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# Phylogenetic Analysis of Low Pathogenicity H5N1 and H7N3 Influenza A Virus Isolates Recovered from Sentinel, Free Flying, Wild Mallards at One Study Site During 2006

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## Abstract

From August 2-October 11, 2006, clusters of low pathogenicity (LP) North American lineage H5N1 and H7N3 avian influenza A viruses (AIV), and other subtypes, were recovered from free-flying, resident, wild mallards used as sentinels at one site. The antigenic subtypes, pathogenicity potential, and Sanger sequencing of the isolates determined the H5N1 and H7N3 isolates were only recovered from samples collected on 8/2/2006 and 9/8/2006, respectively. However, subsequent efforts using next-generation sequencing (NGS) and additional Sanger sequencing found partial H7 segments in other HA-NA virus combinations on 8/2/2006, 9/8/2006 and 10/11/2006. It is well established that over larger geographic areas and years AIVs form transient genomic constellations; this sequential sampling data revealed that over a short period of time the dynamics of AIVs can be active and newer sequencing platforms increase recognition of mixed infections. Both findings provide further insight into the natural history of AIVs in natural reservoirs.

## INTRODUCTION

Low pathogenic (LP), avian-origin influenza A viruses (AIVs) are widely distributed in free-ranging *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (shore birds and gulls) (Fouchier and Munster, 2009; Krauss et al., 2004; Munster and Fouchier, 2009; Olsen et al., 2006; Slemons and Easterday, 1976; Slemons et al., 1974). The LP AIVs recovered from *Anseriformes* and *Charadriiformes* include all known hemagglutinin (HA) subtypes (H1-H16) and all known neuraminidase (NA) subtypes (N1–N9) and the most diverse array of HA-NA combinations maintained in any host groups. Yet after nearly 40 years of AIV surveillance in wild birds, it is not yet clear how this diversity is maintained in these natural

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reservoirs. In wild duck reservoir species, the wild bird-origin LP AIVs cause asymptomatic infections in the respiratory and lower gastrointestinal tracts; the latter infections are associated with a longer period of viral shedding and viruses escaping from the infected hosts in feces (Krauss et al., 2004; Slemons and Easterday, 1977; Slemons and Easterday, 1978). A recent survey of the genetic diversity of duck-origin North American LP AIV genomes recovered over two decades illustrated no clear pattern of gene segment association, which supported the hypothesis that LP AIV in wild birds form 'transient genome constellations' that are continually being reshuffled by reassortment (Dugan et al., 2008).

Most significance given to wild bird-origin LP AIVs maintained in waterfowl stems from the fact that they have been associated with stable host switch events into novel hosts (Taubenberger and Kash, 2010). Adaptation to, and generally mild symptomatic infections of, domestic poultry are the most frequent of these events (Swayne and Pantin-Jackwood, 2006). Once in poultry, strains of poultry-adapted H5 or H7 LP AIV can sporadically evolve into highly pathogenic avian influenza A virus (HP AIV) through acquisition of an insertional mutation resulting in a polybasic amino acid cleavage site within the HA (Garcia et al., 1997; Lee et al., 2005; Pantin-Jackwood and Swayne, 2009; Spackman et al., 2007; Suarez et al., 1999; Suarez, Spackman, and Senne, 2003; Swayne and Suarez, 2000). More recently molecular virology data have provided convincing evidence that wild birds appear to represent major natural reservoirs for influenza A viruses that can subsequently adapt to or donate gene segments through reassortment to novel influenza A viruses infecting horses, and swine, and contributed to the emergence of pandemic influenza A viruses infecting humans (Taubenberger and Morens, 2009; Webster et al., 1992). The last four human influenza pandemic influenza viruses all contained two or more novel gene segments that were very similar to those found in wild birds hosts (Taubenberger and Kash, 2010).

Historically, H5 and H7 LP AIVs have only been sporadically recovered from North American wild birds (Krauss et al., 2004; Spackman et al., 2007; Suarez, Spackman, and Senne, 2003); however, increasing surveillance efforts over the last 10 years have shown that these HA subtypes are most likely circulating in wild ducks, at least in low levels, in combination with any one of several NA subtypes every year. Over a four-month period in 2006, monthly monitoring of a resident, free-flying, wild mallard population at one study site in Maryland resulted in the recovery of clusters of North American lineage H5N1 and H7N3 LP AIV isolates along with AIVs possessing more commonly detected HA-NA combinations during August, September and October. No isolates were recovered during November. The H5N1 and H7N3 isolates were of most interest since, in poultry these subtypes have been associated with the emergence of HP AIVs. To gain insight into the molecular dynamics of these LP AIV isolates, full-length sequences were examined for genetic diversity, and then included in the larger context of North American AIV phylogeny. Three additional H5 isolates recovered from wild ducks at different locations, including one H5N1 isolate recovered in Ohio during 2004, one H5N2 isolate recovered in Maryland earlier in 2006, and one H5N1 isolate recovered in Maryland in 2007 and genomic sequence data from temporally (2005) and spatially (within 30 miles) related LP AIV isolates available in GenBank. The initial goal was to use the sentinel mallard population to monitor for the presence of AIV in local wild birds before the arrival of avian migrants.

