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Original Article

Molecular and phylogenetic analysis of matrix gene of avian influenza viruses isolated from wild birds and live bird markets in the USA

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Background Wild birds are the natural hosts for influenza A viruses (IAVs) and provide a niche for the maintenance of this virus.

Objectives This study was undertaken to analyze nucleotide sequences of the matrix (M) gene of AIVs isolated from wild birds and live bird markets (LBMs) to index the changes occurring in this gene.

Methods M-gene of 229 avian influenza virus (AIV) isolates obtained from wild birds and LBMs was amplified and sequenced. Full-length sequences (~900 nt.) thus obtained were analyzed to identify changes that may be associated with resistance to adamantanes. Phylogenetic analysis of all sequences was performed using clustalw, and evolutionary distances were calculated by maximum composite likelihood method using MEGA (ver. 5.0) software.

Results Twenty-seven different viral subtypes were represented with H3N8 being the most dominant subtype in wild birds and H7N2 being the predominant subtype among isolates from LBMs. Phylogenetic analysis of the M-gene showed a high degree of nucleotide sequence identity with US isolates of AIVs but not with those of Asian or European lineages. While none of the isolates from wild birds had any antiviral resistance–associated mutations, 17 LBM isolates carried polymorphisms known to cause reduced susceptibility to antiviral drugs (adamantanes). Of these 17 isolates, 16 had S31N change and one isolate had V27A mutation.

Conclusions These results indicate independent evolution of M-gene in the absence of any antiviral drugs leading to mutations causing resistance indicating the need for continued active surveillance of AIVs.

Keywords Adamantanes, antiviral resistance, avian influenza virus, live bird markets, matrix gene, wild birds.

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Introduction

Wild birds, belonging to the order Anserifomes (ducks, geese, and swans), are the natural reservoirs and historic source of all influenza A viruses (IAVs).[1,2] They harbor all known hemagglutinin (HA) and neuraminidase (NA) subtypes, and at least 103 of the possible 144 combinations of HA and NA have been found in them.[3,4] These viruses are generally non-pathogenic to wild birds but can cause significant morbidity and mortality in domesticated birds and occasionally in humans.[5,6]

The genome of IAVs consists of eight segments of single-stranded, negative-sense RNA encoding for 11 proteins.[7] Each segment encodes one protein except for the matrix (M), non-structural (NS), and PB-1 genes, each of which encodes two proteins.[8] Of the two proteins encoded by the matrix gene, M1 is important in initiating progeny virus assembly, while M2, an integral membrane protein, acts as a multifunctional ion channel and has a role in virus entry as well as in virus assembly and budding.[9]

The avian influenza virus (AIV) in wild birds is maintained for a long time, and the infection rate can be as high as 30%.[10] One important method of virus transfer from one to the other host is live bird markets (LBMs) where these birds come in close contact with domestic poultry and provide an easy route for their dissemination into the human population through the food chain. Live bird markets provide an ideal environment, whereby several avian

species captured from different sources come together. In the past, LBMs have been linked with outbreak of many diseases, as these markets are the major contact point between humans and live animals and provide optimal conditions for the zoonotic transfer of pathogens.[11,12] In fact, recent outbreaks of influenza in commercial poultry and humans have been linked to birds in LBMs.[13,14] Continual movement of birds into and out of LBMs provides opportunities for introduction and dissemination of AIVs,[15] which necessitates continuous surveillance of these markets to monitor the evolutionary changes in the AIV subtypes.

As virus control measures are difficult to implement in the wild, understanding the ecology of AIV in its natural host is important to develop strategies to control or prevent outbreaks in domestic poultry and humans.[16,17] Despite the importance of gene pool of AIVs in wild birds and the potential that pandemic strains can evolve from viruses from wild birds, knowledge about this gene pool is limited.[18]

Vaccination is the primary method to control influenza infections in humans, but antiviral agents have also been developed to treat infections. Two classes of antiviral drugs, the M2 channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir), are available for treatment of human influenza.[19] Adamantanes were the first class of antiviral drugs approved for treatment of human influenza. Unfortunately, their widespread use has led to the emergence of antiviral resistance against this class of drugs. According to one estimate, >60% of human H3N2 and >90% avian H5N1 viruses are resistant to this drug.[20,21] So far, five mutations, for example, single amino acid substitutions at positions 26, 27, 30, 31, and 34, have been shown to be responsible for resistance to adamantanes.[22] All these mutations are located on the M2 protein, in helix–helix packing interface of the protein.[23] The most commonly observed mutation is at position 31 leading to change from serine to asparagine (S31N).[22]

