Arch Virol (2000) 145: 1399–1419

Archives of Virology

Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States

C. W. Olsen, S. Carey, L. Hinshaw, and **A. I. Karasin**

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin, U.S.A.

Accepted January 21, 2000

Summary. Influenza virus infection in pigs is both an animal health problem and a public health concern. As such, surveillance and characterization of influenza viruses in swine is important to the veterinary community and should be a part of human pandemic preparedness planning. Studies in 1976/1977 and 1988/1989 demonstrated that pigs in the U.S. were commonly infected with classical swine H1N1 viruses, whereas human H3 and avian influenza virus infections were very rare. In contrast, human H3 and avian H1 viruses have been isolated frequently from pigs in Europe and Asia over the last two decades. From September 1997 through August 1998, we isolated 26 influenza viruses from pigs in the north-central United States at the point of slaughter. All 26 isolates were H1N1 viruses, and phylogenetic analyses of the hemagglutinin and nucleoprotein genes from 11 representative viruses demonstrated that these were classical swine H1 viruses. However, monoclonal antibody analyses revealed antigenic heterogeneity among the HA proteins of the 26 viruses. Serologically, 27.7% of 2,375 pigs tested had hemagglutination-inhibiting antibodies against classical swine H1 influenza virus. Of particular significance, however, the rates of seropositivity to avian H1 (7.6%) and human H3 (8.0%) viruses were substantially higher than in previous studies.

Introduction

Influenza is a commonly encountered respiratory disease of pigs throughout the swine-raising regions of the United States. Infections are manifest most commonly as explosive outbreaks of acute respiratory disease with fever, anorexia and weight loss, lethargy, nasal and ocular discharge, coughing and dyspnea [23]. It has been estimated that the clinical signs of influenza in pigs add 2 weeks

to the time that it takes animals to reach market weight (B.C. Easterday, pers. comm.). Therefore, swine influenza may be a substantial economic concern for farmers, and there is growing concern for the impact of synergistic infections with influenza and porcine reproductive and respiratory syndrome viruses [29, 40, 78].

From a public health perspective, influenza virus infections in pigs pose two threats. It is well documented that classical H1N1 swine influenza viruses are zoonotic pathogens. Human infections with swine influenza viruses have been documented in the U.S. [19, 31, 82], Europe [20] and New Zealand [22], including fatal infections [22, 41, 57, 63, 70, 75, 81]. On a broader scale, pigs are susceptible to infection with influenza viruses of both avian and 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 [36]. As such, they have been implicated as the intermediate host for adaptation of avian influenza viruses to mammals [12] and as the "mixing vessels" in which human-avian influenza virus reassortment occurs [64, 65, 80]. The major pandemics of human influenza this century were caused by viruses that were reassortants between pre-existing human and avian viruses [80]. More recently, human-avian influenza virus reassortants have been isolated from commercially-raised pigs in Europe [14] and subsequently from children in the Netherlands [17]. Furthermore, maintenance of older human influenza virus strains in the pig population [3, 39, 49, 51, 54] may allow for re-introduction of antigenic variants back into the human population, and swine influenza viruses may also be transmitted into domestic turkey and wild bird populations [32, 33, 46].

Given the important role that pigs can play in the ecology and evolution of influenza viruses [80], it is critical as part of an overall pandemic preparedness plan to maintain surveillance over the nature of influenza viruses circulating among pigs [71, 79]. Previous serologic surveillance studies conducted during 1976/1977 [31] and 1988/1989 [16] demonstrated that influenza virus infections were common among pigs in the north-central portion of the United States, with seropositivity rates against classical swine H1N1 viruses of 20–47% in 1976/1977 and 51% in 1988/1989. In contrast, serologic evidence of H3 influenza virus exposure was remarkably lower in both studies (1.4% in 1976/1977 and 1.1% in 1988/1989). In 1988/1989, sera were also tested for antibodies to an avian virus, A/Duck/Alberta/16/87, but none of the 2,337 samples tested contained detectable antibodies to this virus.

These surveillance studies clearly demonstrated that classical swine H1 influenza viruses were the predominant subtype circulating among pigs in the United States from 1976 through 1989. Nonetheless, variant H1 viruses have been isolated subsequently from pigs in North America and influenza viruses of other subtypes have been isolated from pigs in Europe and Asia. An H1N1 swine influenza virus with an antigenically and genetically unique hemagglutinin (HA) was isolated in Nebraska in 1992 [53] and a novel H1N1 influenza virus was associated with atypical proliferative and necrotizing pneumonia among pigs in Quebec in 1991 [21, 60]. Outside of North America, avian-like H1N1 viruses

became the predominant influenza virus among pigs on the European continent [58, 66] and avian H1 viruses were also isolated from pigs in the United Kingdom [7, 10] and Asia [30]. A variety of reassortant influenza viruses have also been isolated from pigs. Reassortant H1N2 viruses were isolated from pigs in France in 1987 and 1988 [28], in Japan in 1978 [52] and 1989/1990 [55], and in the United Kingdom since 1994 [6, 8]. In addition, an H1N7 virus containing an HA gene most closely related to human H1 viruses and an NA gene most similar to equine N7 viruses was isolated from pigs in the United Kingdom in 1992 [9], and human/swine H3N2 reassortant viruses have been isolated in southern China [69].