## RESULTS

AIVs were recovered from fecal samples collected at a sentinel wild mallard (*Anas platyrhyncos*) flock location on August 2, September 8, and October 11, 2006, and no isolates were recovered from samples collected on November 24. No AIV were recovered

from the source flock of hatch-year mallards on 6/8/2006, 7/11/2006, 7/31/2006, and 9/8/2006.

On August 2nd, 26 of the 50 fresh fecal samples collected off the ground across the loafing area used by the resident, wild mallards, which were the only birds observed in this immediate area, were positive for type A influenza a virus isolates. Antigenic subtyping identified 24 LP North American lineage H5N1 AIV isolates, one H5N1,4 isolate, and one as H5N2 isolate. However, based on Sanger sequence data, the H5N1,4 and H5N2 isolates were also identified as H5N1 isolates (Table 1). Based on subsequent NGS data, the antigenically subtyped H5N1,4 isolate was also found to contain H7 HA and N3 NA consensus gene sequence fragments of >500 nucleotides.

Seventeen of the 69 September 8, 2006 fecal samples collected at the same location were positive for AIV recovery. Antigenic subtyping identified 14 North American lineage LP AIV H7N3 isolates and three H4N6 isolates, and based on Sanger sequencing data, one of the H4N6 isolates also contained an H7 sequence fragment (Table 1). On October 11, 2006, four of the 50 fecal samples were positive for AIV recovery and antigenic subtyping identified one H3N6 isolate, two H4N6 isolates, and one H6N1 isolate. Sanger sequences obtained from the H3N6 isolate also demonstrated H4 and H7 HA partial gene sequences. All 49 cloacals swabs collected from the sentinel mallard population on November 24 were negative for AIV.

The H5 and H7 influenza A virus isolates genome constellations recovered from the sentinel mallards in August and September 2006 at the study site suggest a single introduction of LP AIV H5N1 and H7N3 virus, respectively. The LP H5N1 AIV isolates were only recovered from samples collected on August 2, 2006; genomic sequence data provided no evidence for the persistence of the H5 HA gene in the sentinel populations, and the genomic constellations of 12 H5N1 isolates from August 2 were virtually identical. This suggests that the H5N1 virus spread quickly through the population, infections peaked, and then this lineage of virus appeared to clear from the population. Even so, there was molecular evidence that the isolate antigenically identified as H5N1,4 was from a mixed infection. Identifying the NA as N1,4 is not uncommon when antigenically subtyping neuraminidases (RDS, unpublished data) and the Sanger sequencing indicted this was an H5N1 isolate. However, the NGS data detected a partial H7 HA gene sequence fragment of 626 nucleotides matching the H7 sequences from the H7N3 isolates from 9/8/2006, and a partial NA N3 gene fragment of 586 nucleotides. Based on these data, it appears the host was experiencing a mixed infection involving H5, H7, N1, and N3 genomic segments. The absence of a detectable N4 segment did not support the N1,4 antigenic subtype, and may indicate an immune cross reaction between these subtypes caused by the N1 and N4 reference antibodies and antigens used in the neuraminidase inhibition test.

The persistence of the H7 HA gene in the sentinel population appears to be different from the H5 genome. Like the H5N1 viruses, the H7N3 viruses were only antigenically identified on one day (September 9, 2006) and the genomic constellations of the sequenced H7N3 isolates were also essentially identical. However, there were sequence data that the identical H7 HA gene was present on August 2, see above, and later on October 11. An October 11 AIV isolate was antigenically subtyped as H3N6 but Sanger sequencing demonstrated at least partial sequences for H3, H4, H7, and N6 genes. Also, Sanger sequencing one of the September 8 isolates identified as H4N6 antigenically demonstrated at least a partial H7 sequence. Thus, the H7 HA gene was maintained at this study site at least at a low frequency over three months (Table 1). While sequences derived from the mixed infections were excluded from the phylogenetic analyses performed next on the genomes of the H5N1 and H7N3 isolates, these results are compatible with previous studies showing a high incidence

of mixed subtype influenza infections in wild birds (Wang et al., 2008), and further supports the evidence of common reassortment among LPAIV in wild birds.

