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Antiviral resistance among highly pathogenic influenza A (H5N1) viruses isolated worldwide in 2002–2012 shows need for continued monitoring

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ABSTRACT

Highly pathogenic (HP) H5N1 influenza viruses are evolving pathogens with the potential to cause sustained human-to-human transmission and pandemic virus spread. Specific antiviral drugs can play an important role in the early stages of a pandemic, but the emergence of drug-resistant variants can limit control options. The available data on the susceptibility of HP H5N1 influenza viruses to neuraminidase (NA) inhibitors and adamantanes is scarce, and there is no extensive analysis. Here, we systematically examined the prevalence of NA inhibitor and adamantane resistance among HP H5N1 influenza viruses that circulated worldwide during 2002–2012. The phenotypic fluorescence-based assay showed that both human and avian HP H5N1 viruses are susceptible to NA inhibitors oseltamivir and zanamivir with little variability over time and ~5.5-fold less susceptibility to oseltamivir of viruses of hemagglutinin (HA) clade 2 than of clade 1. Analysis of available sequence data revealed a low incidence of NA inhibitor–resistant variants. The established markers of NA inhibitor resistance (E119A, H274Y, and N294S, N2 numbering) were found in 2.4% of human and 0.8% of avian isolates, and the markers of reduced susceptibility (I117V, K150N, I222V/T/K, and S246N) were found in 0.8% of human and 2.9% of avian isolates. The frequency of amantadine-resistant variants was higher among human (62.2%) than avian (31.6%) viruses with disproportionate distribution among different HA clades. As in human isolates, avian H5N1 viruses carry double L26I and S31N M2 mutations more often than a single S31N mutation. Overall, both human and avian HP H5N1 influenza viruses are susceptible to NA inhibitors; some proportion is still susceptible to amantadine in contrast to ~100% amantadine resistance among currently circulating seasonal human H1N1 and H3N2 viruses. Continued antiviral susceptibility monitoring of H5N1 viruses is needed to maintain therapeutic approaches for control of disease.

1. Introduction

Highly pathogenic (HP) influenza A (H5N1) viruses predominantly affect avian species but occasionally cross the species barrier and infect humans. Since 2003, outbreaks of H5N1 influenza viruses have been reported in domestic poultry and wild birds in 63 countries/territories (WHO/OIE/FAO, 2012). Human H5N1 virus infections were first reported in 1997, and since the virus reappeared in humans in 2003, it continues to cause sporadic infections of humans in Southeast Asia, the Middle East, Europe, and Africa with a total of >600 human cases and >60% mortality rates (www.who.int; World Health Organization 2012).

To date, multiple clades/subclades of H5N1 influenza viruses have been distinguished by phylogenetic analysis of the hemagglutinin (HA) gene, some of which have a distinct geographical distribution. Clade 1.1 viruses, which evolved from clade 1, continue to circulate in Vietnam and Cambodia. Clade 2 viruses are the most diverse group of H5N1 viruses and can be divided into 5 circulating subclades: 2.1.3 variants circulate in Indonesia, 2.2 viruses circulate in India and Bangladesh, 2.2.1 viruses are found in Egypt, 2.3.2 encompasses viruses found from Southeast and Central Asia to Eastern Europe, and 2.3.4 viruses circulate in Vietnam, Laos, Thailand, and China (Marinova-Petkova et al., 2012). Virus clade 2.3.2 in its various forms is now considered the dominant type in China, although clade 2.3.4 has not disappeared (WHO/OIE/FAO, 2012). Clade 7 viruses are also found in circulation in Vietnam and China. Since 1997, human infections have been caused by H5N1 viruses of clades 0, 1, 2.1, 2.2, 2.3, and 7; however, infections in 2010–2012 have been predominantly caused by viruses of clades 2.3.2 and 2.3.4.

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The current pandemic in poultry and wild birds is a potential threat to human health due to continuous evolution and genetic diversity of circulating avian influenza H5N1 viruses and the contribution of genetic material from avian influenza viruses to the emergence of human pandemic virus. In response to this threat, major efforts have been made to develop immunogenic cross-clade–protective H5N1 vaccines (Subbarao and Luke, 2007; Prieto-Lara and Llanos-Méndez, 2010). In the absence of an effective vaccine, antiviral prophylaxis and treatment can play an important role. The neuraminidase (NA) inhibitors oseltamivir and zanamivir are the recommended antiviral drugs against influenza. Patients infected with H5N1 viruses have been predominantly treated with oral oseltamivir (Writing Committee, 2008; Adisasmito et al., 2010). Analysis of Avian Influenza Registry data from 10 countries showed that the strongest impact on survival among H5N1-infected patients was observed when treatment was initiated $\leqslant$2 days after symptom onset (Chan et al., 2012), and there was an increased likelihood of survival when treatment was initiated as late as 3–5 days after symptom onset but before respiratory failure occurred (Adisasmito et al., 2010).

