A/shorebird/Delaware/274/2009 (H1N1), which did not transmit through airborne routes (Figure 2B and Table 2).

Table 2 Transmission efficiency of avian H1N1 influenza A viruses in ferrets through direct-contact or airborne-contact transmission

H1N1 strains used for infection	Pathogenicity index[a]	Donor	Direct contact	Airborne contact
A/mallard/Alberta/119/1998	4	2/2	1/2	0/2
A/mallard/Minnesota/AI07-3100/2007	4	2/2	2/2	0/2
A/pintail/Alberta/210/2002	4	2/2	1/2	0/2
A/shorebird/Delaware/324/2009	4	2/2	2/2	2/2
A/red-headed duck/Minnesota/Sg-00123/2007	3	2/2	1/2	0/2
A/gull/Delaware/428/2009	3	2/2	2/2	2/2
A/shorebird/Delaware/274/2009	2	2/2	2/2	0/2
A/green-winged teal/Louisiana/Sg-00090/2007	1	2/2	2/2	0/2

[a]Pathogenicity index scores were calculated based on the weight loss and survival scores in the DBA/2J mouse model.[8]

Amino acid differences among the wild-type avian H1N1 viruses

To determine the natural variations in the genomes of wild-type avian H1N1 influenza viruses and their potential importance in pathogenicity and transmissibility, we analyzed the full-genome sequences of the eight avian H1N1 influenza viruses and those of two avian H1N1 viruses studied previously in ferrets.[9] Twenty-three residues differed across the three airborne-transmissible viruses, which were of Charadriiformes origin, and the non-airborne-transmissible viruses, six of which were of Anseriformes origin and one was of Charadriiformes origin (Table 3). The differences were mainly found in the polymerase and nonstructural gene segments (five residues in PB2, three in PB1, nine in PB1-F2, one in PA-X C-terminal, three in NS1, and two in nuclear export protein (NEP)). A/shorebird/274/2009 (H1N1), which did not transmit through the airborne route, differed from the three shorebird viruses that did transmit through the airborne route at each of the 23 amino acid residues observed. The amino acids at those positions in A/shorebird/274/2009 (H1N1) were identical to those found in duck H1N1 viruses (Table 3).

Residue 58 in PB1-F2 appeared to be important for the transmissibility of these viruses. Two Anseriformes-origin viruses, A/mallard/Alberta/119/1998 (H1N1) and A/pintail/Alberta/210/2002 (H1N1), had W58 in their PB1-F2 and showed 50% transmission efficiency to direct-contact ferrets. In contrast, the viruses with L58 showed 100% transmission efficiency to direct-contact ferrets, and the ones with S58 showed 100% efficiency by both routes of transmission.

Rearrangement of gene segments in a non-airborne-transmissible H1N1 influenza virus from shorebirds