As noted above, the genomes of 12 LP H5N1 AIV isolates recovered from samples collected on August 2, 2006 were nearly identical to each other (Tables 2 & 3). Comparing these H5 segments to other H5 HA sequences revealed that they were most closely related to the HA gene of two H5N1 AIV isolates recovered from Canadian mallards in 2005 [A/mallard/MB/458/2005 (H5N1) and A/mallard/ON/499/2005 (H5N1)], an H5N1 isolate recovered from a wild mute swan sampled in Michigan on August 8, 2006, and an H5N1 isolate recovered from a wild mallard sampled at a site approximately 35 miles away in Maryland in 2007 (Table 2; Figure 1).

As stated above the genomes of five sequenced H7N3 virus isolates recovered from samples collected on September 8, 2006 were also nearly identical. Comparing these sequences to other H7 sequences (Table 4, Figure 2) revealed that they were most closely related to LP AIV H7 HAs from North American ducks and Delaware shorebirds and less related to HAs from the 2004 HP AIV H7N3 outbreak in British Columbia (Hirst et al., 2004) and the HA of the H7N2 viruses isolated in New York live bird markets (Spackman et al., 2003). No expanded cleavage sites were observed in either the HA gene sequence in the 2006 Maryland H5N1 LP AIVs or the H7N3 LP AIVs. The MD H7 genomic sequences also lacked the characteristic deletion in the HA1 domain noted in the New York live bird market H7 AIV isolate sequences (Spackman et al., 2003), further supporting their genetic distance from this lineage of poultry-adapted H7 AIVs. Likewise, no stalk deletions were observed in any of the NA sequences of the MD H5N1 and H7N3 AIV isolates; such NA stalk deletions have been associated with AIV adaptation to gallinaceous poultry (Spackman et al., 2003).

Analysis of the genome constellations of the internal gene segments of the Maryland H5N1 and the H7N3 LP AIVs revealed significant differences over time when compared to other H5 and H7 isolates, supporting the non-stable association of LP AIV virus gene segments (Tables 3 and 4). Of the AIV isolates recovered at this study site the two H4N6 isolates, recovered on the September 8, 2006, showed the most diverse reassortment patterns (Table 2), sharing only the NP and HA genes with 98% similarity, and the NA gene with 97.6%. These H4N6 viruses also had a closely related NP gene with an H3N6 virus isolated one month previously. One of these isolates, A/environment/MD/1101/2006 (H4N6), had a PB1 and NS gene that were closely related to the H5N1 reference sequence isolated from the population approximately one month previously, with nucleotide identity at 99.2% and 98.6%, respectively (Table 2). While the immediate source of these genomic segments was not determined, these data strongly support previous analyses that LP AIV gene segments are readily being exchanged via reassortment during mixed infections in wild birds (Dugan et al., 2008).

Further support for the frequent occurrence of genomic reassortment among LP AIVs was revealed from the comparative analysis of the gene segments of the 2006 LP AIV H5N1 environmental samples from Maryland and the viral genomes of other H5 AIV isolates from North American wild birds (Spackman et al., 2007). Phylogenetic analysis of the 2006 H5 HA sequences compared to those of Spackman *et al*. (Spackman et al., 2007) revealed that these isolates corresponded to their Group 4 clade (Figure 1).

In comparison to 13 other complete genomes available from North American H5 subtype LP AIV (Table 3), a large number of viral genome constellations were apparent, indicative of little linkage among the gene segments. In all, thirteen different genome constellations were identified among the thirteen H5 LP AIVs compared with the reference sequence (A/environment/MD/1158/2006/H5N1). Phylogenetic and reassortment patterns showed that

two Canadian wild mallard-origin LP AIV isolates, A/mallard/MB/458/2005 (H5N1) and A/mallard/ON/499/2005 (H5N1) (Spackman et al., 2007), are very closely related to the environmental H5N1 isolates from Maryland for seven segments, but the 2005 Canadian H5N1 isolates share PB2 gene segments that are in a different clade as compared to the 2006 Maryland H5N1 isolates (Table 3, Figure 1). This genome constellation pattern suggests that these isolates are linked both by migration between sites in central Canada and Maryland but limited reassortment occurred along the inter-migratory flyway routes possibly across the south shore of Lake Erie.