Given the wide variety of influenza viruses that have been isolated from pigs around the world during recent years, we sought to determine whether there have also been changes in the nature of the viruses infecting pigs in the United States since the last large-scale surveillance study was conducted in 1988/1989. In this paper, we report the results of a year-long (September 1997–August 1998) virologic and serologic evaluation of influenza virus infections among pigs in the north-central United States. We specifically addressed the hypotheses that antigenic variants of swine H1N1 influenza viruses were circulating among pigs in the United States and that pigs in the United States were being exposed to human H3 and avian influenza viruses to a greater degree than in the past.

Materials and methods

Reference viruses

Three influenza viruses were used as reference strains for serologic testing during this study. A classical swine influenza virus, A/Swine/Indiana/1726/88 (Sw/IND) (H1N1), and an avian H1 virus, A/Duck/Alberta/35/76 (Dk/ALB) (H1N1), were kindly provided by Dr. V. Hinshaw from the Influenza Virus Repository of the University of Wisconsin-Madison. A human H3N2 influenza virus representative of the viruses circulating among people in the U.S. during the two years prior to our study, A/Wuhan/359/95 (A/WUH) (H3N2), was kindly provided by the Influenza Branch of the Centers for Disease Control and Prevention, Atlanta, Georgia.

Sample collections

A total of 2,375 serum samples were obtained from two sources over the period from September 1, 1997 through August 31, 1998. One thousand, one hundred and seventy five samples were selected randomly (approximately 100 samples/month) from sera submitted to the Wisconsin Animal Health Laboratory (Madison, WI) for pseudorabies virus testing. One thousand two hundred samples (50 samples approximately every 2 weeks) were collected from pigs at the time of slaughter at a commercial abattoir. Samples of nasal secretions were collected from these same pigs at slaughter for virus isolation. Dacron swabs were inserted into the nasal passages of the pigs immediately after stunning, but before exsanguination. Swabs were placed in viral transport media (50% glycerol in phosphate-buffered saline [PBS] containing 1000 units Penicillin G, 200 μg streptomycin, 50 units nystatin and 40 μg gentamicin per ml) and maintained at 4 °C overnight until inoculated into eggs for virus isolation. The abattoir at which the samples were collected obtained pigs from southwest Wisconsin, northeast Iowa

and northwest Illinois. However, because it was not possible to trace the origin of each pig sampled, all viruses have simply been designated as Wisconsin isolates.

Virus isolation and antigenic and genetic characterizations

Nasal swab samples were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and virus growth was detected by hemagglutination assay [56] on the allantoic fluid following 3 days of culture at 35 °C. Influenza viruses were identified and subtyped by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays [56] using H1-, H3-, N1- and N2-monospecific sera. The HA proteins of the isolates and H1 reference strains were characterized antigenically by HI assay using a panel of 4 monoclonal antibodies (Mabs) previously shown to recognize 4 epitopes in 3 antigenic sites on swine H1 HA molecules [48, 67]. These assays were conducted using serial 2-fold dilutions (1:100 to 1:204,800) of Mabs in PBS. The monospecific sera and Mabs were kindly provided by Dr. V. Hinshaw, University of Wisconsin-Madison.

The full-length HA genes of 11 isolates representative of each Mab-defined antigenic pattern, the full-length nucleoprotein (NP) genes of these isolates, and the HA and NP genes of our working stock of Sw/IND were amplified by RT-PCR using AMV reverse transcriptase (Promega Corporation, Madison, WI) and Pfu polymerase (Stratagene, La-Jolla, CA). Amplifications were carried out as suggested by the manufacturers, except that the RT reactions were conducted using 1 μg of primer per reaction and reaction conditions of 48.5 °C for 45 min. The HA genes were amplified using primers specific for nucleotides 1-21/forward (5′-AGCAAAAGCAGGGGAAAATAA-3′) and 1747–1771/reverse (5′-CAAGGGTGTTTTTTCTCATGTCTC-3′). The NP genes were amplified using primers specific for nucleotides 1-21/forward (5′-GCAGGGTAGATAATCACTCAC-3′) and 1533–1557/reverse (5′-CAAGGGTATTTTTCTTTAATTGTC-3′) (for isolates 125, 136, 163, 164, 166, 168, 235) or the SZANP+ (5′-CTCGAGAGCAAAAGCAGGGT-3′) and SZANP-(5′-AGTAGAAACAAGGGTATTTTTC-3′) primers of Zou [85] (for isolates 238, 457, 458 and 464). (The later NP genes could not be amplified using the 1–21 and 1533–1557 NP primers used for the other isolates, presumably because of minor sequence differences detected in the 5′ and 3′ non-coding regions of the genes.)