The effectiveness of antiviral drugs will depend on the susceptibility of the pandemic strain, should it emerge. Oseltamivir carboxylate (the active ingredient of oseltamivir) was shown to be active in vitro against human and avian H5N1 influenza viruses (McKimm-Breschkin et al., 2007; Hurt et al., 2007). NA enzyme inhibition assays revealed that clade 2.1 viruses from Indonesia have a naturally occurring 15- to 30-fold lower sensitivity to oseltamivir carboxylate in vitro than clade 1 viruses from Vietnam (McKimm-Breschkin et al., 2007), which was attributed to NA's H252Y amino acid difference between the clades (Rameix-Welti et al., 2006). In contrast, Hurt and colleagues (2007) found little variation in the sensitivity of the NAs of 51 avian H5N1 isolates collected over a similar time in similar regions. Overall, available data on in vitro sensitivity to oseltamivir carboxylate generally show sensitivities to drug concentrations well below the minimum in vivo concentrations achieved during therapy (Le et al., 2008). Susceptibility of H5N1 viruses to zanamivir was not affected (McKimm-Breschkin et al., 2007).

Resistance to oseltamivir in clinically derived seasonal influenza A viruses was associated with H274Y or N294S amino acid substitutions in the N1 NA subtype (N2 numbering used here and throughout the text) and E119V, R292K or N294S substitutions in the N2 NA subtype (McKimm-Breshkin, 2012). Oseltamivir-resistant H5N1 influenza viruses with an H274Y NA mutation were reported in three patients during oseltamivir treatment or prophylaxis (de Jong et al., 2005; Le et al., 2005). One of these patients had a mixed population of wild-type NA and both H274Y and N294S mutations (Le et al., 2005). Our previous studies showed that some Egyptian H5N1 isolates from humans have had an N294S NA mutation, which confers a 12- to 15-fold increase in the IC_{50} value in an NA inhibition assay (Earhart et al, 2009; Kayali et al., 2011). Screening of 29 HP H5N1 viruses of clade 2.3.2 from the Republic of Laos in 2006-2008 identified three outliers with reduced NA inhibitor susceptibility with different mutations (V116A, I222L, and S246N) (Boltz et al., 2010). A minor subpopulation of drug-resistant clones with I117V and E119A NA mutations (the latter being associated with zanamivir resistance in the N2 NA subtype) were detected in human A/Turkey/65-1242/2006 (H5N1) virus (Govorkova et al., 2009).

The worldwide emergence of drug-resistant seasonal H3N2 and H1N1pdm09 influenza variants (Bright et al., 2006; Deyde et al., 2007; Gubareva et al., 2010) restricted the use of another class of specific anti-influenza drugs, M2 ion channel inhibitors (amantadine and rimantadine). Although the antiviral activity of amantadine against HP H5N1 was seen in vitro and in vivo (Ilyushina et al., 2007), the data on its therapeutic effectiveness is lacking. The WHO guidelines from 2007 do not recommend treatment of H5N1 virus–infected patients with amantadine unless the infecting virus is known to be susceptible or other drugs are unavailable (World Health Organization, 2007; Schunemann et al., 2007). Molecular markers of resistance to adamantanes are amino acid substitutions at residues L26, V27, A30, S31, and G34 within the transmembrane domain of the M2 protein (Hay et al., 1986; Pinto et al., 1992; Li et al., 2004). Previous publications reported emergence and geographic diversity in the distribution of amantadine-resistant H5N1 influenza viruses (Cheung et al., 2006; Monne et al., 2008; Tosh et al., 2011). Amantadine-resistant H5N1 viruses were reported in Saudi Arabia, Thailand, Vietnam, Cambodia, Malaysia, Indonesia, China, and India, although the prevalence of resistant viruses varies in different geographical areas (Cheung et al., 2006; Hurt et al., 2007; Monne et al., 2008; Tosh et al., 2011).

There are ongoing concerns that H5N1 viruses may yet cause a pandemic; hence, antiviral surveillance of HP H5N1 is essential for the determination of our options for the control of a pandemic. Here we provide a comprehensive analysis of the antiviral susceptibility of the HP human and avian H5N1 influenza viruses isolated during the past decade (2002–2012) based on phenotypic analysis and determination of amino acid changes occurring at the conserved or semi-conserved NA residues (GenBank data) that may confer either a resistant or reduced susceptibility genotype. The incidence of amantadine resistance among multiple and evolving HA clades of H5N1 viruses was analyzed based on the sequence data generated in the current study and that available in GenBank.

2. Materials and methods

2.1. Viruses

Ten human and 85 avian (originating from wild birds, ducks, geese and chickens) HP H5N1 influenza viruses representative of HA clades 1 and 2 and isolated in 2002–2011 were obtained through the WHO network. The viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35 °C for 40 h. All experiments were conducted in biosafety level 3+ conditions in compliance with applicable laws and guidance.

2.2. Compounds

The NA inhibitors oseltamivir carboxylate (oseltamivir, [*3R,4R,5S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) and zanamivir (4-guanidino-Neu5Ac2en) were provided by Hoffmann-La Roche, Ltd. (Basel, Switzerland). Stocks of oseltamivir and zanamivir were prepared in distillated water, filter-sterilized, and stored in aliquots at −20 °C.

