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Characterization of Low-Pathogenicity H5N1 Avian Influenza Viruses from North America[▽]

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Wild-bird surveillance in North America for avian influenza (AI) viruses with a goal of early identification of the Asian H5N1 highly pathogenic AI virus has identified at least six low-pathogenicity H5N1 AI viruses between 2004 and 2006. The hemagglutinin (HA) and neuraminidase (NA) genes from all 6 H5N1 viruses and an additional 38 North American wild-bird-origin H5 subtype and 28 N1 subtype viruses were sequenced and compared with sequences available in GenBank by phylogenetic analysis. Both HA and NA were phylogenetically distinct from those for viruses from outside of North America and from those for viruses recovered from mammals. Four of the H5N1 AI viruses were characterized as low pathogenicity by standard in vivo pathotyping tests. One of the H5N1 viruses, A/MuteSwan/MI/451072-2/06, was shown to replicate to low titers in chickens, turkeys, and ducks. However, transmission of A/MuteSwan/MI/451072-2/06 was more efficient among ducks than among chickens or turkeys based on virus shed. The 50% chicken infectious dose for A/MuteSwan/MI/451072-2/06 and three other wild-waterfowl-origin H5 viruses were also determined and were between $10^{5.3}$ and $10^{7.5}$ 50% egg infective doses. Finally, seven H5 viruses representing different phylogenetic clades were evaluated for their antigenic relatedness by hemagglutination inhibition assay, showing that the antigenic relatedness was largely associated with geographic origin. Overall, the data support the conclusion that North American H5 wild-bird-origin AI viruses are low-pathogenicity wild-bird-adapted viruses and are antigenically and genetically distinct from the highly pathogenic Asian H5N1 virus lineage.

The H5 hemagglutinin (HA) subtype makes up less than 1% of the avian influenza (AI) virus isolates recovered in North America from wild aquatic birds (8, 22), which are major natural host reservoir species for type A influenza (18). However, H5 isolation rates from wild birds near 10% have been reported in recent surveys in Europe (13, 32). Because of the recent outbreak of Asian-lineage H5N1 highly pathogenic AI (HPAI) in Asia, Europe, and Africa, which has been associated in some cases with wild-bird transmission, a greatly enhanced wild-bird surveillance program for H5 influenza has been conducted in Canada, the United States, and Mexico. Similarly, the Asian-lineage H5N1 HPAI virus has increased the focus on the N1 neuraminidase (NA) subtype, which is common in wild-bird species (7, 9, 16, 22) and has been associated with several outbreaks in poultry (2) as well as being common in swine and human influenza A viruses.

Understanding the genetic and biological characteristics of H5 viruses from wild birds can provide insight into the biology of the H5 HA, the ecology of AI virus, and the ability of wild birds to disseminate influenza viruses. Although much of the current attention to H5 is due to the Asian H5N1 HPAI virus and human health concerns, the H5 subtype has also been considered a priority in animal health for many years because it is one of the two HA subtypes (the other being H7) which can become HPAI in chickens and turkeys (28). Furthermore, because wild aquatic birds are a major reservoir for influenza A viruses and this reservoir has directly or indirectly been identified as a major source of AI viruses infecting poultry, further characterization of wild-bird-origin H5 AI viruses can provide insight into preventing future AIV outbreaks in poultry, understanding of which H5 wild-bird viruses are more likely to cross species barriers and infect poultry, and insight into the identification of optimal vaccine seed strains for use in poultry. Here the basic genetic, biological, and antigenic characteristics of wild-bird-origin AI virus isolates from North America with an H5 HA and/or N1 NA subtype are reported.

MATERIALS AND METHODS

Viruses. North American viruses possessing either the H5 HA subtype or the N1 NA subtype (Table 1) were recovered from cloacal swabs collected from wild birds (primarily ducks) during monitoring programs in Canada and the United States. Samples were either screened with a real-time reverse transcriptase PCR

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TABLE 1. H5 and N1 subtype AI virus isolates from wild birds from North America and for which HA and NA sequence data have not been previously reported in GenBank that were included in this analysis