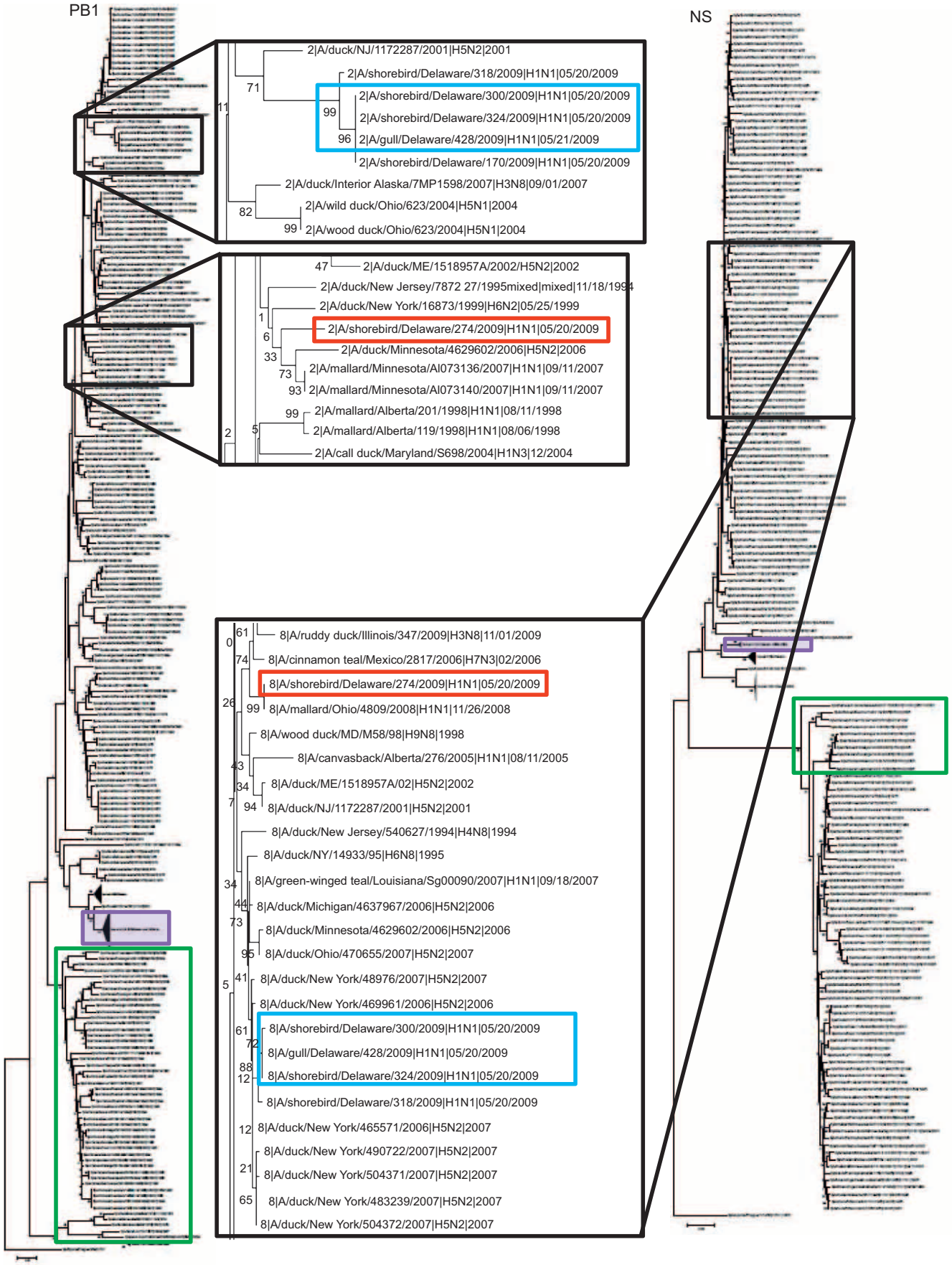
We conducted phylogenetic analysis to identify any evidence of gene reassortment between H1N1 influenza viruses of Anseriformes and Charadriiformes origins. Phylogenetic trees were constructed using the nucleotide sequences from the coding region of each gene segment of the viruses discussed here and avian viruses isolated in the United States, Canada, or Mexico as recent as 2009 that were published in the Influenza Research Database. We limited our analysis of the HA and NA gene segments to viruses of H1 and N1 subtypes, respectively. Additionally, gene segments of the non-airborne-transmissible Charadriiformes virus, A/shorebird/Delaware/274/2009 (H1N1), were compared with those of the following airborne-transmissible Charadriiformes viruses: A/shorebird/Delaware/324/2009 (H1N1), A/gull/Delaware/428/2009 (H1N1), and A/shorebird/Delaware/300/2009 (H1N1). The HA and NA segments of these four viruses clustered together, whilst the other gene segments showed evolutionary distances. The largest differences were observed in PB2, PB1, and nonstructural (NS) phylogenies, where A/shorebird/Delaware/274/2009 (H1N1) clustered with Anseriformes-origin viruses, distant from the other three Charadriiformes viruses, which clustered together (PB1 and NS phylogenies are shown in Figure 3).

A different pattern was observed in the NP and PA trees (Figure 4). In both trees, A/shorebird/Delaware/324/2009 (H1N1) and A/shorebird/Delaware/300/2009 (H1N1) clustered closely, whilst A/shorebird/Delaware/274/2009 (H1N1) and A/gull/Delaware/428/2009 (H1N1) clustered closely with Anseriformes-origin viruses but distant

Table 3 Residues in the genome of wild-type avian H1N1 influenza A viruses that differ between airborne-transmissible viruses and non-airborne-transmissible viruses

	PB2					PB1			PB1-F2									PA-X C-ter	NS1			NEP	
H1N1 strains used for infection[a]	67	152	199	508	649	298	642	667	8	15	23	26	27	31	58	69	75	21	7	213	227	7	70
A/shorebird/Delaware/300/2009[b]	V	S	T	Q	I	I	S	V	Q	R	S	R	I	G	S	R	L	V	L	S	G	L	G
A/shorebird/Delaware/324/2009	V	S	T	Q	I	I	S	V	Q	R	S	R	I	G	S	R	L	V	L	S	G	L	G
A/gull/Delaware/428/2009	V	S	T	Q	I	I	S	V	Q	R	S	R	I	G	S	R	L	V	L	S	G	L	G
A/shorebird/Delaware/274/2009	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	L	Q	H	A	S	P	E	S	S
A/mallard/Alberta/119/1998	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	W	Q	H	A	T	P	E	T	S
A/mallard/Minnesota/AI07-3100/2007	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	L	Q	R	A	T	P	E	T	S
A/pintail/Alberta/210/2002	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	W	Q	R	A	T	P	E	T	S
A/red-headed duck/Minnesota/Sg-00123/2007	I	A	A	R	V	L	N	I	L	H	N	Q	T	E	L	Q	R	A	T	P	E	T	S
A/mallard/Minnesota/Sg-00627/2008[b]	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	L	Q	R	A	S	P	E	S	S
A/green-winged teal/Louisiana/Sg-00090/2007	I	A	A	R	V	L	N	I	P	H	N	Q	T	E	L	Q	R	A	S	P	E	S	S

