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Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia

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Abstract

Here, we report the diversity of amantadine-resistant mutants among avian influenza A viruses with pandemic potential (H5, H6, H7, and H9 hemagglutinin subtypes). Drug-resistant variants were not detected among 1979–83 isolates, whereas 31.1% of H5 and 10.6% of H9 strains from Southeast Asia isolated in 2000–04 carried mutations in M2 protein. In North America, resistant variants occurred among H7 viruses only (16.4% of those tested). H6 viruses were amantadine-sensitive. These findings prompt concern regarding the control of pandemic influenza, the possibility that the next pandemic virus will be amantadine-resistant and the need to monitor the use of the drug in poultry.

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Introduction

Newly emerging avian influenza A viruses pose a continued threat, not only to avian species but also to humans. In 1997, a highly pathogenic H5N1 influenza virus was isolated from 18 humans, demonstrating direct transmission of avian influenza viruses to humans (Claas et al., 1998; Suarez et al., 1998). In 1999, two cases of human infection with avian H9N2 influenza viruses were reported in Hong Kong (Peiris et al., 1999). More recently, an outbreak of highly pathogenic avian H7N7 influenza in the Netherlands in 2003 was associated with conjunctivitis in 349 humans and the death of a veterinarian (Koopmans et al., 2004), and in February 2004, H7N3 viruses were reported to infect humans in Canada (Kermode-Scott, 2004). At the same time, between late 2003 and early

2004, outbreaks of highly pathogenic avian H5N1 influenza occurred among poultry in eight Asian countries (Li et al., 2004), with human cases of H5N1 infection in Vietnam and Thailand associated with a mortality rate approaching 70% (WHO, 2004). In addition, recent avian H6N1 viruses were shown to have internal genes genetically similar to those of human H5N1 and H9N2 influenza isolates, suggesting that H6N1 viruses could become novel human pathogens (Chin et al., 2002). It is worth mentioning that the most recent H5N1 strains isolated in Southeast Asia were resistant to amantadine and rimantadine (Li et al., 2004); a group of antiviral drugs used for treatment and prevention of human influenza A virus infections. These drugs inhibit virus replication during the early stage of infection by blocking the ion channel formed by the M2 protein. Substitution of one of five amino acids (positions 26, 27, 30, 31, and 34) within the transmembrane domain of M2 has been implicated in loss of sensitivity to M2 blockers (Hay et al., 1985; Pinto et al., 1992). However, to our knowledge, there is no information on the frequency of amantadine-resistant variants in field isolates of avian influenza A viruses,

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particularly among strains having hemagglutinin (HA) subtypes with pandemic potential. In this study, we analyzed sequence data on matrix (M) genes of avian influenza viruses of the H5, H6, H7, and H9 HA subtypes that were isolated in North America and Southeast Asia during 1979–83 and 2000–04 and evaluated the frequency of drug-resistant strains.

Results and discussion

The analysis was based on the M gene sequences from 60 viruses isolated in Southeast Asia and 74 viruses from North America and were representative of two time periods (1979–83 and 2000–04) (Table 1). To systematically examine the avian gene pool circulating in both geographical regions, we also included sequence data available in GenBank on 408 strains from various avian hosts. Our analysis showed a diversity in the frequency of amantadine-resistant variants among different HA subtypes, years of isolation, and geographical areas (Table 1, Fig. 1). Interestingly, analysis of viruses isolated during 1979–83 did not reveal H5, H7, or H9 isolates with amino acid substitutions in the transmembrane region of M2 protein corresponding to resistance to adamantanes (amantadine and rimantadine) (Table 1). This finding was a feature of North American as well as Southeast Asian isolates. In contrast, drug-resistant strains occurred among viruses of all three of these HA subtypes obtained during 2000–04 (Table 1). Viruses of the H6 HA subtype were the only exception: analysis of sequence data obtained from H6 strains isolated from both regions did not reveal variants with M2 protein-associated mutations that would confer resistance to amantadine (Table 1, Fig. 1B).

