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Virus Research 103 (2004) 67–73
Virus Research
www.elsevier.com/locate/virusres

Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population

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Available online 24 April 2004

Abstract

Before the isolation of H3N2 viruses in 1998, swine influenza in the United States was an endemic disease caused exclusively by classical-swine H1N1 viruses. In this study we determined the antigenic and phylogenetic composition of a selection of currently circulating strains and revealed that, in contrast to the situation pre-1998, the swine population in the United States is now a dynamic viral reservoir containing multiple viral lineages. H3N2 viruses still circulate and representatives of each of two previously identified phylogenetic groups were isolated. H1N1 and H1N2 viruses were also identified. In addition to the genotypic diversity present, there was also considerable antigenic diversity seen. At least three antigenic profiles of H1 viruses were noted and all of the recent H3N2 viruses reacted poorly, if at all, to the index A/swine/Texas/4199-2/98 H3N2 antiserum in hemagglutination inhibition assays. The influenza reservoir in the United States swine population has thus gone from a stable single viral lineage to one where genetically and antigenically heterogeneic viruses co-circulate. The growing complexity of influenza at this animal–human interface and the presence of viruses with a seemingly high affinity for reassortment makes the United States swine population an increasingly important reservoir of viruses with human pandemic potential.

Keywords: Swine influenza; Reassortment; H1N1; H3N2; H1N2

1. Introduction

Human influenza is a zoonotic disease with the primary viral reservoir residing within the aquatic birds of the world (reviewed by Webster et al. (1992)). Although aquatic birds are the major viral reservoir, swine play an important role in the ecology of human influenza. In addition to the sporadic isolation of swine viruses from humans (reviewed by Brown (2000)), swine have been postulated to be a "mixing vessel" where avian and mammalian viruses reassort (Scholtissek, 1990). Pigs, unlike humans (Beare and Webster, 1991), seem to be readily infected by avian viruses, and most, if not all, avian hemagglutinin (HA) subtypes are capable of replicating in swine (Kida et al., 1994). The susceptibility of swine to both mammalian and avian viruses is due to the presence of receptors for both lineages of virus in the pig trachea (Ito et al., 1998). Human influenza viruses preferentially bind NeuAcα2,6Gal linkages, whereas avian influenza viruses bind NeuAcα2,3Gal linkages (Rogers and Paulson, 1983). Thus, pig tracheal cells can be infected not only by human influenza viruses but also by avian viruses. It has been postulated that pigs may have been the intermediate host responsible for the genesis of the last two human pandemic viruses which were reassortants between human and avian viruses (Kawaoka et al., 1989; Scholtissek et al., 1983).

Influenza in swine is an acute respiratory disease, the severity of which depends on many factors including pig age, virus strain, and secondary infections (Easterday and Hinshaw, 1992). Currently, three main subtypes of influenza virus are circulating in different swine populations throughout the world: H1N1, H3N2, and H1N2. Prior to 1998 swine influenza in the United States had been caused almost exclusively by viruses of the classical-swine H1N1 lineage (Chambers et al., 1991; Hinshaw et al., 1978). In late August 1998, a severe influenza-like illness was observed in pigs on a farm in North Carolina. During November and December of the same year, additional outbreaks among swine herds were reported in Minnesota, Iowa, and Texas. The causative

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0168-1702/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2004.02.015

agents were subsequently identified as influenza viruses of the subtype H3N2. Genetic analysis of these H3N2 viruses showed that at least two different genotypes were present. The initial North Carolina isolate (double reassortant) contained gene segments similar to those of the human (HA, NA, PB1) and classical-swine (NS, NP, M, PB2, PA) lineages, whereas the isolates from Minnesota, Iowa, and Texas (triple reassortant) contained genes from the human (HA, NA, PB1), swine (NS, NP, M), and avian (PB2, PA) lineages (Zhou et al., 1999). By the end of 1999 viruses antigenically and genetically related to the triple reassortant lineage were widespread in the US swine population (Karasin et al., 2000; Webby et al., 2000).