[a] Viruses are ordered based on their transmissibility and origin of host (Charadriiformes versus Anseriformes)
[b] The pathogenicity and transmission of these two viruses were previously studied.[8]


PB1
2|A/duck/NJ/1172287/2001|H5N2|2001
2|A/shorebird/Delaware/318/2009|H1N1|05/20/2009
2|A/shorebird/Delaware/300/2009|H1N1|05/20/2009
2|A/shorebird/Delaware/324/2009|H1N1|05/20/2009
2|A/gull/Delaware/428/2009|H1N1|05/21/2009
2|A/shorebird/Delaware/170/2009|H1N1|05/20/2009
2|A/duck/Interior Alaska/7MP1598/2007|H3N8|09/01/2007
2|A/wild duck/Ohio/623/2004|H5N1|2004
2|A/wood duck/Ohio/623/2004|H5N1|2004
2|A/duck/ME/1518957A/2002/H5N2|2002
2|A/duck/New Jersey/7872 27/1995mixed|mixed|11/18/1994
2|A/duck/New York/16873/1999|H6N2|05/25/1999
2|A/shorebird/Delaware/274/2009|H1N1|05/20/2009
2|A/duck/Minnesota/4629602/2006|H5N2|2006
2|A/mallard/Minnesota/AI073136/2007|H1N1|09/11/2007
2|A/mallard/Minnesota/AI073140/2007|H1N1|09/11/2007
2|A/mallard/Alberta/201/1998|H1N1|08/11/1998
2|A/mallard/Alberta/119/1998|H1N1|08/06/1998
2|A/call duck/Maryland/S698/2004|H1N3|12/2004
NS
8|A/ruddy duck/Illinois/347/2009|H3N8|11/01/2009
8|A/cinnamon teal/Mexico/2817/2006|H7N3|02/2006
8|A/shorebird/Delaware/274/2009|H1N1|05/20/2009
8|A/mallard/Ohio/4809/2008|H1N1|11/26/2008
8|A/wood duck/MD/M58/98|H9N8|1998
8|A/canvasback/Alberta/276/2005|H1N1|08/11/2005
8|A/duck/ME/1518957A/02|H5N2|2002
8|A/duck/NJ/1172287/2001|H5N2|2001
8|A/duck/New Jersey/540627/1994|H4N8|1994
8|A/duck/NY/14933/95|H6N8|1995
8|A/green-winged teal/Louisiana/Sg00090/2007|H1N1|09/18/2007
8|A/duck/Michigan/4637967/2006|H5N2|2006
8|A/duck/Minnesota/4629602/2006|H5N2|2006
8|A/duck/Ohio/470655/2007|H5N2|2007
8|A/duck/New York/48976/2007|H5N2|2007
8|A/duck/New York/469961/2006|H5N2|2006
8|A/shorebird/Delaware/300/2009|H1N1|05/20/2009
8|A/gull/Delaware/428/2009|H1N1|05/20/2009
8|A/shorebird/Delaware/324/2009|H1N1|05/20/2009
8|A/shorebird/Delaware/318/2009|H1N1|05/20/2009
8|A/duck/New York/465571/2006|H5N2|2007
8|A/duck/New York/490722/2007|H5N2|2007
8|A/duck/New York/504371/2007|H5N2|2007
8|A/duck/New York/483239/2007|H5N2|2007
8|A/duck/New York/504372/2007|H5N2|2007

←

Figure 3 The PB1 and NS1 genes of the non-airborne-transmissible virus cluster separately from those of the three airborne-transmissible viruses. On the PB1 and NS1 trees, A/shorebird/Delaware/274/2009 (H1N1), which did not transmit through the airborne route in ferrets (red boxes), clustered separately from the viruses that did transmit through the airborne route, i.e., A/shorebird/Delaware/300/2009 (H1N1), A/gull/Delaware/428/2009 (H1N1), and A/shorebird/Delaware/324/2009 (H1N1) (blue boxes), which clustered together. These viruses did not cluster near human pandemic H1N1 viruses isolated in Mexico (purple shaded boxes) or viruses isolated in chickens in Mexico (green boxes). Phylogenetic trees were constructed under Kimura-2-parameter model with the assumption of uniform rates among sites using nucleotide sequences of avian and human viruses isolated in North America or Mexico as recently as 2009 that were published in the Influenza Research Database.

from the other two viruses and from each other (Figure 4). These data indicate that the non-airborne-transmissible Charadriiformes virus, A/shorebird/Delaware/274/2009 (H1N1), was more Anseriformes-like, mainly in PB2, PB1, and NS gene segments.

