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Influenza A viruses of swine circulating in the United States during 2009–2014 are susceptible to neuraminidase inhibitors but show lineage-dependent resistance to adamantanes

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Abstract

Antiviral drug susceptibility is one of the evaluation criteria of pandemic potential posed by an influenza virus. Influenza A viruses of swine (IAV-S) can play an important role in generating novel variants, yet limited information is available on the drug resistance profiles of IAV-S circulating in the U.S. Phenotypic analysis of the IAV-S isolated in the U.S. (2009–2011) (n=105) revealed normal inhibition by the neuraminidase (NA) inhibitors (NAIs) oseltamivir, zanamivir, and peramivir. Screening NA sequences from IAV-S collected in the U.S. since 1930 showed 0.03% (1/3396) sequences with clinically relevant H274Y-NA substitution. Phenotypic analysis of IAV-S isolated in the U.S. (2009–2011) confirmed amantadine resistance caused by the S31N-M2 and revealed an intermediate level of resistance caused by the I27T-M2. The majority (96.7%, 589/609) of IAV-S with the I27T-M2 in the influenza database were isolated from pigs in the U.S. The frequency of amantadine-resistant markers among IAV-S in the U.S. was high (71%), and their distribution was M-lineage dependent. All IAV-S of the Eurasian avian M lineage were

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amantadine-resistant and possessed either a single S31N-M2 substitution (78%, 585/747) or its combination with the V27A-M2 (22%, 162/747). The I27T-M2 substitution accounted for 43% (429/993) of amantadine resistance in classic swine M lineage. Phylogenetic analysis showed that both S31N-M2 and I27T-M2 emerged stochastically but appeared to be fixed in the U.S. IAV-S population. This study defines a drug-susceptibility profile, identifies the frequency of drug-resistant markers, and establishes a phylogenetic approach for continued antiviral-susceptibility monitoring of IAV-S in the U.S.

Keywords

Porcine influenza virus; Swine influenza virus; Antiviral resistance; Neuraminidase inhibitors; Oseltamivir; Zanamivir; Peramivir; Amantadine; Adamantanes

1. Introduction

Influenza A viruses of swine (IAV-S) can be zoonotic pathogens. The respiratory tract of swine possesses both types of sialic acid receptors recognized by influenza viruses: avian-like (α -2, 3-linked receptors) and human-like (α -2, 6-linked receptors) (Nelli et al., 2010). Therefore, pigs are permissive to infections with both avian-like and human-like influenza A viruses, and some consider pigs as “mixing vessels” in which gene segments from evolutionarily diverse swine, avian, and human influenza viruses can reassort (Scholtissek et al., 1985; Ito et al., 1998). Thus, pigs can contribute to the emergence of a pandemic influenza virus. The first influenza pandemic of the 21st century was caused by 2009 H1N1 influenza virus (H1N1pdm09) which possessed genes from IAV-S (Garten et al., 2009; Vijaykrishna et al., 2010).

Antiviral treatment susceptibility is one of the 10 criteria included in the Influenza Risk Assessment Tool (IRAT) that seeks to assess the potential risk posed by influenza A viruses that currently circulate in animals (Trock et al., 2012). The neuraminidase (NA) and matrix (M) proteins are the targets of the two classes of FDA-approved anti-influenza drugs, NA inhibitors (NAIs) and M2 inhibitors, respectively. Amino acid substitutions in these proteins confer drug resistance (Nguyen et al., 2012; Gu et al., 2013). Phenotypic NAI susceptibility of influenza A viruses is evaluated based on the fold-change in IC₅₀ values (the concentration of drug required to inhibit a standardized amount of NA activity by 50%) compared to that in susceptible virus from the same NA subtype/lineage: “normal inhibition” (10-fold change); “reduced inhibition” (10–100 fold change); “highly reduced inhibition” (100-fold change) (WHO, 2012).

H1N1, H1N1pdm09, H1N2, and H3N2 are endemic subtypes of IAV-S in the U.S. (Vincent et al., 2014). The evolutionary pattern and ecology of IAV-S circulating in the U.S. differ from those circulating in Europe or Asia (Vincent et al., 2014). Since 1998, most of the IAV-S isolated in the U.S. have possessed a triple-reassortant internal genes (TRIG) constellation (Vincent et al., 2008; Webby et al., 2000). IAV-S with TRIG carry human PB1, avian PB2 and PA genes, and classic swine NP, M, and NS genes; they are also characterized by efficient replication in pigs (Pascua et al., 2012). Since 2005, human HA (from H1 and H3 subtypes) and NA (from N1 and N2 subtypes) segments have reassorted

with IAV-S carrying TRIG and established a distinct lineage in IAV-S in the U.S. Notably, IAV-S with these NA genes that share 99% identity at the nucleotide and protein levels with those circulated in humans in the 1990s and early 2000s are still endemic in swine. In 2011, the introduction of the M gene from the pandemic H1N1pdm09 viruses (Eurasian avian lineage) to the H3N2 IAV-S (Ducatez et al., 2011) generated the H3N2 variant viruses (H3N2v), which infect humans and have pandemic potential (Lindstrom et al., 2012; Nelson et al., 2012).