Once established in the swine population, the H3N2 viruses started to evolve through reassortment with both human and swine viruses. In 1999, viruses of the triple reassortant genotype were isolated that had acquired two further H3 molecules from viruses circulating in the human population (Webby et al., 2000). In addition to further reassortment with human viruses, reassortment with classical-swine H1N1 viruses was documented. These reassortant viruses were H1N2 viruses containing seven genes from the H3N2 viruses and the HA from the classical-swine H1N1 viruses (Karasin et al., 2000). Subsequent studies have shown that these H1N2 viruses have also become widespread (Choi et al., 2002; Karasin et al., 2002).

The increase in genetic diversity in the US swine population has implications for both veterinary and human health sectors. As described, swine populations act as reservoirs of viruses with proven ability to infect humans. As such, it is critical to determine the amount of genetic diversity of viruses within the swine reservoir. In this study we characterized a number of recent viruses isolated from swine in the United States.

2. Materials and methods

2.1. Virus isolation and propagation

Viruses were isolated from tissue samples submitted to the Veterinary Diagnostic Medicine Laboratory, University of Minnesota, St. Paul, MN and the Rollins Animal Disease Diagnostic Laboratory, Rollins, NC. All viruses were isolated and cultured in MDCK cells in the presence of 1 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin.

2.2. Antigenic analysis

The antigenic relationships among the viruses were determined by using hemagglutination inhibition (HI) tests as previously described (Palmer et al., 1975). All sera were pretreated with the receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo) to abolish interference by non-specific serum inhibitors.

2.3. RNA extraction, RT-PCR, and DNA sequencing

Viral RNA was extracted from cell culture supernatants by using the RNeasy kit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions. Reverse transcription and PCR were carried out under standard conditions by using influenza-specific primers (Hoffmann et al., 2001). PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Template DNA was sequenced by using rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaqDNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc. [PE/ABI], Foster City, CA), and synthetic oligonucleotides. Samples were subjected to electrophoresis, detection and analysis on PE/ABI model 373 or model 377 DNA sequencers.

2.4. Sequence analysis

DNA sequences were compiled and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments were made by using CLUSTAL W (Thompson et al., 1994) and phylogenetic analysis done by using the PHYLIP software package (Felsenstein, 1993).

3. Results

3.1. Antigenic analysis

Ten viruses were chosen for characterization. Of these 10 viruses, 6 were identified as H1 and 4 identified as H3 using hemagglutination inhibition assays.

3.2. H1 viruses

The six H1 viruses were tested using antiserum against early classical-swine viruses, two recent field strains (taken from naturally infected animals in the field), and a contemporary human virus (Table 1). Of the six H1 viruses tested there was considerable diversity in reactivity to these sera. Although most recent swine isolates reacted poorly to A/swine/Iowa/30 antiserum, all but A/swine/Arkansas/2976/02 reacted well to A/New Jersey/67 antiserum. The isolates were also inhibited to different degrees by the serum from two recent field infections, highlighted by one of these sera recognizing only the A/swine/Minnesota/37866/99 antigen. A/swine/Arkansas/2976/02 was not inhibited to any large extent by any of the tested sera. None of the swine isolates showed any reactivity to A/Caledonia/20/99, a contemporary human H1N1 isolate.

Table 1
Antigenic comparison of H1 swine influenza viruses using hemagglutination inhibition (Sw, swine)

Virus	Genotype	Titre of antisera to virus				
		Sw/IA/30	A/NJ/76	Field sera[a] #1	Field sera #2	A/New Caledonia/20/99[b]
Sw/MN/37866/99	H1N1	80	1280	640	320	<10
Sw/NC/18161/02	H1N1	<10	320	<10	80	<10
Sw/MN/26194/01	H1N1	40	640	<10	640	<10
Sw/MN/7002/01	H1N1	<10	320	<10	320	<10
Sw/AR/2976/02	H1N2	40	<10	<10	20	<10
Sw/MN/29404/00	H1N2	40	640	<10	640	<10
A/NJ/76	H1N1	160	**640**	<10	40	<10
Sw/IA/30	H1N1	**320**	640	<10	20	<10

[a] Antisera collected from diseased animals in 2002. Titers in bold are homologous reactions.
[b] This serum reacts to titers of 5124 with the homologous antigen.