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 conducted using the Multiple Alignment Construction & Analysis Workbench program (Version 2.0.5, Win32I). The phylogenetic relationships among the sequenced virus isolates and selected reference strains were estimated by the method of maximum parsimony (PAUP, Version 4.0b2, Dr. David Swofford, Smithsonian Institution), using the tree-bisection-reconnection branch swapping algorithm and with the MULTREES option in effect. The GenBank accession numbers for the reference virus sequences used in the phylogenetic analyses are listed in Table 1.

Serologic testing

The 2,735 serum samples were tested by HI assay [56] for the presence of antibodies recognizing 3 reference viruses: Sw/IND (swine H1); Dk/ALB (avian H1); and, A/WUH (human H3). Prior to conducting the assays, the serum samples were treated with receptor-destroying enzyme (RDE) (Denka Siken Company, Tokyo) at 37 °C for 18 h, followed by heat inactivation at 56 °C for 30 min. All sera were screened at a dilution of 1:40. Positive and negative serum controls were included with each set of sera tested. In addition, each serum sample was tested against chicken RBCs in the absence of virus to rule out induction of non-specific hemagglutination.

Table 1. Reference virus gene sequences employed in phylogenetic analyses of the HA and NP genes of the H1N1 swine influenza viruses isolated during this study

Virus	GenBank accession no.	Ref.
HA genes		
A/USSR/90/77	K01330	[18]
A/Taiwan/1/86	D00407	[61]
A/Bayern/7/95	n.a.[a]	[34]
A/Wuhan/359/95	AF038268	[44]
A/Swine/Iowa/15/30	X57492	[73]
A/Swine/New Jersey/11/76	K00992	[4]
A/Swine/Ehime/1/80	X57494	[73]
A/Swine/Germany/2/81[b]	Z30276	[46]
A/Swine/QC/81	U03720	[60]
A/Swine/Indiana/1726/88	M81707	[48]
A/Swine/QC/91	U03719	[60]
A/Swine/Germany/8533/91[b]	Z46434	[47]
A/Swine/England/195852/92[b]	U72667	[10]
A/Swine/Nebraska/1/92	L09063	[53]
A/Swine/England/283902/93	U72668	[10]
A/Duck/Alberta/35/76	D10477	[2]
A/Duck/Hong Kong/196/77	D00839	[37]
A/Duck/Wisconsin/1938/80	L25071	[35]
NP genes		
A/Singapore/1/57	M63752	[26]
A/Victoria/5/68	M63753	[26]
A/Udorn/307/72	M14922	[11]
A/Hong Kong/5/83	M22577	[25]
A/Ohio/4/83	M59334	[62]
A/Memphis/8/88	L07370	[68]
A/Beijing/337/89	L07374	[68]
A/Guangdong/38/89	L07373	[68]
A/Shanghai/6/90	L07357	[68]
A/Swine/Wisconsin/1/67	M76607	[1]
A/Swine/Tennessee/24/77	M30748	[27]
A/Swine/Germany/2/81[b]	M22579	[24]
A/Swine/Ontario/2/81	M63767	[26]
A/Swine/Hong Kong/126/82[b]	M63771	[26]
A/Swine/Indiana/1726/88	L46849	[50]
A/Swine/Iowa/17672/88	M63768	[26]
A/Swine/Wisconsin/1915/88	M76608	[1]
A/Swine/Italy/839/89[b]	M63772	[26]
A/Swine/England/195852/92[b]	L40332	[10]
A/Swine/Nebraska/1/92	L11164	[53]
A/Shearwater/Australia/72	M27298	[24]

Table 1 (*continued*)

Virus	GenBank accession no.	Ref.
A/Duck/Bavaria/2/77	M22574	[25]
A/Turkey/England/647/77	M76603	[1]
A/Turkey/Minnesota/833/80	M30769	[27]
A/Duck/Australia/749/80	M63783	[26]
A/Teal/Iceland/29/80	M63784	[26]
A/Mallard/Astrakhan/244/82	M30764	[27]
A/Equine/Prague/1/56	M63748	[26]
B/Lee/40	K01395	[5]