IAV-S have been monitored through active and passive surveillance conducted in the U.S. (Webby et al., 2000; Olsen et al., 2000; Anderson et al., 2013; Corzo et al., 2013; Feng et al., 2014), though antiviral surveillance was not part of the overall efforts. A limited number of H1N1 IAV-S isolated in the U.S. during 2005–2009 were susceptible to NAIs (Stoner et al., 2010). The frequency of amantadine-resistant IAV-S in the U.S. remains elusive.

In this study we addressed this gap by characterizing phenotypic and genotypic susceptibility of IAV-S circulating in the U.S. (2009–2011) to NAIs and amantadine. Additionally, we determined the frequency of drug-resistant markers among IAV-S in the U.S. (1930–2014) by screening NA- and M-sequence data available in the influenza research database (IRD) (Squires et al., 2012). We also applied the phylogenetic approach to elucidate the origins of the M gene of IAV-S and assess the distribution of amino acid substitutions associated with antiviral resistance among different lineages.

2. Materials and methods

2.1. Viruses and cells

Nasal swabs were collected from pigs at 33 farms during active surveillance from June 2009 to December 2011, in Iowa, Illinois, Indiana, and Minnesota. IAV-S were isolated from nasal swabs by inoculation of Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) (Corzo et al., 2013). The 105 IAV-S were randomly selected for phenotypic NAI-susceptibility testing and for NA- and M-gene sequencing. (H1N1, 15 strains; H1N1pdm09, 17 strains; H1N2, 62 strains; and H3N2, 11 strains).

2.2. Susceptibility to NAIs

Stocks of oseltamivir carboxylate (oseltamivir), zanamivir, and peramivir were prepared in distilled water, filter-sterilized, and stored in aliquots at -20°C . Susceptibility to NAIs was assessed in a fluorescence-based assay using 100 μM fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) (Sigma-Aldrich, St. Louis, MO) (Govorkova et al., 2013). IC_{50} values were calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). To define the NAI susceptibility of IAV-S, we used the established criteria based on the fold-change of their IC_{50} value compared to those of reference viruses of the same NA subtype (WHO).

NA sequences of the 105 IAV-S generated in this study and the 3291 IAV-S available in the IRD from the U.S. (accessed 10/23/2014) were screened for the presence of known molecular markers (N2 numbering) of NAI resistance that demonstrated clinical relevance in human influenza A viruses of N1 (D198N, I222R, H274Y, N294S) or N2 (E119V, R292K,

N294S) subtypes (WHO, 2012), and for NA markers reported in surveillance studies or in recombinant viruses of N1 (V116A, I117V, E119V, Q136L/K, V149A, Y155H, I222V/M/K, S246N/G) or N2 (E119I, Q136K, D151E/V, S246P) subtypes (Nguyen et al., 2012; Sleeman et al., 2014). Additionally, we screened N1 IAV-S sequences for permissive substitutions that maintained full NA function in the presence of the H274Y-NA (Bloom et al., 2010; Duan et al., 2014; Butler et al., 2014).

2.3. Susceptibility to adamantanes

Stocks of amantadine hydrochloride (amantadine) (Sigma-Aldrich, St. Louis, MO) were prepared in distilled water. Phenotypic susceptibility was assessed using plaque size–reduction (Abed et al., 2005) and biological assays in MDCK cells (Bright et al., 2005). The frequency of genetic markers of resistance to amantadine at positions 26, 27, 30, 31, and 34 (Gu et al., 2013) was assessed by screening the M sequences of 105 IAV-S from the U.S. (2009–2011) generated in this study and available in the IRD (n=1635, 1930–2014, accessed 10/23/2014).

2.4. Phylogenetic analysis of the M-gene segment of IAV-S

All available full-length M-gene sequence data from IAV-S isolated worldwide (1930–2014) were downloaded from the IRD and aligned. Detailed methods for phylogenetic analysis are described in the Supplementary Data.

