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Alexander I. Karasin

University of Wisconsin – Madison

Melissa M. Schutten

University of Wisconsin – Madison

Lynn A. Cooper

Influenza Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA

Catherine B. Smith

Influenza Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA

Kanta Subbarao

Influenza Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA

See next page for additional authors

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Authors

Alexander I. Karasin, Melissa M. Schutten, Lynn A. Cooper, Catherine B. Smith, Kanta Subbarao, Gary A. Anderson, Suzanne Carman, and Christopher W. Olsen

Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes

Alexander I. Karasin^a, Melissa M. Schutten^a, Lynn A. Cooper^b,
Catherine B. Smith^b, Kanta Subbarao^b, Gary A. Anderson^c,
Suzanne Carman^d, Christopher W. Olsen^{a,*}

^a *Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin — Madison, 2015 Linden Drive West, Madison, WI 53706, USA*

^b *Influenza Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA*

^c *ImmTech Biologics, LLC, 8600 West 239th Street, Bucyrus, KS 66013, USA*

^d *Animal Health Laboratory, University of Guelph, Box 3612, Guelph, Ont., Canada N1H 6R8*

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Abstract

Since 1998, H3N2 viruses have caused epizootics of respiratory disease in pigs throughout the major swine production regions of the U.S. These outbreaks are remarkable because swine influenza in North America had previously been caused almost exclusively by H1N1 viruses. We sequenced the full-length protein coding regions of all eight RNA segments from four H3N2 viruses that we isolated from pigs in the Midwestern U.S. between March 1998 and March 1999, as well as from H3N2 viruses recovered from a piglet in Canada in January 1997 and from a pig in Colorado in 1977. Phylogenetic analyses demonstrated that the 1977 Colorado and 1997 Ontario isolates are wholly human influenza viruses. However, the viruses isolated since 1998 from pigs in the Midwestern U.S. are reassortant viruses containing hemagglutinin, neuraminidase and PB1 polymerase genes from human influenza viruses, matrix, non-structural and nucleoprotein genes from classical swine viruses, and PA and PB2 polymerase genes from avian viruses. The HA proteins of the Midwestern reassortant swine viruses can be differentiated from those of the 1995 lineage of human H3 viruses by 12 amino acid mutations in HA1. In contrast, the Sw/ONT/97 virus, which did not spread from pig-to-pig, lacks 11 of these changes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Influenza A virus; H3N2; Pig; Reassortment; Phylogenetic analysis

* Corresponding author. Tel.: +1-608-2658681; fax: +1-608-2630438.

E-mail address: olsenc@svm.vetmed.wisc.edu (C.W. Olsen).

1. Introduction

H1N1 influenza viruses were first isolated from pigs in the U.S. by Shope in 1930 (Shope, 1931), although influenza-like illness was recognized clinically among American pigs in the late summer and fall of 1918 (Koen, 1919; Easterday and Hinshaw, 1992) during the H1N1 ‘Spanish flu’ human pandemic (Murphy and Webster, 1996). Recent evidence indicates that the 1918 human viruses and the 1930 swine viruses are closely related (Taubenberger et al., 1997; Reid et al., 1999), although it remains unclear whether these early H1N1 viruses appeared first in people and then spread to pigs, or vice versa (Reid and Taubenberger, 1999; Webster, 1999). In either case, interspecies transmission of influenza viruses between pigs and people has continued to occur since that time. Zoonotic transmission of H1N1 viruses from pigs has been documented repeatedly in the U.S. (Hinshaw et al., 1978; Dasco et al., 1984; Wentworth et al., 1997), Europe (de Jong et al., 1988) and New Zealand (Eason and Sage, 1980), in some cases resulting in the death of the people involved (Smith et al., 1976; Top and Russell, 1977; Patriarca et al., 1984; Rota et al., 1989; Wentworth et al., 1994; Kimura et al., 1998). Conversely, human H3N2 influenza viruses have been isolated regularly from pigs in Europe and Asia (Shortridge et al., 1977, 1979; Nakajima et al., 1982; Ottis et al., 1982; Mancini et al., 1985; Castrucci et al., 1994; Katsuda et al., 1995).

Beyond serving as sources for direct interspecies transmission of influenza viruses to people, pigs have also been suggested to be the ‘mixing vessel’ host in which human and avian virus co-infection can produce reassortant influenza viruses of novel genotypes (Scholtissek et al., 1985; Scholtissek and Naylor, 1988; Webster et al., 1992). Pigs are susceptible to infection with influenza viruses of avian (Castrucci et al., 1993; Kida et al., 1994; Campitelli et al., 1997; Brown et al., 1998) as well as mammalian origin because their tracheal epithelium contains virus receptor sialyloligosaccharides with both 2,3- (preferred by avian influenza viruses) and 2,6- (preferred by mammalian influenza viruses) *N*-acetylneuraminic acid–galactose linkages (Ito et al., 1998). The pandemics of hu-

man influenza in 1957 and 1968 were caused by viruses that were human/avian virus reassortants (Webster et al., 1992), and more recently, human–avian influenza virus reassortants have been isolated from commercially raised pigs in Europe (Castrucci et al., 1993) and, thereafter, from children in the Netherlands (Claas et al., 1994). In addition, wholly avian H1N1 viruses entered the swine population in Europe in 1979–1980 and have subsequently become the dominant H1N1 virus among European pigs (Pensaert et al., 1981; Scholtissek et al., 1983; Webster et al., 1992).