Similarly, reassortment analysis between the 2006 LP AIV H7N3 environmental samples and the 10 available North American LP AIV H7N3 viral genomes revealed frequent reassortment and very little evidence of gene segment linkage (Table 4). A total of nine genome constellations among the ten H7N3 isolates were identified in comparison to the reference sequence [A/environment/MD/261/2006 (H7N3)]. Analysis of the NA genes from the environmental H7N3 isolates revealed that they are closely related to the A/blue wing teal/OH/658/2004 (H7N3) NA gene sequence and to the NA gene from A/shorebird/DE/22/2006 (H7N3). Interestingly, excluding the expanded cleavage site, the H7 HA of the HP AIV H7N3 viruses isolated in British Columbia in 2004 are closely related to the HA of the 2006 Maryland H7N3 isolates, and shared 96% homology with the reference sequence (Figure 2). However, no other closely related gene segments were observed between the 2004 British Columbia HPAIV H7N3 isolates and the 2006 Maryland LP AIV H7N3 isolates (Table 3). These results further support both frequent reassortment but also the rapid movement of influenza viruses between flyways in North America (Chen and Holmes, 2009; Slemons et al., 2003).

## DISCUSSION

Relatively recent molecular analyses of avian-origin influenza A viruses (AIV) recovered from wild birds sampled at widely separated geographic areas over three decades have increased the general understanding of the natural history of these viruses in their natural reservoirs (Dugan et al., 2008; Obenauer et al., 2006). In this study, evidence was found for introductions of LP AIV H5N1 and H7N3 isolates within a population of hatch-year, free-flying, resident, wild mallard ducks at one site in Maryland during the summer of 2006 and also for genomic segment reassortment among recent LP AIV isolates, including H5N1 and H7N3 subtype viruses, from North America. The initial introduction of LP AIV H5N1, possibly followed by LP AIV H7N3 viruses into this sentinel population showed the mixing of gene segments over a relatively short period of time with evidence of mixed infections and probable reassortment within one season at one sampling site in 2006. These observations are consistent with a previous large-scale LP AIV genomics study demonstrating frequent reassortment between viral isolates recovered from 1986–2005 (Dugan et al., 2008; Obenauer et al., 2006). Extending the genomic analyses from among the LP AIV genomes obtained during the surveillance period itself to those of the North American AIVs from the past 30 years available in GenBank, strongly supports the lack of stable genome constellations associated with particular HA-NA subtype combinations, and also supports the movement of AIVs (and individual gene segments via reassortment) across the flyways of the North American continent, for example the H7 HA from these H7N3 isolates in Maryland were closely related to the H7 sequences from the 2004 British Columbia HPAIV H7N3 isolates.

Reassortment was further supported by the detection of several discrepancies between serologic (antigenic) and genomic subtyping and genotypic evidence of mixed infection in several of the isolates from this study (Table 1), especially using sensitive NGS technologies. Moreover, the discrepancies between antigenic and genomic subtyping and

evidence of mixed infection are suggestive of potential reassortment opportunities in progress at this local site over a relatively short period of time (Wang et al., 2008).

These studies support the hypothesis that AIV in wild birds exists as a vast pool of genomic segments that form transient genome constellations (Dugan et al., 2008). While this was established using AIV genomic data collected over a large geographic area and many years, the sequential data from this single surveillance site provide further evidence that the genomic dynamics of AIV can be very active over a short period of time. Also, it was interesting, although not surprising that newer sequencing technologies refined our awareness of the frequency of mixed infections. Both findings provide further insight into the natural history of AIV in their natural host reservoirs. Although the direction of gene flow cannot be ascertained, it is unlikely that H5N1 and H7N3 LP AIV are maintained as stable genome constellations in wild ducks and the adaptation of H5 and H7 LP AIV into domestic gallinaceous poultry historically produced a predominantly unidirectional host switch events (Swayne, 2007), although the current HP AIV H5N1 Asian lineage viruses have shown a bidirectional flow (Li et al., 2010). Additional surveillance at other sites is needed in the future to better ascertain the epizootology and viral ecology of LP AIV in North American wild birds.