2.5. Nucleotide sequence accession numbers

Sequences generated in this study were deposited in the GenBank database with the accession numbers: KP100813-KP101000; KP412321-KP412342.

2.6. Statistical analyses

GraphPad Prism 5 software (GraphPad Software, Inc.) was used for all statistical analyses. Two-way analysis of variance (ANOVA) was used to compare groups. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Phenotypic susceptibility of IAV-S to NAIs

The NAI susceptibility of 105 IAV-S of 4 HA/NA subtypes are shown in Table 1. N1 and N2 IAV-S displayed normal inhibition by oseltamivir, zanamivir, and peramivir (IC₅₀-fold increase <10 when compared with N1 and N2 reference human influenza viruses). Of interest, IC₅₀ values of 3 H1N1 IAV-S with the I117V-NA were on average 7.3-fold higher for oseltamivir than those of the susceptible control (individual IC₅₀ values are shown in Table 2). NAI susceptibility over the 3-year study remained stable from year to year (data not shown).

3.2. Frequency of molecular markers of NAI resistance among IAV-S

Sequence analysis of the NA genes from the 105 IAV-S collected in the U.S. (2009–2011) and 3291 NA sequences available in the IRD for IAV-S in the U.S. (1930–2014) revealed a

single N1 sequence that contained the clinically relevant H274Y-NA (Table 3). H274Y-NA in human H1N1 influenza viruses is known to decrease the number of the NA expressed on the cell surface and attenuate virus replication in vitro and in vivo, as well as restrict airborne transmission between ferrets (Butler et al., 2014; Duan et al., 2014; Ives et al., 2002). Of the 1034 N1 sequences from IAV-S in the U.S. (1930–2014), more than 99% possessed permissive NA substitutions that abolish the deleterious effect of H274Y; 37% to 46% of N1 sequences of the H1N1pdm09 in swine harbored substitutions that confer robust fitness on recent human H1N1pdm09 viruses (Table 4). Screening for markers of NAI resistance reported in surveillance or experimental studies revealed 0.38% (13/3396) sequences with the I117V-NA (including 3 IAV-S from this study), 0.24% (8/3396) with the Y155H-NA, and 0.09% (3/3396) with the E119K-NA among N1; 0.24% (8/3396) sequences with the V149A-NA, 0.15% (5/3396) with the I222V-NA, and 0.06% (2/3396) with the Y155H-NA among the N2 IAV-S (Table 3).

3.3. Frequency of molecular markers of amantadine resistance among IAV-S

The frequency of IAV-S sequences with substitutions in M2 varied by HA/NA subtype: 33.4% (136/407) H1N1, 100% (747/747) H1N1pdm09, 62.2% (191/307) H1N2, and 57.0% (159/279) H3N2 carried M2 inhibitor resistance-conferring substitutions (Fig. 1a). The origin of the M gene was limited to two lineages: 993 isolates were from classic swine and 747 isolates were from Eurasian avian lineages (Fig. 1b). The S31N-M2 accounted for 78% (585/747) of resistant sequences alone and 22% (162/747) in combination with the V27A-M2 in the Eurasian avian lineage. The frequency of the I27T-M2 was 49% (486/993) in the classic swine lineage (Fig. 1b).

To evaluate the role of swine as the host for influenza A viruses harboring the I27T-M2, we analyzed sequences with this substitution that were available in the IRD: 96.7% (589/609) genes were of swine origin, and 97.3% (573/609) were reported from the U.S., suggesting that viruses with the I27T-M2 were predominantly circulating in swine populations (data not shown). The U.S. performs 10 times more influenza surveillance in swine than any other country (Dr. M. Culhane, personal communications), and thus IAV-S sequences with the I27T-M2 from the U.S. could be overrepresented in the databases. Despite the epidemiological data on the presence of the I27T-M2 in IAV-S and human influenza viruses, the role of this substitution on the susceptibility to amantadine is not well defined.

3.4. Phenotypic susceptibility of IAV-S to amantadine

To evaluate the role of the I27T-M2 on the phenotypic susceptibility of IAV-S to amantadine, we tested representative isolates harboring this substitution (Table 5). The mean EC₅₀ values for viruses with the I27T-M2 were 50 μ M (range 34.5–62.6 μ M); these values were 7.6-fold and 1.3-fold higher than the average (6.6 μ M) and maximum (39.7 μ M) plasma concentrations of amantadine in humans, respectively (Hayden FG, 1996). Because the EC₅₀ values of the viruses with I27T-M2 were higher than the susceptible ones (<1 μ M) but lower than the resistant ones (>100 μ M), we propose that the I27T-M2 confers intermediate resistance to amantadine.