In contrast to Europe and Asia, human H3 and avian influenza virus infections have historically been very uncommon among pigs in North America. Surveillance studies conducted in 1976/1977 (Hinshaw et al., 1978) and 1988/1989 (Chambers et al., 1991) documented widespread classical H1 swine influenza virus infection of pigs in the North-Central U.S., with H1 seropositivity rates of up to 47% in 1976/1977 and 51% in 1988/1989. Seropositivity to human H3 influenza viruses, on the other hand, was very limited in both 1976/1977 (1.4%) and 1988/1989 (1.1%), and only three H3 viruses are reported to have been isolated from pigs in North America between 1977 and 1996 (Hinshaw et al., 1978; Bikour et al., 1994, 1995). In the 1988/1989 survey, swine sera were also tested for antibodies to an avian virus, A/Duck/Alberta/16/87 (H3N8), but none of the 2337 samples contained detectable antibodies to this virus. Therefore, throughout this period, classical swine H1 viruses were clearly the predominant influenza viruses infecting pigs in the U.S.

In the last 3 years, the epidemiologic pattern of swine influenza in North America has changed. In 1997–1998, we noted distinct increases in seropositivity against human H3 and avian viruses during influenza surveillance in Wisconsin, Iowa and Illinois (Olsen et al., 2000). Specifically, 7.6% of the pigs had hemagglutination-inhibiting (HI) antibodies against an avian H1N1 virus, A/Duck/Alberta/35/76, and 8% of the pigs had HI antibodies against a human H3N2 virus, A/Wuhan/359/95 (WUH/95). During this same time period, we also began to isolate H3N2 viruses from North American pigs. We isolated an H3N2 virus from a piglet in Canada in 1997 and four H3N2 viruses

from pigs in the Midwestern U.S. in 1998–1999 (Table 1). With the exception of the isolate from Canada, these viruses were collected during extensive outbreaks of respiratory illness in the herds-of-origin. Additionally, one of the viruses (Sw/MN/99) was also associated with abortions in pregnant sows.

The purpose of the present study was to genetically characterize these H3N2 viruses isolated from pigs in North America between 1997 and 1999, and to compare them to an H3N2 virus isolated from a pig in Colorado in 1977, A/Swine/Colorado/1/77 (Sw/CO/77) (H3N2) (Hinshaw et al., 1978). We determined the full-length protein coding region sequences for all eight viral RNA segments from each virus and subjected the gene sequences to phylogenetic analyses to determine the likely evolutionary origin of these viruses, as well as their relationships to each other. During the final analysis of these data, Zhou et al. (1999) described four additional H3N2 viruses isolated from pigs in the U.S. between September and December 1998, three from the Midwest (Iowa, Minnesota and Texas) and one from North Carolina. To provide the most complete evaluation possible, we have also included sequence data from these viruses in our analyses. Our results indicate that the H3N2 viruses that have infected pigs in North America since 1977 are of multiple genotypes and separate sublineages within those genotypes.

2. Materials and methods

2.1. Viruses

The Sw/ONT/97, Sw/NB/98, Sw/IA533/99, Sw/IA569/99 and Sw/MN/99 viruses were isolated as described in Table 1. The Sw/ONT/97 virus was subsequently cultivated in embryonated chicken eggs after three passages in primary pig kidney cells, although it could not be isolated in eggs from the original inoculum. The Sw/NB/98, Sw/IA533/99, Sw/IA569/99, Sw/MN/99 viruses were each passaged once in Madin–Darby canine kidney (MDCK) cells after isolation in Madin–Darby bovine kidney (MDBK) cells. The

Sw/CO/77 virus (Hinshaw et al., 1978) was kindly provided by V. Hinshaw as an allantoic fluid stock from the Influenza Virus Repository at the University of Wisconsin — Madison.

2.2. Virus subtype and genetic characterization

The subtype of each virus was confirmed to be H3N2 by HI and neuraminidase-inhibition assays (Palmer et al., 1975), using subtype-monospecific sera. The full-length protein coding regions of all eight viral RNA segments from each of the six viruses were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using AMV reverse transcriptase (Promega Corporation, Madison, WI) and *Pfu* polymerase (Stratagene, LaJolla, CA). Amplifications were conducted as suggested by the enzyme suppliers, except that the RT reactions were performed using 1 µg of primer per reaction, and reaction conditions of 49.5°C for 60 min for the polymerase genes and 48.5°C for 45 min for the remaining genes. Amplifications of the neuraminidase (NA), nucleoprotein (NP), matrix (M), non-structural (NS) and PB1 polymerase genes were accomplished using the SZANA +/–, SZANP +/–, SZAM +/–, SZANS +/– and SZAPB1 +/– primers, respectively, developed by Zou (1997). Amplifications of the hemagglutinin (HA) and the PB2 and PA polymerase genes were conducted using the following primers: H3HA-30F (5'-atgaatactatcattgct-3') and H3HA-1727R (5'-tcaaatgcaaatgtg-3'); PB2-43F (5'-gaactaagagatcta-3') and PB2-2341R (5'-agtagaaacaaggtcgt-3'); PA-55F (5'-aaggcaatgaaagaata-3') and PA-2152R (5'-tgtgtgagggaaggagtt-3'). (F denotes a forward primer and R a reverse primer.)

The sequences of the amplified genes were determined from the PCR products by cycle sequencing (ABI Big Dye, PE Applied Biosystems, Foster City, CA). Sequence comparisons at the nucleotide and deduced amino acid levels were made using DNASTAR software (Version 4.0 for Win32). The phylogenetic relationships among the virus isolates and selected reference strains were estimated from their nucleotide sequences by the