## MATERIALS AND METHODS

### Study population

The sentinel host population in this investigation consisted of hatch-year, free flying, wild, resident mallards obtained from a source flock of hatch-year mallards. On three separate occasions during the two months prior to August 2, 2006, fifty cloacal swabs from the source flock had tested negative for the presence of recoverable AIVs. The last sampling occurred on July 31, just two days before August 2, 2006. Also, all 35 hatch-year mallards remaining in the source flock were negative for virus recovery on September 8, 2006 approximately one month after the LP H5N1 AIVs were recovered from the sentinels and on the same day the LP H7N3 AIV positive samples were recovered from the sentinels. In addition, hatch year mallards from the source flock premise had tested negative for recoverable AIV on two or three occasions during the summer in each of the prior three years (RDS, unpublished data). The sentinel mallard flocks were established for the detection of AIVs circulating in local wild birds before the arrival of migratory waterfowl and for the introduction of AIVs into the general area after migrant waterfowl arrived. The most visible wild birds visiting the study site of this investigation (MD-2) in June-July before the waterfowl migrants arrived mainly included gulls, shorebirds, and a few Canada Geese and diving ducks. While the free-flying, sentinel ducks could visit nearby fields and bodies of water, this activity was limited during June-August. This study site is part of a larger collaborative surveillance effort that started in 2001 by conscientious and progressive thinking private landowners, government representatives, and researchers from The Ohio State University (Slemons et al., 2003).

### Viral surveillance

Fifty cloacal swabs for virus isolation were collected from mallards in the source flock on 6/8/2006, 7/11/2006, 7/31/2006, and 35 samples were collected on 9/8/2006. Fifty fresh fecal samples were also collected off the ground across the loafing area used by the sentinel mallards on 8/2/2006, 69 collected on 9/8/2006, 50 on 10/11/2006, and 49 cloacal swabs were collected on 11/24/2006. Each cloacal swab or fecal sample was placed in an individual cryovial containing virus-transport medium and antibiotics (Slemons et al., 1974) and stored temporarily on dry ice in the field. Within three days of collection, samples were transferred to a −80°C freezer and virus recovery attempts were accomplished using

inoculation of 10-day-old embryonating chicken eggs (Beard et al., 1980). Hemagglutinating agents were recovered from embryonating eggs were confirmed as type A influenza viruses using a commercially available diagnostic assay (Avian Influenza Virus Type A Antigen Test Kit (Synbiotics, San Diego, CA)). The HA and NA serotypes and pathogenicity of the AIV isolates were determined at the USDA, APHIS, National Veterinary Services Laboratories (NVSL, Ames, IA) using standard hemagglutinin inhibition and NA testing procedures, nucleotide sequencing, and, when appropriate, the intravenous pathogenicity index test.

### Isolates used for this study

Twelve 12 H5N1s (11 NIH & 1 JCVI) and 5 H7N3 (2 NIH & 3 JCVI) isolates used in the sequence analysis were recovered at a single site (MD-2) from very fresh fecal samples collected from the environment. In addition, three isolates (A/wood duck/Ohio/2004 (H5N1), A/mallard/Maryland/2006 (H5N2), and A/mallard/Maryland/2007 (H5N1)), recovered from wild, free-flying ducks sampled at other locations on other dates, were also sequenced and included in the comparative genomic analyses. An additional 15 isolates recovered from environmental or wild duck samples collected in Maryland and Delaware during 2005 (RDS and JMN) and sequenced as part of the NIH Influenza Sequencing Project (sequenced at the J. Craig Venter Institute) which had been posted in GenBank were also included in the analyses. These newly generated sequences were compared with an additional 436 published AIV genomes in GenBank for the overall phylogenetic analyses.

### Sequence amplification, sequencing, and phylogenetic analyses

Viral RNA was isolated from allantoic fluid using Trizol® Reagent (Invitrogen Corp., Carlsbad, CA) and transcribed into 20 μl of cDNA for use in a PCR assay followed by Sanger DNA sequencing as previously described (Dugan et al., 2008). For next-generation sequencing (NGS), viral isolates were prepared as previously described (Djikeng et al., 2008; Zhou et al., 2009) and sequenced with 454/Roche GS-FLX and Solexa/Illumina Genome Analyzer IIx platforms to generate a consensus assembly representing a mix of both technologies (Ghedin et al., 2011). Sanger sequences were assembled and edited with the Sequencher software package version 4.6 (Gene Codes Corp, Ann Arbor, MI) and aligned with an existing set of 423 AIV genomes from the Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) using MUSCLE (Edgar, 2004). Sequence alignments consisted of the following coding regions for each segment: PB2 (2,277 nt), PB1 (2271 nt), PA (2148 nt), HA (1218 nt) sequences, NP (1494 nt) sequences, NA (1410 nt), M1/2 (1002 nt), and NS1/2 (831 nt). Sequence alignments are available upon request. Gene sequences have been deposited in GenBank (accession numbers JF758661–JF758823).