3.5. Evolution of amantadine-resistance markers among IAV-S circulating in the U.S. (1976–2012)

Phylogenetic analysis of the M gene of IAV-S isolated worldwide showed two major lineages that correspond to the classic swine and Eurasian avian lineages. These lineages diverged in 1933.1–1948.7 [mean, 1941.2; 95% Bayesian credibility intervals (BCI)] (data not shown). Our analysis of the M-gene sequences in the IRD for IAV-S circulating in the U.S. identified alterations only at residues 27 and 31 of the M2 protein; thus, we concentrated our analysis on those positions to determine the diversity of amino acid substitutions that occur there, the time of appearance of those substitutions, and whether they emerged stochastically or were fixed in the population.

We observed six substitutions at position 27 of M2 protein (V27I, I27V, I27T, V27S, V27F, and V27A) among IAV-S isolated in the U.S. (1976–2013). Valine at position 27 is the ancestral state for most variants isolated in the U.S. Two amino acid substitutions (I27V and I27T) were present in the IAV-S of classic swine lineage, and four were identified in the viruses of Eurasian avian lineage (V27I, V27A, V27S, and V27F) (Fig. 2). Although I27T-M2 emerged independently at least four times, it was fixed only three times in the classic swine lineage (Fig. 2). I27T-M2 in the classic swine lineage occurred in the A, B, and C sublineages in 2002.7, 2008.2, and 2003.7, respectively.

Most currently circulating IAV-S with an M gene of classic swine lineage possessed this substitution. In contrast, position 31 of the M2 protein of all contemporary isolates showed only two amino acids (serine and asparagine). Asparagine at position 31 is typical of Eurasian avian isolates, and serine at this position characterizes the IAV-S of classic swine lineage. Our analysis suggested that the fixation of the S31N mutation in the IAV-S population occurred as early as 1998.8, but it was not detected in the IAV-S population until 2009. S31N-M2 occurred sporadically in the IAV-S of the classic swine lineage, suggesting that this mutation emerged randomly. Regardless, our results show that the emergence and rapid spread of the S31N-M2 amantadine-resistant IAV-S in the U.S. was facilitated by reassortment events that most likely occurred after the mutation was already fixed in the IAV-S of Eurasian avian lineage. By tracing the history of this particular position, we found that the S31N-M2 represents 83% of all changes that occur at this position across the phylogeny, suggesting that this mutation has a high probability of emerging randomly. The V27A-M2 occurred independently at least twice in 2009 [2009.9 (BCI 2010.20–2009.9) lineage D and 2009.50 (BCI 2010.0–2009.1) lineage E] (Fig. 3D-E). This finding and the observation that V27A-M2 is present only in combination with S31N-M2 suggests that the emergence of the amantadine-resistant double mutant (S31N-M2 + V27A-M2) in the Eurasian avian lineage of IAV-S in the U.S. occurred after the S31N-M2 IAV-S became established in the swine population.

4. Discussion

Given the expanding diversity of IAV-S, both geographically and genetically, and the risk of their role in the genesis of pandemic influenza viruses, it is of concern that so little information is available about the frequency of drug-resistant variants circulating in pigs. To address this question, we used two approaches. First, we applied phenotypic and genotypic

methods to examine the susceptibility of IAV-S that have circulated in the U.S. to FDA-approved drugs. Second, we screened NA- and M-gene sequences of IAV-S isolated in the U.S. and available in the IRD for markers of antiviral drug resistance. This broad screening demonstrated that naturally occurring NAI resistance among IAV-S is rare (0.03%) and confirmed the high frequency of amantadine resistance (71%). We identified the I27T-M2 as the amino acid substitution that confers an intermediate level of resistance to amantadine in IAV-S of classic swine M lineage. The temporally structured M-gene phylogenetic tree showed that S31N-M2 and I27T-M2 emerged stochastically but appeared to be fixed in the U.S. IAV-S population.