Table 1
Virus isolates described in this report

Virus name	Date of isolation	Sample	Cells used for primary isolation	Epidemiological and clinical characteristics of disease on the farm of origin
A/Swine/Ontario/00130/97 (Sw/ONT/97)	January 15, 1997	Lung tissue	primary pig kidney cells	Isolated from a 1-week-old piglet that died of pneumonia. This was the only sick animal in the herd ^b .
A/Swine/Nebraska/209/98 (Sw/NB/98)	March 19, 1998	Nasal swab	MDBK ^a cells	Isolated during an outbreak of widespread illness among nursery-age piglets (3–8 weeks of age). Affected animals exhibited nasal discharge, coughing, lethargy and temperatures up to 41.7°C.
A/Swine/Iowa/533/99 (Sw/IA533/99)	January 27, 1999	Nasal swab	MDBK cells	Isolated during an outbreak of widespread illness among nursery-age piglets. Affected animals exhibited sneezing, severe coughing, nasal discharge, lethargy and temperatures up to 41.7°C ^b .
A/Swine/Iowa/569/99 (Sw/IA569/99)	February 17, 1999	Nasal swab	MDBK cells	Isolated during an outbreak of widespread illness among feeder pigs (3–4 months of age). Affected animals exhibited conjunctivitis, severe coughing, lethargy and temperatures up to 41.1°C.
A/Swine/Minnesota/593/99 (Sw/MN/99)	March 2, 1999	Nasal swab	MDBK cells	Isolated during an outbreak of widespread illness among adult pigs. Affected animals exhibited sneezing, coughing, nasal discharge, lethargy and temperatures up to 41.1°C, as well as abortions among pregnant sows.

^a MDBK, Madin–Darby bovine kidney cells.

^b The vaccination history for the herds from which Sw/ONT/97 and Sw/IA533/99 were isolated is unknown, but the herds-of-origin for Sw/IA569/99, Sw/NB/98 and Sw/MN/99 all used a conventional, commercially available H1N1 swine influenza virus vaccine.

method of maximum parsimony (PAUP software v.4.0b2, David Swofford, Smithsonian Institution), using the tree-bisection–reconnection branch swapping algorithm and with the MULTREES option in effect. For the HA genes, although we determined the full-length protein-coding region sequences of our isolates, only the HA1 sequences were employed for the phylogenetic analyses because of insufficient HA2 sequence data in GenBank™ for reference viruses. For the other genes, the full-length coding region sequences for each of our isolates were analyzed in relation to the available reference virus sequences in GenBank™, with the ‘gaps treated as missing’ PAUP rule in effect.

The HA and NS sequences of Sw/CO/77 were published previously (Bean et al., 1992; Kawaoka et al., 1998), but the sequences used in this study were re-derived from our own virus stocks. Our Sw/CO/77 HA sequence varied at five nucleotides from that in GenBank™, but these translated into only three conservative amino acid changes (210/I > S, 242/V > I and 428/V > L). Our NS gene segment sequence was identical to that in GenBank™.

2.3. GenBank™ accession numbers

The GenBank™ numbers assigned to the sequences determined during this study are as follows: AF251 389–AF251 394 (A/Swine/Colorado/1/77); AF251 395–AF251 402 (A/Swine/Ontario/00130/97); AF251 403–AF251 410 (A/Swine/Nebraska/209/98); AF251 411–AF251 418 (A/Swine/Iowa/533/99); AF251 419–AF251 426 (A/Swine/Iowa/569/99); and, AF251 427–AF251 434 (A/Swine/Minnesota/593/99). The GenBank™ accession numbers for the reference virus sequences used in the phylogenetic analyses are available upon request.

3. Results

3.1. Phylogenetic analyses

The results of the phylogenetic analyses indicate that the HA, NA and PB1 genes of Sw/CO/

77 and all of the H3N2 viruses isolated from pigs since 1997 are most closely related to the corresponding genes from human influenza viruses (Fig. 1), although of different sublineages. The HA genes of Sw/ONT/97, Sw/NB/98, Sw/IA/533/99, Sw/IA/569/99, Sw/MN/99 and the viruses isolated by Zhou et al. (1999) are most closely related to the HA genes from H3 human viruses isolated after 1995. In contrast, the HA gene of Sw/CO/77, as expected from its year of isolation, was derived from the older human H3 lineage typified by A/Victoria/3/75. Interestingly, this older lineage also includes an H3N2 virus isolated from a pig in Canada in 1990, A/Swine/Ange-Gardien/150/90 (referred to as A/Swine/St-Hyacinthe/150/90 by Bikour et al., 1995). In the NA and PB1 gene phylogenetic trees, the Sw/CO/77 virus also diverges at earlier nodes than the more recent H3N2 isolates from pigs.

The results of phylogenetic analyses of the remaining internal viral protein genes clearly differentiate the genotypes of Sw/CO/77 and Sw/ONT/97 from the other H3N2 swine isolates. The M, NP and NS genes (Fig. 2) of Sw/CO/77 and Sw/ONT/97 are of human virus origin, and in each case, these viruses are related most closely to human viruses isolated during approximately the same years. In contrast, the M, NP and NS genes of the viruses isolated from pigs in the U.S. since 1998 are most closely related to the classical H1N1 swine viruses. In the case of the M and NS genes, the swine lineages also include H1N1 swine viruses isolated from people (A/Wisconsin/3523/88 and A/Wisconsin/4754-4755/94: M and NS genes) and turkeys (A/Turkey/North Carolina/1780/88: M gene). The phylogenetic analyses of the PA and PB2 genes (Fig. 3) further distinguish the genotypes of the swine H3N2 isolates. The PA and PB2 genes of Sw/CO/77 and Sw/ONT/97 are again most closely related to those of human viruses, the genes from the 1998 and 1999 Midwestern swine isolates are from the avian influenza virus lineage and those of Sw/NC/98 (Zhou et al., 1999) are derived from the swine virus lineage.

3.2. HA gene sequence analyses

The phylogenetic analyses characterized the overall genotypes of the swine H3 viruses as either wholly human, human/swine virus reassortant or human/swine/avian virus reassortant. We sought, however, to more specifically define the evolutionary origin of the HA genes of the 1997–1999 swine H3 viruses. To do so, we first analyzed the amino acid sequences deduced from their HA1 nucleotide sequences for the presence of the signature mutations that define the A/Johannesburg/33/94 (JOH/

94), WUH/95 and A/Sydney/05/97 (SYD/97) lineages of human H3 influenza viruses (Table 2). Signature amino acids are residues that identify a genetic sublineage and are conserved among all members of the group. At each of these signature residues, the HA genes from all of the swine H3 viruses isolated since 1997 encode the amino acids typical of the Johannesburg/33 lineage that appeared in 1994 or the Wuhan/359 lineage that appeared in 1995. None of the swine viruses contain the amino acid signature mutations of the Sydney/05 lineage that emerged in 1997.