From an evolutionary point of view, IAVs are broadly divided into two lineages: North American and Eurasian. Genetic diversity of HA and NA sequences of isolates from wild birds has been well established[24,25,] but studies on the allelic variation among internal proteins such as the M-gene are scarce. It has been suggested that the M-gene is highly conserved and that it has evolved into host-specific lineages.[26,27] Recent studies have suggested that migratory birds might play an important role in the spread of highly pathogenic avian influenza (HPAI) viruses.[1,28] This necessitates continuing surveillance of AIV evolution in its natural host so that information on the emerging HPAI and potential human pandemic strains can be obtained.

This study was undertaken to compare nucleotide sequences of the matrix gene of IAVs from wild birds and those from LBMs to index the extent of allelic variations in this segment as well as identify any changes that might be associated with antiviral drug resistance. In this study, we sequenced M-gene as amino acid changes in this gene can significantly impact the pathogenesis and host range of AIV infection.

Materials and methods

Virus isolation and subtyping

Avian influenza virus isolates were obtained from the University of Georgia, Athens, GA, USA. All AIVs were isolated from cloacal swabs of wild birds collected during 2007–2008. Virus isolation was carried out by inoculation of embryonated chicken eggs via the allantoic route. Real-time RT-PCR[29] was used to confirm the presence of AIVs in harvested allantoic fluids, and viral subtyping was carried out using the previously published methods.[30,31] The AIV isolates from LBMs ($n = 128$) were purchased from the National Veterinary Services Laboratory (NVSL), Ames, Iowa. All AIVs were isolated from samples collected from LBMs located in northeastern United States during 1994–2004 and were subtyped at NVSL.

RT-PCR and nucleotide sequencing of M-gene

Viral RNA was extracted from infected allantoic fluids using QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA, USA). RT-PCR was performed to amplify the entire open reading frame of M-gene, using previously described primers.[32] The reaction conditions were as follows: initial reverse transcription step at 50°C for 30 minutes followed by initial denaturation at 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 43°C for 30 seconds, and extension at 72°C for 7 minutes, and a final extension step at 72°C for 10 minutes. The PCR products were separated by electrophoresis on 1·2% agarose gel and purified using PCR product purification kit (Qiagen). Purified products were submitted to the University of Minnesota's Advanced Genomic Analysis Center (AGAC) for sequencing. Both strands (forward and reverse) were sequenced using the primers used for amplification. To obtain full-length sequence, forward and reverse sequences of a given isolate were aligned using sequencer (ver 4·8) software (Gene Codes, Ann Arbor, MI, USA).

Phylogenetic analysis

The nucleotide sequences of all isolates were aligned by clustalW (Tempe, AZ, USA), and evolutionary distances were calculated by maximum composite likelihood method using MEGA ver 5.0 (www.megasoftware.net) software.[33]. Phylogenetic tree was constructed by aligning nucleotide sequences of all isolates with reference sequences obtained from GenBank, using the neighbor-joining (NJ) method and bootstrap analysis (500 replicates).

Antiviral resistance

Sequences were analyzed using annotation tools available at http://www.ncbi.nlm.nih.gov, to identify any antiviral drug resistance–associated mutations in the M-gene.

Results

Amplification of M-gene

In this study, matrix gene of 229 AIV isolates was amplified and sequenced. In total, 27 different combinations of HA and NA subtypes were identified, with H3N8 being the dominant subtype from wild birds and H7N2 from LBMs. List of subtypes isolated from different hosts is listed in Table 1. A sequence of approximately 900 nucleotides (nt) in length, encoding for two proteins, that is, M1 (26–784 nt) and M2 (26–51, 740–1000) for each isolate was obtained. No insertions or deletions were observed in any of the sequences.