Isolate[a]	Subtype		GenBank accession no. for:	
	HA	NA	HA	NA
A/BlackDuck/NC/674-694/06	H5	N1	EF607853	EF607895
A/Duck/PA/454069-9/06	H5	N1	EF607854	EF607896
A/Mallard/MB/458/05	H5	N1	EF205596	EF426681
A/Mallard/ON/499/05	H5	N1	EF405825	EF564746
A/MuteSwan/MI/451072-2/06	H5	N1	EF607855	EF607897
A/WoodDuck/OH/623/04	H5	N1	EF607856	EF607898
A/BlueGoose/WI/711/75	H5	N2	EF607857	
A/Mallard/MD/790/02	H5	N2	EF607858	
A/Mallard/MD/865/02	H5	N2	EF607859	
A/Mallard/MD/185/03	H5	N2	EF607860	
A/Mallard/MD/252/01	H5	N2	EF607861	
A/Mallard/MD/258/01	H5	N2	EF607862	
A/Mallard/MD/302/01	H5	N2	EF607863	
A/Mallard/MD/786/02	H5	N2	EF607864	
A/Mallard/MD/789/02	H5	N2	EF607865	
A/Mallard/MD/791/02	H5	N2	EF607866	
A/Mallard/MD/792/02	H5	N2	EF607867	
A/Mallard/MD/795/02	H5	N2	EF607868	
A/Mallard/MD/866/02	H5	N2	EF607869	
A/RuddyTurnstone/NJ/1608/01	H5	N2	EF607870	
A/Mallard/MN/113/00	H5	N2	EF607871	
A/Mallard/MN/133/98	H5	N2	EF607872	
A/Mallard/MN/166/00	H5	N2	EF607873	
A/Mallard/MN/168/00	H5	N2	EF607874	
A/Mallard/MN/410/00	H5	N2	EF607875	
A/Mallard/MN/6/00	H5	N2	EF607876	
A/Mallard/OH/345/88	H5	N2	EF607877	
A/Pheasant/MD/4457/93	H5	N2	EF607878	
A/Pheasant/NJ/9804563/98	H5	N2	EF607879	
A/RuddyTurnstone/DE/313/03	H5	N2	EF607880	
A/RuddyTurnstone/DE/371/03	H5	N2	EF607881	
A/NorthenPintail/FL/598/07	H5	N2	EF607882	
A/CanadaGoose/AK/44075-058/06	H5	N2	EU099317	
A/TundraSwan/AK/44049-168/06	H5	N2	EF607884	
A/Mallard/MN/382/00	H5	N3	EF607885	
A/Mallard/MN/479/00	H5	N3	EF607886	
A/Mallard/WI/169/75	H5	N3	EF607887	
A/Mallard/MN/105/00	H5	N5	EF607888	
A/ShoreBird/DE/1346/01	H5	N7	EF607889	
A/RuddyTurnstone/NJ/1148676/04	H5	N7	EF607890	
A/RuddyTurnstone/NJ/1698/01	H5	N7	EF607891	
A/RuddyTurnstone/NJ/1851/01	H5	N7	EF607892	
A/RuddyTurnstone/DE/2046/01	H5	N8	EF607893	
A/Mallard/MD/185/03	H5	?[b]	EF607894	
A/Mallard/MD/11/03	?	N1		EF607899
A/Mallard/MD/13/03	?	N1		EF607900
A/Blue-WingedTeal/LA/B228/86	H1	N1		EF607901
A/Duck/NJ/7717-70/95	H1	N1		EF607902
A/Duck/NY/13152-13/94	H1	N1		EF607903
A/Duck/NY/15024-21/96	H1	N1		EF607904
A/Green-WingedTeal/LA/213GW/87	H1	N1		EF607905
A/Mallard/MD/321/02	H1	N1		EF607906
A/Quail/IN/38685/93	H1	N1		EF607907
A/Mallard/MD/163/02	H1	N1		EF607908
A/Mallard/MD/168/02	H1	N1		EF607909
A/Mallard/MD/170/02	H1	N1		EF607910
A/Mallard/MD/181/02	H1	N1		EF607911
A/Mallard/MD/307/02	H1	N1		EF607912
A/Mallard/MD/322/02	H1	N1		EF607913
A/Mallard/MD/334/02	H1	N1		EF607914
A/Mallard/MD/352/02	H1	N1		EF607915
A/Mallard/MD/369/02	H1	N1		EF607916
A/Mallard/MD/382/02	H1	N1		EF607917
A/Mallard/MD/390/02	H1	N1		EF607918
A/Mallard/MD/403/02	H1	N1		EF607919
A/Mallard/MD/304/02	H1	N1		EF607920
A/Mallard/MD/350/02	H1	N1		EF607921
A/Duck/PA/69	H6	N1		EF607922
A/Mallard/MD/1037/02	H6	N1		EF607923
A/Mallard/MD/852/02	H6	N1		EF607924
A/Mallard/MD/851/02	H6	N1		EF607925
A/Rhea/NC/39482/93	H7	N1		EF607926

[a] Canadian provinces and U.S. states are designated by their standard two-letter postal codes.

[b] "?" indicates that the subtype is unknown.

TABLE 2. CID_{50}s by virus isolate in 4- to 5-week-old White Leghorn chickens

Virus	Subtype	CID_{50}
A/Mallard/MD/791/02	H5N2	$>10^{5.3}$
A/Duck/PA/454069-9/06	H5N1	$10^{7.5}$
A/RuddyTurnstone/NJ/1148676/04	H5N7	$>10^{7.5}$
A/MuteSwan/MI/451072-2/06	H5N1	$10^{6.3}$

(RT-PCR) test targeting the influenza matrix gene (20), with positive samples being processed for virus isolation in embryonating chicken eggs (ECE), or else directly processed for virus isolation in ECE per standard procedures (30).