Oseltamivir-resistant human H1N1 influenza viruses that emerged 2007–2009 restricted therapeutics options in humans (Holmes, 2010) and emphasized the importance of monitoring influenza viruses for the presence of drug-resistance markers and markers that predict such viruses will emerge. Our extensive screening of the NA IAV-S sequences identified one IAV-S sequence that possesses the H274Y-NA, a known marker of clinically relevant NAI resistance. Two IAV-S with the H274Y-NA were reported from a farm in Canada (Nfon et al., 2011), where humans were infected with a reassortant influenza A virus (HA/NA from human H1N1 and internal genes from swine TRIG IAV) (Bastien et al., 2010). Even with the worldwide circulation of the oseltamivir-resistant human H1N1 viruses during 2007–2009, the NA gene from human H1N1 viruses with the H274Y-NA were not introduced into the IAV-S populations. This finding highlights the need for more studies to understand the factors that restrict swine-human transmission of influenza viruses. Our data on the low frequency of NAI-resistant IAV-S in North America support data on NAI susceptibility of IAV-S in Europe (Bauer et al., 2007; Bauer et al., 2012) and suggest that the prevalence of NAI-resistant IAV-S globally is low. Although the overall frequency of NAI-resistance markers among IAV-S was low (0.03%; 1/3396), the vast majority of N1 sequences possessed NA substitutions that compensated for the diminished fitness normally associated with H274Y-NA in human seasonal influenza A (H1N1) viruses. Because the NA gene in IAV-S circulating in the U.S. originated from human seasonal influenza viruses of N1 subtype, there is a potential risk of fit oseltamivir-resistant IAV-S emerging. Moreover, we found a number of IAV-S sequences with NA markers that were expected to reduce inhibition by NAIs, urging the importance of continuous monitoring of NAI resistance among IAV-S.

Previous studies described reduction in susceptibility to oseltamivir in human H5N1 and H1N1pdm09 viruses with the I117V-NA (Takano et al., 2013; Hurt et al., 2012; Hurt et al., 2007; Ilyushina et al., 2010). Our phenotypic analysis of H1N1 IAV-S with the I117V-NA showed less than 10-fold decreased inhibition by oseltamivir, suggesting normal inhibition by NAIs using recent WHO criteria (WHO, 2012). The studies are needed to re-evaluate the I117V-NA marker, as well as other markers reported to reduce susceptibility to NAIs (e.g., E119K, Y155H) based on the recent WHO criteria, and to understand whether variants harboring these substitution alone or in combination with the clinically-relevant H274Y-NA would alter antiviral susceptibility and fitness in IAV-S.

This study identified high levels of amantadine-resistant markers among IAV-S sequences. Despite the widespread amantadine resistance among currently circulating IAV-S, the M2

protein remains an important target for anti-influenza drugs; novel compounds with antiviral activity against amantadine-resistant viruses with the S31N-M2 have been described (Jizhou Wang et al., 2013). Most of the IAV-S analyzed possessed the S31N-M2, which is associated with amantadine resistance in human H1N1, H3N2, H5N1, and H7N9 influenza viruses (Jun Wang et al., 2013; Gao et al., 2013). Three independent events of the emergence of the S31N-M2 have been documented among IAV-S worldwide. The Eurasian avian lineage of IAV-S acquired the S31N-M2 in the mid-1980s, and the emergence and spread of these viruses have been described among IAV-S in Europe (Krumbholz et al., 2009). The IAV-S circulating in the U.S. acquired the S31N-M2 around 1999, but it was not detected until 2009, providing a 10-year window during which the S31N-M2 was acquired by IAV-S circulating in North America. The V27A-M2, which also confers resistance to adamantanes, and S31N-M2 were detected in the IAV-S of Eurasian avian lineage, and IAV-S with dual S31N-M2 and V27A-M2 emerged after the S31N-M2 IAV-S were established in the swine population in the U.S. The biological significance of this event can be tested in future studies.

The I27T-M2 was the second-most commonly identified substitution among IAV-S isolated in the U.S. The effect of this substitution on the susceptibility of influenza viruses to amantadine was controversial. Although the susceptibility of the I27T-M2 protein was similar to that of wild-type protein (Holsinger et al., 1994), the I27T-M2 virus showed decreased susceptibility to amantadine in vitro, comparable pathogenicity in mice (Watanabe et al., 2001) and transmissibility between chickens (Bean et al., 1989). In this study, we confirmed that the I27T-M2 causes an intermediate level of resistance to amantadine in IAV-S in vitro and that pigs in the U.S. possess influenza viruses with this substitution. Recently, two human influenza cases were caused by the IAV-S with the I27T-M2 in the U.S. (Dawood et al., 2011; Balish et al., 2013). Together, these data suggest that the possible spread of variant viruses carrying an M gene of classic swine origin, of which 43% possess the I27T-M2 drug-resistant marker, should be taken into consideration.