Table 1. List of avian influenza virus subtypes isolated from live bird markets (LBMs) and wild birds

Source	Species	Subtype
Live bird markets	Unknown avian species (n = 50)*	H5N2 (6*); H5N8 (1); H7N2 (43)
	Chicken (n = 48)	H2N2 (14); H7N2 (34)
	Gallus gallus domesticus	
	Duck (n = 8)	H2N2 (1); H2N3 (1); H5N2 (1); H7N2 (5)
	Environment (n = 3)	H2N2 (1); H7N2 (2)
	Guinea fowl (n = 11)	H2N2 (5); H2N3 (1); H7N2 (5)
	Quail (n = 2)	H7N2 (2)
	Turkey (n = 5)	H7N2 (5)
	Meleagris gallopavo	
	Waterfowl (n = 1)	H7N2 (1)
	Total	128
Wild birds	Blue-winged teal (n = 9)	H3N8 (3); H4N5 (1); H4N6 (1); H4N8 (4)
	Anas discors	
	Green-winged teal (n = 4)	H6N2 (2); H6N8 (1); H11N9 (1)
	Anas crecca	
	Northern shoveler (n = 22)	H1N1 (2); H3N1 (1); H3N8 (16); H4N4 (1); H4N6 (1); H8N4 (1)
	Anas clypeata	
	Mallard (n = 65)	H1N1 (3); H1N3 (1); H1N9 (1);
	Anas platyrhynchos	H2N3 (1); H3N4 (1); H3N6 (1); H3N8 (24); H4N6 (5); H4N8 (4); H6N1 (3); H6N2 (1); H6N8 (1); H8N4 (9); H10N6 (1); H10N3 (2); H10N7 (4); H11N2 (1); H11N9 (1); H12N9 (1)
	Ruddy-turnstone (n = 1)	H3N2 (1)
	Arenaria interpres	
	Total	101
Grand total		229

*Numbers in parenthesis indicate number of isolates.

Phylogenetic analysis of M-gene

Phylogenetic analysis showed that M-gene from all isolates in this study belonged to North American avian lineage (Figure 1). Within this lineage, all isolates were divided into two major groups, I and II, with most of the isolates belonging to group I with nucleotide homology between the isolates ranging from 93% to 100%. There was no distribution of isolates in the phylogenetic tree on the basis of host, year of isolation, or subtype. All AIVs in the sublineages I and II clustered with other AIVs of North American avian-like lineage separate from those of Eurasian avian lineage (Figure 1).

Analysis of the M1 and M2 protein sequences

For further analysis of M-gene, amino acid sequences of M1 and M2 proteins were analyzed separately. The predicted amino acid sequences of M1 and M2 proteins were compared with that of A/turkey/Canada/1963(H6N8) (GU052877). For M1, a region between amino acids 10–252, and for M2, a region between amino acids 10–92, was analyzed. Phylogenetic analysis of M-gene on the basis of amino acid sequences showed considerable difference from that done on the basis of nucleotide sequences. Phylogenetic analysis on the basis of M1 and M2 amino acid sequences showed little variation among different lineages as compared to nucleotide-based tree, and there were few sublineages (Figures 2 and 3).

Comparison of predicted amino acid sequence of M1 protein from all isolates with the reference sequence [A/turkey/Canada/1963(H6N8); GU052877] and substitutions at 16 amino acid positions were observed over a span of 230 amino acid residues, with 1–2 substitutions per sequence (Table 2). In contrast to M1 protein, more variation was observed in M2 protein with substitutions observed at 26 positions as compared to the reference sequence (Table 2). Most of the isolates had one or two substitutions over a span of 70 amino acids. Most of the amino acid changes were observed in LBM isolates as compared to WB isolates.

Antiviral resistance

All sequences were analyzed using the annotation tools available online, to determine any mutations that might confer resistance to adamantanes. While none of the wild bird isolates had any mutation, 17 of the 128 isolates from LBMs (13·3%) were found to have mutations that are known to confer adamantine resistance. Of these 17 isolates, 16 had S31N change and one isolate had V27A mutation (Table 2).