Glycan array analysis of airborne-transmissible avian H1N1 influenza virus

Avian influenza viruses classically bind to sialic acids with α2,3 linkages, whereas human influenza viruses classically bind to sialic acids with α2,6 linkages.[15–17] Three of four viruses of Charadriiformes origin transmitted to the contact ferrets through the airborne route; thus, we investigated the glycan-binding specificities of wild-type A/shorebird/Delaware/300/2009 (H1N1) and the virus isolates obtained from donor, direct-contact, and airborne-contact ferrets infected with this virus. The wild-type virus showed a mixed binding preference to sialic acids, with α2,3 and α2,6 linkages, which is unusual for an avian virus. This mixed binding preference was maintained in viruses isolated from a donor ferret and a direct-contact ferret (Figure 5). Virus isolated from an airborne contact ferret did not show mixed binding preference. Instead, it showed a strong binding preference for sialic acids with α2,6 linkages, with minimal binding to sialic acids with α2,3 linkages (Figure 5). Sialic acids with α2,6 linkages were the same as those to which wild-type A/shorebird/Delaware/300/2009 (H1N1) bound. Therefore, viruses isolated from an airborne contact ferret did not acquire new binding specificities but merely lost binding specificities for sialic acids with α2,3 linkages (Figure 5). These data suggest that binding to sialic acids with α2,6 linkages is particularly important for airborne transmission of these viruses but less so for direct-contact transmission.