3.3. H3 viruses

The four H3N2 viruses were tested against a panel of antiserum to A/swine/Texas/4199-2/98 and A/swine/North Carolina/35922/98, two of the index 1998 viruses, and to A/Wuhan359/95, A/Sydney/5/97, and A/Panama/2007/99, three contemporary human vaccine strains (Table 2). Antisera to A/Sydney/5/97 and A/Panama/2007/99 displayed the only significant HI activity against the recent H3N2 strains. A/swine/North Carolina/7940/02 and A/swine/North Carolina/28373/02 reacted with both the 1997 and 1999 human virus antisera whereas A/swine/North Carolina/50270/01 and A/swine/North Carolina/46482/02 reacted with A/Sydney/5/97 but not A/Panama/2007/99. The lack of cross-reactivity between the A/swine/Texas/4199-2/98 antiserum and A/swine/North Carolina/50270/01 was despite a nucleotide similarity of 97.7% between the HA gene sequence of these two viruses (see below).

3.4. Genetic analysis

Three genotypes of influenza A viruses have been described as currently circulating in US swine populations, classical-swine H1N1, H3N2 and H1N2. In order to determine the subtypes of the currently circulating viruses we partially sequenced the HA and neuraminidase (NA) genes of the 10 selected swine viruses. The swine viruses could be separated into each of the three distinct subtypes based on the composition of these gene components. Four viruses (A/swine/North Carolina/7940/02, A/swine/North Carolina/50270/01, A/swine/North Carolina/28373/02, and A/swine/North Carolina/46482/02) were H3N2 viruses, two viruses (A/swine/Arkansas/2976/02 and A/swine/Minnesota/29404/00) were H1N2 viruses, and four viruses (A/swine/Minnesota/37866/99, A/swine/North Carolina/18161/02, A/swine/Minnesota/26194/01, and A/swine/Minnesota/7002/01) were H1N1 viruses. Details of each of the genotypes is given below.

3.5. H3N2 viruses

Six-hundred-and-six nucleotides (corresponding to positions 4–610 of the open reading frame) of the HA gene were sequenced and compared to database sequences. The four H3N2 viruses could be separated into two major HA groups corresponding to two of the three phylogenetically distinct groups previously described (Webby et al., 2000) (Fig. 1). A/swine/North Carolina/50270/01 had 97.7% nucleotide identity to the index A/swine/Texas/4199-2/98 isolate but less than 93% identity to the other three viruses sequenced.

Table 2
Antigenic comparison of H3 swine influenza viruses using hemagglutination inhibition

Virus	Titre of antisera to virus				
	A/Sw/TX/4199-2/98	A/Sw/NC/35922/98	A/Wuhan359/95[a]	A/Sydney/5/97[a]	A/Panama/2007/99[a]
A/Sw/NC/7940/02	<20	<20	<20	1280	320
A/Sw/NC/50270/01	20	<20	<20	160	<20
A/Sw/NC/28373/02	<20	<20	<20	1280	320
A/Sw/NC/46482/02	<20	20	<20	320	<20
A/Sw/TX/4199-2/98	**640**	20	N/D[b]	N/D	N/D
A/Sw/NC/35922/98	<20	**360**	N/D	N/D	N/D

Titers in bold are homologous reactions.
[a] These sera react to titers of 5124 with the homologous antigen.
[b] Not done.

