Long-term surveillance of H7 influenza viruses in American wild aquatic birds: are the H7N3 influenza viruses in wild birds the precursors of highly pathogenic strains in domestic poultry?

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The emergence of influenza A virus (IAV) in domestic avian species and associated transmissions to mammals is unpredictable. In the Americas, the H7 IAVs are of particular concern, and there have been four separate outbreaks of highly pathogenic (HP) H7N3 in domestic poultry in North and South America between 2002 and 2012, with occasional spillover into humans. Here, we use long-term IAV surveillance in North American shorebirds at Delaware Bay, USA, from 1985 to 2012 and in ducks in Alberta, Canada, from 1976 to 2012 to determine which hemagglutinin (HA)–neuraminidase (NA) combinations predominated in Anseriformes (ducks) and Charadriiformes (shorebirds) and whether there is concordance between peaks of H7 prevalence and transmission in wild aquatic birds and the emergence of H7 IAVs in poultry and humans. Whole-genome sequencing supported phylogenetic and genomic constellation analyses to determine whether HP IAVs emerge in the context of specific internal gene segment sequences. Phylogenetic analysis of whole-genome sequences of the H7N3 influenza viruses from wild birds and HP H7N3 outbreaks in the Americas indicate that each HP outbreak was an independent emergence event and that the low pathogenic (LP) avian influenza precursors were most likely from dabbling ducks. The different polybasic cleavage sites in the four HP outbreaks support independent origins. At the 95% nucleotide percent identity-level phylogenetic analysis showed that the wild duck HA, PB1, and M sequences clustered with the poultry and human outbreak sequences. The genomic constellation analysis strongly suggests that gene segments/virus flow from wild birds to domestic poultry.

Keywords: American wild birds; Anseriforme; avian influenza; Charadriiforme; H7; highly pathogenic avian influenza; surveillance

INTRODUCTION

While there is general acceptance for the zoonotic origins of influenza A viruses (IAVs) from reservoirs in wild aquatic birds, limited information is available about whether peaks of influenza activity in wild birds correlate with virus spread to other species and about the potential predictive value of surveillance. In the aquatic bird reservoir, the IAVs show a cyclic dominance with peaks of activity for one season followed by low levels of detection. Long-term surveillance of IAVs in Anseriformes (ducks) in Alberta, Canada, from 1976 to 2012 and in Charadriiformes (shorebirds and gulls) at Delaware Bay in New Jersey and Delaware, USA, from 1985 to 2012 provides a large dataset that has contributed to the general understanding of influenza in the reservoir species and has established some of the ecological principles of influenza in nature, but these studies have not been evaluated for their predictive value. Of the 16 hemagglutinin (HA) subtypes of IAVs known to be circulating in the aquatic bird reservoir, two – H5 and H7 – have the unique ability to become highly pathogenic (HP) in domestic gallinaceous poultry.

Between 2002 and the present there have been four outbreaks of HP H7N3 influenza in poultry in the Americas, as represented by A/Chicken/Chile/4322/2002, A/Chicken/Canada/AVF2/2004, A/Chicken/Saskatchewan/HR00011/2007, and A/Chicken/Ialisco/CPA1/2012. Each of these outbreaks of HP H7N3 IAVs caused high mortality in gallinaceous poultry. These viruses acquired additional basic amino acids juxtaposed to the cleavage site in the HA, which is necessary for the HP phenotype. Generally HPAI HA gene segments acquire these basic amino acids through insertion of short stretches of adenosine and guanosine (e.g., AGAAAAAAAAGA) nucleotides and if these insertions are in frame, they will be translated to lysine/arginine. In one case, it appears that these basic residues were acquired by...
non-homologous recombination.12 The molecular mechanism by which HPAI typically evolves has not been clearly delineated, and we hypothesized that specific polymerase gene segments/constellations may favor the evolution of HPAI. Additionally, the HP H7N3 IAV from chickens in British Columbia in 2004 was associated with mild respiratory disease and unilateral conjunctivitis in two humans,13 and the Mexican HP H7N3 from 2012 was isolated from conjunctivitis in humans.14 Multiple outbreaks of low pathogenic (LP) H7N2 influenza occurred in chickens and turkeys between 1996 and 2004 in the eastern United States from South Carolina to Massachusetts, including Pennsylvania, New Jersey, and Ohio (reviewed in the ref. 15). In turkeys, the H7N2 viruses caused respiratory disease, egg production drop, lethargy, and depression,16 but there was usually limited mortality. In chickens, the H7N2 viruses were characterized by rapid spread and multi-causal respiratory disease.17 During 1996–2004, H7N2 IAVs were frequently isolated from live-bird markets along the east coast of the United States, which contributed to their spread and evolution. The spread and evolution of H7 viruses also lead to infrequent zoonosis such as: isolation of a LP H7N2 virus from the respiratory tract of an immunocompromised patient with mild respiratory disease;18 the isolation of a LP H7N7 IAV from Phoca vitulina (harbor seals) from a human with conjunctivitis after an experimentally infected seal sneezed in the face of the handler.19