The phylogenetic approach is a well-established tool for monitoring the evolution of influenza viruses. Incorporating drug-resistant markers into this analysis allowed us to enhance the tool's ability to predict the natural evolutionary pathway of drug-resistant IAV-S in different lineages. The antiviral-susceptibility profile is a crucial element of IRAT. The comparative genetic risk-assessment approach established here allows monitoring of the evolutionary dynamics of genes with drug resistance. NAIs appear to be an appropriate choice for stockpiling in anticipation of the emergence of a swine-origin influenza virus in humans; however, continued monitoring is needed to predict the likelihood of this event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCI	Bayesian credibility interval
NA	neuraminidase
NAI	neuraminidase inhibitor
IRAT	influenza risk-assessment tool
IRD	influenza research database
MDCK	Madin-Darby canine kidney
IAV-S	influenza A virus of swine
TRIG	triple reassortant internal genes

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Highlights

- Susceptibility to FDA-approved antivirals was evaluated among 105 influenza A viruses of swine (IAV-S) from the U.S.
- 3396 neuraminidase and 1740 matrix IAV-S sequences from the U.S. (1930–2014) were screened for markers of drug resistance.
- The frequency of neuraminidase inhibitor-resistant IAV-S in the U.S. was low (0.03%).
- The frequency of amantadine-resistant IAV-S in the U.S. was high (71%).
- Phylogenetic analysis of matrix genes showed that amantadine resistance-conferring substitutions emerged stochastically.

Fig. 1. Frequency of amantadine-resistant markers in different HA/NA subtypes (a) and M lineages (b) of IAV-S circulated in the U.S. (1930–2014). The frequency was calculated by dividing the number of times the substitution of interest was observed in an IAV-S HA/NA subtype or M lineage by the total number of relevant sequences of that subtype or lineage available in the IRD on October 2014 (Squires et al., 2012). The numbers correspond to the number of amantadine-resistant variants per total number of viruses.

Fig. 2.

Temporally structured maximum clade-credibility phylogenetic tree of the M gene of IAV-S. The timeline of amino acid substitutions at position 27 of the M2 protein is shown. Ancestral-state reconstruction of tested amino acids at position 27 of the M2 protein is annotated on the tree backbone to indicate when specific mutations were fixed in the population. X-axis shows the time scale in years. The bars labeled A-E indicative of clades.

Fig. 3. Temporally structured maximum clade-credibility phylogenetic tree of the M gene of IAV-S. The timeline of amino acid substitutions at position 31 of the M2 protein is shown. Ancestral-state reconstruction of tested amino acids at position 31 of the M2 protein is annotated on the tree backbone to indicate when specific mutations were fixed in the population. X-axis shows the time scale in years.

Table 1
Susceptibility of IAV-S isolated in the U.S. (2009–2011) to NAIs by the NA enzyme inhibition assay

NAI	IAV-S of NA subtype						Reference human influenza virus of NA subtype, mean IC ₅₀ ± SD, nM ^b	
	N1			N2				
	mean IC ₅₀ ± SD, nM (fold change) ^a			mean IC ₅₀ ± SD, nM (fold change)			N1	N2
	Average N1 (n=32)	H1N1 (n=15)	H1N1pdm09 (n=17)	Average N2 (n=73)	H1N2 (n=62)	H3N2 (n=11)		
Oseltamivir	1.26 (2.38)	2.20 ± 0.20 (4.15)	0.31 ± 0.05 (0.58)	0.17 (1.21)	0.18 ± 0.02 (1.29)	0.15 ± 0.01 (1.07)	0.53 ± 0.02	0.14 ± 0.01
Zanamivir	0.26 (0.67)	0.26 ± 0.03 (0.67)	0.26 ± 0.03 (0.67)	0.40 (0.51)	0.43 ± 0.03 (0.55)	0.36 ± 0.04 (0.46)	0.39 ± 0.18	0.78 ± 0.03
Peramivir	0.22 (1.69)	0.34 ± 0.16 (2.62)	0.09 ± 0.01 (0.69)	0.14 (0.54)	0.14 ± 0.03 (0.54)	0.14 ± 0.01 (0.54)	0.13 ± 0.01	0.26 ± 0.01

^aThe concentration of NAI that reduced NA activity by 50% relative to a reaction mixture containing virus but no inhibitor. Values are the mean ± SD from 3 independent experiments. Fold change in comparison to susceptible reference human influenza virus of the same NA subtype is shown in parentheses: normal inhibition (<10-fold increase), reduced inhibition (10- to 100-fold increase) and highly reduced inhibition (>100-fold increase) by NAIs (WHO, 2012).