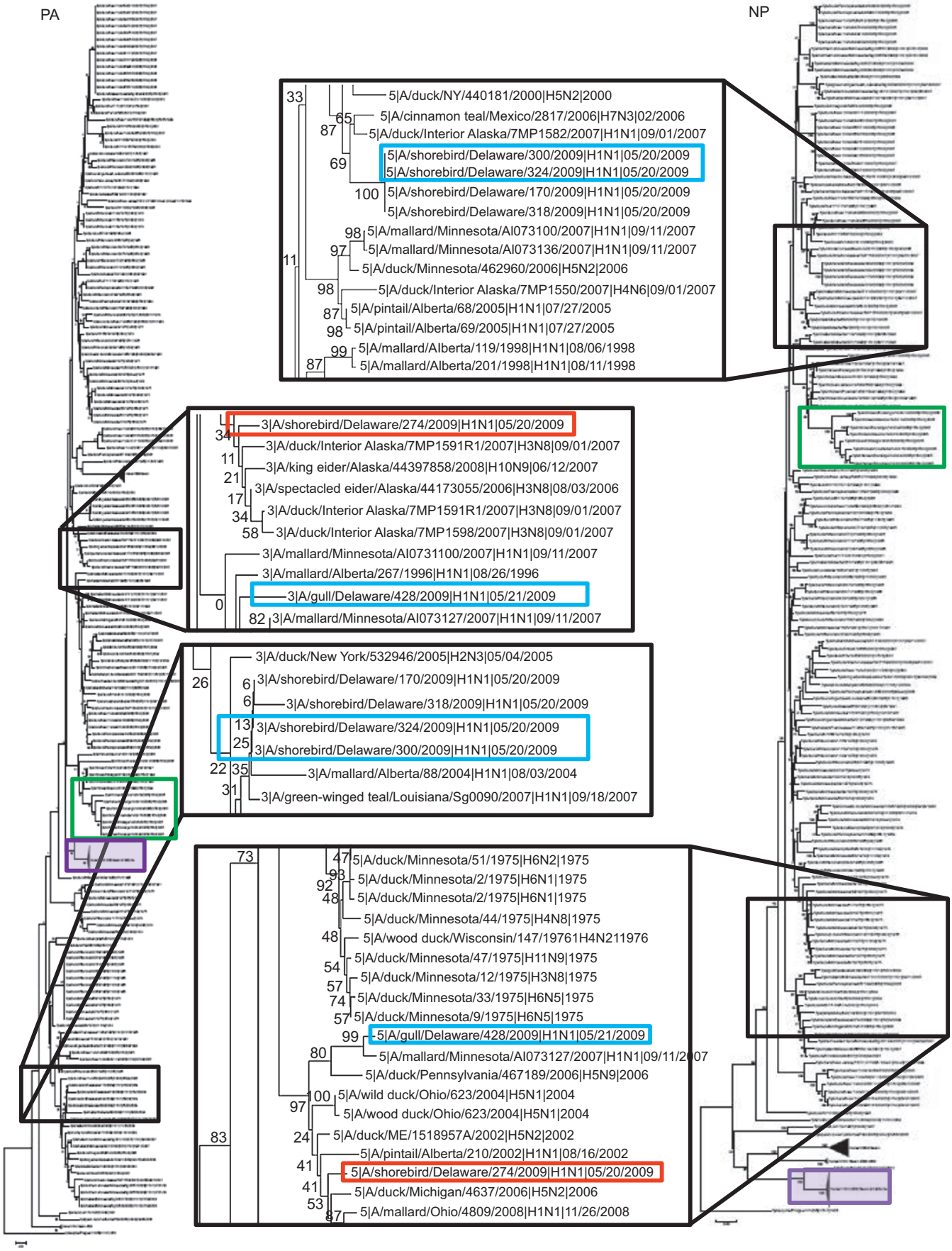
DISCUSSION

H1N1 influenza viruses have caused at least two pandemics in humans during the past century. This raises the possibility that those viruses in the aquatic bird reservoir have a unique ability to transmit to mammals and cause pandemics in humans. In the present study, we showed that H1N1 influenza viruses are perpetuated in Anseriformes and Charadriiformes species with non-overlapping cycles of dominance. Previous studies of the pathogenic potential of 31 North American H1N1 viruses from migratory ducks and shorebirds in DBA/2J mice indicated that H1N1 viruses from these birds were more pathogenic in mice than other subtypes from ducks and shorebirds, including H2N2 and H3N2 influenza viruses.[9] Preliminary studies of two of the viruses showed that both replicated in donor ferrets and transmitted to direct-contact ferrets but only the shorebird H1N1 virus transmitted through the airborne route to ferrets in separate cages. In the present study, we demonstrated that two of the three shorebird and gull H1N1 isolates transmitted to ferrets through the airborne route, whereas five H1N1 isolates from ducks did not. Additionally, we showed that the one shorebird influenza virus that did not transmit through the airborne route, A/shorebird/Delaware/274/2009 (H1N1), possessed multiple gene segments (i.e., PB2, PB1, and NS) reassorted from duck H1N1 influenza viruses. This result was consistent with the genomic comparison between airborne-transmissible and non-airborne-transmissible viruses. Therefore, gene reassortment between viruses of duck and shorebird origins probably contributed to the lack of transmission observed, though more work is needed to test this hypothesis.

Transmission of influenza viruses through the airborne route to ferrets is considered an important assay in assessing the risk of influenza viruses and their potential for human infection.[18] The present findings that H1N1 influenza viruses isolated from Charadriiformes species at Delaware Bay in 2009 transmit through the airborne route indicates that these viruses have a higher ''risk potential'' than H1N1 viruses from Anseriformes origin. Transmissibility, like pathogenicity, is a very complex property, dependent on both the virus and the host.[19] The viral gene products determining transmissibility include the surface glycoproteins (HA and NA), the polymerase complex (PB2, PB1, PA), and immune modulation by NS1. Virus replication in the upper respiratory tract of donor animals is also a primary requirement. In the present study, the viral loads of the Anseriformes-origin virus A/red-headed duck/Minnesota/Sg-00123/2007 (H1N1) detected in donor ferrets were as high as those of A/gull/Delaware/428/2009 (H1N1); however, it failed to transmit efficiently even to direct-contact ferrets. In contrast, A/gull/Delaware/428/2009 (H1N1) transmitted efficiently to both direct-contact and airborne-contact animals. Thus, virus load alone is insufficient to explain transmissibility. Characterization of the glycan-binding specificities of A/shorebird/Delaware/300/2009 (H1N1), one of the shorebird viruses that transmitted efficiently to ferrets, revealed that the airborne-transmitted virus had a strong binding preference for α2,6-linked sialic acids, which is classically found in human influenza viruses. Thus, airborne transmission in ferrets selected for α2,6 sialic acid-binding viruses, whereas direct-contact transmission was caused by a mixture of α2,3- and α2,6-linked sialic acid-binding viruses, similar to the wild-type virus.

The role of the polymerase gene products in replication and pathogenicity of influenza viruses is well established.[19] Specific mutations in the PB2 gene have been associated with host-range transmission of avian influenza viruses, including residues 627 and 701.[20,21] Airborne transmission of avian influenza viruses to guinea pigs is promoted by D301N and E627K, when 701N compensates for the lack of 627K.[19,22] Both duck and shorebird H1N1 viruses have avian-type amino acids at these residues in PB2 (i.e., E627 and D701). However, five naturally occurring amino acid differences across the PB2s of all three shorebird influenza viruses that transmit through the airborne route in ferrets and the six duck viruses that do not (I67V, A152S, A199T, R508Q, and V649I) indicate that these residues function in transmission. These five residues are associated with pathogenicity of the viruses in mice by residue effect meaning that observed residue variations affected the pathogenicity of the viruses in mice,[23] and four PB2 residues (67, 152, 199, and 508) are associated with pathogenicity by the host effect.[23]

The role of PB1 and PB1-F2 in host-range transmission is not well resolved. PB1-F2 shows dramatic differences between avian and