Understanding the evolution of LP and HP H7 IAVs is important to animal health and pandemic preparedness. Because LP H7N9 influenza viruses have recently emerged in humans in China and have caused high mortality20 we determined the presence of H7N9 in wild aquatic birds and domestic poultry in America. In addition, because HP H7 influenza viruses have emerged multiple times in poultry in the Americas and have occasionally transmitted to humans and LP H7N2 viruses have spread in domestic poultry for several years in the United States, we examined our long-term surveillance data to evaluate its value as a predictive tool to forecast H7 outbreaks and gain a better understanding of the emergence of HP IAV. The present study utilizes long-term surveillance data on influenza in migratory birds to determine: (i) the patterns of point prevalence of H7 influenza in wild birds in the Americas, (ii) which HA–neuraminidase (NA) combinations have predominated, (iii) whether Anseriformes (ducks) and Charadriiformes (shorebirds) have similar or different HA-NA combinations, (iv) if there is concordance between peaks of prevalence and transmission of H7 in wild aquatic birds and the emergence of H7 IAVs in poultry and people, and (v) if a particular lineage of RNA polymerase gene segments/constellations have repeatedly given rise to HPAI viruses. Phylogenetic and genomic constellation analyses were used to infer the precursors of the HP influenza outbreaks and their estimated dates of emergence, as well as to determine whether HP viruses emerge in the context of specific internal gene segment sequences.

MATERIAL AND METHODS
Surveillance sites and sampling
Wild ducks. Surveillance was conducted at various lakes throughout Alberta, Canada at various timepoints spanning July through August from 1976 to 2012. Cloacal or paired cloacal and oropharyngeal swabs from hatch-year and after hatch-year ducks were collected from birds at pre-migration staging areas. Details about collection sites, species sampled, sample collection procedures, and transportation of specimens have been reported.1,21 A total 3693 IAVs were isolated from 17 369 samples (21.3%) representing 15 989 birds (3575 influenza positive birds (22.4%).

Shorebirds. Fecal samples, along with a limited number of cloacal swabs, were collected from migrating shorebirds at their stopover site at Delaware Bay, USA during May from 1985 to 2012. Fecal samples from gull species were also obtained. Samples came primarily from beaches in New Jersey, but from 1985 to 1990, a few beaches in Delaware also served as collection sites. Details about collection sites, species sampled, sample collection procedures, and transportation of specimens have been reported previously.1,22 A total of 1085 IAVs were isolated from 10 430 samples (10.4%).

Isolation and identification procedures
Viruses were isolated in 10-day-old embryonated hen’s eggs according to the protocol of Palmer et al. (1975).23 The HA and NA subtypes of the virus isolates were identified by hemagglutination inhibition (HAI)23 and neuraminidase inhibition (NAI)24 assays, respectively. In instances where the subtype could not be determined by HAI or NAI, we employed reverse transcription-polymerase chain reaction (RT-PCR) to amplify the HA and/or NA gene,25 followed by nucleotide sequencing and BLAST comparisons with GenBank records.