[a] *n.a.* Not available – This sequence has not been deposited in GenBank

[b] These strains are avian-like swine viruses

GenBank accession numbers

The GenBank accession numbers for the HA genes sequenced as part of this study are as follows: A/Swine/WI/125/97 (AF222026), A/Swine/WI/136/97 (AF222027), A/Swine/WI/163/97 (AF222028), A/Swine/WI/164/97 (AF222029), A/Swine/WI/166/97 (AF222030), A/Swine/WI/168/97 (AF222031), A/Swine/WI/235/97 (AF222032), A/Swine/WI/238/97 (AF222033), A/Swine/WI/457/98 (AF222034), A/Swine/WI/458/98 (AF222035), A/Swine/WI/464/98 (AF222036). The GenBank accession numbers for the NP genes sequenced as part of this study are as follows: A/Swine/WI/125/97 (AF222768), A/Swine/WI/136/97 (AF222769), A/Swine/WI/163/97 (AF222770), A/Swine/WI/164/97 (AF222771), A/Swine/WI/166/97 (AF222772), A/Swine/WI/168/97 (AF222773), A/Swine/WI/235/97 (AF222774), A/Swine/WI/238/97 (AF222775), A/Swine/WI/457/98 (AF222776), A/Swine/WI/458/98 (AF222777), A/Swine/WI/464/98 (AF222778).

Results

Virus isolation rates

A total of 26 influenza viruses were isolated during the course of this study, giving an overall rate of virus recovery of 2.2% of the pigs sampled. However, a distinct seasonal pattern was noted, with a substantially higher rate of virus shedding during the fall and early winter months of the year. Specifically, 24 of the 26 isolates were obtained between October and January, with virus shedding rates of up to 16% of the pigs tested during this time period.

Antigenic analysis of the H1 virus isolates

All of the virus isolates were defined as H1N1 subtype viruses by HI and NI assays. However, reactivity by HI assay with a panel of 4 H1-specific Mabs differed substantially among the isolates. Using a greater than 4-fold difference (either decrease or increase) in HI titer to conservatively define variations compared to our prototype classical H1 swine virus, Sw/IND, 7 different reactivity patterns were evident (Table 2). Despite this antigenic variability, however, all of the viruses reacted to the same titer as Sw/IND (1:512) with polyclonal sera collected

Table 2. Hemagglutination-inhibition titers of four H1 HA Mabs against the H1N1 swine influenza viruses isolated during this study and reference avian, human and swine H1 viruses

Virus	Mab 2-15F1	Mab 7B1b	Mab 1-6B2	Mab 3F2c
H1N1 reference viruses				
A/Swine/Indiana/1726/88	12,800	102,400	102,400	400
A/Duck Alberta/35/76	***<100***	25,600	102,400	***<100***
A/Bayern/7/95	***<100***	***<100***	***<100***	***<100***
H1N1 viruses isolated during this study				
A/Swine/Wisconsin/125,127, 129,130,134,135,136,137/97	12,800–51,200	***1,600–3,200***	102,400–204,800	200–1,600
A/Swine/Wisconsin/163/97	***1,600***	***12,800***	25,600	***6,400***
A/Swine/Wisconsin/164/97	***1,600***	25,600	***<100***	***204,800***
A/Swine/Wisconsin/168/97	3,200	25,600	204,800	***204,800***
A/Swine/Wisconsin/166, 167,246/97 A/Swine/Wisconsin/303,1005/98	12,800– 51,200	102,400– 204,800	204,800	***204,800***
A/Swine/Wisconsin/238/97	***204,800***	204,800	204,800	***204,800***
A/Swine/Wisconsin/235,247/97	***1,600***	204,800	204,800	***25,600– 204,800***
A/Swine/Wisconsin/457,458, 460,463,464,470,1003/98	***400–800***	51,200– 102,400	51,200– 204,800	***25,600– 204,800***

Greater than 4-fold differences (decreases or increases) in HI titer compared to Sw/IND are indicated in bold, italic font. Dashed lines separate groups of viruses with different Mab profiles as defined by those differences

from pigs that had received 2 doses of the commercially-available H1N1 influenza virus vaccine for pigs (MaxiVac-Flu, Syntrovet, Lenexa, KS, serial #07205) as part of a previous experimental study [43].