^bThe panel of human influenza A viruses for assessment of resistance to NAIs was obtained from the Antiviral Group, International Society for Influenza and Other Respiratory Virus Diseases: A/Mississippi/03/2001 (H1N1) – NAI susceptible; A/Mississippi/03/2001 (H1N1) – NAI-resistant H274Y-NAI; A/Fukui/20/2004 (H3N2) – NAI susceptible; A/Fukui/45/2004 (H3N2) – NAI-resistant E119V-NA.

Table 2

IC₅₀ values of NAIs against IAV-S with the I117V-NA substitution^a

H1N1 IAV-S (NA accession number)	NAI		
	Oseltamivir	Peramivir	Zanamivir
A/Swine/Indiana/28-0705/2011 (KP100839)	5.70 ± 0.10	0.13 ± 0.01	0.41 ± 0.04
A/Swine/Indiana/28-0715/2011 (KP100841)	8.03 ± 0.56	0.81 ± 0.13	0.22 ± 0.01
A/Swine/Indiana/28-0726/2011 (KP100997)	8.21 ± 0.65	0.97 ± 0.06	0.23 ± 0.01
Average	7.31	0.64	0.29

^a Inhibitory concentration (IC₅₀) values are expressed as the mean ± SD (nM).

Table 3

NA amino acid substitutions identified among IAV-S circulating in the U.S. (1930–2014)^a

NA subtype/clinical relevance of the NA substitution	NA amino acid position	Substitution	Number of sequences	Frequency, %	NA lineage ^b	Accession number
N1 subtype						
Reported to cause clinically relevant resistance	H274	Y	1	0.03	1A	CY158379
	I117 ^c	V	13	0.38	1B	JN652437, JN652438, CY108936, JQ922363, JX092340, CY114913, JX080563, KC571610, KC571604, KJ161862; this study: KP100839, KP100841, KP100997
Reported in surveillance or experimental studies	E119	K	3	0.09	1B	CY122325, KC189018, KC167164
	Y155	H	8	0.24	1B	CY040560, JF812294, JF812295, JX092323, JX092324, CY122325 (also E119K), KJ452351, KM503167
N2 subtype^d						
	V149	A	8	0.24	2A	JX099415, CY089402, CY089405, JX983101, KC405493, KC800972, CY158041, KJ880050
	Y155	H	2	0.06	2A	AF251389 (1977), CY009310 (1980)
	I222	V	5	0.15	2A	KF888361, JX657822, JX908498, KC167152, KC4055023

^a A total of 3396 NA sequences were analyzed (3291 NA sequences available in the IRD (Squires et al., 2012) as of October 24, 2014 and 105 NA sequences from this study).

Amino acid substitutions V116A-NA, Q136L/K-NA, V149A-NA, D198N-NA, I222R/V-NA, N294S-NA were not detected in the N1 sequences. E119K-NA, R292K-NA, N294S-NA (reported in clinical samples) and E119V/I-NA, Q136K-NA, D151E/V-NA, S246P-NA, N294S-NA were not detected in the N2 sequences.

^b Assessed by influenza A genotype tool, which is available at <http://www.flugenome.org/index.php>. The numbers represent the NA subtype, and the letters correspond to the genotype: A – swine, B – human.

^c IC₅₀ values of the I117V-NA (H1N1) IAV-S in this study were on average 7.3-, 0.6-, and 0.3-fold higher for oseltamivir, zanamivir, and peramivir, respectively, than those of the susceptible control; thus, they did not meet the WHO criteria for reduced susceptibility by NAIs (WHO, 2012).

^d None of the substitutions were reported for N2 viruses.

Table 4

Frequency of the NA substitutions permissive for oseltamivir-resistant human H274Y-NA influenza viruses

Group of the NA-permissive substitutions	NA substitution	Number of detected N1 IAV-S sequences (n=1034) ^a	
		N1pdm09 sequences (n=145)	N1pdm09 sequences excluded (n=889)
Restores full NA function in human H1N1 viruses with H247Y-NA ^{b, c}	233M	1 (0.7%)	0
	345N	145 (100%)	888 (99.9%)
	357G	144 (99.3%)	886 (99.7%)
Confers robust fitness in recent human H1N1pdm09 viruses with H274Y-NA ^d	240I	54 (37.2%)	218 (24.5%)
	372K	67 (46.2%)	17 (1.9%)

^a Available in the IRD (n=1034) as of October 29, 2014 (Squires et al., 2012). The frequency represents the incidence of a variant in the N1pdm09 or N1 sequences in the U.S. swine population (1930–2014). The frequency is calculated by dividing the number of times the substitution of interest is observed in a population by the total number of sequences of the NA subtype at that particular amino acid position.