Sequencing. The HA genes from a total of 44 H5 subtype viruses and the NA genes from a total of 34 N1 subtype viruses (including 6 viruses that were H5N1) from wild birds in North America were sequenced. All viruses were sequenced after no more than three passages in ECE. RNA was extracted with TRIzol LS reagent (Invitrogen, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions or with the Ambion MagMax AI/NDV RNA extraction kit (Ambion Inc., Austin, TX). Individual influenza virus genes were amplified by RT-PCR as previously described (26). The amplicons were purified from agarose gels with a QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA) and directly sequenced. Two isolates, A/Mallard/ON/499/05 (H5N1) and A/Mallard/MB/458/05 (H5N1), were cloned prior to sequencing as follows: after amplification by RT-PCR, the amplicons were purified directly and cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI).

Cycle sequencing was performed with a BigDye Terminator kit (Applied Biosystems, Foster City, CA) and run on an ABI 3730 (Applied Biosystems, Foster City, CA) and assembled with SeqMan 7.0 (DNAStar, Madison, WI).

Phylogenetic and sequence analysis. Sequences were aligned with Clustal V (Lasergene 7.0; DNAStar, Madison, WI). Phylogenetic analysis was performed with PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, MA) using the maximum parsimony tree-building method, with a heuristic search and 500 bootstrap replicates. For the HA genes, separate phylogenetic groups were defined by more than 96.0% identity among the genes. Analysis of the HA and NA genes was done with the North American wild-bird-origin viruses alone and with genes from viruses that were phylogenetically representative of other lineages based on this analysis and previous reports, including genes from viruses from all possible sources: wild birds, poultry from outbreaks, and mammals from North America, Europe, and Asia.

Phylogenetic analysis of the HA gene was done with the HA1 gene including bases 1 to 1040 of the coding region and included genes from a total of 62 isolates collected from wild birds between 1975 and 2007, including sequences from 18 isolates in GenBank. The entire coding region of the NA gene was used for phylogenetic analysis and included a total of 66 North American wild-bird-origin viruses collected between 1969 and 2006, of which 32 sequences were obtained from GenBank.

Animal studies. All animal studies were conducted in BSL-2Ag or BSL-3Ag laboratory space, as required for work with AI virus. All animal care was provided as directed by the Institutional Animal Care and Use Committees of the institutions where the work was performed. For all studies, the birds were housed with ad libitum access to feed and water.

Pathotype testing. In vivo pathogenicity studies were performed with the following selected H5 AI virus isolates: A/MuteSwan/MI/451072-2/06 (H5N1), A/Duck/PA/454069-9/06 (H5N1), A/Mallard/ON/499/05 (H5N1), and A/Mallard/MB/458/05 (H5N1). Pathogenicity testing was performed in accordance with the World Animal Health Organization (OIE) procedures (1). The HA proteolytic cleavage sites for all H5 subtype isolates were also evaluated for amino acid identity with highly pathogenic viruses as defined by the OIE (1).

CID_{50}. Four North American wild-waterfowl-origin H5 low-pathogenicity AI (LPAI) viruses were selected to determine the 50% chicken infectious dose (CID_{50}) (Table 2). These four isolates were selected primarily because they were recent, and it is assumed that using isolates with four different NA subtypes and three different species of origin may increase the diversity within H5 LPAI viruses. Each virus was evaluated with three concentrations as follows: undiluted amnioallantoic fluid (AAF) and AAF diluted 1:10 and 1:100 (dilutions were made in brain heart infusion broth). The AAF was titrated at the time of dilution to determine an accurate challenge dose. With the exception of A/Mallard/MD/791/02 (H5N2), where 5 chickens were used per treatment group, for each dilution of each virus, 10 specific-pathogen-free 4- to 5-week-old White Leghorn chickens were inoculated with 0.1 ml by the intranasal route. The chickens were observed daily for clinical signs. At 14 days postinoculation (p.i.), serum was

FIG. 1. Nucleotide phylogenetic tree of the H5 HA1 from selected North American-origin wild aquatic bird isolates (from this report and previously reported) and selected isolates from avian and mammalian virus species rooted to A/WhooperSwan/Mongolia/244/05. Isolates used for antigenic characterization are in boldface, and isolates used in animal studies are underlined. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, a heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree). Canadian provinces and U.S. states are abbreviated by their standard two-letter postal codes.

collected from each chicken and tested for AI virus antibody by standard agar gel immunodiffusion assay (31). Chickens that were positive for AI virus antibody were considered to have been infected.