Survey of LP H7 in domestic poultry
Information on the detection of H7 avian influenza in domestic poultry in the Americas between 1976 and 2013 was obtained from reports to the World Animal Health Association for the years 2005–2013,26 reports to the US Animal Health Association for the years 1976–2013 (Supplementary Table S1) and reports made at the International Symposium on Avian Influenza for years 1986–2005.27–30

Statistical analysis
A chi-square test was used to compare the frequency of detection of H7N3 versus any other H7-NA combination. This test was also used to compare the frequency of detection of any H7 subtype or H7N3 between ducks and shorebirds. PASW (SPSS) 18 software was used (IBM, Armonk, NY).

Viral sequencing
Sequencing and genome assembly for the majority of viruses was done using a next-generation sequencing (NGS) pipeline at J. Craig Venter Institute that used the Roche 454 GS-FLX and the Illumina HiSeq 2000. Viral RNA was isolated and subjected to multi-segment RT-PCR (M-RTPCR),31,32 which simultaneously and specifically amplicons, (iv) if there is concordance between peaks of prevalence and transmission of H7 IAVs in poultry and people, and (v) if a particular lineage of RNA polymerase gene segments/constellations have repeatedly given rise to HPAI viruses. Phylogenetic and genomic constellation analyses were used to infer the precursors of the HP influenza outbreaks and their estimated dates of emergence, as well as to determine whether HP viruses emerge in the context of specific internal gene segment sequences.
were then used for reference-based assembly using CLC Bio’s clc_ref_assemble_long program.

**Phylogenetic and constellation analyses**

**Sequence collection and curation.** All complete avian and human H7 genomes isolated in North and South America were downloaded from GenBank, including historical references and equine H7N7 genomes. We also included South American H7 viruses known to have produced a HP outbreak whose genomes were not fully sequenced. Because our initial dataset included duplicated genomes due to variable strain names, we deduplicated the dataset by standardizing case, spelling, and abbreviations in the strain names for all segments. This yielded 467 genomes for the initial phylogenetic and constellation analyses.

**Maximum likelihood (ML) analysis.** Multiple alignment using fast Fourier transform (MAFFT) v7 was used to construct an HA alignment, which was checked and trimmed by hand to the coding region. jModelTest 2.4 was used to determine that the most appropriate nucleotide substitution model for our HA data was a general time reversible (GTR) model with a gamma rate distribution and invariant sites (GTR-IG). We performed a ML phylogenetic analysis for the HA nucleotide sequences using the GARLI 2.0 web service. We performed a ML phylogenetic analysis for the HA nucleotide sequences using the GARLI 2.0 web service. The resultant tree was colored based on the taxonomic Order of the host species (e.g., Anseriformes, Galliformes, etc.) using in-house PERL scripts.

**Bayesian analysis.** Using our initial ML and constellation analysis results, we chose a subset of our total genomes for a Bayesian analysis of HA nucleotide sequences by selecting viral strains with unique phylogenetic and reassortant histories while ensuring that host diversity, available NA subtypes, and historical reference strains were retained. To determine if our subsampled data exhibited temporal qualities, we performed an exploratory analysis with Path-O-Gen (available at http://tree.bio.ed.ac.uk/software/pathogen/) to measure root-to-tip divergence for a subsampled HA ML tree constructed using MEGA6 with a GTR-IG substitution model. The results supported the use of a molecular clock model, so we proceeded with our time-based Bayesian analysis using BEAST v1.8 on the Cyber Infrastructure for Phylogenetic Research Science Gateway (available at http://www.phylo.org/). Using a Bayes factor test to compare the use of a strict versus lognormal relaxed molecular clock for our data, we determined that a lognormal relaxed clock best fit our data. The resultant tree was colored based on the taxonomic Order of the host species (e.g., Anseriformes, Galliformes, etc.) using in-house PERL scripts.

**Genome constellation analysis.** Genomic variation and reassortant histories while ensuring that host diversity, available NA subtypes, and historical reference strains were retained. To determine if our subsampled data exhibited temporal qualities, we performed an exploratory analysis with Path-O-Gen (available at http://tree.bio.ed.ac.uk/software/pathogen/) to measure root-to-tip divergence for a subsampled HA ML tree constructed using MEGA6 with a GTR-IG substitution model. The results supported the use of a molecular clock model, so we proceeded with our time-based Bayesian analysis using BEAST v1.8 on the Cyber Infrastructure for Phylogenetic Research Science Gateway (available at http://www.phylo.org/). Using a Bayes factor test to compare the use of a strict versus lognormal relaxed molecular clock for our data, we determined that a lognormal relaxed clock best fit our data. The resultant tree was colored based on the taxonomic Order of the host species (e.g., Anseriformes, Galliformes, etc.) using in-house PERL scripts.