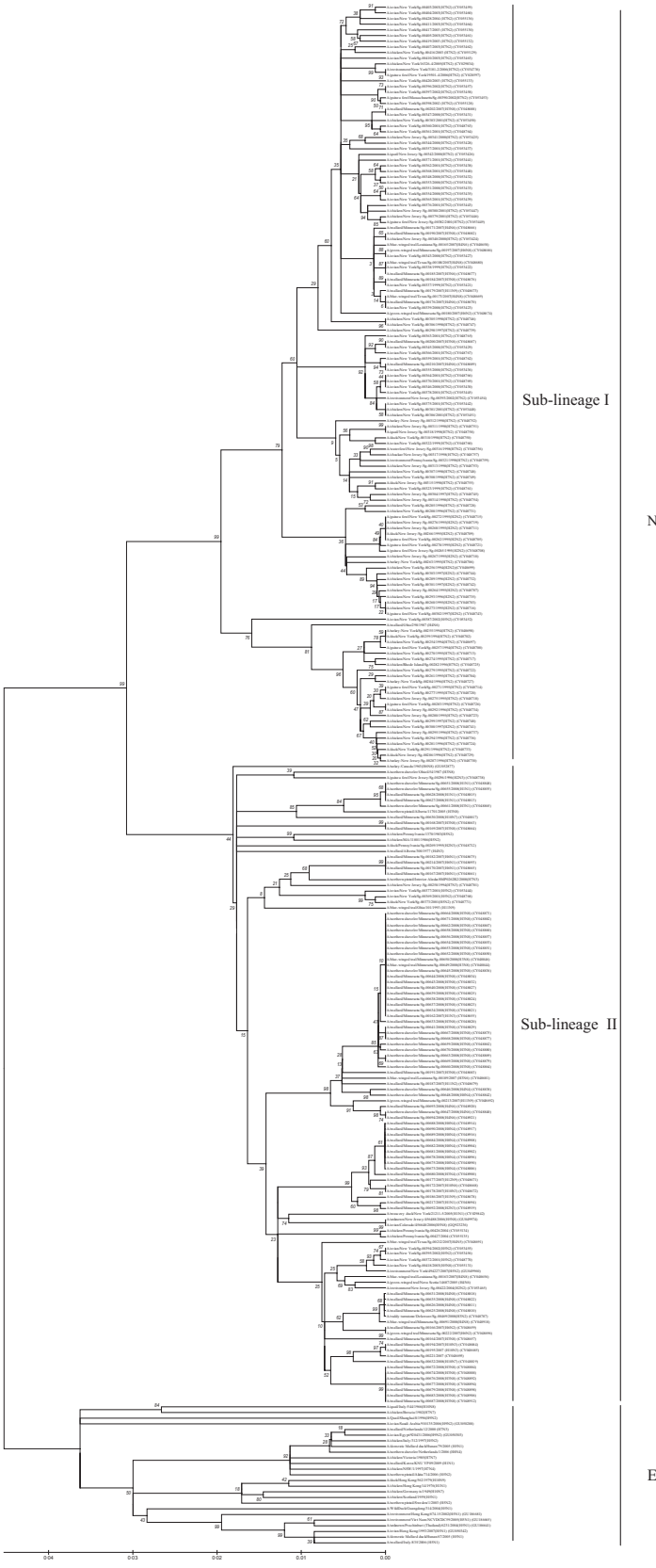

Figure 1. Phylogenetic analysis of matrix gene of avian influenza isolates inferred using the neighbor-joining method and 500 bootstrap replicates (values shown on tree). The evolutionary distances were computed using the number of differences method, and evolutionary analyses were performed in MEGA ver 5.0.