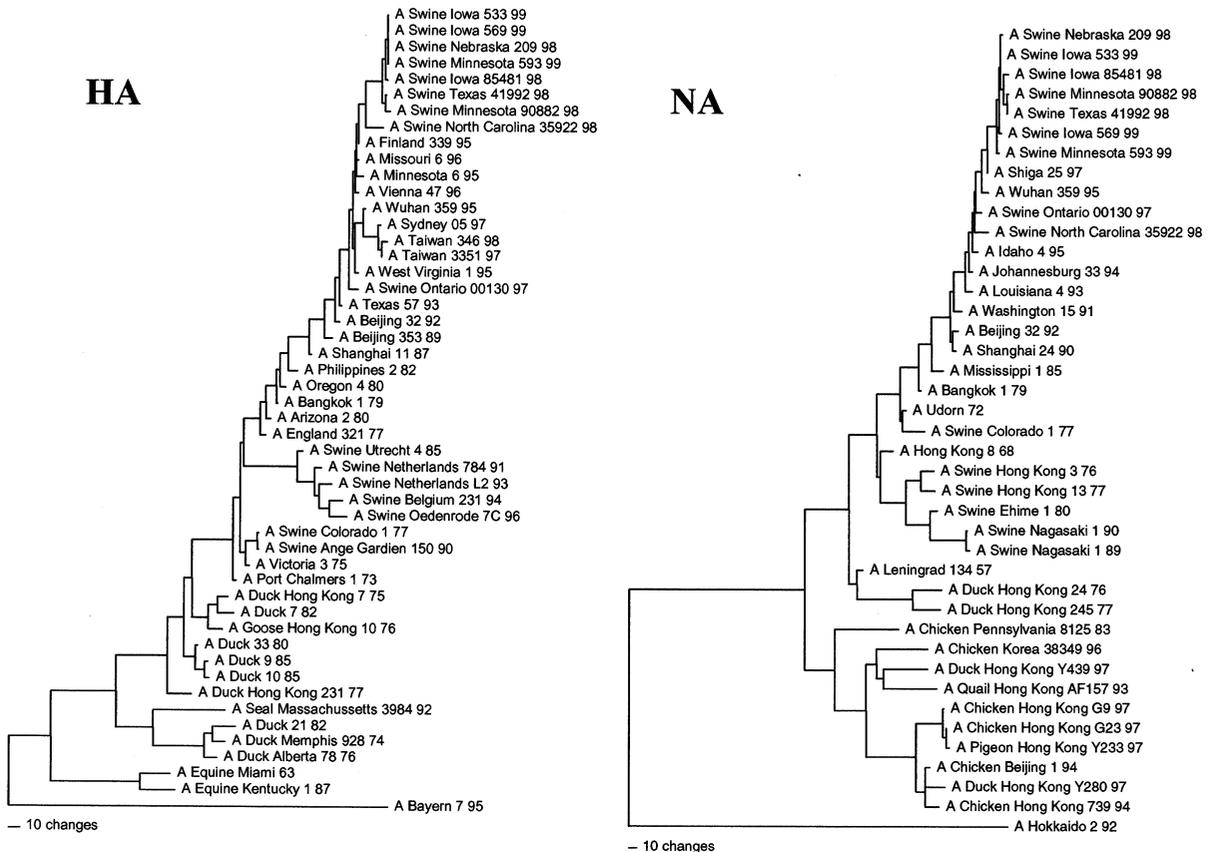


Fig. 1. Nucleotide phylogenetic trees for the HA, NA and PB1 genes of H3N2 influenza viruses isolated from pigs in North America. The evolutionary relationships were estimated by the method of maximum parsimony as described in the Section 2. The trees shown represent the best of numerous rearrangements that were generated. The scores and number of rearrangements for each tree are as follows: HA (score 1595/258 582 rearrangements), NA (score 1998/438 796 rearrangements) and PB1 (score 2968/252 792 rearrangements). Horizontal line distances are proportional to the minimum number of nucleotide changes needed to join nodes and gene sequences. The vertical lines are simply for spacing the branches and labels. The HA and NA trees are rooted to influenza A viruses of different subtypes (H1 and N1), whereas the PB1 tree is rooted to an influenza B virus. The GenBank™ accession numbers for the genes of the viruses isolated during this study are listed in Section 2. The accession numbers for the reference virus sequences used in these analyses are available upon request.

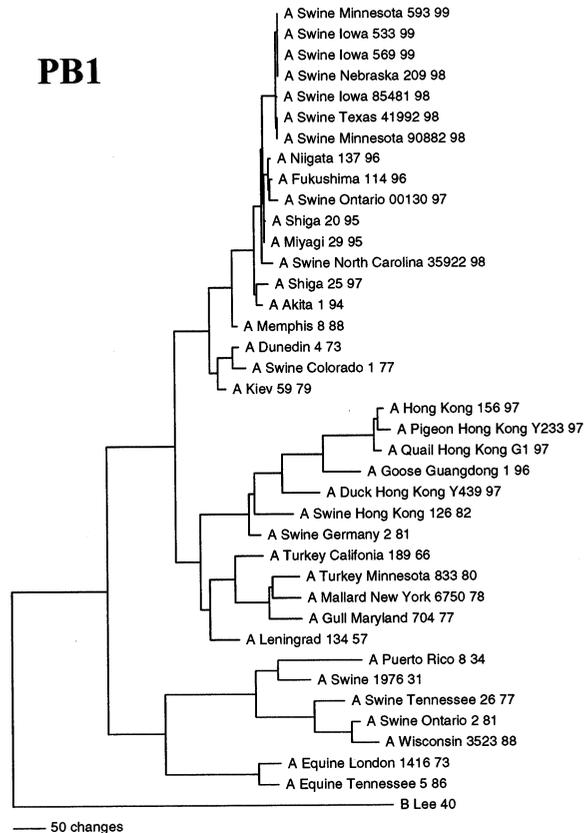


Fig. 1. (Continued)