Pathogenicity and transmission of A/MuteSwan/MI/45072-2/06 in ducks, chickens, and turkeys. Ducks were housed in cages, while chickens and turkeys were housed in Horsfall isolators. Groups of seven or eight specific-pathogen-free chickens (SPAFAS, Norwich, CT) (4 weeks of age), OARDC-developed Eggline turkeys (3 weeks of age), or Pekin ducks (2 weeks of age) were inoculated with 10^6 50% egg infective dose (EID_{50})/0.2 ml by the intranasal route. Three or four contact exposure birds of the same species were placed into the same cage or isolator as the inoculated birds 1 day after inoculation (this was day 0 for the contact birds). A final group of four birds for each species served as uninoculated and unexposed controls. These controls remained negative for virus isolation and antibodies throughout the experiment.

Birds were monitored daily for clinical signs, and oropharyngeal and cloacal swab samples were collected at days 2, 4, 7, and 14 p.i. from the inoculated birds and at days 1, 3, 6, and 13 postexposure from the contact exposure birds. Virus shed titers were determined by quantitative real-time RT-PCR using the USDA matrix gene protocol (11, 20). RNA from titrated stocks of A/MuteSwan/MI/451072-2/06 was used to establish the standard curve. Samples with high cycle threshold values, indicating low concentrations of virus, were retested by conventional RT-PCR with H5 gene-specific primers to rule out false positives or contamination.

Serology was used to confirm virus replication. Sera were collected at 14 days

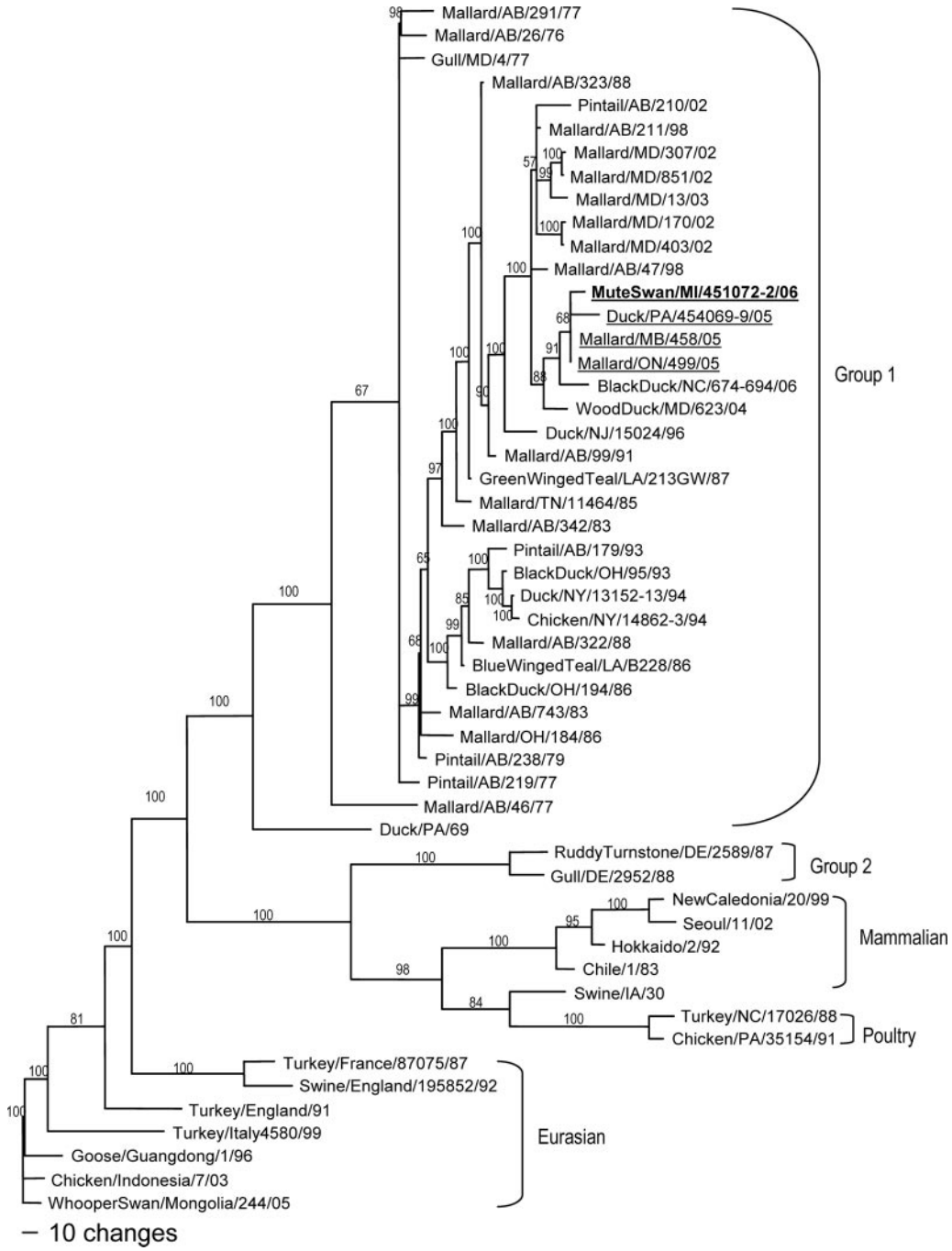

FIG. 2. Nucleotide phylogenetic tree of N1 NA from selected North American-origin wild aquatic bird isolates (from this report and previously reported) and selected isolates from avian and mammalian virus species rooted to A/WhooperSwan/Mongolia/244/05. The isolate used for antigenic characterization is in boldface, and isolates used in animal studies are underlined. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, a heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree). Canadian provinces and U.S. states are abbreviated by their standard two-letter postal codes.