The frequency of emergence of drug-resistant strains varied among HA subtypes (H5, H7, and H9) during 2000–04 (Table 1). In the present study, we did not identify M2-ion channel resistant viruses among the H5 and H9 influenza A viruses circulating in North American region (Table 1, Figs. 1A, D). However, sequence analysis

determined 2 resistant H7 variants from northeastern United States among the 8 avian viruses from this region characterized in the present study. In total, 9 of the 55 available North American H7 isolates were amantadine-resistant (Table 1), corresponding to an overall frequency of about 10% from 2000 to 2004 (Fig. 1C). Previously, it was shown that 7 of 9 H7 influenza A viruses that were associated with disease outbreaks in commercial poultry in the United States had the V27A and S31N amino acid substitutions in the M2 protein, and the amino acid sequence of the HA cleavage site of these 7 viruses fulfilled the molecular criteria for highly pathogenic avian influenza viruses (Spackman et al., 2003). Our finding is consistent with the idea that H7 viruses circulating in poultry could provide an opportunity for random selection of variants with amino acid changes in M2 transmembrane sequences. The likelihood of such generation could be high due to accumulation in the viral genome of random point mutations caused by the viral transcriptase, a situation that can be enhanced by rapid spread of the highly pathogenic viruses. On the other hand, the percentage of H7 drug-resistant variants (16.4%) could be explained due to the limited number of available isolates.

The pattern of frequency of resistant strains circulating in Southeast Asia differed from that in North America. Sequence analysis of Asian isolates identified 8 H5 and 1 H9 amantadine-resistant influenza variants and included 5 viruses isolated from chickens, 3 viruses isolated from silky chickens, and 1 virus isolated from a pheasant (Tables 1 and 2). Importantly, the largest proportion of Asian drug-resistant avian viruses of H5 and H9 subtypes occurred in China. In contrast, avian H7 influenza viruses from Asia were sensitive to M2 inhibitors (Table 1, Fig. 1C). Taking into account that H9N2 viruses were the most prevalent HA subtype in the live-poultry markets in southeastern China between 2001 and 2004 and that these viruses exhibited increasing genetic and biologic diversity (Choi et al., 2004), our identification of a frequency of 10.6% for amantadine resistance among H9 strains might be explained by the diverse nature of the gene pool. Further, the percentage of

Table 1
Amantadine-resistant variants among avian influenza A viruses isolated in North America and Southeast Asia

Viruses	Year of isolation	Hemagglutinin subtypes							
		H5		H6		H7		H9	
		North America	Southeast Asia	North America	Southeast Asia	North America	Southeast Asia	North America	Southeast Asia
From GenBank	1979–83	1	1	0	0	0	0	1	2
	2000–04	17	114	16	7	47	0	0	35
Sequenced in this study	1979–83	10	6	14	6	6	2	4	3
	2000–04	6	21 (8) ^a	15	10	8 (2)	0	11	12 (1)
Number of resistant variants (%)	1979–83	0	0	0	0	0	0	0	0
	2000–04	0	42 (31.1)	0	0	9 (16.4)	0	0	5 (10.6)

Amantadine resistance-associated mutations present in the M2 gene of viruses of the H5 HA subtype were at positions V27A (21% of resistant variants), A30S (17%), and S31N (62%); of the H7 HA subtype were V27A (11%), A30S (22%), and S31N (67%); and of the H9 HA subtype was S31N (100%).

^a In parenthesis, the number of drug-resistant variants identified among viruses sequenced in the present study.