2.3. NA inhibition assay

NA activity of the H5N1 influenza viruses was measured in a fluorescence-based assay using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) (Sigma-Aldrich, St. Louis, MO) (Potier et al., 1979). Fluorometric determinations were quantified with a Synergy 2 multi-mode microplate reader (BioTek Instruments, Winooski, VT) based on the release of the fluorescent product 4-methyl-umbelliferone using excitation and emission wavelengths of 360 and 460 nm, respectively. Viruses were standardized to equivalent NA enzyme activity in the linear range of the curve and were mixed with various concentrations of inhibitor in 96-well flat-bottom black opaque plates (Corning Costar, NY). The final reaction mixture concentrations of the NA inhibitors ranged from 0.05 nM to 5000 nM. The virus-inhibitor mixture was incubated at 37 °C for

30 min prior to the addition of MUNANA substrate and then incubated at 37 °C for 30 min. The reaction was terminated by the addition of the stop solution (0.1 M glycine in 25% ethanol, pH 10.7). The concentration of NA inhibitor that reduced NA activity by 50% relative to a control mixture with no NA inhibitor (IC_{50}) was determined by plotting the percent inhibition of NA activity as a function of the compound concentrations calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). IC_{50} values were recorded as the means of 2–3 independent determinations. Reference oseltamivir-susceptible and -resistant A/Mississippi/3/2001 (H1N1) influenza strains (mean IC_{50}, 0.53 nM and 299.58 nM, respectively) were obtained from the Antiviral Group, International Society for Influenza and Other Respiratory Virus Diseases, and were included in each assay and showed an IC_{50} variability of 8% over six separate assays.

2.4. Data analysis

The obtained IC_{50} values were analyzed for oseltamivir and zanamivir to determine statistical cutoffs for identification of potentially resistant viruses (outliers) using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Quantile box-and-whisker plots were used to display the distribution of log-transformed IC_{50} values. The bottom and the top of the box marked the 10th and 90th percentiles, and the line across the box marked the 50th percentile or median. Whiskers were added to identify potential outliers and extended above and below the box by 1.5 times the interquartile range. Mild outliers had IC_{50} values above the statistical cutoff, which was >3-fold but <10-fold greater than the mean IC_{50} for the drug. The isolates with $\geqslant$10-fold of the mean IC_{50} value were considered extreme outliers and were excluded from statistical analysis of the overall population.

2.5. Susceptibility to the adamantanes

Genetic analysis of the transmembrane region of the M2 protein was conducted, and substitutions of five residues (L26, V27, A30, S31, and G34) were used to screen for molecular markers of adamantine resistance. RNA extraction was performed using the RNeasy kit (QIAGEN, Valencia, CA), and RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. DNA sequences were completed and edited using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

In addition, the alignments using ClustalW were performed in BioEdit 7 (Ibis Biosciences, Carlsbad, CA), which was also used to scan all of the above-mentioned mutations. The 28 M2 gene sequences from this study as well as 208 human and 1433 avian sequences of HP H5N1 viruses obtained from the GenBank database (www.ncbi.nlm.nih.gov as of October 2012) were included in the analyses.

2.6. NA sequence analysis

All viruses with elevated IC_{50} values (in this study, mild outliers with IC_{50} >3-fold but <10-fold greater than the mean IC_{50} for oseltamivir) were subject to sequence analysis and characterization of individual virus clones (28–30 clones) by using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). The position of the identified NA mutations in relations to the proximity to the residues located within catalytic site and within 3 Å of the drug bound sites was analyzed as described elsewhere (Maurer-Stroh et al., 2009). In addition, the NA gene sequences of 287 human and 1716 avian HP H5N1 viruses (those with full-length NA sequences and those covering amino acids from 116 to 345 of the NA obtained from the GenBank database were analyzed for the presence of NA mutations associated with either NA inhibitor–resistant genotype in different NA subtypes (E119V/I/A/G, H274Y, R292K, and N294S) or reduced susceptibility genotype to NA inhibitors (V116A, I117V, K150N, D198N/G/E/Y, I222V/T/R/K/M, S246N, and E276D). The HAs of all H5N1 influenza viruses included in the analysis had a multibasic amino acid motif (R-X-R/K-R) in the HA connecting peptide.

2.7. Nucleotide sequence accession numbers

The NA (two viruses, accession numbers KC436119 and KC436121) and M gene (30 viruses; accession numbers KC436109–KC436118, KC436120, KC436122–KC436138) sequences determined in this study were deposited into the GenBank database.

2.7.1. HA clade determination

The corresponding HA sequences of H5N1 influenza viruses with M2 protein resistance markers were obtained from GenBank, and H5N1 viruses representative of the HA clades were obtained from the WHO website (WHO/OIE/FAO, 2012). The human, avian, and reference HA sequences were edited using BioEdit 7 and aligned using MUSCLE (Edgar 2004). Neighbor-joining phylogenetic trees were generated using MEGA software (MEGA v5.05) (Tamura et al., 2011), and the HA clades were determined for human and avian amantadine-resistant viruses.