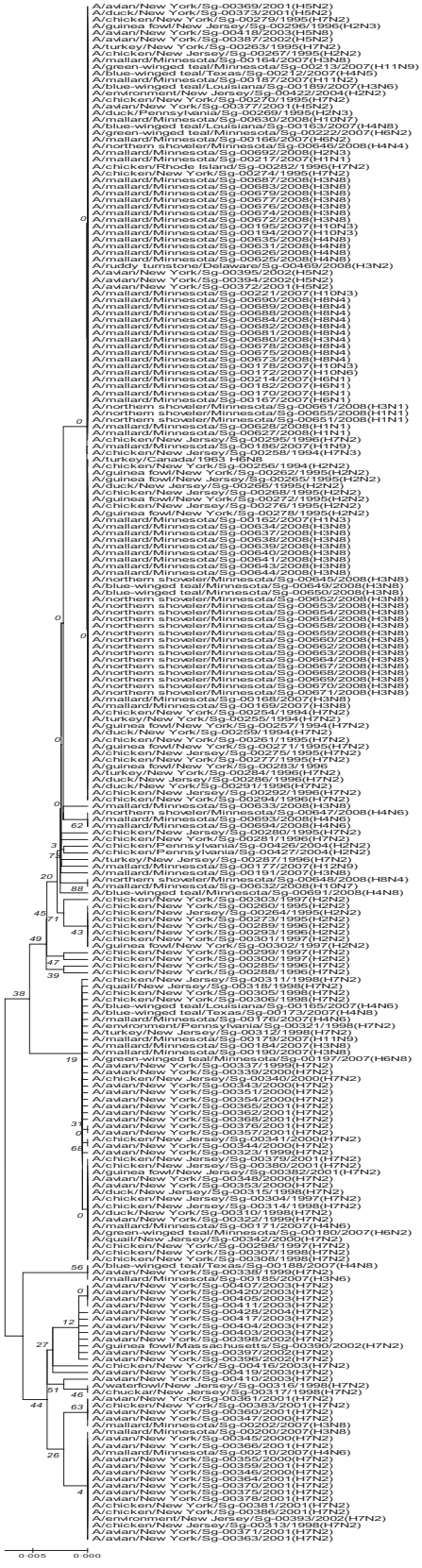

Figure 2. Phylogenetic analysis of M1 gene of avian influenza isolates inferred using the neighbor-joining method and 100 bootstrap replicates (values shown on tree). The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Evolutionary analyses were performed in MEGA ver 5.0.

Discussion

Wild birds are the natural hosts of IAVs, where these viruses are known to be in evolutionary stasis[26,34] but evolve rapidly once they cross the species barrier. AIVs from wild birds are known to switch to new hosts leading to emergence of novel influenza A lineages transmissible to new hosts.[35] Adaptation to domestic poultry is frequent, and LBMs provide an ideal condition for this transmission because different species of birds are kept in close proximity to each other, where this virus can adapt itself to domestic poultry. In the past, a few outbreaks of influenza in commercial poultry and humans have been linked to LBMs.[13,14] Thus, surveillance of wild birds and LBMs is important to understand the evolution of AIVs.

Matrix gene is an important structural gene encoding for both matrix and membrane proteins and has multiple functions.[26] A number of studies are available on the genetic analysis of HA and NA genes of AIVs in its natural host,[25,36] but little information is available on the evolution of the M-gene. This study was undertaken to analyze evolution and allelic variation in the M-gene of AIVs from wild birds and LBMs. Recent identification of zoonotic transmission of swine H3N2 carrying the 2009 pandemic matrix gene emphasizes that forces behind interspecies transmission transcend beyond those captured by HA or NA subtyping alone.

Overall sequence comparison of M-gene from all AIV isolates used in this study with isolates from other locations showed clear segregation of AIVs in clusters with respect to geographic location, for example, North American being separate from Eurasian lineage. These results are similar to those of Manzoor *et al.*[37], who observed that 65 of 67 isolates from free-flying birds belonged to Eurasian non-gull lineage, one to Eurasian gull-like, and one to the North American non-gull-avian lineage, suggesting independent evolution of M-gene along geographic lines. The assortment of M-gene into sublineages was independent of host or subtype or year of isolation. As previously reported by Widjaja *et al.*[17] and Manzoor *et al.*,[37] distribution of M-gene into sublineages varies according to number of sequences analyzed and is independent of isolation history, indicating multiple lines of evolution.[17]

In this study, we did not find any M-gene sequence that matched closely with those from European or Asian avian-like lineages. These results are similar to those reported by Widjaja *et al.*,[17] who conducted phylogenetic analysis of the

Figure 3. Phylogenetic analysis of M2 gene of avian influenza isolates inferred using the neighbor-joining method and 100 bootstrap replicates (values shown on tree). The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Evolutionary analyses were performed in MEGA ver 5.0.

M-gene and found that all gene sequences from mallard ducks grouped together with those of North American avian-like lineage. Although mixing of North American and Eurasian lineages has been reported in wild birds, it was not observed in this study. This is probably due to the fact that all the birds sampled in this study were ducks and not shorebirds. It has previously been reported that overlap of lineages is profound in shorebirds in Bering Strait during migration.[38]

Phylogenetic analysis of amino acid sequences of M1 and M2 proteins showed that M2 protein is under greater positive selection pressure in comparison with M1 protein and that both proteins have evolved independently. These results are similar to those observed by Widjaja *et al.*[17] and Furuse *et al.*[26] Since M1 protein plays an important role in virus assembly and budding, any change in M1 can have deleterious effect on virus survival, whereas M2 protein is integral membrane protein forming pH-gated channels in the viral lipid membrane.[23] More sites in the M2 protein under positive selection are located in the extracellular domain, which is recognized by the host immune response system. Thus, to evade host immune response, M2 protein is more susceptible to mutations.[26,39,40] Also, higher rate of amino acid variations in M1 and M2 proteins was observed in LBM isolates as compared to WB. This indicates that chances of random selection of variants are higher in LBMs because of close proximity of the birds. These findings are consistent with those reported by Ilyushina *et al.*[41] These authors also reported that the likelihood of random selection of variants because of accumulation of random point mutations was higher in H7 viruses circulating in poultry.