We also investigated whether there are amino acids in the HA1 proteins that are unique to the triple reassortant human/swine/avian viruses that have spread widely throughout the pig population of the Midwestern U.S., compared to the 1995 lineage of human H3 viruses (Table 3). Twelve amino acid sites were found in which each of these reassortant swine viruses differ in sequence from the WUH/95 lineage. Mutation at one of these amino acids (246:N > S) would result in the loss of a potential glycosylation site in the swine viruses, and three of the other amino acids (135, 138 and 190) are among those previously defined as forming part of the receptor-binding site of the HA (Weis et al., 1988; Lamb and Krug, 1996; Zhou et al., 1999). Circulation of a 1995-lineage human H3 influenza virus among pigs in the United States between 1995 and 1998 may have

provided the opportunity for these mutations to develop. Interestingly, the non-reassortant Sw/ONT/97 virus, which was isolated from a single sick pig in 1997, lacks 11 of these 12 amino acid changes.

4. Discussion

Influenza virus infection is an important cause of respiratory disease among pigs throughout the swine producing regions of the world. Classical H1N1 swine influenza virus infections typically present with clinical signs including fever, anorexia, weight loss, lethargy, nasal and ocular discharge, coughing and dyspnea (Easterday and Hinshaw, 1992). As such, the respiratory signs described for the animals/herds from which the

Table 2
Analysis of signature amino acids in the HA1 portion of H3 HA proteins that define the A/Johannesburg/33/94, A/Wuhan/359/95 and A/Sydney/05/97 lineages

Amino acid position	Human reference viruses		Swine viruses isolated during this study										Swine viruses isolated by Zhou et al. (1999)													
	JOH/94 ^a	WUH/95 ^a	SYD/97 ^a	Sw/ONT/97 ^a	Sw/NB/98 ^a	Sw/MN/99 ^a	Sw/IA533/99 ^a	Sw/IA569/99 ^a	Sw/TX/98 ^a	Sw/NC/98 ^a	Sw/MN/98 ^a	Sw/IA/98 ^a	JOH/94 ^a	WUH/95 ^a	SYD/97 ^a	Sw/ONT/97 ^a	Sw/NB/98 ^a	Sw/MN/99 ^a	Sw/IA533/99 ^a	Sw/IA569/99 ^a	Sw/TX/98 ^a	Sw/NC/98 ^a	Sw/MN/98 ^a	Sw/IA/98 ^a		
47	P ^b	S ^b	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
62	K	E ^b	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
124	N ^b	G ^b	G	G	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
144	V	I ^b	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
156	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
158	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
172	G ^b	D ^b	D	G	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
196	A	V ^b	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
197	R ^b	Q ^b	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
216	D ^b	N ^b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
219	Y ^b	S ^b	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
226	Q ^b	I ^b	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
233	Y	Y	H ^b	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
276	N	N	K ^b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

^a Abbreviations: JOH/94, A/Johannesburg/33/94; WUH/95, A/Wuhan/359/95; SYD/97, A/Sydney/05/97; Sw/ONT/97, A/Swine/Ontario/00130/97; Sw/NB/98, A/Swine/Nebraska/209/98; Sw/MN/99, A/Swine/Minnesota/593/99; Sw/IA533/99, A/Swine/Iowa/533/99; Sw/IA569/99, A/Swine/Iowa/569/99; Sw/TX/98, A/Swine/Texas/4199-2/98; Sw/NC/98, A/Swine/North Carolina/35922/98; Sw/MN/98, A/Swine/Minnesota/9088-2/98; Sw/IA/98, A/Swine/Iowa/8548-1/98

^b Indicates an amino acid that defined the beginning of that virus lineage among human H3N2 influenza viruses.

Sw/NB/98, Sw/IA/533/99, Sw/IA/569/99, Sw/MN/99 and Sw/ONT/97 H3N2 viruses were isolated (Table 1) were not unique. However, abortions also occurred on the farm-of-origin of Sw/MN/99 and in association with H3N2 virus infections of pigs in North Carolina (Zhou et al., 1999). Reproductive disorders have been reported only very infrequently in association with classical H1N1 swine influenza virus infections (Young and Underdahl, 1949; Madec et al., 1989) and, to our knowledge, there is only a single report in the literature identifying an H1N1 influenza virus in an aborted fetus following a naturally acquired infection of the sow (Madec et al., 1989). It will, therefore, be important to determine experimen-

tally whether the reassortant H3N2 viruses infect the placenta and/or fetuses of pregnant sows to any greater degree than classical H1N1 swine influenza viruses. Alternatively, the abortions associated with H3N2 influenza virus infections may simply be due to high fevers or other metabolic disturbances in the sows.