p.i. from inoculated birds and at day 13 p.i. from the contact exposure birds. Standard hemagglutination inhibition (HI) assays (31) were done using red blood cells from the species being tested and homologous antigen.

Antigenic comparison. Standard HI assay was performed with seven viruses selected from seven phylogenetic groups (Fig. 1). The HA1 regions of the viruses included in the antigenic comparison had between 82.6 and 96.8% amino acid identity with all other isolates included (see Table 5). Antigen was prepared from beta-propriolactone-inactivated AAF. With the exception of A/MuteSwan/MI/451-72-2/06, antibody was produced in chickens with beta-propriolactone inactivated oil-adjuvanted (24) AAF from the same virus preparations used for the HI assay antigen source. Chickens were used as the source of antibody both because of the practical implications of chicken immune recognition for vaccination and because the use of chicken sera is common with avian isolates. Inactivated oil-adjuvanted preparations were used because consistently high antibody titers are produced regardless of the replication characteristics of the virus in chickens; for example, many wild-

TABLE 3. Virus titers at 2, 4, 7, and 14 days p.i.[a]

Species	Swab	Log_{10} EID_{50} of virus ± SD per 0.2 ml (no. of positive birds/no. tested) on indicated day p.i.[b]			
		2	4	7	14
Chicken	Oropharyngeal	3.5 ± 0.4 (3/8)	2.4 ± 0.4 (5/8)	4.2 ± 0.0 (1/8)	ND[c]
	Cloacal	2.9 ± 0.0 (1/8)	4.3 ± 1.2 (2/8)	0 (0/7)	ND
Turkey	Oropharyngeal	1.7 ± 0.3 (7/7)	1.3 ± 0.0 (1/7)	2.6 ± 0.0 (1/7)	ND
	Cloacal	2.0 ± 0.8 (4/7)	2.4 ± 0.7 (3/7)	0 (0/7)	ND
Duck	Oropharyngeal	2.8 ± 1.1 (4/8)	2.0 ± 0.3 (3/8)	1.5 ± 0.3 (4/8)	0 (0/8)
	Cloacal	3.2 ± 1.5 (5/8)	4.5 ± 0.8 (8/8)	2.8 ± 0.6 (8/8)	0 (0/8)

[a] Virus titers were determined by quantitative real-time RT-PCR from oropharyngeal and cloacal swabs from 4-week-old chickens, 3-week-old turkeys, and 2-week-old ducks inoculated by the intranasal route with 10^6 EID_{50} of MuteSwan/MI/451072-2/06.
[b] A zero indicates that all birds were negative for virus detection by real-time RT-PCR.
[c] ND, not done.

bird-origin AI viruses do not replicate adequately in chickens to produce good titers, and the highly pathogenic viruses will normally kill the chickens too quickly to get good antibody titers.

Chickens were inoculated with 0.5 ml of the adjuvanted antigen preparation at 4 and 7 weeks of age by the subcutaneous route. Serum collected 3 weeks after the second immunization was used for the reference antibody. Reference antibody for A/MuteSwan/MI/451072-2/06 was collected from chickens in the pathotyping study 3 weeks postexposure.

Nucleotide sequence accession numbers. GenBank accession numbers for new sequences are given in Table 1.

RESULTS

Sequencing and phylogenetic analysis. The H5 HA genes of 62 North American wild-bird-origin AI virus isolates and 66 N1 NA genes were analyzed phylogenetically. Analysis was done with the North American wild-bird viruses alone and with H5 subtype viruses which were phylogenetically representative of viruses from other lineages based on this analysis and previous reports (Fig. 1). Among the wild-bird isolates from North America, there were five clades based on a minimum of 4.0% difference in identity. A sixth group consisted of chicken isolates from Mexico. One isolate, A/Mallard/OH/556/87, had 96 to 97% identity with isolates from both groups 1 and 2. Other than viruses of group 3, which was comprised entirely of isolates from ruddy turnstones from New Jersey or Delaware, the viruses did not clearly group by location of sample collection, time of collection, or species of origin. In relation to H5 HA genes from poultry and from other species from Europe and Asia, grouping was geographic. Phylogenetic analysis with amino acid sequences revealed similar relationships (data not shown).