The best-fit GTR+I+$\Gamma_4$ model of nucleotide substitution was determined using ModelTest 3.7 (Posada and Crandall, 1998), and resulting parameter estimates were imported into PAUP* (Swofford, 2003) to create maximum likelihood (ML) trees through TBR branch-swapping (parameter values available upon request) for each gene segment. To assess the individual nodes and trace for reassortment, we employed the neighbor-joining (NJ) bootstrap method with 1,000 replications to the inferred ML phylogenetic trees. Identification of viral clades sharing a common ancestor was supported by a bootstrap value >70% for all eight gene segments. In addition, to further distinguish viral clades, nucleotide percent identities were calculated using DNAStar software (Lasergene 7.2, DNAStar, Madison, WI). Two sequences, A/Environment/MD/1158/2006 (H5N1) and A/Environment/MD/261/2006 (H7N3), were arbitrarily used as reference sequences to calculate nucleotide percent identities for 24 whole genome LPAIV sequences isolated from MD-2 in 2006 (Table 1), and for additional selected AIV genomic sequences.

Although multiple clades exist within the individual gene segment trees containing AIV sequences (see Supplemental Figures 1–6), thirteen unique clades were distinguished to trace reassortment events of the 2006–2007 LP AIV genomes. These thirteen clades are represented by the following viruses (see Table 2–4 legend): A/duck/MN/1525/1981 (H5N1), A/ruddy turnstone/NJ/65/1985 (H7N3), A/widgeon/OH/379/1988 (H5N2), A/wood duck/OH/623/2004 (H5N1), A/shorebird/DE/101/2004 (H5N7), A/blue-winged teal/OH/658/2004 (H7N3), A/environment/MD/261/2006 (H7N3), A/shorebird/DE/22/2006 (H7N3), A/environment/MD/2103/2006 (H3N6), A/mallard/MD/182/2006 (H5N2), A/environment/MD/1158/2006 (H5N1), A/mallard/MD/1235/2006 (H3N6), A/green-winged teal/CA/609/2007 (H5N2). These thirteen unique clades were color-coded to show individual gene segment reassortment events among the 2006–2007 samples of thirty-seven sequences, and are summarized in Tables 2–4. For those sequences isolated from MD-2 where there was a discrepancy between subtype and genotype, the sequences were excluded from the reassortment analysis. Table 2 tabulates the reassortment events of internal gene segments sampled from various isolates in the 2006 season from the same surveillance site (MD-2) in Maryland (isolates from other sites, MD-1 and MD-3 were excluded from the reassortment analysis), and Tables 3 and 4 illustrate the reassortment events with the other North American H5 and H7 sequences, respectively. Gene segments that did not fall within these designated clades were labeled as other (orange), indicating extensive reassortment within the individual gene segment trees (see Table 2–4 legend and phylogenetic trees in the Supplemental Figures 1–6). Isolates that were serologically typed as multiple (mixed) infections (Table 1) were excluded from the phylogenetic analyses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Figure 1.**
Maximum likelihood tree with bootstrap values (>70 %) of avian H5 sequences (78 HA gene segments). Horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. The tree is rooted by an H5 Eurasian avian sequence (A/Muscovy duck/Vietnam/57/2007/H5N1). The unique reference sequences used to trace reassortment patterns across gene segments are color-coded.

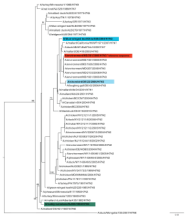

**Figure 2.**
Maximum likelihood tree with bootstrap values (>70 %) of avian H7 sequences (50 HA gene segments). Horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. The tree is rooted by an H7 Eurasian avian sequence (A/duck/Mongolia/720/2007/H7N6). The unique reference sequences used to trace reassortment patterns across gene segments are color-coded.

**Table 1**

Antigenic subtype and genotype results of LP AIV collected from wild ducks in Maryland (sites MD 1–3) in 2006.