The results of our genetic analyses demonstrate that during the past 22 years, pigs in North America have been infected with H3N2 viruses of two different genotypes. The Sw/CO/77 and Sw/ONT/97 viruses are wholly human viruses, all of whose genes are most closely related phylogenetically to those of influenza viruses circulating in the human population during approximately the

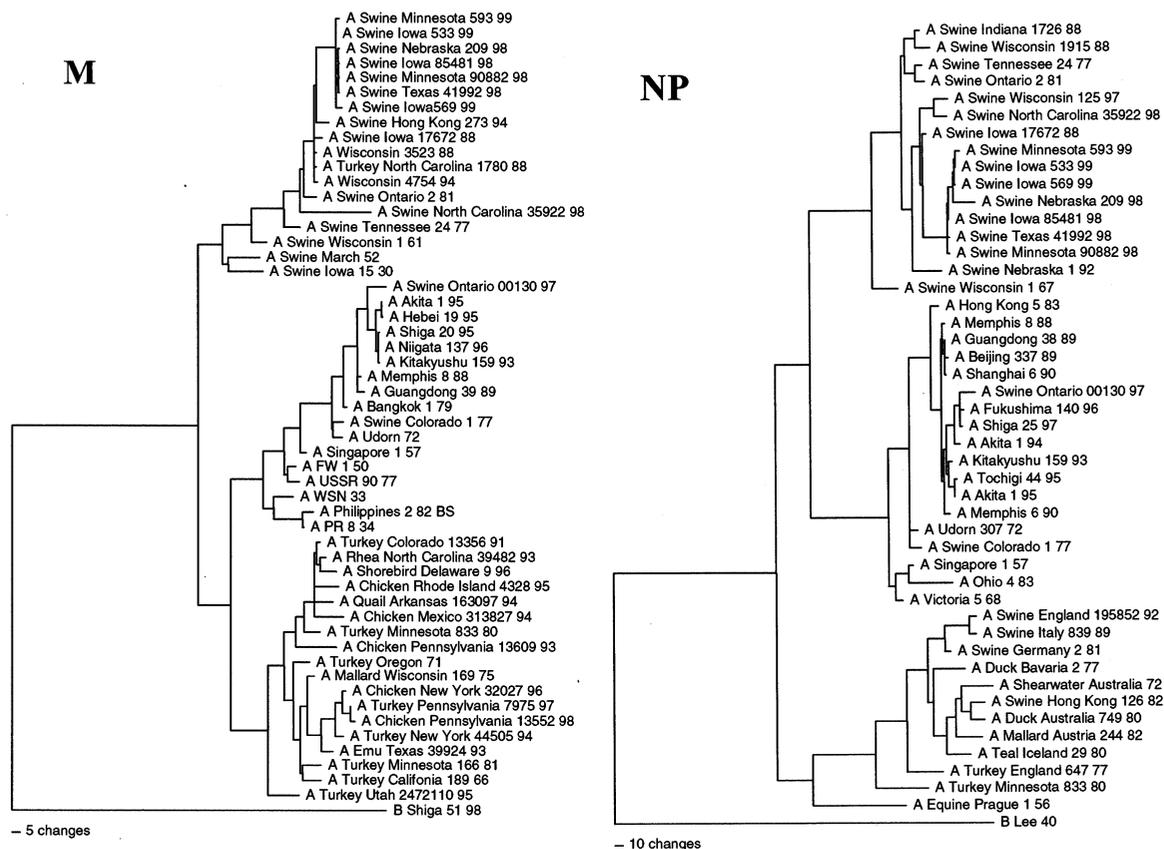


Fig. 2. Nucleotide phylogenetic trees for the M, NP and NS genes of H3N2 influenza viruses isolated from pigs in North America. The evolutionary relationships were estimated and the phylogenetic trees were constructed as described in Section 2 and in the legend for Fig. 1. The scores and number of rearrangements for each tree are as follows: M (score 975/11 077 024 rearrangements), NP (score 2092/482 611 rearrangements) and NS (score 1271/402 217 rearrangements). All three of the trees are rooted to influenza B viruses.

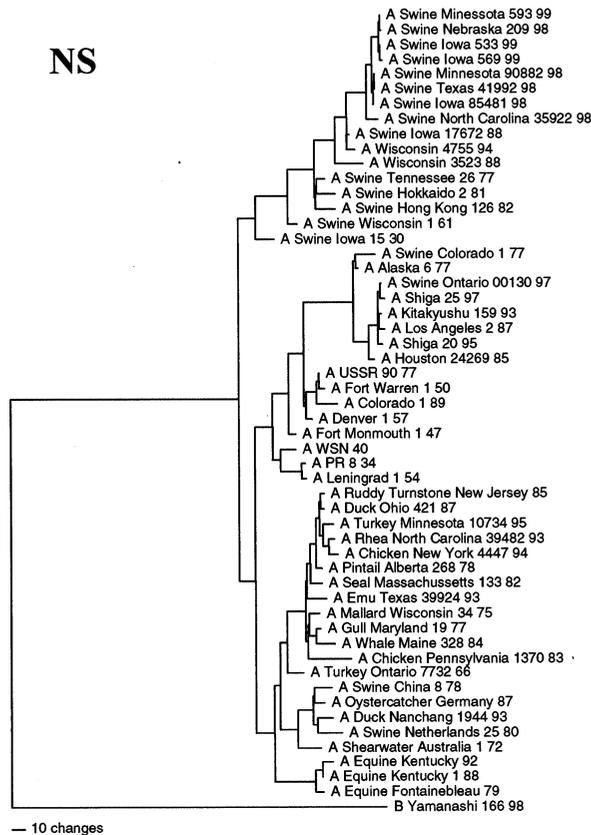


Fig. 2. (Continued)

years of their isolations (Figs. 1–3). In contrast, the viruses isolated from pigs in the U.S. since 1998 are reassortant viruses containing HA, NA and PB1 genes of human influenza virus origin, NP, M and NS genes of classical swine virus origin, and PA and PB2 polymerase genes from avian or swine viruses (Figs. 1–3). As such, it is immediately clear that Sw/ONT/97 was not the direct source for the subsequent H3N2 virus infections in the U.S., despite the fact that it appeared only 14 months earlier in nearby Ontario, Canada.