PA
NP
5|A/duck/NY/440181/2000|H5N2|2000
5|A/cinnamon teal/Mexico/2817/2006|H7N3|02/2006
5|A/duck/Interior Alaska/7MP1582/2007|H1N1|09/01/2007
5|A/shorebird/Delaware/300/2009|H1N1|05/20/2009
5|A/shorebird/Delaware/324/2009|H1N1|05/20/2009
5|A/shorebird/Delaware/170/2009|H1N1|05/20/2009
5|A/shorebird/Delaware/318/2009|H1N1|05/20/2009
5|A/mallard/Minnesota/AI073100/2007|H1N1|09/11/2007
5|A/mallard/Minnesota/AI073136/2007|H1N1|09/11/2007
5|A/duck/Minnesota/462960/2006|H5N2|2006
5|A/duck/Interior Alaska/7MP1550/2007|H4N6|09/01/2007
5|A/pintail/Alberta/68/2005|H1N1|07/27/2005
5|A/pintail/Alberta/69/2005|H1N1|07/27/2005
5|A/mallard/Alberta/119/1998|H1N1|08/06/1998
5|A/mallard/Alberta/201/1998|H1N1|08/11/1998
3|A/shorebird/Delaware/274/2009|H1N1|05/20/2009
3|A/duck/Interior Alaska/7MP1591R1/2007|H3N8|09/01/2007
3|A/king eider/Alaska/44397858/2008|H10N9|06/12/2007
3|A/spectacled eider/Alaska/44173055/2006|H3N8|08/03/2006
3|A/duck/Interior Alaska/7MP1591R1/2007|H3N8|09/01/2007
3|A/duck/Interior Alaska/7MP1598/2007|H3N8|09/01/2007
3|A/mallard/Minnesota/AI0731100/2007|H1N1|09/11/2007
3|A/mallard/Alberta/267/1996|H1N1|08/26/1996
3|A/gull/Delaware/428/2009|H1N1|05/21/2009
3|A/mallard/Minnesota/AI073127/2007|H1N1|09/11/2007
3|A/duck/New York/532946/2005|H2N3|05/04/2005
3|A/shorebird/Delaware/170/2009|H1N1|05/20/2009
3|A/shorebird/Delaware/318/2009|H1N1|05/20/2009
3|A/shorebird/Delaware/324/2009|H1N1|05/20/2009
3|A/shorebird/Delaware/300/2009|H1N1|05/20/2009
3|A/mallard/Alberta/88/2004|H1N1|08/03/2004
3|A/green-winged teal/Louisiana/Sg0090/2007|H1N1|09/18/2007
5|A/duck/Minnesota/51/1975|H6N2|1975
5|A/duck/Minnesota/2/1975|H6N1|1975
5|A/duck/Minnesota/2/1975|H6N1|1975
5|A/duck/Minnesota/44/1975|H4N8|1975
5|A/wood duck/Wisconsin/147/19761H4N211976
5|A/duck/Minnesota/47/1975|H11N9|1975
5|A/duck/Minnesota/12/1975|H3N8|1975
5|A/duck/Minnesota/33/1975|H6N5|1975
5|A/duck/Minnesota/9/1975|H6N5|1975
5|A/gull/Delaware/428/2009|H1N1|05/21/2009
5|A/mallard/Minnesota/AI073127/2007|H1N1|09/11/2007
5|A/duck/Pennsylvania/467189/2006|H5N9|2006
5|A/wild duck/Ohio/623/2004|H5N1|2004
5|A/wood duck/Ohio/623/2004|H5N1|2004
5|A/duck/ME/1518957A/2002|H5N2|2002
5|A/pintail/Alberta/210/2002|H1N1|08/16/2002
5|A/shorebird/Delaware/274/2009|H1N1|05/20/2009
5|A/duck/Michigan/4637/2006|H5N2|2006
5|A/mallard/Ohio/4809/2008|H1N1|11/26/2008

←

Figure 4 The PA and NP genes of the non-airborne-transmissible virus cluster separately from the three airborne-transmissible viruses. On the PA and NP trees, the virus A/shorebird/Delaware/274/2009 (H1N1), which did not transmit through the airborne route in ferrets (red boxes), clustered away from those of the viruses that did transmit through the airborne route, i.e., A/shorebird/Delaware/300/2009 (H1N1), A/gull/Delaware/428/2009 (H1N1), and A/shorebird/Delaware/324/2009 (H1N1) (blue boxes). A/shorebird/Delaware/300/2009 (H1N1) and A/shorebird/Delaware/324/2009 (H1N1) clustered together on both the PA and NP trees, whilst A/gull/Delaware/428/2009 (H1N1) clustered away from the other three viruses on both trees. These viruses did not cluster near human pandemic H1N1 viruses isolated in Mexico (purple boxes) or viruses isolated in chickens in Mexico (green boxes). Phylogenetic trees were constructed under Kimura-2-parameter model with the assumption of uniform rates among sites using nucleotide sequences of avian and human viruses isolated in North America or Mexico as recently as 2009 that were published in the Influenza Research Database.

mammalian strains. For example, avian influenza viruses encode full-length PB1-F2 gene products, but swine and human viruses encode mostly truncated products.[24] PB1-F2 is associated with increased pathogenicity of H5N1 and H1N1 Spanish influenza,[24,25] but its role in host-range transmission is unresolved. The largest number of amino acid differences across the three shorebird viruses that transmitted to ferrets through the airborne route and the six duck H1N1 influenza that did not were in the PB1-F2 protein. Four of the residues (H15R, N23S, T27I, and H75L) are associated with pathogenicity in mice by residue effect, and six residues (8, 15, 23, 27, 69, and 75) are associated with pathogenicity by host effect.[23] Thus, PB1-F2 substantially contributes to the transmissibility of shorebird H1N1 viruses to ferrets. PB1-F2 and PA-X also modulate host gene expression and may decrease pathogenicity.[26] The single difference detected in the C-terminal of PA-X, A21V, is associated with the pathogenicity by host–residue interactions,[23] which implicates the possible role of this gene product in the transmissibility of shorebird viruses in ferrets.