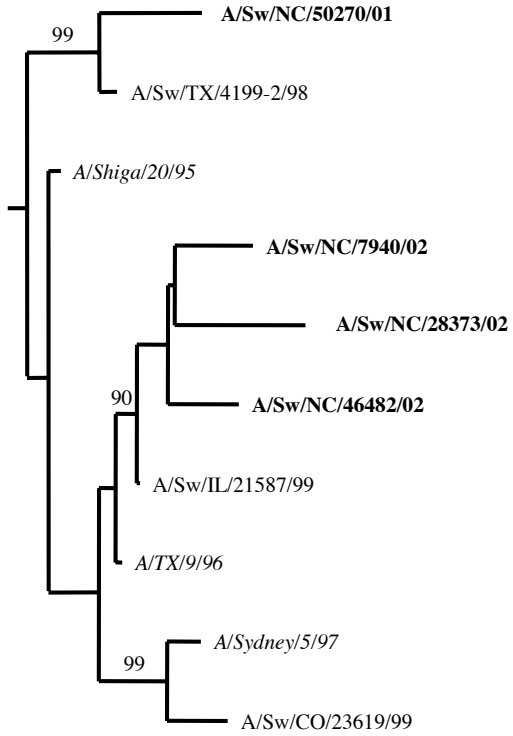

Fig. 1. A distance phylogenetic tree based on the partial nucleotide sequences of the HA genes of selected H3N2 influenza viruses. The bold isolates are swine viruses sequenced in this study and the italicized isolates are representative human viruses. Horizontal distances are proportional to genetic distance, and the numbers above the nodes are bootstrap scores out of 100 replicates. The tree is rooted to A/England/878/69.

The other three viruses had >95% nucleotide identity and were most similar to A/Illinois/21587/99 (96.4–97.7% identity).

3.6. H1N1 viruses

Five-hundred-and-three nucleotides of HA nucleotide sequence (corresponding to positions 12–515 of the open reading frame) were analyzed from each virus. Nucleotide identities over this region ranged from 97.8% between A/swine/North Carolina/18161/02 and A/swine/Minnesota/7002/01 to 92.4% between A/swine/Minnesota/26194/01 and A/swine/North Carolina/18161/02. A/swine/North Carolina/18161/02, A/swine/Minnesota/26194/01, and A/swine/Minnesota/7002/01 had >97% nucleotide identity to North American H1N2 isolates in the Influenza Sequence Database (Macken et al., 2001). The HA of A/swine/Minnesota/37866/99 had the highest identity to A/swine/Hong Kong/273/94 (99%), a virus of the classical-swine lineage.

3.7. H1N2 viruses

Two H1N2 viruses were identified in this study. These viruses contained the HA of the classical-swine lineage, but the NA gene of the H3N2 virus lineage. These viruses were 92.6% identical over the 503 base pairs analyzed. The most similar HA genes in the Influenza Sequence Database belonged to H1N2 viruses recently isolated from North American swine.

4. Discussion

Prior to 1998 swine influenza in the US had a simple etiology. Apart from a single reported isolation of a wholly human H3N2 virus from Colorado in 1977 (Karasin et al., 2000), the only influenza viruses isolated belonged to the classical-swine H1N1 virus lineage. Within Asia and Europe H3N2 viruses had entered the swine population about the same time as they had appeared in humans (reviewed by Scholtissek et al. (1998)). In addition, a wholly avian H1N1 that was antigenically and genetically distinct from the classical H1N1 virus entered and established in the European swine population in the late 1970s (Pansaert et al., 1981). The emergence of reassortant H3N2 viruses in 1998 changed the complexity of swine influenza in the US (Olsen, 2002). The results of this study have shown that the US swine population has gone from a reservoir for a single virus lineage to one where at least three genotypes of influenza virus co-circulate (Fig. 2).