The H3N2 viruses isolated from pigs in the Midwestern U.S. by Zhou et al. (1999) are of the same human/swine/avian reassortant genotype as that of our four Midwestern viruses. When the gene sequences of all seven of these viruses were examined together, we found that the Midwestern

H3N2 swine viruses share very high degrees of sequence identity with each other. At the amino acid level, the percents identity among these viruses are 98.8–100% for HA, 97.6–100% for NA, 97.0–100% for NP, 95.3–98.2% for NS, 98.1–99% for M, 99.0–100% for PA, 99.5–100% for PB1 and 99.4–100% for PB2. In addition, these viruses consistently form their own phylogenetic clades for each gene segment (Figs. 1–3). Therefore, it is most plausible that these Midwestern H3N2 viruses were all derived from a single reassortant progenitor virus that subsequently spread from state-to-state, rather than as the products of multiple, independent reassortment events. Given the frequent transport of pigs throughout the country, spread of a reassortant progenitor virus could easily have occurred via the movement of infected animals.

Since Sw/NC/98 (Zhou et al., 1999) is a reassortant between only human and swine viruses, whereas the Midwestern swine H3N2 viruses are 3-way human/swine/avian virus reassortants, one might hypothesize that Sw/NC/98 was an intermediary in a sequential process of reassortment. For instance, human and swine viruses might have reassorted to produce a Sw/NC/98-like virus, followed by reassortment between that virus and an avian virus to produce the Midwestern viruses. Our recovery of a 3-way reassortant virus from a pig in Nebraska 5 months prior to the isolation of Sw/NC/98 in North Carolina argues against this, although dates of isolation may not necessarily reflect ancestry. More definitively, however, the results of our phylogenetic analyses indicate that

the human and swine virus progenitors of Sw/NC/98 were distinct from the progenitors of the Midwestern swine H3N2 viruses. The NA, PB1, NP and M phylogenies (Figs. 1 and 2) each demonstrate that Sw/NC/98 diverged from progenitor human (NA, PB1) and swine (NP and M) viruses at different points than the Midwestern clade of viruses. In addition, the sequence identity of Sw/NC/98 to the Midwestern viruses is lower for each of these gene segments (NA/96.3–97.2, NP/97.2–98.8%, M/90.7–91.4% and PB1/98.7–99.0%) than the identity of the Midwestern isolates to each other (see above). Thus, Sw/NC/98 appears to be the result of a completely unique reassortment event that was independent of the reassortment that gave rise to the Midwestern viruses.

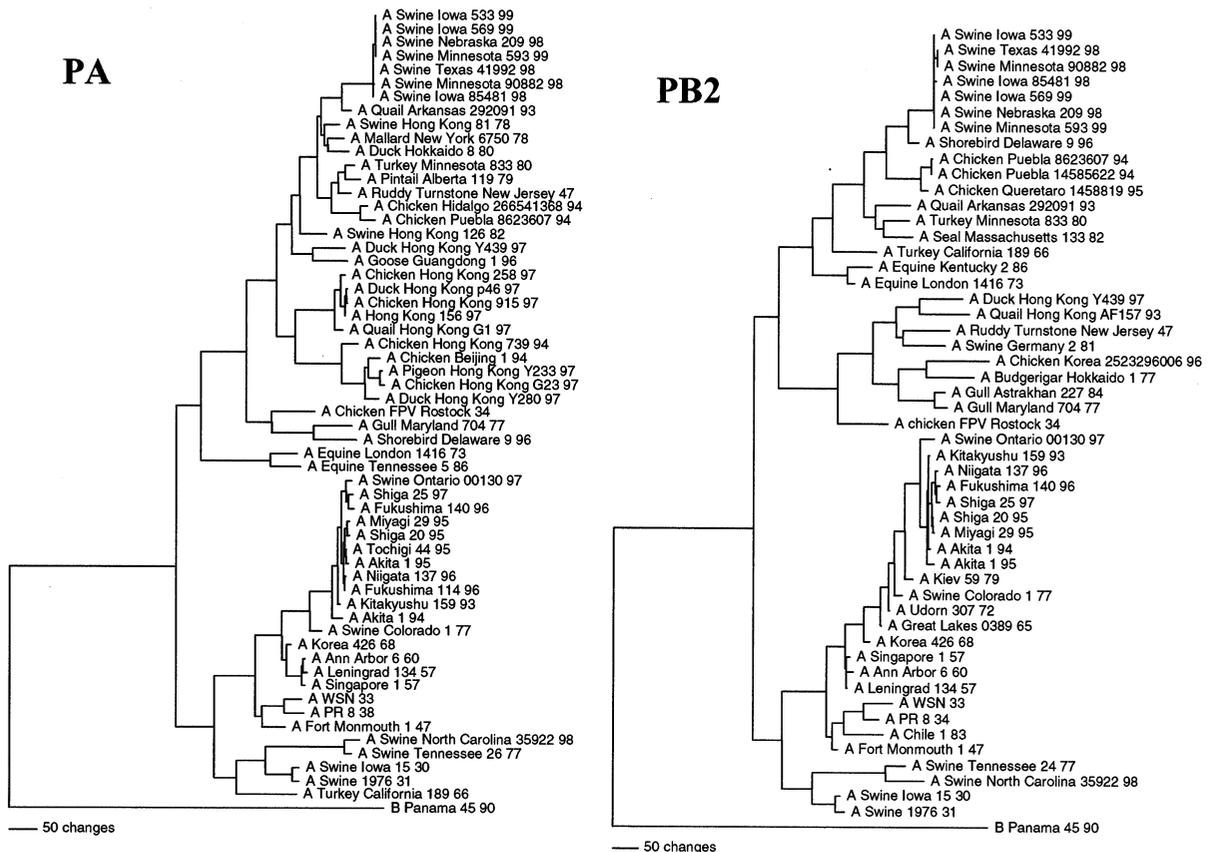


Fig. 3. Nucleotide phylogenetic trees for the PA and PB2 genes of H3N2 influenza viruses isolated from pigs in North America. The evolutionary relationships were estimated and the phylogenetic trees were constructed as described in the Section 2 and in the legend for Fig. 1. The scores and number of rearrangements for each tree are as follows: PA (score 3823/740 428 rearrangements) and PB2 (score 4207/110 568 rearrangements). Both trees are rooted to influenza B viruses.