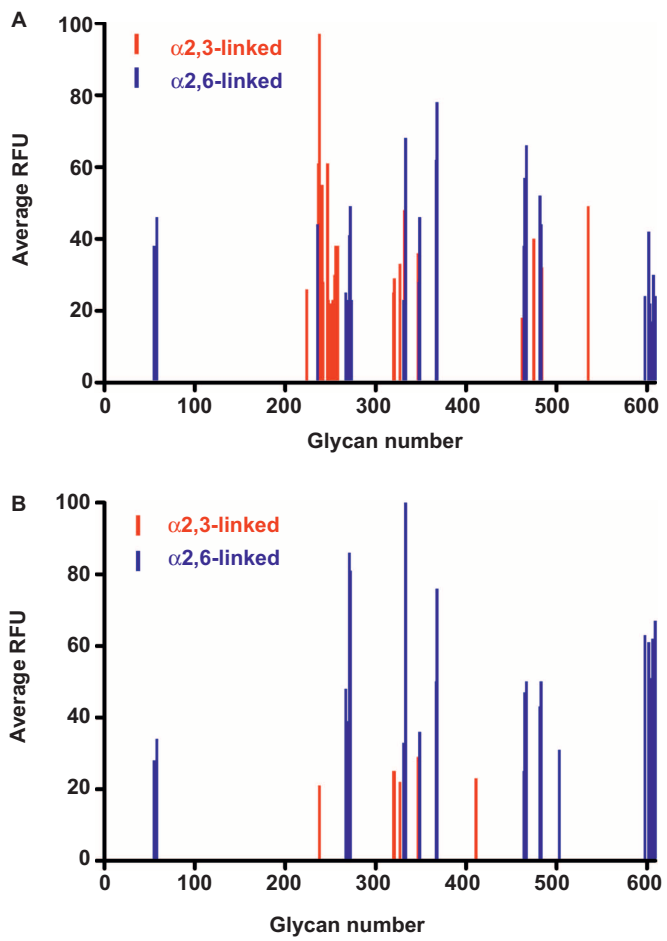


Figure 5 Altered glycan-binding specificities of A/shorebird/Delaware/300/2009 (H1N1) after airborne transmission in ferrets. (**A**) Viruses shed from a donor ferret showed mixed specificities for α2,3-linked (red plot) and α2,6-linked (blue plot) sialic acids. (**B**) In contrast, viruses shed from an airborne-contact ferret show stronger binding to α2,6-linked sialic acids and reduced binding to α2,3-linked sialic acids. RFU, relative fluorescent unit.

NS1 is a multifunctional protein that modulates viral RNA replication and protein synthesis and inhibits the innate immune response of the host.[19] Amino acid differences between the shorebird and duck influenza viruses, three in NS1 and two in NEP, imply that both of these genes are involved in airborne transmissibility. Most of these residues are associated with pathogenicity in mice by residue effect (S/T7L and E227G in NS1 and S/T7L and S70G in NEP) and by host effect (227 in NS1 and 70 in NEP).[23] The transmissibility of the three shorebird viruses to ferrets through the airborne route is a multigenic property involving residues in PB2, PB1, PB1-F2, PA-X, NS1, NEP, virus load, and the selection of the optimal receptor-binding characteristics from mammals (α2,6-linked sialic acid). The reassortant shorebird influenza virus A/shorebird/Delaware/274/2009 (H1N1) contained "duck-type" amino acids at those residues and failed to transmit through the airborne route, which also supports this hypothesis.

All three shorebird H1N1 viruses that transmitted through the airborne route were isolated in North America during 2009; the H1N1 influenza viruses isolated from shorebirds in previous years (1994, 2002, 2006) remain to be studied, as do H1N1 isolates from shorebirds and gulls from other parts of the world. Current evidence suggests that the influenza virus gene pools in North American ducks and shorebirds are probably not separated.[27–29] However, although the present study supports the idea that the H1N1 viruses in different hosts do reassort, some host-specific amino acids are not shared between the duck and shorebird gene pools. These findings support the notion that influenza viruses from North American Charadriiformes origin are species specific,[30] but additional studies of H1N1 viruses isolated from Charadriiformes species during earlier years and in other geographic regions are needed to confirm these findings.

We used PI scores determined in mice[9] as a basis for selecting viruses to use in ferret experiments. However, due to differences in the mouse and ferret models, we did not see mortality in ferrets, as we did in mice. However, the least pathogenic virus in mice[9] also replicated the least efficiently in ferrets.