3. Results

3.1. Susceptibility of H5N1 viruses to NA inhibitors

This study was undertaken to evaluate the antiviral susceptibility of the HP H5N1 influenza viruses isolated during past decade (2002–2012) to NA inhibitors, and to determine the incidence of drug-resistance associated mutations among these rapidly evolving viruses. A total of 95 H5N1 viruses (10 human and 85 avian) representing HA clades 1 and 2 were screened in a phenotypic fluorescence-based assay and were found to be fully susceptible to oseltamivir and zanamivir, although the IC_{50} values for oseltamivir were ~2-fold higher than those for zanamivir (mean IC_{50}, 1.48 nM and 0.65 nM, respectively). The human and avian isolates were equally susceptible to oseltamivir (mean IC_{50}, 1.53 nM and 1.43 nM, respectively), although the number of human isolates tested was limited to 10 strains. No differences were observed in the susceptibility to zanamivir. Analysis was also undertaken by year of H5N1 virus isolation to determine whether the distribution of IC_{50} values varied in different years and whether a significant increase in mean IC_{50} values could be observed (Fig. 1A and B). The H5N1 viruses isolated in 2005–2007 had the highest IC_{50} values to both NA inhibitors, but we did not see a significant linear tread to sensitivity over time.

Although the mean IC_{50} values varied little, there were variant outliers with sensitivities both slightly higher and lower than the means. Only a few viruses had IC_{50} values above the upper quantile limit for oseltamivir (4 of 95) and zanamivir (2 of 95). Our phenotypic assay identified three mild outliers for oseltamivir among avian H5N1 viruses and none for zanamivir (Table 1). The subsequent clonal analysis of NA genes found that 4–5 individual clones among 28–30 sequenced had different single NA amino acid substitutions. These NA substitutions were random and did not occur in the positions reported previously to be associated with either NA inhibitor resistance or reduced susceptibility phenotype.

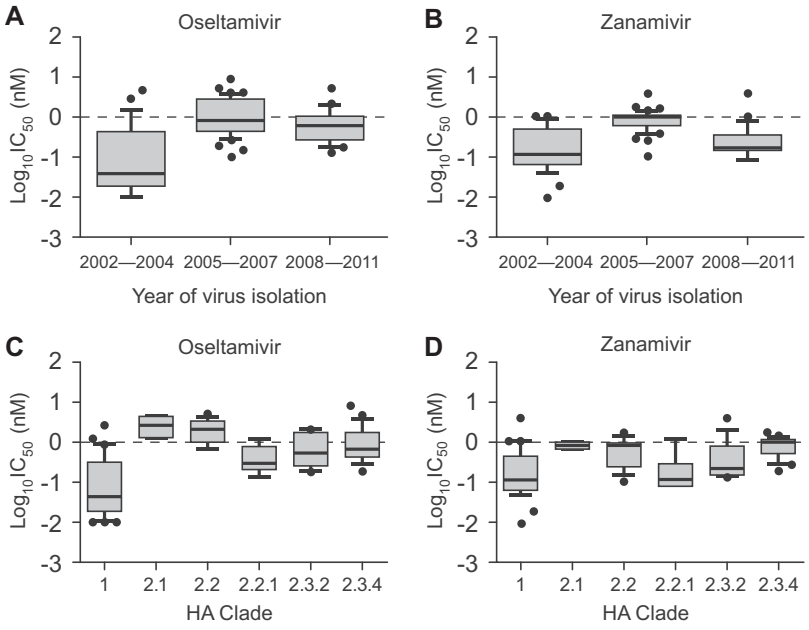

Fig. 1. Plots showing the IC_{50} (nM) ranges of oseltamivir and zanamivir for human and avian influenza H5N1 viruses tested by a phenotypic fluorescence-based NA inhibition assay. Panels A and B show quantile box plots illustrating the mean IC_{50} values for oseltamivir (A) and zanamivir (B) for H5N1 viruses isolated in different years. Panels C and D show quantile box plots illustrating the mean IC_{50} values for oseltamivir (C) and zanamivir (D) for H5N1 viruses representative of different HA clades.

Table 1
Avian influenza A (H5N1) viruses with elevated IC_{50} values detected in this study.