Several residues, 182, 190, 192, and 225, in H5 HA1 have been reported to be associated with binding either the α2,3- or α2,6-linked sialic acid linkages (23, 34). Of the 62 North American wild-bird-origin viruses, all had residues associated with α2,3 binding at sites 182 (Asn), 190 (Glu), and 192 (Gln). At site 225, 62 of the isolates had Gly, which is associated with α2,3 binding, and 1 isolate, A/RuddyTurnstone/NJ/1608/01, had Arg, for which the specificity has not been reported.

Two HA proteolytic cleavage site sequences were observed in the wild-bird-origin viruses: 56 isolates had the sequence RETR/G, and 6 isolates (A/Ruddy Turnstone/NJ/1851/01, A/Ruddy Turnstone/DE/2046/01, A/ShoreBird/DE/1346/01, A/Ruddy Turnstone/NJ/1698/01, A/Ruddy Turnstone/NJ/1608/01, and A/Mallard/ON/499/05) had the sequence RGTR/G.

The N1 subtype NA genes from 66 North American wild-bird-origin isolates were analyzed phylogenetically (Fig. 2). Among the North American wild-bird-origin isolates, the lowest nucleotide identity observed was 78.8%, which was between that of the group with only gull and ruddy turnstone isolates (group 2) and that of the rest (group 1). Within group 1, nucleotide identity ranged from 89.8 to 99.8%. The other major phylogenetic groups of N1 NA genes, the Asian H5N1 HPAI viruses, European poultry viruses, North American swine viruses, and human viruses all had approximately 75 to 87% nucleotide identity with the North American wild-bird viruses. With the exception of A/RuddyTurnstone/DE/2589/87 and A/Gull/DE/2952/88, the N1 NA genes from North American wild birds could not be divided into distinct groups but rather were continuous in nucleotide identities. Phylogenetic analysis of amino acid sequences revealed similar relationships (data not shown). None of the wild-bird isolates had NA stalk deletions, but several of the poultry isolates did.

Pathotype testing. Four North American wild-bird-origin viruses (A/MuteSwan/MI/451072-2/06, A/Duck/PA/454069-9/06, A/Mallard/MB/458/05, and A/Mallard/ON/499/05) were tested for pathogenicity by standard OIE procedures, which classify a virus as highly pathogenic if it causes 75% or higher mortality in chickens with an intravenous challenge. All viruses were classified as LPAI virus (intravenous pathogenicity index = 0.0). No mortality or clinical signs were observed with any of the viruses tested. Additionally, all North American H5 wild-bird-origin viruses had HA proteolytic cleavage sites that were consistent with LPAI virus.

CID_{50}. Four North American H5 AI virus isolates were evaluated for their infectivities to poultry by determining the CID_{50} following simulated natural exposure, i.e., intranasal inoculation. The titer of viruses needed to infect at least 50% of the White Leghorn chickens at 4 to 5 weeks of age was at a minimum $10^{5.3}$ EID_{50} to $10^{7.5}$ EID_{50} (Table 2). For two viruses, A/Mallard/MD/791/02 and A/RuddyTurnstone/NJ/1148676/04, a CID_{50} could not be determined because the maximal growth titers in ECE of $10^{5.3}$ and $10^{7.5}$ EID_{50}, respectively, were insufficient to produce a 50% infection rate. No morbidity or mortality was observed with any isolate.

Pathogenicity and transmission of A/MuteSwan/MI/45072-2/06 in ducks, chickens, and turkeys. Following a high intranasal challenge dose (10^6 EID_{50}), all three species shed virus at 2 and 4 days p.i. by the oropharyngeal and cloacal routes and from the oropharynx at 7 days p.i. (Table 3). However, from the cloacal route at 7 days p.i., only the ducks were shedding

TABLE 4. Virus titers at 1, 3, 5 and 13 days postexposure[a]

Species	Swab	Log_{10} EID_{50} of virus ± SD per 0.2 ml (no. of positive birds/no. tested) on indicated day p.i.[b]			
		1	3	5	13
Chicken	Oropharyngeal	2.6 ± 0.4 (1/4)	0 (0/4)	2.4 ± 0.0 (1/4)	ND[c]
	Cloacal	0 (0/4)	0 (0/4)	0 (0/4)	ND
Turkey	Oropharyngeal	1.7 ± 0.4 (3/4)	0 (0/4)	2.8 ± 0.0 (1/7)	ND
	Cloacal	0 (0/4)	3.0 ± 2.4 (2/4)	0 (0/4)	ND
Duck	Oropharyngeal	0 (0/4)	2.0 ± 0.0 (1/3)	2.2 ± 0.0 (1/3)	0 (0/3)
	Cloacal	0 (0/4)	3.8 ± 1.4 (3/3)	3.5 ± 0.8 (3/3)	0 (0/3)