**RESULTS**

**Distribution of H7 influenza viruses and HA-NA subtype diversity in wild aquatic birds at North American surveillance sites**

Long-term surveillance of IAVs in migratory ducks in Alberta, Canada, from 1976 to 2012 and in shorebirds and gulls at Delaware Bay, United States, from 1985 to 2012 reveals differences in the frequency of NA subtypes that have been detected in combination with the H7 HA (Table 1). There was a statistically significant (P value < 0.001) predominance of H7N3 over all other H7-NA combinations in the sampled species (29 duck virus isolates and 44 shorebird virus isolates). The H7 subtype was more likely to occur in shorebirds and gulls (6.18%) rather than ducks (1.11%) (P value < 0.001). Similarly, H7N3 was more frequently detected in shorebirds and gulls (4.06%) than ducks (0.79%) (P value < 0.001). In ducks, N8 was the next most frequent NA subtype (four virus isolates), followed by N1 (three virus isolates), N5 and N9 (two virus isolates each), and N2 (one virus isolate). In ducks, N4, N6, and N7 were noticeably absent. In shorebirds, eight of the nine NA subtypes found in wild birds were detected in combination with an H7 HA; the most frequent NA subtype after N3 was N7 (seven virus isolates), followed by N4 (five virus isolates), N2 (four virus isolates), N5 (three virus isolates), N8 (two virus isolates), and N1 and N9 (one virus isolate each). As was the case for ducks, N6 was not detected in shorebirds.

**Temporal distribution of the H7N3 subtype in aquatic birds at North American surveillance sites**

Of all H7-NA combinations in both migratory ducks and shorebirds, H7N3 was the most common. The H7N3 combination occurred 12 times in ducks over 36 years of surveillance and seven times in shorebirds over 27 years of surveillance. H7N3 was detected much more frequently than any other H7-NA combination, sometimes occurring in both ducks and shorebirds within the same year (e.g., 1985 and 1996) or in consecutive years (e.g., 2001/2002 and 2010/2011) (Figure 1). Since 2000, there have been three major peaks of H7N3 activity: one in ducks in 2001 that contributed 25% of the total isolates for the year, one in shorebirds in 2006–2007 that constituted 55% of...
the total isolates those years, and another in 2011 in shorebirds that made up 40% of the total isolates that year.

**Reports of LP H7 IAVs in domestic poultry in the Americas**

To determine the incidence of H7 IAVs in domestic poultry in the United States from 1976 to 2013 (Table 2), we used USDA reports from live bird market influenza surveillance,27–30 reports of LP viruses on domestic poultry farms from the World Organisation for Animal Health (OIE),26 and reports from the United States Animal Health Association (Supplementary Table S1). H7N2 was detected most frequently, with 17 reports from markets and 22 reports from poultry farms; H7N3 was the next most frequently reported subtype (2 market outbreaks, 14 farm outbreaks), followed by H7N9 (6 farm outbreaks). The frequency of isolation of H7N1 and H7N7 was surprisingly lower, with only four and two reports, respectively. It is notable that four NA subtypes (N4, N5, N6, N8) were not detected in combination with H7 in domestic poultry, and two of these subtypes were not isolated from ducks (N4 and N6) or shorebirds and gulls (N6) in our surveillance studies.

It is noteworthy that LP H7N9 was the third most frequently detected H7–NA combination reported in commercial poultry (Table 2). These H7N9-associated outbreaks of respiratory disease in poultry occurred in Nebraska turkeys in 2007, Minnesota turkeys in 2009 and 2011, and Kentucky chickens in 2009; in total, these outbreaks involved over 300,000 birds.26 However, H7N9 was detected in only three sampling periods during our long-term surveillance in wild ducks and shorebirds: two duck isolates from 1999 and 2004 and one shorebird isolate from 1995. It was not detected in live bird markets from 1979 to 2013.