| ANTIGENIC SUBTYPE (NO. OF ISOLATES) | COLLECTION DATE | HA-NA GENOTYPE | LOCATION | ISOLATES |
|---|---|---|---|---|
| H5N2 (1) | 1/21/06 | H5N2 | MD-3 | A/mallard/MD/182/2006 (H5N2) |
| H11N9 (1) | 5/3/06 | H11N9 | MD-1 | A/environment/MD/763/2006 (H11N9) |
| H5N1 (12) | 8/2/06 | H5N1 | MD-2 | A/environment/MD/1158/2006 (H5N1)*, 1149, 1153, 1154, 1156, 1159, 1162, 1165, 1170, 1171, 1172, 1189 |
| H5N1,4 (1)§ | 8/2/06 | H5,7N1,3 | MD-2 | A/environment/Maryland/1152/2006 (H5,7N1,3) |
| H5N2 (1) | 8/2/06 | H5N1 | MD-2 | A/environment/Maryland/1192/2006 (H5N1) |
| H3N6 (1) | 8/7/06 | H3N6 | MD-1 | A/mallard/MD/1235/2006 (H3N6) |
| H4N6 (2) | 9/8/06 | H4N6 | MD-2 | A/environment/MD/1101/2006 (H4N6); 1655 |
| H4N6 (1)§ | 9/8/06 | H4,7,N6 | MD-2 | A/environment/Maryland/2114/2006 (H4,7N6) |
| H7N3 (5) | 9/8/06 | H7N3 | MD-2 | A/environment/MD/261/2006/H7N3*, 267, 1630, 1637, 1631 |
| H3N6 (1)§ | 10/11/06 | H3,4,7N6 | MD-2 | A/environment/Maryland/2103/2006 (H3,4,7N6) |

§Indicates isolates with evidence of mixed infections by genotypic and/or serologic subtyping that were excluded from the reassortment analysis.

*Indicates the designated reference H5 and H7 sequences for the reassortment analysis (A/environment/MD/1158/2006/H5N1 and A/environment/MD/261/2006/H7N3). The location and year of isolation are the same for identical H5N1 and H7N3 sequences used in the analysis, thus isolate numbers are listed only.

## Table 2

The genome constellations (internal gene segments only) of 19 LP AIV recovered from ducks sampled at the same location in Maryland, USA in 2006 without evidence of mixed infection. The different colors reflect segments whose sequences fall into different major clades as defined by strong bootstrap support (>80%) in each internal gene segment tree (see legend). A/environment/MD/1158/2006/H5N1* and A/envroinment/MD/261/2006 (H7N3) were used as the reference sequence to calculate nucleotide percent identity to identify thirteen unique clades. Shared segments are shaded in the same color (see Tables 2–4 legend for explanation of color codes).

| ISOLATE | PB2 | PB1 | PA | NP | MP | NS | Date of Isolation |
|---|---|---|---|---|---|---|---|
| **environment/MD/1158/2006 (H5N1)*** | | | | | | | **8/2/06** |
| **environment/MD/261/2006 (H7N3)*** | | | | | | | **9/8/06** |
| **environment/MD/1101/2006 (H4N6)** | | | | | | | **9/8/06** |
| **environment/MD/1655/2006 (H4N6)** | | | | | | | **9/8/06** |

ξIndicates that eleven of the environmental H5N1 and four of the H7N3 collected from a single locale in the Eastern Shore of Maryland were virtually identical to the reference sequences, and were not included in the table for the sake of redundancy.

**Tables 2–4 Legend.** Color-coded reference sequences representing thirteen unique clades were used to trace reassortment patterns across all eight segments. Gene segments outside of these clades were designated other (orange). *A/environment/MD/1158/2006 (H5N1) and A/environment/MD/261/2006 (H7N3) were used as the reference sequences in the analyses.

| Legend |
|---|
| A/duck/MN/1525/1981 (H5N1) |
| A/ruddy turnstone/NJ/65/1985 (H7N3) |
| A/widgeon/OH/379/1988 (H5N2) |
| A/wood duck/OH/623/2004 (H5N1) |
| A/shorebird/DE/101/2004 (H5N7) |
| A/blue wing teal/OH/658/2004 (H7N3) |
| A/environment/MD/261/2006 (H7N3)* |
| A/shorebird/DE/22/2006 (H7N3) |
| A/environment/MD/2006 (H3N6) |
| A/mallard/MD/182/2006 (H5N2) |
| A/environment/MD/1158/2006 (H5N1)* |
| A/mallard/MD/1235/2006 (H3N6) |

## Table 3

The genome constellations of 25 LP H5 AIVs by year of isolation collected from wild birds in North America. The different colors reflect segments whose sequences fall into different major clades as defined by strong bootstrap support (>80%) in each internal gene segment tree (see legend). A/environment/MD/1158/2006/H5N1* was used as the reference sequence to calculate nucleotide percent identity to identify thirteen unique clades. Gene segments outside of these clades were designated other (orange). Shared segments are shaded in the same color. A comparison of the reassortment patterns of N1, N2, and N3 could not be made; therefore, the reassortment analysis for the NA gene segment was excluded.