Genetic analyses of the HA genes of the virus isolates

Eleven isolates, representative of each of the Mab-defined antigenic profiles, were chosen for genetic analyses. The full-length HA genes of these viruses were sequenced and their deduced amino acid sequences are presented in Fig. 1 in comparison to our reference swine H1 influenza virus, Sw/IND. Pairwise sequence analysis of the HA1 segments indicated that all 11 HA genes are more closely related to the HA of a classical swine H1 virus, Sw/IND (95–97% nucleotide identity and 95–98% amino acid identity), then the HAs of a recent human H1 virus, A/Bayern/7/95 (74% nucleotide identity and 71–72% amino acid identity),

```
A/SW/IN/1726/88    1 MKAILLVLLYTFTAANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDRHNGKLCK  60
A/SW/WI/125/97       -----V-------T-----------------------------------N--------
A/SW/WI/136/97       -----V-------T-----------------------------------N--------
A/SW/WI/163/97       ------------------------------------------------------------
A/SW/WI/164/97       ------------------------------------------------------------
A/SW/WI/166/97       ------------------------------------------------------------
A/SW/WI/168/97       ------------------------------------------------------------
A/SW/WI/235/97       -----V-------T------------------------------------------------
A/SW/WI/238/97       -----V-------T------------------------------------------------
A/SW/WI/457/98       ------------------------------------------------------------
A/SW/WI/458/98       -----V----A--T------------------------------------------------
A/SW/WI/464/98       -----V----A--T------------------------------------------------

A/SW/IN/1726/88   61 LRGVAPLHLGKCNIAGWLLGNPECELLFTASSWSYIVETSNSDNGTCYPGDFINYEELRE 120
A/SW/WI/125/97       -K------------------------------------------------------------
A/SW/WI/136/97       -K------------------------------------------------------------
A/SW/WI/163/97       ---I--------------------------------------------------------
A/SW/WI/164/97       ---I--------------------------------------------------------
A/SW/WI/166/97       ---I--------------------------------------------------------
A/SW/WI/168/97       ---I--------------------------------------------------------
A/SW/WI/235/97       -K-I--------------------------------------------------------
A/SW/WI/238/97       -K-I--------------------------------------------------------
A/SW/WI/457/98       ---I--------------------------------------------------------
A/SW/WI/458/98       -K------------------------------------------------------------
A/SW/WI/464/98       -K------------------------------------------------------------

A/SW/IN/1726/88  121 QLSSVSSFERFEIFPKASSWPNHETNRGVTAACPYAGANSFYRNLIWLVKKGNSYPKLSK 180
A/SW/WI/125/97       ----------------N------------S------------------------------
A/SW/WI/136/97       ----------------N------------S------------------------------
A/SW/WI/163/97       ------------------------------------------------------------
A/SW/WI/164/97       --------------------D---------------------------------------
A/SW/WI/166/97       ------------------------------------------------------------
A/SW/WI/168/97       ------------------------------------------------------------
A/SW/WI/235/97       ----------------------------S-------------------------------
A/SW/WI/238/97       ----------------------------S-------------------------------
A/SW/WI/457/98       ------------------------------------------------------------
A/SW/WI/458/98       ----------------------------S----S--------------------------
A/SW/WI/464/98       ----------------------------S----S--------------------------

A/SW/IN/1726/88  181 SYVNNKEKEVLVLWGIHHPPTSTDQQSLYQNADAYVFVGSSKYNKKFKPEIATRPKVRGQ 240
A/SW/WI/125/97       --I---G--------------N-------------------------M------------
A/SW/WI/136/97       --I---G--------------N-------------------------M------------
A/SW/WI/163/97       --I-------------------------------------------------S-------
A/SW/WI/164/97       --I-------------------------------------------------S-------
A/SW/WI/166/97       --I-------------------------------------------------S-------
A/SW/WI/168/97       --I-------------------------------------------------S-------
A/SW/WI/235/97       --I---G-K--------------------------------------M------------
A/SW/WI/238/97       --I---G-K--------------------------------------M------------
A/SW/WI/457/98       --I-------------------------------------------------S-------
A/SW/WI/458/98       --I---G----------------------------------------M------------
A/SW/WI/464/98       --I---G----------------------------------------M------------

A/SW/IN/1726/88  241 AGRMNYYWTLVEPGDTITFEATGNLVVPRYAFAMKRGSGSGIIISDTPVHDCNTTCQTPK 300
A/SW/WI/125/97       ----------I--------------A-------E------------------A------
A/SW/WI/136/97       ----------I--------------A-------E------------------A------
A/SW/WI/163/97       ----D-------------------------------------------------------
A/SW/WI/164/97       ----D-------------------------------------------------------
A/SW/WI/166/97       ----D-------------------------------------------------------