From the sequencing of H3N2 viruses in 1999 we have previously shown that these viruses had acquired three phylogenetically and temporally distinct H3 genes from viruses circulating in the human reservoir (Webby et al., 2000). The first of these HA molecules were similar to the index swine cases and to human viruses circulating in 1995. The second group contained HA genes derived from human viruses circulating in 1996, and the third contained HA genes similar to the A/Sydney/5/97-like drift variants that

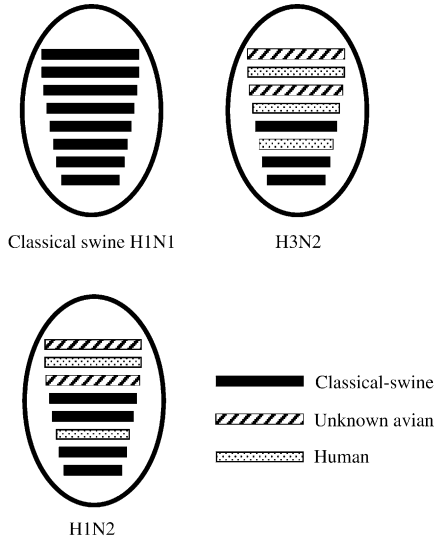

Fig. 2. A schematic diagram representing the influenza virus genotypes currently circulating in the United States swine population.

emerged in the human population in 1997. In the present study we were able to identify viruses belonging to two of these groups. One virus was phylogenetically most similar to the index A/swine/Texas/4199-298 isolate (1995-like human HA) and the other three to viruses containing 1996-like human HA molecules. In this limited study we did not identify any further A/Sydney/5/97-like variants. The small number of viruses sequenced does not preclude the presence of A/Sydney/5/97-like viruses in the US swine reservoir and a more intense surveillance is needed to determine the degree of diversity within currently circulating H3N2 viruses.

Considerable amounts of diversity were seen in the HA genes of the H1 viruses. A/swine/Minnesota/37866/99, a H1N1 virus, was at least 5% divergent to the other H1 genes sequenced but was 99% identical to A/swine/Hong Kong/273/94, a classical-swine H1N1 virus. The similarity of A/swine/Minnesota/37866/99 to the 1994 classical virus and the decreased homology with the other 2001/2002 viruses suggests that the different H1 lineages in US swine are evolving independently and that antigenic drift is occurring.

Sequence analysis of HA genes has shown that antigenic drift does occur in both European H1N1 and H3N2 viruses of swine (Brown et al., 1997; de Jong et al., 1999) raising concerns from some investigators that vaccines in swine may need to be continually updated as in human populations. Heinen et al. (2001) have shown, however, that vaccination with A/Port Chalmers/1/73 (H3N2) was sufficient to stop the development of fever and transmission upon challenge with a recent European field strain. The vaccine was not able, however, to completely stop viral shedding from the challenged animal. Similar studies with the European H1N1 viruses have revealed similar results in that heterologous virus vaccination can protect from clinical disease and reduce viral load although not viral replication (Van Reeth et al., 2001). The continued diversity of influenza in the US swine reservoir does, however, suggest that similar studies should be carried out with current US strains. The antigenic characterization of the H1 and H3 viruses supports the need for cross-protection studies. The contemporary H1 viruses displayed at least three different patterns of reactivity to the tested antisera (Table 1). Although there was no correlation between the viral genotype and antigenicity or phylogeny within the H1 viruses, the antigenic and phylogenetic similarities were consistent (Fig. 3). The inability of sera produced against the index A/swine/Texas/4199-2/98 and A/swine/North Carolina/35922/98 H3N2 viruses to react with contemporary viruses suggest that H3N2 viruses are also drifting in swine populations. The reactivity of the swine H3N2 strains with recent human virus antiserum would suggest that these viruses as an entity possess limited human pandemic potential due to cross-reactive immunity. As these viruses continue to evolve, however, their threat as human pathogens increases correspondingly.