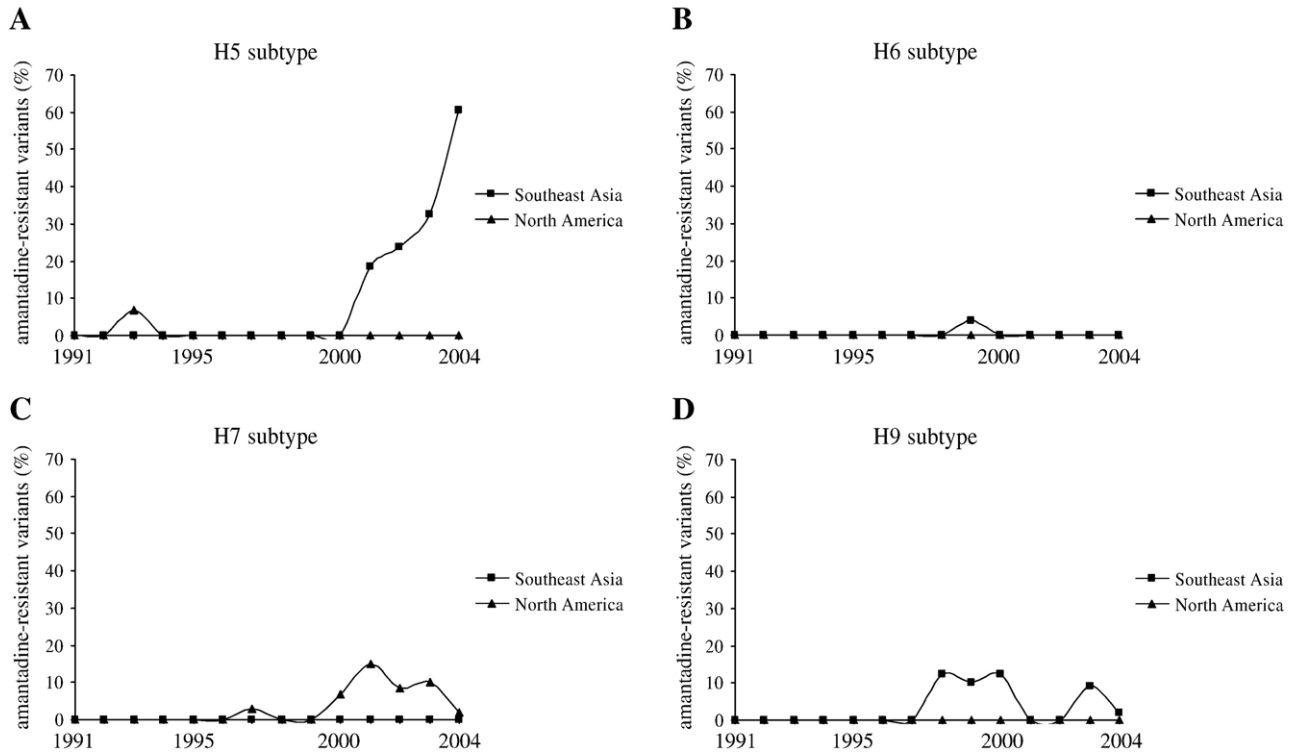


Fig. 1. Appearance of amantadine-resistant variants among avian influenza viruses of H5, H6, H7, and H9 HA subtypes between 1991 and 2004. Sequence analysis (GenBank) revealed 1 drug-resistant variant of the H5, 2 of the H6, 1 of the H7, and 3 of the H9 HA subtypes among viruses isolated during 1991–99. Amantadine-resistant variants isolated in 2000–04 are presented in Table 1.

resistant H5 viruses (31.1%) is particularly high and in contrast to the number of resistant variants among H6, H7, and H9 HA subtypes (Table 1). One of the possible explanations that we were not able to identify any drug-resistant H6 and H7 viruses in Asia could be due to the limited number of available isolates. The distribution of the mutations in the M2 genes of H5 HA suggests that they were independently acquired rather than having descended from a single lineage of amantadine-resistant M2 genes. In

addition, the rate of mutation in the M2 gene was expected not to be quiet high among all HA subtypes: about 11×10^{-3} substitutions per nucleotide site per year (Rodgers and Swofford, 1998). Therefore, there is no direct evidence, but in light of the genetic stability of this particular gene segment (Trampuz et al., 2004), we speculate that H5 avian viruses with amino acid residues in the M2 protein, that confer resistance to the amantadine, have a selective advantage in poultry in Asia and would not occur naturally.