Since 2003, regulatory changes by the USDA have mandated the culling of all LP, as well as HP, H5, and H7 viruses. Thus, the LP H7N9 outbreaks of influenza in domestic poultry were stamped out. The timeline for the introductions of LP H7N2, H7N3, and H7N9 into domestic poultry is shown in Figure 2.

**Reports of HP H7 IAVs in the Americas**

Since the late 1950s, the only HP H7 IAV detected in the Americas has been H7N3.15 Prior to that time, HP IAVs were characterized based on

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**Table 1 Influenza H7-NA subtypes isolated during surveillance studies in wild aquatic birds in Alberta, Canada, and Delaware Bay, USA, between 1976 and 2012**

|          | N1 | N2 | N3 | N4 | N5 | N6 | N7 | N8 | N9 | Total NO of H7 isolates | Total NO of all influenza isolates | % of H7 isolates |
|----------|----|----|----|----|----|----|----|----|----|------------------------|-----------------------------------|-----------------|
| Ducks    | 3  | 1  | 29 | 0  | 2  | 0  | 0  | 4  | 2  | 41                     | 3693                                             | 1.1             |
| Shorebirds | 1  | 4  | 44 | 5  | 3  | 0  | 7  | 2  | 1  | 67                     | 1085                                            | 6.2             |
| Total    | 4  | 5  | 73 | 5  | 5  | 0  | 7  | 6  | 3  | 108                    | 4778                                            | 2.3             |

*P value < 0.001; a chi-square test was used to compare the frequency of detection of H7N3 in ducks versus the frequency in shorebirds.

*P value < 0.001; a chi-square test was used to compare the frequency of detection of any H7-NA subtype between ducks and shorebirds.

*P value < 0.001; a chi-square test was used to compare the frequency of detection of H7N3 versus any other H7-NA combination.

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**Figure 1 H7N3 HPAI domestic poultry outbreaks in the Americas relative to H7N3 wild bird isolates in North America between 1976 and 2012.**

H7N3 isolates obtained for ducks (red) and shorebirds (blue) is given as a percentage of the total number of annual influenza virus isolates sampled from each wild bird taxonomic Order. The four HPAI domestic poultry outbreaks that occurred in the Americas during this time period are marked with arrows.
disease signs in poultry and collectively referred to as “fowl plague”; the H5 and H7 subtypes were not specified, and the NA was not characterized. The emergence of a single HP subtype, H7N3, in the Americas contrasts with Eurasia and Australasia where multiple HP subtypes – H7N1, H7N3, H7N4, and H7N7 – have been sporadically reported in domestic poultry.\textsuperscript{15} In the Americas, HP H7N3 emerged in chickens in Chile in 2002 (A/Chicken/Chile/4322/2002); in chickens in British Columbia, Canada in 2004 (A/Chicken/Canada/AVF2/2004); in chickens in Saskatchewan, Canada in 2007 (A/Chicken/Saskatchewan/HR00011/2007); and in chickens in Jalisco, Mexico in 2012 (A/Chicken/Jalisco/CPA1/2012) (Figure 1). There was general concordance between the major peaks of LP H7N3 influenza detected in wild birds and outbreaks of HP H7N3 in domestic gallinaceous poultry.

Gene flow from wild to domestic birds in the Americas

Phylogenetic analysis of the H7 gene from both LP and HP viruses from wild and domestic avian species revealed that the H7 genes of viruses isolated from wild ducks and shorebirds are closely related to the LP and HP H7 genes of viruses isolated from North and South American poultry (Figure 3 and Supplementary Figure S1). The four HP poultry outbreaks in the Americas arose from polyphyletic strains (the green and red strain names within red boxes in Figure 3), indicating that each HP outbreak was an independent emergence event. Therefore, each event represents a separate introduction of LP avian influenza into poultry from the wild aquatic bird reservoir, most likely from dabbling ducks. H7 sequences from each H7N3 domestic poultry outbreak are most closely related to H7 sequences isolated from wild ducks during the same year as the outbreak and/or in the years immediately preceding the outbreak (dark blue strain names in Figure 3 representing Anseriformes). An alignment of the HP HA from each outbreak indicated that the polybasic cleavage site sequence varied among the four outbreak strains (Figure 4), further supporting their independent origins. The South American LP and HP strains form a distinct H7 lineage from the North American strains, which diverged around 1955 (with a 95% highest posterior density (HPD) range from 1938 to 1969) (Figure 3). All modern American H7 lineages diverged from historic European H7 strains around 1877 (95% HPD range from 1836 to 1911).