A/SW/WI/168/97       ----D-------------------------------------------------------
A/SW/WI/235/97       ----------I----------------------E--Y----------------------
A/SW/WI/238/97       ----------I----------------------E--Y----------------------
A/SW/WI/457/98       ----D-------------------------------------------------------
A/SW/WI/458/98       ----------I--------------A-------E--------------Y----A-----
A/SW/WI/464/98       ----------I--------------A-------E--------------Y----A-----

A/SW/IN/1726/88  301 GAINTSLPFQNIHPVTIGECPKYVKSTKLRMATGLRNIPSIQSRGLFGAIAGFIEGGWTG 360
A/SW/WI/125/97       -----N--------------------R--------------------------------
A/SW/WI/136/97       -----N--------------------R--------------------------------
A/SW/WI/163/97       ------------------------------------------------------------
A/SW/WI/164/97       ------------------------------------------------------------
A/SW/WI/166/97       ------------------------------------------------------------
A/SW/WI/168/97       ------------------------------------------------------------
A/SW/WI/235/97       ------------------------------------------------------------
A/SW/WI/238/97       ------------------------------------------------------------
A/SW/WI/457/98       ------------------------------------------------------------
A/SW/WI/458/98       ------------------------------------------------------------
A/SW/WI/464/98       ------------------------------------------------------------
```

```
A/SW/IN/1726/88  361 MIDGWYGYHHQNEQGSGYAADRKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNHLEKR 420
A/SW/WI/125/97       ------------------------------------------------------------
A/SW/WI/136/97       ------------------------------------------------------------
A/SW/WI/163/97       --------------------------------V---------------------------
A/SW/WI/164/97       --------------------------------V---------------------------
A/SW/WI/166/97       --------------------------------V---------------------------
A/SW/WI/168/97       --------------------------------V---------------------------
A/SW/WI/235/97       ------------------------------------------------------------
A/SW/WI/238/97       ------------------------------------------------------------
A/SW/WI/457/98       --------------------------------V---------------------------
A/SW/WI/458/98       ------------------------------------------------------------
A/SW/WI/464/98       ------------------------------------------------------------

A/SW/IN/1726/88  421 IENLNKKVDDGFLDVWTYNAELLVLLENERTLDYHDSNVKNLYEKVRSQLKNNAKEIGNG 480
A/SW/WI/125/97       -K----------------------------------------------R---------
A/SW/WI/136/97       ------------------------------------------------R---------
A/SW/WI/163/97       ------------------------------------------------------------
A/SW/WI/164/97       ------------------------------------------------------------
A/SW/WI/166/97       ------------------------------A-----------------------------
A/SW/WI/168/97       ------------------------------------------------------------
A/SW/WI/235/97       ------------------------------------------------------------
A/SW/WI/238/97       ------------------------------------------------------------
A/SW/WI/457/98       ------------------------------------------------------------
A/SW/WI/458/98       -------------N----------------------------------------------
A/SW/WI/464/98       -------------N----------------------------------------------

                                        *
A/SW/IN/1726/88  481 CFEFYHKCDDTCMESVKNGTYDYPNYSEESKLNREEIDGVKLESTRIYQILAIYSTVASS 540
A/SW/WI/125/97       ---------------I-------------A--T-----------------------I---
A/SW/WI/136/97       ---------------I-------------A--T-----------------------I---
A/SW/WI/163/97       ------------------------------------------------------------
A/SW/WI/164/97       ------------------------------------------------------------
A/SW/WI/166/97       ------------------------------------------------------------
A/SW/WI/168/97       ------------------------------------------------------------
A/SW/WI/235/97       ------------------------------A---------------------------I---
A/SW/WI/238/97       ------------------------------A---------------------------I---
A/SW/WI/457/98       ------------------------------------------------------------
A/SW/WI/458/98       ------------------------------AR--------------------------I---
A/SW/WI/464/98       ------------------------------AR--------------------------I---

A/SW/IN/1726/88  541 LVLSVSLGAISFWMCSNGSLQCRICI 566
A/SW/WI/125/97       ---L----------------------
A/SW/WI/136/97       ---L----------------------
A/SW/WI/163/97       -I-L----------------------
A/SW/WI/164/97       -I-L----------------------
A/SW/WI/166/97       -I-L----------------------
A/SW/WI/168/97       -I-L----------------------
A/SW/WI/235/97       ---L----------------------
A/SW/WI/238/97       ---L----------------------
A/SW/WI/457/98       -I-L----------------------
A/SW/WI/458/98       ---L----------------------
A/SW/WI/464/98       ---L----------------------
```