In conclusion, we showed that H1N1 influenza viruses isolated from shorebirds at Delaware Bay in 2009 have the potential to transmit to ferrets through the airborne route in the absence of ferret adaptation. This finding supports the general consensus of an avian origin of mammalian influenza viruses. We also established the multigenic basis for host-range transmission, but many unanswered questions persist. Are the 2009 H1N1 influenza viruses from Delaware Bay unique, or are the molecular requirements of host-range transmission generally applicable?

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1 Jernigan D, Cox N. Human influenza: one health, one world. In: Webster R, Monto A, Braciale T, Lamb R (eds.) *Textbook of influenza*. 2nd ed. West Sussex, UK: Wiley Blackwell, 2013: 3–19.
2 Garten RJ, Davis CT, Russell CA *et al*. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009; **325**: 197–201.
3 Fouchier R, Guan Y. Ecology and evolution of influenza viruses in wild and domestic birds. In: Webster R, Monto A, Braciale T, Lamb R (eds.) *Textbook of influenza*. 2nd ed. West Sussex, UK: Wiley Blackwell, 2013: 175–189.
4 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992; **56**: 152–179.
5 Tong S, Li Y, Rivailler P *et al*. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A* 2012; **109**: 4269–4274.
6 Wang T, Palese P. Emergence and evolution of the 1918, 1957, 1968 and 2009 pandemic virus strains. In: Webster R, Monto A, Braciale T, Lamb R (ed.) *Textbook of influenza*. 2nd ed. West Sussex, UK: Wiley Blackwell, 2013: 218–230.
7 Gregg MB, Hinman AR, Craven RB. The Russian flu. Its history and implications for this year's influenza season. *JAMA* 1978; **240**: 2260–2263.
8 Krauss S, Walker D, Pryor SP *et al*. Influenza A viruses of migrating wild aquatic birds in North America. *Vector Borne Zoonotic Dis* 2004; **4**: 177–189.
9 Kocer ZA, Krauss S, Stallknecht DE, Rehg JE, Webster RG. The potential of avian H1N1 influenza A viruses to replicate and cause disease in mammalian models. *PLoS One* 2012; **7**: e41609.
10 Gulati S, Smith DF, Cummings RD *et al*. Human H3N2 influenza viruses isolated from 1968 To 2012 show varying preference for receptor substructures with no apparent consequences for disease or spread. *PLoS One* 2013; **8**: e66325.
11 Kocer ZA, Obenauer J, Zaraket H *et al*. Fecal influenza in mammals: selection of novel variants. *J Virol* 2013; **87**: 11476–11486.
12 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725–2729.
13 Krauss S, Pryor SP, Raven G *et al*. Respiratory tract versus cloacal sampling of migratory ducks for influenza A viruses: are both ends relevant? *Influenza Other Respir Viruses* 2013; **7**: 93–96.
14 Belser JA, Katz JM, Tumpey TM. The ferret as a model organism to study influenza A virus infection. *Dis Model Mech* 2011; **4**: 575–579.
15 Connor RJ, Kawaoka Y, Webster RG, Paulson JC. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 1994; **205**: 17–23.
16 Rogers GN, Paulson JC. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 1983; **127**: 361–373.
17 Suzuki Y, Ito T, Suzuki T *et al*. Sialic acid species as a determinant of the host range of influenza A viruses. *J Virol* 2000; **74**: 11825–11831.
18 Cox NJ, Trock SC, Burke SA. Pandemic preparedness and the Influenza Risk Assessment Tool (IRAT). *Curr Top Microbiol Immunol* 2014; **385**: 119–136.
19 Klenk H, Garten W, Matrosovich M. Pathogenesis. In: Webster R, Monto A, Braciale T, Lamb R (ed.) *Textbook of influenza*. 2nd ed. West Sussex, UK: Wiley Blackwell, 2013: 157–174.
20 Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 2001; **293**: 1840–1842.
21 Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol* 1993; **67**: 1761–1764.
22 de Jong MD, Simmons CP, Thanh TT *et al*. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006; **12**: 1203–1207.
23 Kocer ZA, Fan Y, Huether R *et al*. Survival analysis of infected mice reveals pathogenic variations in the genome of avian H1N1 viruses. *Sci Rep* 2014; **4**: 7455.
24 McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. *PLoS Pathog* 2010; **6**: e1001014.
25 Schmolke M, Manicassamy B, Pena L *et al*. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. *PLoS Pathog* 2011; **7**: e1002186.
26 Jagger BW, Wise HM, Kash JC *et al*. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 2012; **337**: 199–204.
27 Dugan VG, Chen R, Spiro DJ *et al*. The evolutionary genetics and emergence of avian influenza viruses in wild birds. *PLoS Pathog* 2008; **4**: e1000076.
28 Krauss S, Obert CA, Franks J *et al*. Influenza in migratory birds and evidence of limited intercontinental virus exchange. *PLoS Pathog* 2007; **3**: e167.
29 Widjaja L, Krauss SL, Webby RJ, Xie T, Webster RG. Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses. *J Virol* 2004; **78**: 8771–8779.
30 Hanson BA, Luttrell MP, Goekjian VH *et al*. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? *J Wildl Dis* 2008; **44**: 351–361.