A genomic constellation analysis where each segment was clustered at a 90% nucleotide identity cutoff demonstrated that the HP H7 segments emerged in the context of different internal gene segment combinations (Figure 3). The three HP H7N3 lineages that emerged in North America possessed similar HA, NA, PB2, PB1, NP, and M genes at a 90% nucleotide identity cutoff, but they differed in their PA and NS genes, while the 2002 HP outbreak in Chile was generated by precursor viruses distinct in most gene segments from LP avian influenza strains found in North America. Using a more stringent evolutionary cutoff (95% nucleotide identity) (Figure 4), each HP H7N3 outbreak in domestic poultry and human spillover contains almost fully unique genome constellations, as evidenced by the different colors for strains from each outbreak within a column that represents one of the eight gene segments (Figure 4). The only exceptions are the 2012 Mexican and 2004 British Columbian outbreaks that had NS segments that share 95% nucleotide identity, M segments that are within 95% nucleotide identity for all three North American outbreaks, and the 2007 Saskatchewan and 2012 Mexican outbreaks that had PB1 segments that share 95% identity. When multiple sequences are available from a single outbreak, their genome constellations are identical at 95% nucleotide identity.

Table 2 Influenza H7-NA subtypes reported in domestic poultry in the Americas between 1976 and 2013

| NA  | N1 | N2 | N3 | N4 | N5 | N6 | N7 | N8 | N9 | Total NO of H7 reports |
|-----|----|----|----|----|----|----|----|----|----|------------------------|
| Commercial farms   | 4  | 22 | 14 | 0  | 0  | 0  | 2  | 0  | 6  | 48                     |
| Live bird markets  | 0  | 17 | 2  | 0  | 0  | 0  | 0  | 0  | 0  | 19                     |
| Total             | 4  | 39 | 16 | 0  | 0  | 0  | 2  | 0  | 6  | 67                     |

Figure 2 Outbreaks of low pathogenic H7 influenza virus subtype in domestic poultry in the Americas between 1976 and 2013.
Figure 3  Avian influenza virus H7 subtype evolution in the Americas and the emergence of HP viruses in a whole-genome context. For all sampled wild bird sequences, all available highly pathogenic H7N3 strains for which the majority of internal genes were also sequenced, and a number of reference genomes, an HA nucleotide phylogeny was inferred using the GTR-IG substitution method, a lognormal relaxed clock, and a skygrid coalescent model in BEAST v1.8 with a chain length of 100 million. Runs were evaluated in Tracer to ensure reasonable effective sample size scores, and a maximum clade credibility tree was constructed using Tree Annotator. Estimates for the most recent common ancestor with the outgroup sequences (1877) and between the North and South American viruses (1955) are provided, along with the 95% HPD ranges (also indicated as horizontal blue bars). A genome constellation analysis was performed by generating gene clusters for each segment using a 90% nucleotide identity cutoff. Cluster assignments are represented by one colored box for each segment, creating a genome constellation for each virus. Coloring between columns is independent, and the total number of colors in a column reflects the number of clusters generated for that gene segment. Black indicates that the gene sequences were unavailable. Strain names are colored by taxonomic Order. Human and/or poultry outbreak strains are boxed in red, and HP strains are marked with red circles based on their HA cleavage site sequences.