| ISOLATE | PB2 | PB1 | PA | H5 | NP | MP | NS |
|---|---|---|---|---|---|---|---|
| A/duck/MN/1516/1981 (H5N2) | | | | | | | |
| A/duck/MN/1525/1981 (H5N1) | | | | | | | |
| A/black duck/NY/184/1988 (H5N2) | | | | | | | |
| A/widgeon/OH/379/1988 (H5N2) | | | | | | | |
| A/herring gull/NJ/402/1989 (H5N3) | | | | | | | |
| A/mallard/MD/789/2002 (H5N2) | | | | | | | |
| A/shorebird/DE/101/2004 (H5N7) | | | | | | | |
| A/wood duck/OH/623/2004 (H5N1) | | | | | | | |
| A/mallard/MB/458/2005 (H5N1) | | | | | | | |
| A/mallard/ON/499/2005 (H5N1) | | | | | | | |
| A/mallard/MD/182/2006 (H5N2) | | | | | | | |
| A/environment/MD/1158/2006 (H5N1)*[ξ] | | | | | | | |
| A/mallard/MD/802/2007 (H5N1) | | | | | | | |
| A/green wing teal/CA/609/2007 (H5N2) | | | | | | | |

[ξ]Indicates that eleven of the environmental H5N1 collected from a single locale in the Eastern Shore of Maryland were virtually identical to the reference sequence, and were not included in the table for the sake of redundancy.

**Tables 2–4 Legend.** Color-coded reference sequences representing thirteen unique clades were used to trace reassortment patterns across all eight segments. Gene segments outside of these clades were designated other (orange). *A/environment/MD/1158/2006 (H5N1) and A/environment/MD/261/2006 (H7N3) were used as the reference sequences in the analyses.

| Legend |
|---|
| A/duck/MN/1525/1981 (H5N1) |
| A/ruddy turnstone/NJ/65/1985 (H7N3) |
| A/widgeon/OH/379/1988 (H5N2) |

## Table 4

The genome constellations of 12 H7N3 AIVs by year of isolation collected from wild birds in North America. The different colors reflect segments whose sequences fall into different major clades as defined by strong bootstrap support (>80%) in each gene segment tree (see legend). A/environment/MD/261/2006/H7N3* was used as the reference sequence to calculate nucleotide percent identity to identify thirteen unique clades. Gene segments outside of these clades were designated other (orange). Shared segments are shaded in the same color.

| ISOLATE | PB2 | PB1 | PA | H7 | NP | NA | MP | NS |
|---|---|---|---|---|---|---|---|---|
| **A/widgeon/ALB/284/1977 (H7N3)** | | | | | | | | |
| **A/ruddy turnstone/NJ/65/1985 (H7N3)** | | | | | | | | |
| **A/green-winged teal/ALB/228/1985 (H7N3)** | | | | | | | | |
| **A/mallard/OH/322/1998 (H7N3)** | | | | | | | | |
| **A/black duck/OH/415/2001 (H7N3)** | | | | | | | | |
| **A/blue-wing teal/OH/658/2004 (H7N3)** | | | | | | | | |
| **A/chicken/British Columbia/2004 (H7N3)** | | | | | | | | |
| **A/environment/MD/261/2006 (H7N3)*[ξ]** | | | | | | | | |
| **A/shorebird/DE/22/2006 (H7N3)** | | | | | | | | |

[ξ]Indicates that four of the H7N3 collected from a single locale in the Eastern Shore of Maryland were virtually identical to the reference sequence, and were not included in the table for the sake of redundancy.

**Tables 2–4 Legend.** Color-coded reference sequences representing thirteen unique clades were used to trace reassortment patterns across all eight segments. Gene segments outside of these clades were designated other (orange). *A/environment/MD/1158/2006 (H5N1) and A/environment/MD/261/2006 (H7N3) were used as the reference sequences in the analyses.

| Legend |
|---|
| A/duck/MN/1525/1981 (H5N1) |
| A/ruddy turnstone/NJ/65/1985 (H7N3) |
| A/widgeon/OH/379/1988 (H5N2) |
| A/wood duck/OH/623/2004 (H5N1) |
| A/shorebird/DE/101/2004 (H5N7) |
| A/blue wing teal/OH/658/2004 (H7N3) |
| A/environment/MD/261/2006 (H7N3)* |
| A/shorebird/DE/22/2006 (H7N3) |
| A/environment/MD/2006 (H3N6) |
| A/mallard/MD/182/2006 (H5N2) |
| A/environment/MD/1158/2006 (H5N1)* |
| A/mallard/MD/1235/2006 (H3N6) |
| A/green wing teal/CA/609/2007 (H5N2) |
| Other |