Table 2
Amantadine sensitivity assay of influenza viruses in MDCK cells

Virus	Subtype	M2 mutation that confers resistance to amantadine	IC ₅₀ (mean ± SD, μM) ^a
A/Duck/Jiangxi/6151/2003	H5N3	– ^b	0.10 ± 0.02
A/Chicken/Pennsylvania/143586/2002	H7N2	–	0.10 ± 0.04
A/Chicken/Hong Kong/WF208/2001	H9N2	–	0.49 ± 0.07
A/Chicken/Hong Kong/SF131/2003	H5N1	V27A	≥100
A/Chicken/Hong Kong/SSP94/2003	H5N1	V27A	≥100
A/Silky Chicken/Hong Kong/YU238/2003	H5N1	V27A	≥100
A/Chicken/Hong Kong/YU250/2003	H5N1	S31N	≥100
A/Silky Chicken/Hong Kong/YU316/2003	H5N1	V27A	≥100
A/Silky Chicken/Hong Kong/SSP7/2003	H5N1	S31N	≥100
A/Chicken/Hong Kong/SSP139/2003	H5N1	V27A	≥100
A/Pheasant/Hong Kong/NT23/2003	H5N1	V27A	≥100
A/Chicken/CT/9407/2003	H7N2	A30S	33.2 ± 2.6
A/Chicken/NY/116124/2003	H7N2	A30S	44.1 ± 3.7
A/Chicken/Hong Kong/FY313/2000	H9N2	S31N	≥100

^a Susceptibility to amantadine was determined by plaque reduction assay in MDCK cells. Results are the mean values (IC₅₀, μM) of two experiments ± standard deviation (SD).

^b No mutations that confer resistance to amantadine.

In support of this possibility, it is worth mentioning that the overall frequency of resistant H5 strains was 33.8% during 2000–04 (Fig. 1A) and that ~30% to 80% of patients usually shed resistant strains when amantadine or rimantadine is used for therapy of influenza virus infections among untreated adults (Saito et al., 2003).

In the present study, we identified 11 drug-resistant variants of H5, H7, and H9 HA subtypes among all 134 viruses sequenced. These strains possessed amino acid substitutions in the M2 protein at three positions – V27A, A30S, and S31N (Table 2) – reported previously to confer resistance to amantadine (Hay et al., 1985; Pinto et al., 1992). Among the characterized resistant viruses, 62% of the H5, 67% of the H7, and 100% of the H9 strains demonstrated the S31N substitution (Table 1). We verified the susceptibility to amantadine of all 11 strains by plaque reduction assay in MDCK cells, using as controls representatives of each subtype that lacked the M2 changes associated with resistance to amantadine. IC₅₀ values for H7 mutants were ~350-fold higher than those for H7 drug-sensitive virus (Table 2). Amantadine concentrations as high as 100 μM failed to inhibit replication of H5 and H9 drug-resistant viruses. In contrast, A/Duck/Jiangxi/6151/2003 (H5N3) virus could be inhibited almost completely by amantadine at concentration as low as 0.1 μM. With A/Chicken/Hong Kong/WF208/2001 (H9N2) virus, a 50% reduction was seen with a drug concentration of 0.49 μM (Table 2). It was reported, that the mutations that confer amantadine resistance are located in the transmembrane domain of the M2 protein at one of five amino acid positions: 26, 27, 30, 31, and 34. These residues differ in their effects on binding of amantadine: viruses with S31N or A30T(S) amino acid substitutions no longer bind the blocker, which therefore can no longer exert its inhibitory function, whereas viruses with mutations at residue 26 or 27 retain binding of the blocker, but function of the M2 protein is not inhibited (Astrahan et al., 2004). In our study we confirmed that H5, H7, and H9 avian influenza A viruses with V27A, A30S, or S31N amino acid substitutions in their M genes exhibited high-level resistance to amantadine, with ≥100-fold reduction in susceptibility compared with that of sensitive viruses of similar HA subtype (Table 2).