Avian H5N1 virus	HA clade	NA enzyme inhibition assay (mean IC_{50} ± SD, nM)[a]				No. mutant clones/no. total (NA mutation)[c]
		Oseltamivir	Fold change[b]	Zanamivir	Fold change[b]	
A/chicken/Jogjakarta/BBVET/IX/2004	2.1.3	4.87 ± 1.30	2.95	0.69 ± 0.04	1.21	5/30 (G31A, E228D, T383A, P420S, F422L)
A/muscovy duck/Vietnam/56/2007	2.3.4	9.25 ± 0.33	5.61	0.56 ± 0.05	0.98	4/28 (P73S, S90F, V267A, G414D)
A/goose/Guandong/1051/2008	2.3.4	5.34 ± 0.14	3.24	0.57 ± 0.07	1.0	5/30 (I122T, S125P, P162S, G210S, I374 N)
Susceptible viruses (n = 92)	All clades	1.48 ± 0.19	N/A	0.65 ± 0.07	N/A	N/A

N/A – not applicable: Amino acid numbering is based on N2 NA.
[a] The concentration of NA inhibitor that reduced NA activity by 50% relative to a reaction mixture containing virus but no inhibitor. Values are the mean ± SD from three independent experiments.
[b] Compared with H5N1 susceptible viruses.
[c] TOPO TA cloning was performed using PCR products obtained by amplification of the virus inoculum. Individual virus clones were analyzed by sequencing. Each clone possesses a single NA mutation.

The location of E228D NA mutation was close to the NA enzyme active site and may be important for future analysis.

Analysis of the susceptibility distribution among HP H5N1 viruses representative of different HA clades showed that viruses of clade 1 were ~5.5-fold more sensitive to oseltamivir and ~1.7-fold more sensitive to zanamivir (mean IC_{50}, 0.31 ± 0.26 nM and 0.40 ± 0.04 nM, respectively, 30 viruses tested) than viruses of HA clade 2 (mean IC_{50}, 1.72 ± 0.32 nM and 0.69 ± 0.07 nM, respectively, 65 viruses tested) (Fig. 1C and D). The viruses of clades 2.3.2 and 2.3.4, which are predominant H5N1 variants circulating in poultry in Southeast Asia (WHO/OIE/FAO, 2012), were susceptible to both NA inhibitors. Overall, our data revealed that most H5N1 viruses retained susceptibility to oseltamivir and zanamivir with little variability over time, although with some variability among divergent HA genetic groups.

3.2. NA molecular markers of resistant or reduced susceptibility phenotype

Analysis of N1 NA sequences of 287 human HP H5N1 viruses isolated in 2002–2012 identified seven viruses (2.4%) with NA inhibitor–resistant NA mutations (Table 2), although A/Hanoi/30408/2005 (H5N1) virus had clones carrying both the H274Y and N294S NA mutations (Le et al., 2005) and was counted as a single NA inhibitor–resistant variant. In addition, seven human H5N1 viruses (2.4%) had I117V, K150N, or I222V/T/K NA mutations.

Analysis of N1 NA sequences of 1716 avian HP H5N1 viruses identified 13 strains (0.8%) with either oseltamivir-resistant (H274Y and N294S) or zanamivir-resistant (E119A) NA mutations (Table 2). Two H5N1 viruses isolated in 2008 had both I117V and E119A NA mutations. The NA mutations reported to reduce the susceptibility of influenza viruses to NA inhibitors (V116A, I117V, K150N, D198N, I222V/T/M, and S246N) were identified in 50 avian viruses (2.9%) (Table 2). Double mutant viruses with I117V and D198N, I222T and S246N, and K150N and S246N NA mutations were isolated in 2005, 2006, and 2007, respectively. No NA inhibitor resistance–associated mutations were detected among human and avian H5N1 viruses isolated in 2010–2012, although the number of isolates was low.

Thus, available N1 NA sequence data suggest a low incidence of mutations that affect susceptibility to NA inhibitors among both human and avian H5N1 viruses.

Table 2
Incidence of NA inhibitor–resistant mutants among human and avian influenza A (H5N1) viruses isolated in 2002-2012.

Origin/year of isolation	Number of H5N1 viruses with molecular markers of NA inhibitor resistance associated with[a]								
	Resistant genotype				Reduced susceptibility genotype				
	No. of mutant/no. of isolates (%)	E119A	H274Y	N294S	No. of mutant/no. of isolates (%)	I117V	K150N	I222V/T/K	S246N
Human isolates									
2002–2004	0/44 (0)	–[b]	–	–	2/44 (4.5)	–	1	1	–
2005–2007	6/173 (3.5)	–	4[c]	3[c]	3/173 (1.7)	–	–	3	–
2008-2012	1/70 (1.4)	–	–	1	2/70 (2.9)	1	–	1	–
2002-2012	7/287 (2.4)	–	4[c]	4[c]	7/287 (2.4)	1	1	5	–
Avian isolates									
2002–2004	2/387 (0.5)	–	1	1	7/387 (1.8)	4	–	3	–
2005–2007	4/953 (0.4)	–	3	1	35/953 (3.7)[d]	9	11	8	11
2008–2012	7/376 (1.9)	4[e]	–	3	8/376 (2.1)	4[e]	3	1	2
2002–2012	13/1716 (0.8)	4[e]	4	5	50/1716 (2.9)[d]	17[e]	14	12	13