[a] Virus titers were determined by quantitative real-time RT-PCR from oropharyngeal and cloacal swabs from 4-week-old chickens, 3-week-old turkeys, and 2-week-old ducks exposed to MuteSwan/MI/451072-2/06 by contact with birds inoculated by the intrachoanal route 24 h after inoculation.
[b] A zero indicates that all birds were negative for virus detection by real-time RT-PCR.
[c] ND, not done.

virus. No bird shed virus at 14 days p.i. All three species had mean shed titers which did not exceed $10^{4.5}$ EID_{50} per ml. Overall, a higher proportion of ducks shed virus longer than what was seen for either chickens or turkeys. The chickens and turkeys exposed by contact were intermittently positive at 1, 3, and 5 days postexposure (Table 4), with shedding primarily by the oropharyngeal route. However, all of the contact exposure ducks shed virus at days 3 and 5 postexposure and shed the highest levels of virus of all the contact exposure groups in this study. All directly inoculated and contact exposed birds were positive for antibodies to AI virus at 13 (contact birds) or 14 (directly inoculated birds) days postexposure.

Antigenic characterization. Seven H5 HA subtype AI virus isolates representing seven major H5 HA phylogenetic groups were selected for antigenic characterization (Table 5). Taking into account that two of the reference sera had low homologous titers and some had relatively high homologous titers, in most cases the phylogenetic and antigenic relationships (based on HI titer) were similar: North American viruses had lower HI titers with the Eurasian-lineage viruses as the antigen than with other North American viruses used for the antigen. One exception was A/Shorebird/DE/1346/00, which did not cross-react with the A/MuteSwan/MI/451072-2/06 North American wild-bird isolate.

DISCUSSION

Although the H5 HA subtype is one of the less common HA subtypes isolated from wild birds in North America (8, 22), increased monitoring and surveillance for AI viruses has been initiated to focus on the early detection of potential transmission of the Asian lineage H5N1 HPAI viruses into North America. As a result, isolations of H5 subtype viruses from wild birds in North America have increased. The N1 subtype has been identified from wild birds relatively frequently, although the N2, N6, and N8 subtypes have been reported to be more common (9). Since the HA and NA subtypes are independent of each other, few viruses with the H5N1 combination have previously been isolated from wild birds in North America (19). Upon sequencing, several isolates which had been reported to be H5N1 (17) or either H5 or N1 based on serology (unpublished data), had to be excluded, as they turned out to be different HA or NA subtypes. This could be due to mixed infections in wild birds, which have been reported to be fairly frequent (7, 16), or because of the cross-reactivity of the serological reagents. Since subtype-specific primers were used for sequencing in this case, the latter is likely. However, when there is a mixed infection, one or more egg passages may preferentially select one of the subtypes over another, thus biasing the sequence results.

Between GenBank accessions and recent wild-bird-monitoring efforts in North America, 62 H5 viruses and 66 N1 viruses have been reported since 1975 and since 1969, respectively, and these were included in this genetic analysis. In this study, we selected several recent NA H5 viruses for in vivo characterization and antigenic comparison with other H5 isolates. Genetic characterization showed that the North American wild-bird H5 and N1 viruses are separate from viruses from outside of North America and all mammalian-origin influenza isolates. Some H5 North American poultry isolates grouped phylogenetically near the North American wild-bird isolates, which is consistent with wild birds being the reservoir of viruses

TABLE 5. HI titers and percents HA1 gene amino acid identity for selected H5 viruses[a]

Antibody	HI titer (% HA1 gene amino acid identity) to indicated antigen[b]						
	MS/MI/451072-2/06	AV/NY/31855-3/00	SB/DE/1346/00	TK/WI/68	CK/Hidalgo/232/94	CK/Indonesia/7/03	CK/Scotland/59
MS/MI/451072-2/06	**64**	128 (96.8)	<4 (95.7)	16 (96.1)	16 (89.9)	8 (88.5)	32 (90.6)
AV/NY/31855-3/00	1,024	**2,048**	128 (95.6)	1,024 (96.1)	512 (91.5)	32 (88.0)	1,024 (90.6)
SB/DE/1346/00	512	512	**2,048**	1,024 (95.2)	512 (89.9)	32 (86.9)	1,024 (89.5)
TK/WI/68	2,048	1,024	256	**4,096**	1,024 (90.5)	128 (82.6)	2,048 (86.3)
CK/Hidalgo/232/94	2,048	2,048	1,024	2,048	**2,048**	512 (82.6)	2,048 (86.3)
CK/Indonesia/7/03	128	64	16	32	32	**64**	64 (89.2)
CK/Scotland/59	256	256	32	256	256	32	**2,048**

[a] Abbreviations: AV, avian; CK, chicken; MS, mute swan; SB, shorebird; TK, turkey.
[b] The titer is the reciprocal of the highest dilution of serum which completely inhibited hemagglutination. Homologous titers appear in boldface.

for poultry. However some poultry isolates, like those from Mexico, grouped separately, probably indicating either that wild-bird isolates in this lineage are rare and not represented in the available isolates or that the poultry origin viruses had already drifted sufficiently from the original wild-bird viruses as part of the adaptation process, thus obscuring the origins of the Mexican H5N2 viruses. A similar situation is observed with the A/Chicken/PA/1/83 lineage (27).