In this study, M gene sequence analysis of viruses distributed among four HA subtypes revealed that the percentage of drug-resistant avian H7 and H9 viruses between 2000 and 2004 was ~10–15% and that the appearance of H6 amantadine-resistant mutants was a rare event (Figs. 1B, C, D). This finding can be explained by the idea that under the described epidemiological conditions, such mutants may appear. The high frequency of H5 amantadine-resistant influenza viruses and its tendency to increase in recent years is in sharp contrast to the levels of resistance among other HA subtypes (Fig. 1). Furthermore, the M gene phylogenetic tree revealed that there was no clear separation of H5 amantadine-resistant variants into a particular distinct lineage; conversely, they belonged to

multiple sublineages of Eurasian avian-like lineage (the M gene phylogenetic trees of H5, H7, and H9 viruses that include the drug-resistant variants are available online at <http://www.stjuderesearch.org/data/amantadine/>). This observation prompts concern not only about the possibility that a putative pandemic strain will be resistant to amantadine, but also about the fact that it might not occur randomly because of the limited mutation rate per nucleotide per year. Although the high replication efficiency of highly pathogenic viruses may favor the emergence of mutations, but the resistant variants do not appear to be more virulent, genetically stable, or capable of competing with wild, drug-sensitive strains (Bean et al., 1989; Saito et al., 2003). Therefore, the primary emerged drug-resistant mutants have no survival advantages. Furthermore, high accumulation of random mutations likely would not take place because of the high mortality rate among infected hosts and rapid spread of the highly pathogenic viruses. The single worrisome alternative regarding selection of resistant strains is that the poultry farmers in Asia are adding amantadine to chicken food to protect birds during H5 influenza virus outbreaks in domestic birds. Our findings that H6 viruses had no resistance-related mutations might be explained by the fact that H6 viruses cause unapparent infections of domestic chickens and thus there would be no need to treat the poultry with amantadine.

This study is the first attempt to evaluate the frequency of amantadine-resistant variants among potentially pandemic avian influenza viruses circulating in North America and Southeast Asia. Our findings highlight the necessity of monitoring the susceptibility of avian strains to antiviral drugs and, moreover, the urgent need to control the use of these drugs during outbreaks of highly pathogenic influenza viruses in poultry.

Materials and methods

Virus isolates

We characterized 134 avian influenza viruses representative of H5, H6, H7, and H9 HA subtypes that we obtained from the St. Jude Children's Research Hospital repository (a list of the influenza viruses characterized in this study is available on request). The viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs.

RNA isolation, PCR amplification, and sequencing

Viral RNA was isolated from virus-containing allantoic fluid by using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription of viral RNA and subsequent PCR was performed using primers specific for the M gene segment, as described previously (Hoffmann et al., 2001). PCR products were purified with QIAquick PCR purification kit (Qiagen)

according to the manufacturer's protocol. The sequencing reaction was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The DNA template was sequenced by using rhodamine or dRhodamine dye terminator cycle-sequencing Ready Reaction kits with AmpliTaqDNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were analyzed in a Perkin-Elmer Applied Biosystems model 373 or model 377 DNA sequencer. DNA sequences were completed and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers DQ107406–DQ107518.

Susceptibility to amantadine

Amantadine sensitivity was determined by plaque reduction assay on Madin–Darby canine kidney cells (MDCK, ATCC, Manassas, VA), as described previously (Hayden et al., 1980). Six-well plates were inoculated with virus diluted in minimal essential medium (MEM) to give 80 to 100 plaques per well. Cells were incubated for 1 h at 37 °C and then overlaid with MEM containing 0.9% agar, 4% bovine serum albumin, 1 µg/ml L-1-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Diagnostics, Freehold, NJ), and amantadine (1-aminoadamantane hydrochloride, Sigma-Aldrich, Inc., St. Louis, MO) at different concentrations (0.01 to 100 µM). After 3 days of incubation at 37 °C, plaques were visualized by staining with 0.1% crystal violet containing 10% formaldehyde. The percentage inhibition of plaque formation relative to untreated controls was calculated for each drug concentration. Two independent experiments were performed to determine the drug concentration resulting in a 50 % reduction of the plaque number (IC₅₀). Three amantadine-sensitive viruses of the H5, H7, and H9 subtypes [A/Duck/Jiangxi/6151/2003 (H5N3), A/Chicken/Pennsylvania/143586/2002 (H7N2), and A/Chicken/Hong Kong/WF208/2001 (H9N2)] were used to compare the susceptibility of amantadine-resistant strains with that of those that lacked changes in M2 protein.

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