Table 3

Amino acids in the HA1 proteins that are uniquely different among the triple reassortant H3N2 viruses isolated from pigs in the Midwestern U.S. between March 1998 and March 1999, compared to the 1995 lineage of H3 human viruses

Amino acid position	Swine viruses ^a	Human viruses ^b
88	I ^c	V
106	S ^c	A
124	D ^c	G
135	G	K/T
138	S ^c	A
190	E ^c	D
199	I ^c	S
208	S ^c	R
246	S ^c	N
278	Y ^c	N
307	K ^c	R
312	K ^c	N

^a Analysis includes A/Swine/Iowa/533/99, A/Swine/Iowa/569/99, A/Swine/Minnesota/593/99 and A/Swine/Nebraska/209/98 isolated as part of this study, as well as A/Swine/Texas/4199-2/98, A/Swine/Minnesota/9088-2/98 and A/Swine/Iowa/8548-1/98 characterized by Zhou et al. (1999).

^b Analysis includes A/Wuhan/359/95 and A/Finland/339/95.

^c At each of these residues, A/Swine/Ontario/00130/97 contains the amino acids characteristic of the human viruses.

There is very little genetic variation among the HA genes of the Sw/NB/98, Sw/IA/533/99, Sw/IA/569/99 and Sw/MN/99 viruses that were isolated within a 12-month period of time. The HA1 amino acid sequences of all four of these viruses were identical, and their HA2 sequences differed at only two sites (382/N > D in Sw/MN/99 and 383/R > W in Sw/IA569/98). Additionally, these viruses share a high degree of HA1 sequence identity with the Midwestern H3N2 swine viruses isolated between September and December 1998 by Zhou et al. (1999). The only differences among the later viruses were in A/Swine/Minnesota/9088-2/98 (56/H > Q, 142/E > G, 209/S > N) and A/Swine/Texas/4199-2/98 (142/E > G). These differences are not at sites previously found to be highly variable in H3 HA molecules or related to antigenic drift or host range determination (Bean et al., 1992), nor do they correspond to potential glycosylation sites. It will, however, be of interest to examine rates of antigenic drift and genetic evolution of H3N2 swine viruses that are isolated

over the next several years. By comparison, after avian H1N1 viruses crossed the species barrier to infect pigs in Northern Europe in 1979–1980, they underwent a period of accelerated evolution that persisted through the early 1990s (Ludwig et al., 1995; Scholtissek, 1996). This rapid evolution has been attributed to either the presence of a mutator mutation in their polymerase proteins (Ludwig et al., 1995; Scholtissek, 1996) or positive selection of variants during adaptation to their new host (Stech et al., 1999). The emergence of the reassortant H3N2 viruses in the U.S. provides a second opportunity to examine virus evolution following the introduction of a novel virus into an immunologically naive swine population.

The H3N2 viruses isolated in this study may also offer a unique opportunity to directly address molecular determinants of influenza virus infectivity and replication efficiency in pigs. The triple reassortant H3N2 viruses have spread widely among pigs throughout the Midwestern U.S., whereas the wholly human Sw/ONT/97 virus, although able to cross the species barrier to pigs, did not spread efficiently from pig-to-pig. The Sw/ONT/97 virus was isolated from a single sick pig on one farm. The members of that farm family do not remember whether or not they were sick at the time, but there was no influenza outbreak among the other pigs on the farm. In addition, through November 1999, no additional H3N2 viruses have been isolated from pigs in the province of Ontario at the University of Guelph Animal Health Laboratory. This information strongly supports and extends the hypothesis (Zhou et al., 1999) that the specific constellation of genes present in the reassortant viruses provides a selective advantage for infectivity of pigs. Host–range restriction among influenza viruses is considered to be a polygenic trait and evidence exists for contributions by several genes (Webster et al., 1992; Murphy and Webster, 1996). However, the HA is thought to be a major contributor to host range since it is the viral protein responsible for receptor binding. In this regard, it is of particular interest that the Sw/ONT/97 virus lacks 11 of the 12 amino acid mutations in the HA that differentiate the triple reassortant swine viruses from the 1995 lineage human H3N2 viruses. With

the recent development of powerful new reverse genetics techniques for influenza viruses (Fodor et al., 1999; Neumann et al., 1999), it should be possible to engineer viruses that will enable us to examine the impact of these HA mutations, as well as specific constellations of the other virus genes, on influenza virus infectivity and replication efficiency in pigs.

The widespread appearance of H3N2 influenza viruses among pigs in the U.S. constitutes a significant change in the epidemiology of swine influenza in this country and an antigenic shift from the pre-existing classical H1N1 swine influenza viruses. It is not known whether both H1N1 and H3N2 viruses will continue to co-circulate among American pigs indefinitely, or whether one will eventually come to dominate. It is also not known whether these reassortant H3N2 viruses pose any enhanced threat to human health. Pigs have been shown to serve as reservoirs for older human H3 influenza viruses (Nakajima et al., 1982; Ottis et al., 1982; Mancini et al., 1985; Katsuda et al., 1995). As such, pigs in the U.S. may maintain these reassortant viruses and serve as a source for infection of people in the future. This would be of particular concern for children born after the disappearance of this lineage of H3 viruses from the human population. Finally, it also remains to be determined whether the specific constellation of swine and avian internal protein genes in these viruses will alter their virulence for humans. We have, therefore, begun active surveillance for this reassortant genotype among H3N2 viruses isolated from human beings in the Midwest during the 1999–2000 influenza virus season.

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