Comparison of wild bird sequences that shared the closest cophenetic distance to the H7N3 poultry and human outbreak sequences in the H7 Bayesian phylogeny showed that the duck HA, PB1, and M sequences usually clustered with the poultry and human outbreak sequences at 95% nucleotide identity. In addition, when N3 strains were available for comparison, the NA duck sequences also clustered with the poultry and human outbreak sequences at 95% nucleotide identity. The remaining segments usually differed between the most cophenetic wild bird sequences and the poultry and human outbreak sequences, demonstrating that those wild bird segments were not closely related to the outbreak sequences. This is likely a reflection of the fact that wild bird sampling for influenza surveillance is not performed at a resolution across the Americas that allows for the identification of the matching precursor wild bird strains that emerge in poultry. Collectively, the genomic constellation analysis strongly suggests that gene segments/viruses flow from wild birds to domestic poultry and demonstrates that HP H7 poultry outbreaks represent independent emergence events arising from different polymerase gene lineages.

The New York live bird market H7N2 isolates collected in the 2000s form their own distinct HA phylogenetic lineage (Figure 3 and Supplementary Figure S1), with no clear origin from the North American wild bird populations that were sampled in this study. This is also evidenced by these viruses belonging to their own unique HA cluster at a 90% nucleotide identity cutoff (Figure 3, purple boxes in the first column of the genome constellations).

DISCUSSION

The available evidence on the emergence of HP avian IAVs supports the hypothesis that they emerge from a reservoir of LP IAVs in aquatic birds. Transmission of viruses between wild aquatic birds and domestic poultry may occur in a number of ways. When migratory birds scavenge food, there is direct contact between migratory waterfowl and free-range domestic species including ducks, geese, and “backyard” poultry. Additionally, there is indirect contact between migratory waterfowl and domestic poultry through contaminated water supplies because the majority of the IAVs in migratory waterfowl replicate in the intestinal tract and are transmitted by fecal–oral transmission through environmental contamination, which includes contaminated, untreated water.

After introduction into domestic poultry from wild migratory birds, IAVs are spread by humans either directly by carrying fomites or through the poultry trade, as birds are moved from hatcheries to poultry farms.

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**Figure 4** Genome constellation analyses at a 95% nucleotide identity cutoff further demonstrate the uniqueness of each HP H7N3 outbreak in the Americas. A genome constellation analysis using a 95% nucleotide identity cutoff was performed on a subset of strains, including the human and poultry outbreak strains and some of the wild bird strains (all from the Order Anseriformes) having the closest cophenetic distances in the Bayesian HA phylogeny to the outbreak strains. Cluster assignments are represented by one colored box for each segment, creating a genome constellation for each virus. Coloring between columns is independent, and the total number of colors in a column reflects the number of clusters generated for that gene segment. Black indicates that the gene sequences were unavailable. Strain names are colored by taxonomic Order, and HP strains are marked with red circles based on their HA1/HA2 cleavage site sequences, which are provided on the left. Critical basic residues are colored in red.
Additionally, live poultry markets are an optimal place to amplify and spread IAVs, and the number of such markets in the major cities on both coasts of the United States is higher than most people appreciate. Live bird markets have been described as the breeding grounds for IAVs and are an optimal site for surveillance for IAVs in domestic poultry.

The emergence of IAVs in domestic avian species and the associated transmissions to mammals, including swine and humans, are currently unpredictable. Of current concern are the H7 IAVs, particularly with H7N3 IAVs repeatedly becoming HP for poultry in the Americas and occasionally spilling over into humans. In addition, LP H7N9 viruses in poultry have emerged in China and are HP in humans. Here, we demonstrate that long-term surveillance for influenza in aquatic bird reservoir species can serve as an early warning system for determining when LP and HP poultry outbreaks are likely to occur for certain subtypes. While there was general concordance between the emergence of HP H7N3 with peaks of virus prevalence in shorebirds and ducks, there were no predictable peaks of activity in wild birds for either LP H7N2 or H7N9 in domestic poultry. For H7N3, there were multiple independent introductions into domestic poultry that may have occurred by direct or indirect contact between wild birds and free-ranging poultry. While the Chilean and Canadian HP H7N3 outbreaks were stamped out, the Mexican outbreak was not initially eradicated and spread widely in poultry in seven states in Mexico, sometimes causing conjunctivitis in humans. Adoption of poultry vaccinations has reduced the severity of disease in chickens in Mexico but has not reduced transmission. There is also concern that transmission to humans may be more frequent than reported.