[a] Analysis is based on GenBank data. Amino acid numbering is based on N2 NA. Additionally one avian H5N1 virus isolated in 2007 had V116A and two viruses isolated in 2003 and 2005 had D198N NA mutations (results not shown). Percentage was determined by the number of resistant isolates per the total number of viruses isolated during the particular time period.
[b] No. NA mutations were detected.
[c] A/Hanoi/30408/2005 (H5N1) virus (Le et al, 2005) or the same strain designated as A/Vietnam/HN30408/2005 (Sleeman et al., 2010) had clones with either H274Y or N294S NA mutations. They were counted once in resistant phenotype as H274Y.
[d] Indicates existence of viruses carrying double NA amino acid mutations. One H5N1 virus isolated in 2006 had both I222T and S246N NA mutations, Three H5N1 viruses isolated in 2007 had both K150N and S246N NA mutations. They were counted in reduced susceptibility phenotype only.
[e] Indicates existence of viruses with double NA amino acid mutations. Two H5N1 influenza viruses isolated in 2008 had NA mutations at positions E119A and I117V. They were counted in resistant phenotype only.

3.3. Susceptibility to M2 inhibitors

A total of 1669 M2 gene sequences (208 human and 1433 avian sequences obtained from GenBank database and 28 generated in this study) were analyzed for the presence of molecular markers of amantadine resistance. The incidence of amantadine resistance among human H5N1 influenza viruses varied in different time periods (Fig. 2A). The majority (36/37, 97.3%) of human H5N1 viruses isolated in 2002–2004 were amantadine-resistant; however, the number of amantadine-resistant isolates decreased to 58.2% (82/141) and 38.7% (12/31) among viruses isolated in 2005–2007 and 2008–2012, respectively. The amantadine-resistant phenotype of human H5N1 influenza viruses was predominantly caused by either a single V27A or S31N amino acid substitution or double L26I + S31N or V27A + S31N M2 mutations. We did not identify human H5N1 viruses with amino acid substitutions at positions 26, 30, and 34 previously reported to be associated with resistance. Although most drug-resistant human seasonal A (H1N1) and A (H3N2) viruses contain an S31N substitution, only 10.1% of amantadine-resistant human H5N1 viruses contained this substitution (Fig. 2A). The strong association of L26I and S31N M2 mutations reported previously in H5N1 viruses continues to be present in viruses isolated in 2008–2012 and was detected at a similar frequency as a single S31N substitution. Overall, double L26I + S31N M2 mutations were more predominant among human HP H5N1 influenza viruses isolated during 2002–2012 than a single S31N substitution.

Among avian H5N1 influenza viruses, the incidence of amantadine resistance markers was lower than that in human viruses. The frequency of isolation of amantadine-resistant variants was the highest (193/388, 49.7%) among viruses circulated in 2002–2004 and decreased in the following years (197/825, 23.9% and 71/247, 28.7% in 2005–2007 and 2008–2012, respectively, Fig. 2B). As in human isolates, avian H5N1 viruses isolated during 2002–2012 had double L26I + S31N M2 mutations at a higher frequency than a single S31N mutation, although viruses with single S31N and V27A mutations were identified (Fig. 2).

Interestingly, the distribution of amantadine-resistant variants among human H5N1 viruses was restricted to HA clade 1 during 2002–2004 (36/36, 100%), a diverse distribution pattern in

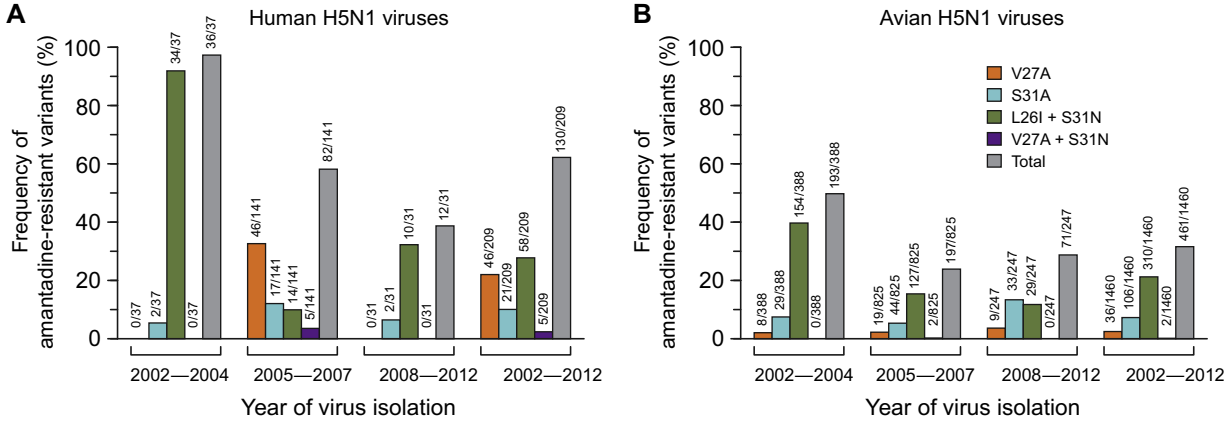

Fig. 2. Prevalence of amantadine-resistant variants among highly pathogenic human and avian influenza H5N1 viruses isolated in 2002–2012. The distribution of amantadine-resistant variants with either single or double M2 mutations among human (A) and avian (B) HP H5N1 influenza viruses. The numbers correspond to the number of amantadine-resistant variants per total number of viruses isolated during different time periods.