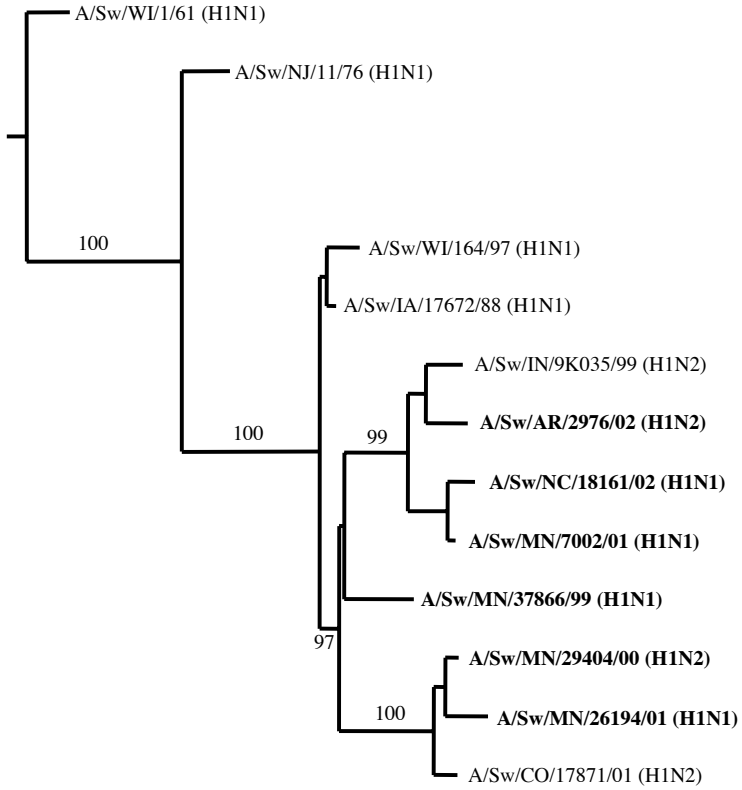

Fig. 3. A distance phylogenetic tree based on the partial nucleotide sequences of the HA genes of selected H1 influenza viruses. The bold isolates are swine viruses sequenced in this study. Horizontal distances are proportional to genetic distance, and the numbers above the nodes are bootstrap scores out of 100 replicates. The tree is rooted to A/swine/Iowa/15/30.

There have been numerous reports of human infection with swine influenza viruses (reviewed by Brown (2000)) and as such any activity in swine virus reservoirs is of concern for human health. Indeed, both the H1N2 and a novel H1N1 swine influenza virus have shown the capacity to cross the species barrier. A H1N2 virus of the same lineage as described in this study has been isolated from a turkey breeder hen (Suarez et al., 2002). Additionally, and of some concern, is the report of human infection by a H1N1 reassortant swine virus (Cooper et al., 2003, personal communication). This virus contained the surface glycoproteins of the classical-swine H1N1 virus but the remaining genes from the H3N2 swine viruses. Although not causing severe disease, the infected person presented with influenza-like illness and the event demonstrated the capacity of this viral genotype to replicate in the human host. The non-reactivity of the recent swine H1 viruses with the reference human sera in HI assays (Table 1) suggests that contemporary human vaccines would afford little protection in the event of the widespread dissemination of such viruses in humans. The serological evidence of swine H1 virus infection of swine farm residents and workers (17 of 74 samples positive) recently reported by Olsen et al. (2002) would also argue that these swine to human interspecies transfer events are not uncommon.

The US swine population has become a reservoir of a much more diverse array of influenza viruses since the arrival of the H3N2 viruses. The replicative gene constellation of the H3N2 viruses has the capacity to reassort with both swine and human viruses and has a demonstrated ability to replicate in swine, human, and avian hosts. The implications of the increased diversity and promiscuous nature of these viruses impact on both swine and human health. Despite recent events it is unlikely that swine vaccines will need to be updated as frequently as human vaccines, but there is no certainty at the moment that a single vaccine will afford complete across the board protection. The bottom line is that now, more than ever, a formal surveillance system for swine influenza is needed in the US. It is only through such a system that novel reassortants and interspecies transfer events will be identified in a timely fashion.

Acknowledgements

These studies were supported by grants AI95357 from the National Institute of Allergy and Infectious Disease, by Cancer Center Support (CORE) grant CA-21765 from the National Institutes of Health, and by the American Lebanese Syrian Associated Charities (ALSAC).

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