As was previously described, when comparing the H5 genes from wild birds, a range of genetic sequences that cluster into multiple lineages are present (5). These genetic differences do translate into antigenic differences. Although some clustering of viruses is seen based on virus isolation, the bias in sampling both by time and by location makes it impossible to draw definitive conclusions about viral drift in wild birds. The sequence analysis provides additional confirmation of the paradigm of separation of H5 AI viruses from the Americas (New World) and from Europe, Asia, Africa, and Australia (Old World) (6, 25). This geographic separation of influenza viruses contrasts with the known overlap in summer breeding grounds or migratory pathways of some North American wild birds with European and Asian wild birds. While the potential for movement of H5N1 HPAI viruses from Europe and Asia to North America remains unknown, the movement of LPAI virus from Europe and Asia to North America is a rare event (33). However, because influenza viruses can change so rapidly, there is concern that the Asian H5N1 HPAI virus may mutate in a way that facilitates transmission between wild birds, which may allow them to spread to the American continent. The Asian H5N1 HPAI viruses are already known to have phenotypic properties unusual for HPAI viruses, such as the ability to cause clinical disease and death in both wild and domestic ducks (3).

Interestingly, the genetic and antigenic outliers for the H5 gene were shorebird isolates, and those for the N1 gene were shorebird and gull isolates. Similar results were seen for other HA subtypes, including H2, suggesting that the separate HA and NA groupings are most likely due to the separation of shorebird and gull viruses due to different host ecologies (12, 14). The HA genes from ruddy turnstone isolates from the mid-Atlantic region from 2001 assorted to a separate phylogenetic group and antigenically seemed to be different from other North American wild-bird isolates. To determine if there was a possible species-associated residue in the HA, gene sequences from the nine ruddy turnstone isolates and selected mallard isolates representing the different clades were aligned (data not shown). Amino acid differences did not appear to be species related and may be random, due to sample bias, or due to other evolutionary pressures. Further work needs to be done to evaluate nucleotide and amino acid differences due to host adaptation between shorebirds, gulls, and ducks.

The biological and genetic data for virulence appear to correlate, as the viruses had HA proteolytic cleavage sites consistent with LPAI viruses (1, 15), and all the viruses tested in vivo were of low pathogenicity. No clinical disease signs were observed for any of the inoculated or contact birds in any of the experiments. Additionally, studies with chickens, turkeys, and ducks to evaluate transmission and virus shed and to establish the CID_{50} showed that the recent H5 viruses selected for these studies, which are assumed to be representative of viruses in the lineage, are not well adapted to chickens or turkeys. The transmission study and the shedding study appeared to show that ducks were more susceptible to the A/MuteSwan/MI/451072-2/06 virus than were chickens and turkeys, but because of the small size of the study groups, definitive conclusions cannot be made. On a genetic level, no NA stalk deletions, which have been associated with transmission to poultry (10), were observed for the wild-bird isolates. However, it should be noted that many biological traits are also associated with the internal protein genes as well, and these genes need to be evaluated to fully understand the virus-host interaction.

Finally, the antigenic relatedness of the H5 viruses as evaluated by HI assay was not entirely consistent with virus geographic origin or percent amino acid identity, reinforcing that amino acid identity is only an approximation for antigenic relatedness. Viruses such as A/Shorebird/DE/1346/01, which had the lowest cross-HI titers for the North American viruses, show that substantial antigenic variation can occur among wild-bird isolates. The relatively high cross-HI titers of A/Turkey/WI/68 and A/Chicken/Hidalgo/232/94 with the Eurasian isolates suggest that vaccines produced from North American viruses can be efficacious for challenge with viruses from Eurasian lineages (29) if adequate antibody levels are attained. The fact that comparisons of the amino acid identities of the entire HA1 protein were not entirely reliable for predicting the antigenic relatedness of the isolates demonstrates the importance of having biological data as well as sequence information for vaccine strain selection.

Although considerable data, often targeting a specific HA subtype, have been reported in recent years (4, 12, 21), more data on wild-bird-origin AI virus biology and genetics needs to be compiled in order to attain a good understanding of AI virus ecology and virus factors involved in avian host adaptation and virulence. Further work needs to be done to analyze the viral internal proteins and their genes to complete the evaluation. Here we report that the basic biological and genetic features of North American H5 subtype wild-bird viruses are clearly distinct from the Asian-lineage H5N1 HPAI viruses that have been isolated from more than 50 countries in Asia, Europe, the Middle East, and Africa since 1997. The study also demonstrates the great variation in pathotypes as well as the antigenic and genetic relatedness that can occur within the H5 and N1 subtypes of AI virus.

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