Fig. 1. Multiple sequence alignment (Multiple Alignment Construction & Analysis Workbench program, Version 2.0.5, Win32I) of the predicted HA amino acid sequences of 11 H1N1 influenza viruses isolated from pigs in the north-central United States, September 1997 to August 1998, compared to a reference classical swine H1 virus, Sw/IND. Amino acid mutations in each isolate compared to Sw/IND are shown, whereas dashed lines indicate regions of sequence identity to Sw/IND. (* When the HA gene of the Sw/IND virus stock used for this study was sequenced, a single nucleotide difference from the sequence present in GenBank was noted, predicting an amino acid change from N>K at position 505.)

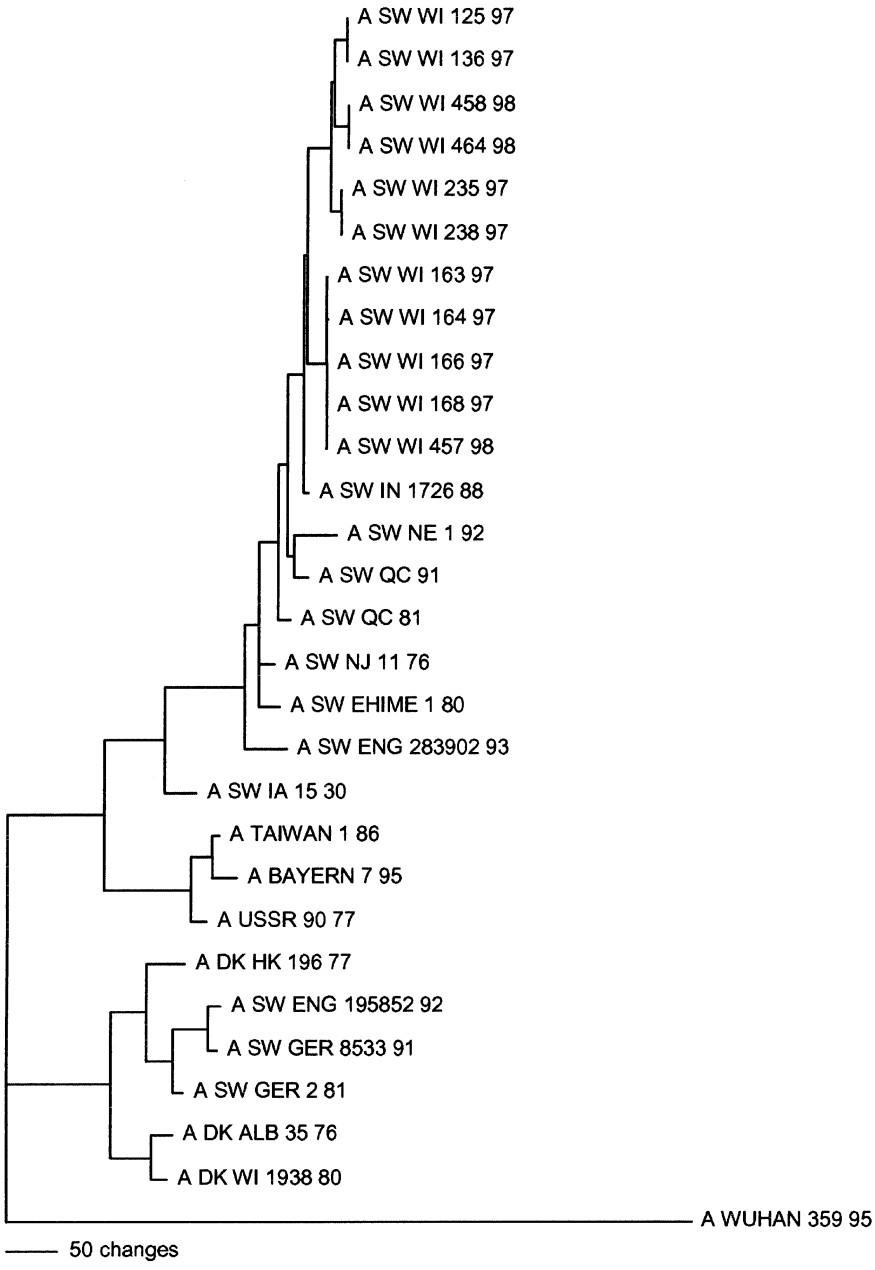

Fig. 2. Phylogenetic tree of the HA gene nucleotide sequences of 11 H1N1 influenza viruses isolated from pigs in the north-central United States, September 1997 to August 1998, compared to selected swine, human and avian reference strains. The tree was generated by the method of maximum parsimony (PAUP, Version 4.0b2, Dr. David Swofford, Smithsonian Institution), using the tree-bisection-reconnection branch swapping algorithm and with the MULTREES option in effect. The tree shown represents the best (score 1,717) of 9,737 rearrangements tried. Horizontal line distances are proportional to the minimum number of nucleotide changes needed to join nodes and HA sequences. The vertical lines are simply for spacing branches and labels. The tree is rooted to the HA gene of A/Wuhan/359/95 (H3N2). The GenBank accession numbers for the HA genes of the viruses isolated during this study are listed in the Materials and methods. The accession numbers for the reference virus HA sequences used in this analysis are shown in Table 1