Overall for wild bird H7 IAVs, shorebirds carried the majority of possible HA–NA combinations; eight of the nine NA subtypes found in aquatic birds were detected in shorebirds, while only six of the nine NA subtypes were found in ducks. The most frequent combination was H7N3, which was found in both shorebird and duck species, but more frequently in shorebirds. However, our genomic analyses suggest that H7 poultry strains in the Americas are most closely related to H7 strains in ducks, rather than shorebirds. This suggests that dabbling ducks are the primary source of the H7 domestic poultry viruses, and broadening surveillance to additional sites would provide finer resolution for the evolution and transmission dynamics of H7 gene flow between shorebirds, ducks, and domestic poultry.

Our surveillance studies in shorebirds were conducted each May when the birds stopover in Delaware Bay to refuel on horseshoe crab eggs en route to their breeding grounds in Northern Canada. At this time, the shorebirds have migrated directly from South America and are shedding high levels of IAVs of most subtype combinations. Delaware Bay has been recognized as a “hot spot” for IAV isolation. Interestingly, all H7 viruses detected in these Delaware Bay shorebirds match North American strains and are phylogenetically distinct from available poultry and duck South American H7 strains. However, no H7 sequences from South American shorebirds were available for comparison. In the future, surveillance and genomic sequencing from South American wild bird species is important to understand long-range intercontinental transmission dynamics of H7 viruses. There appears to have been a single introduction of H7N2 from its wild reservoir into domestic poultry in 1994, and this subtype has since been maintained in live poultry markets and poultry houses in the mid-Atlantic and Northeastern United States. The detection of multiple H7N9 viruses in domestic poultry (primarily turkeys), and only three H7N9 viruses detected during 36 years of wild bird surveillance, suggests that these viruses are rarely sampled in the current surveillance or were generated within poultry and/or have a preference for domestic poultry.

These H7N9 viruses probably arose through direct contact between free-range reared birds and wild birds.Stamping-out protocols presumably eliminated the H7N9 viruses from domestic turkeys and chickens, and the virus did not enter the live poultry market system. While the HP phenotype is a polygenic property, the HA is considered the most critical determinant because cleavage activation of the polybasic HA1/HA2 cleavage site by ubiquitous cellular protease(s) leads to systemic spread. There appears to be two molecular mechanisms by which HP viruses are generated from LP precursors. One mechanism involves stuttering of the polymerase during RNA replication, whereas an atypical mechanism involved the insertion of a series of basic amino acids in the connecting peptide of the HA via insertion of a fragment of another viral RNA. The genomic analysis conducted illustrates that HP H7 poultry outbreaks represent independent emergence of HP HA cleavage site acquisition events, which arose within the context of different polymerase gene segment lineages. One might anticipate that the RNA polymerase gene constellations that give rise to stuttering versus recombination mediated HP HA would stem from different lineages. However, it also appears the multiple HP genotypes generated via stuttering-like mechanism are also generated from different RNA polymerase gene segment lineages.

Although surveillance of influenza in wild aquatic birds has provided information on the ecology and origins of pandemic IAVs, its overall predictive value remains limited to specific subtypes. Our surveillance indicates that for H7N3 viruses there are correlates between peaks of activity in wild birds and outbreaks of HP influenza in domestic poultry. While it appears that H7N3 activity in wild aquatic birds is a precursor for outbreaks in poultry, this could not be validated statistically in our study due to a small number of occurrences and potential unreported outbreaks. However, forewarning of the circulation of H7N3 IAV in wild birds and increased biosecurity on poultry farms could potentially prevent the emergence of HP avian influenza outbreaks. The detection and spread of LP H5 or H7 in gallinaceous poultry is considered indicative of the emergence of a HP strain and is therefore stamped out, typically by depopulation. Changes in turkey husbandry, from open range to indoor production, and the culling of all LP and HP H5- or H7-infected birds have already contributed to reducing the emergence of HP influenza in the Americas. Early warning of potential HP H5 or H7 outbreaks, at least in the Americas, may be possible using wild bird influenza surveillance, especially at hot spots on each flyway.

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