Table 3
Incidence of amantadine-resistant mutants among human and avian influenza A(H5N1) viruses isolates in 2002–2012 based on HA clade.

Origin/year of isolation	No. of isolates	No. of amantadine-resistant H5N1 variants (%) representative of HA clade[a]								
		1	1.1	2.1.1	2.1.2	2.1.3	2.2	2.3.2	2.3.4	Others[b]
Human isolates										
2002–2004	36	36 (100.0)	–[c]	–	–	–	–	–	–	–
2005–2007	82	13 (15.9)	1 (1.2)	–	15 (18.3)	52 (63.4)	–	–	1 (1.2)	–
2008–2012	12	–	10 (83.3)	–	–	–	–	–	2 (16.7)	–
2002–2012	130	49 (37.7)	11 (8.5)	–	15 (11.5)	52 (40.0)	–	–	3 (2.3)	–
Avian isolates										
2002–2004	193	162 (83.9)	–	5 (2.6)	–	–	–	3 (1.6)	–	23 (11.9)
2005–2007	200	84 (42.0)	44 (22.0)	–	5 (2.5)	13 (6.5)	8 (4.0)	5 (2.5)	24 (12.0)	17 (8.5)
2008–2012	74	14 (18.9)	18 (24.3)	–	–	2 (2.7)	18 (24.3)	7 (9.5)	11 (14.9)	4 (5.4)
2002–2012	467	260 (55.7)	62 (13.3)	5 (1.1)	5 (1.1)	15 (3.2)	26 (5.6)	15 (3.2)	35 (7.5)	44 (9.4)

[a] Clade determination based on phylogenetic analysis of HA gene from amantadine-resistant mutants with WHO reference clade (WHO 2012). Percentage was determined by the number of resistant isolates per the total number of viruses isolated during the particular time period.
[b] Other include HA clades 0, 2.5, 3–9.
[c] No amantadine-resistant viruses detected.

2005–2007, and back to predominant isolation from clade 1.1 (10/12, 83.3%) in recent years (Table 3). The proportion of amantadine-resistant avian influenza H5N1 viruses from clade 1 has reduced over time from 83.9% in 2002–2004 to 43.2% in 2008–2012, with clade 2 representing 51.4% of isolates in 2008–2012. Taken together, the amantadine-resistant human variants isolated from 2002–2012 belong equally to HA clades 1 and 2 (46.2% and 53.8%), but avian variants were most frequently isolated from clade 1 (~70%). Continued diversification of avian HP H5N1 virus is also evidenced by the increasing number of clades from which amantadine-resistant avian variants were isolated, from four clades in 2002–2004 to seven clades in 2008–2012.

4. Discussion

The acquisition of NA inhibitor resistance by H5N1 influenza viruses is a serious public health concern and monitoring the susceptibility of these viruses to available drugs is an important part of surveillance studies and an informative aspect of risk assessment for a pandemic. In this study, we focused on the set of questions regarding antiviral susceptibility and resistance in the highly pathogenic H5N1 influenza viruses. We addressed what is the level of susceptibility to NA inhibitors and adamantanes over time and among different HA clades, what is the level of antiviral resistance among human and avian H5N1 viruses, and whether there is evidence for an increased selection of antiviral resistance in recent years. Our phenotypic analysis showed that human and avian H5N1 viruses are highly susceptible to NA inhibitors oseltamivir and zanamivir. Comparison of the HA clade distribution of susceptibility revealed ~5.5-fold higher susceptibility of viruses of clade 1 than those of clade 2, and this difference was more pronounced (~10-fold) when we compared clade 1 and clade 2.1 viruses. Although the number of clade 2.1 viruses was limited, we confirmed the data reported previously (McKimm-Breshkin, 2012) about the lower susceptibility of viruses of clade 2 to NA inhibitors in a phenotypic assay. However, this difference in susceptibility is attributed to a specific subclade of clade 2, and currently circulating 2.3.2 and 2.3.4 viruses are more susceptible to NA inhibitors than viruses of clade 2.1. The observed differences in the susceptibility in different years also most likely is correlated with the predominant HA clade circulating in this particular season, and the higher level of susceptibility in 2002–2004 is correlated with predominant circulation of viruses of clade 1 (WHO/OIE/FAO, 2012).