While none of the AIV isolates from wild birds in this study carried resistance-associated mutations in M-gene, 13·3% (n = 17) of isolates from LBMs were found to have mutation associated with adamantine resistance. Substitution at position 31 (S31N) in M2 protein was the most commonly observed substitution with 16 of the 17 isolate having this mutation, whereas only one isolate had V27A change. Emergence of resistance in LBM isolates of AIVs is a rare occurrence. In the only previous study, Lee *et al.*[42] reported on detection of resistant isolates from live poultry market and wild birds. High prevalence of S31N substitution in this study is in line with those observed earlier, as this substitution is considered to be the most common mutation conferring resistance to adamentanes[20], whereas V27A mutation is rare in frequency (1·6%). High

Table 2. Comparative analysis of predicted amino acid sequence of M1 and M2 proteins of matrix gene

Protein	Amino acid position	Reference*	Change	
			LBM	WB
M1	15	V	I (67)**, L (1)	I (6), L (1)
	42	L	I (4)	I (1)
	94	D	ND	G (3)
	113	K	R (3)	ND
	116	A	S (2)	S (2)
	118	S	G (3)	ND
	142	V	G (72)	G (14)
	144	F	L (2)	ND
	165	M	I (2)	ND
	167	T	A (4)	ND
	192	M	V (73)	V (6)
	203	M	I (2)	ND
	204	E	D (8)	ND
	227	A	T (1)	T (1)
	230	K	R (36)	R (3)
	248	M	L (15), V (1)	ND
M2	11	T	I (44)	I (17), P (1)
	13	N	K (15), S (3), D (1)	S (16)
	14	G	E (2)	E (2)
	18	K	R (14), N (15)	R (38)
	20	S	N (2)	ND
	21	D	G (3)	ND
	23	S	N (9)	ND
	25	P	ND	L (3)
	27	**V**	**A** (**1**), F (2), I (2)	ND
	29	A	T (1)	V (2)
	31	**S**	**N** (**16**)	ND
	32	I	V (2), T (1)	ND
	33	I	V (37)	ND
	35	I	V (1)	ND
	43	L	I (1)	S (1)
	45	R	P (2)	P (1)
	51	I	V (104)	V (17)
	52	Y	C (2)	C (1)
	54	R	H (5)	H (1), P (3)
	55	L	F (50)	F (4)
	56	K	R (1)	R (1)
	58	G	E (3)	ND
	61	R	K (1), G (5)	ND
	70	E	K (17)	ND
	77	R	Q (2)	Q (2)
	87	D	T (3)	ND

Values in bold face indicate amino acid position showing mutation responsible for adamantine resistance.
ND, not detected.
*Matrix gene sequence of A/turkey/Canada/1963(H6N8) (GenBank accession # GU052877).
**Numbers in parenthesis indicate number of isolates showing change.

susceptibility of wild bird isolates to adamantanes is not surprising as these isolates in the avian niche do not experience a selective pressure because of drug use. These antiviral drugs are exclusively used to treat human infections, and the frequency of spontaneous mutations leading to resistance against these drugs is likely very low. However, detection of resistant isolates from LBMs shows possibility of de novo substitution leading to the emergence of resistant AIV strains. These results suggest that adamantine resistance arises as independent point mutations rather than re-assortment or co-variation. This highlights the need to routinely screen M-gene sequences of AIVs to understand the frequency of naturally occurring mutations which might confer resistance to adamantanes.

Results of this study indicate there are variations in the M-gene and that overall there is a significant similarity between gene sequences from different bird species. These results further confirm the evolution of M-gene into distinct North American avian-like lineage in wild birds. Detection of antiviral resistant mutants suggests importance of surveillance studies in understanding the evolution of IAVs and to provide information about emergence of new subtypes which might cause outbreaks in domestic avian species or humans.

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Competing interests

The authors have no competing interests.

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