^b V233M and R222Q amino acid substitutions (N2 numbering) restore full NA function in the presence of the H274Y-NA, which confers high inhibition by oseltamivir (Bloom et al., 2010). The 222Q-NA substitution was not detected in any of the IAV-S N1 sequences.

^c V233M, R222Q, K329E, D345N, and D357G amino acid substitutions maintain full NA function in the presence of the H274Y-NA by altering protein accumulation or enzyme affinity/activity (Duan et al., 2014). The 329E-NA substitution was not detected in any of the IAV-S N1 sequences.

^d V240I and N372K were compensatory for the H274Y in the H1N1pdm09 viruses, which correlated with enhanced surface expression and enzymatic activity of the A(H1N1)pdm09 NA protein (Butler et al., 2014).

Table 5

Susceptibility of IAV-S isolated in the U.S. (2009–2011) to amantadine in cell culture-based assays

Subtype of IAV - S / M gene lineage	Influenza virus (M gene accession number)	Amino acid substitution at the M2 protein position ^a		Plaque-size reduction assay		Biological assay ^b		Phenotype
		27	31			Extended range ^c		
						Standard range		
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H1N1								
M gene of classic swine lineage								
	A/Swine/Iowa/11-0909/2010 (KP412336)	I	S	<1		<1	NT	S
	A/Swine/Iowa/11-0918/2010 (KP412324)	I	S	<1		<1	NT	S
	A/Swine/Iowa/9-0805/2009 (KP412330)	T	S	36.6±6.7		100	31.6	I
	A/Swine/Iowa/9-0814/2009 (KP412342)	T	S	38.2±3.2		100	31.6	I
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H1N1pdm09								
M gene of Eurasian avian lineage								
	A/Swine/Illinois/21-1230/2009 (KP412332)	V	N	>100		>100	>100	R
H1N2	A/Swine/Minnesota/34-0801/2011 (KP100834)	I	S	<1		<1	NT	S
	A/Swine/Minnesota/34-0818/2011 (KP100966)	I	S	<1		<1	NT	S
	A/Swine/Indiana/29-0404/2010 (KP412338)	T	S	62.6±5.4		100	100	I
	A/Swine/Indiana/29-0408/2010 (KP412322)	T	S	58.4±2.5		100	56.2	I
	A/Swine/Indiana/24-0218/2010 (KP412328)	T	S	>100		100	>100	R
<hr/>								
H3N2								
	A/Swine/Iowa/13-0505/2010 (KP412334)	I	S	<1		<1	NT	S
	A/Swine/Iowa/13-0515/2010 (KP412340)	I	S	<1		<1	NT	S
	A/Swine/Indiana/27-1102/2010 (KP100956)	T	S	68.3±3.1		100	100	I
	A/Swine/Indiana/27-1112/2010 (KP100884)	T	S	34.5±4.3		100	31.6	I
	A/Swine/Iowa/10-0628/2010 (KP412326)	I	N	>100		100	>100	R
<hr/>								
Reference human viruses ^d								
H1N1	A/Brisbane/59/2007	V	S	<1		<1	<0.6	S
H3N2	A/Perth/16/2009	V	N	>100		>100	>100	R

Abbreviations: S, Susceptible; I, Intermediate; R, Resistant; NT, not tested.

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^p All tested viruses possessed L26, A30, and G34 residues at the M2 protein.

^b Performed in accordance with the CDC protocol (Bright et al., 2005).

^c Amantadine susceptibility was determined with an extended range of drug concentrations (100, 56.2, 31.6, 17.7, 10.0, 5.6, 3.1, 1.8, 1.0, 0.6, 0.3, and 0.0 μ M).

^d Influenza A/Perth/16/2009 (H3N2) virus harboring the S31N-M2, which confers resistance to amantadine ($EC_{50} > 100 \mu$ M), and A/Brisbane/59/2007 (H1N1) virus without any markers of amantadine resistance ($EC_{50} < 1 \mu$ M) were included in the assays as reference viruses. Susceptibility to amantadine was defined by the following criteria: $EC_{50} = 1 \mu$ M – susceptible; 30μ M $< EC_{50} < 100 \mu$ M – intermediate level of resistance; $EC_{50} = 100 \mu$ M – resistant (Hayden FG, 1996).