Even though some NA mutations, such as H252Y, can increase the affinity of NA for oseltamivir and thus lead to increased antiviral susceptibility (Rameix-Welti et al., 2006; McKimmm-Breschkin et al., 2007), the emergence of other mutations can potentially decrease susceptibility. We therefore analyzed the available NA sequence data for the presence of NA mutations that were previously reported to decrease susceptibility to NA inhibitors. The screening revealed similarity between human and avian H5N1 influenza viruses and a low level of NA mutations associated with NA inhibitor resistance. The H274Y NA mutation is the most frequent mutation in human oseltamivir-resistant isolates of the N1 NA subtype (Dharan et al., 2009; Weinstock and Zuccottim, 2009). This mutation was detected in four human and four avian H5N1 viruses isolated in 2002-2012. Only four human and five avian isolates contained the N294S NA mutation. The zanamivir-related mutation E119A was detected in four avian isolates from 2008 and confirmed the finding by Govorkova et al. (2009) that this mutation can be stably maintained in the N1 NA background. The amino acid substitutions at some of the targeted residues in NA (e.g., V116, I117, K150, D198, I222, and S246) have previously been linked to reduced drug susceptibility in avian and human N1 viruses (Hurt et al., 2009; Ilyushina et al., 2010). However, most of the studies were conducted with recombinant viruses, and their relevance to clinical resistance still needs to be proven. Interestingly, one of the most predominant substitutions in both human and avian H5N1 influenza viruses was at residue 222. This mutation alone did not confer a resistant phenotype in seasonal H1N1 viruses (Ison, 2011). However, in combination with mutation H274Y, the IC_{50} increased almost 2000 times for one H5N1 strain (Matrosovich and Klenk, 2003). The NA I222R mutation was identified in the clinical isolates of patients receiving oseltamivir treatment, and this mutation alone can to a small degree reduce the H1N1pdm09 virus susceptibility to all three NA inhibitors (oseltamivir carboxylate, zanamivir and peramivir) (Nguyen et al., 2010). The I222R + H274Y dual mutations further enhanced the resistance level to oseltamivir and caused moderate resistance to zanamivir (van der Vries et al., 2010; Pizzorno et al., 2011). Amino acid mutations at framework residues such as I222 may interfere with the correct binding of NA inhibitors, thus disrupting the natural susceptibility of influenza viruses to these agents. The wide spread of drug-resistant variants is connected to the fitness of these viruses. The selected drug-resistant mutations that can provide viral fitness benefits can be maintained without direct selection for the mutation. The available reports on the fitness of HP oseltamivir-resistant H5N1 viruses are focused on viruses of the two HA clades that are causing infection in humans: clade 1 and clade 2.2 (Yen et al., 2007; Govorkova et al., 2010; Ilyushina et al., 2010; Kiso et al., 2011). Experimental evidence suggests that a particular NA inhibitor resistance–associated marker can cause different effects on fitness in different H5N1 virus genetic and virulence backgrounds. Deficiency in NA function caused by an NA inhibitor

resistance mutation may not be deleterious for HP H5N1 viruses because of the extremely efficient replication of these viruses. Overall, it appears that in the H5N1 population, oseltamivir resistance is not yet undergoing positive selection. This might be because of the limited number of H5N1-infected patients treated with oseltamivir (Adisasmito et al., 2010) and the fact that the NA gene in H5N1 influenza viruses is evolutionarily constrained at a majority (84%) of positions (Hill et al., 2009). This highlights that H5N1 is still primarily a disease of birds. Monitoring of the natural selection of additional NA mutations at the conserved and semi-conserved NA residues in addition to established molecular markers of resistance is warranted.

Our data revealed the disproportional levels of amantadine resistance observed in H5N1 viruses and human seasonal H3N2 and H1N1pdm09 viruses in recent years (Deyde et al., 2007; Zaraket et al. 2010; Zhou et al., 2011). Importantly, the susceptibility of H5N1 influenza viruses to amantadine has increased in recent years (2010–2012). More likely, the emergence and spread of amantadine-resistant H5N1 viruses occurs through positive drug selection pressure in avian species. This conclusion correlates with the data reported previously that there has been prophylactic use of amantadine in poultry production in some parts of China (He et al., 2008). The application of phylogenetic methods based on analysis of multiple gene segments, molecular evolutionary analyses, and geographic visualization also concluded that amantadine use is driving selection for antiviral resistance in the global H5N1 population (Wallace and Fitch, 2008; Hill et al., 2009). Our findings suggest that amantadine-resistant lineages from other subtypes have limited introduction into the H5N1 subtype, although more sophisticated analysis is required to understand the frequency of possible reassortment events with other influenza subtypes. Our data suggest that some residues (L26I + S31N) in the M2 protein of H5N1 viruses are under stronger drug selection pressure. The predominance of double M2 mutations among human and avian H5N1 viruses also suggests a different pattern of selection than with seasonal H3N2 and H1N1pdm09 viruses, which are characterized by single S31N M2 mutations (Bright et al. 2006; Barr et al., 2008). Interestingly, we observed disproportional distribution of amantadine-resistant variants among avian and human viruses isolated in different years, most likely associated with a particular HA clade circulating in a given geographic area (Barr et al., 2008; Le et al., 2008).

In conclusion, given the expanding diversity of H5N1 viruses both geographically and phylogenetically, continued surveillance of HP H5N1 viruses is needed to determine the incidence of drug-resistant strains in both humans and avian species and to identify molecular markers underlying these changes. These measures would allow maintenance of therapeutic and possibly prophylactic regimens for antiviral control of disease.

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