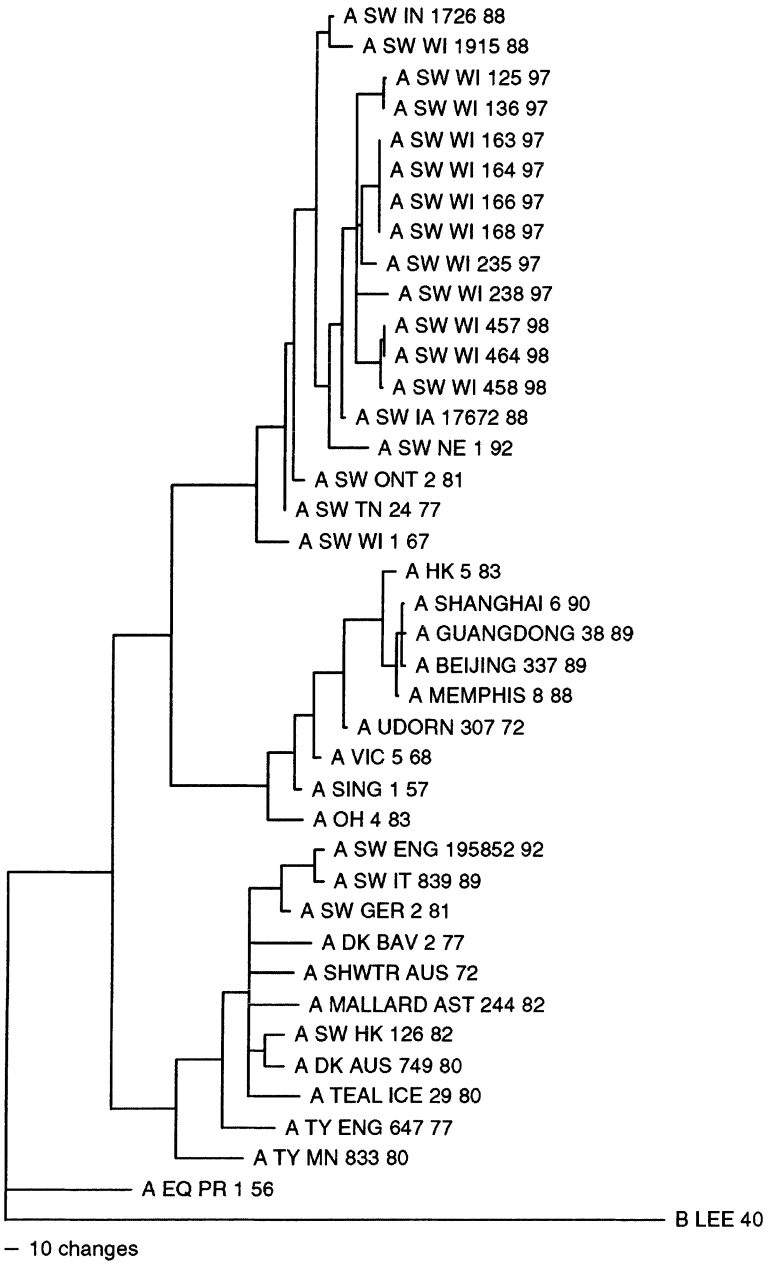

Fig. 3. Phylogenetic tree of the NP gene nucleotide sequences of 11 H1N1 influenza viruses isolated from pigs in the north-central United States, September 1997 to August 1998, compared to selected swine, human and avian reference strains. The tree was generated by the method of maximum parsimony (PAUP, Version 4.0b2, Dr. David Swofford, Smithsonian Institution), using the tree-bisection-reconnection branch swapping algorithm and with the MULTREES option in effect. The tree represents the best (score 1970) of 382,645 rearrangements tried. Horizontal line distances are proportional to the minimum number of nucleotide changes needed to join nodes and NP sequences. The vertical lines are simply for spacing branches and labels. The tree is rooted to the NP gene of B/Lee/40. The GenBank accession numbers for the NP genes of the viruses isolated during this study are listed in the Materials and methods. The accession numbers for the reference virus NP sequences used in this analysis are shown in Table 1

or an avian H1 virus, Dk/ALB (76–77% nucleotide identity and 76–77% amino acid identity). The swine virus origin of these HA genes is further supported by the results of their phylogenetic analysis (Fig. 2). The HA gene phylogenetic tree clearly shows that all 11 isolates evolutionarily segregate with the classical swine H1 viruses, and are distinct from human, avian and avian-like H1 swine viruses. (Note: Despite the fact that the sequences of the full-length HA genes were determined for the viruses isolated during this study, these genetic analyses included only the HA1 sequences, because HA2 sequence information was not available in GenBank for most of the reference strains.)

Genetic analyses of the NP genes of the virus isolates

To provide additional information regarding the genetic background of these viruses, their full-length NP gene sequences were also determined. Nucleotide and amino acid pairwise sequence comparisons indicated that the NP genes of each of the 11 viruses are more closely related to the NP of a classical swine influenza virus, Sw/IND (95–97% nucleotide identity and 98–99% amino acid identity), than the NPs of a human virus, A/Ohio/4/83 (85% nucleotide identity and 90–91% amino acid identity), or an avian virus, A/Duck/Australia/749/80 (82–83% nucleotide identity and 95% amino acid identity). The swine virus origin of these NP genes was also confirmed by phylogenetic analysis (Fig. 3).

Serologic surveillance

We used a conservative HI titer cut-off of 1:40 to define seropositivity, as was done in previous swine influenza virus serosurveys [16, 31]. Based on this definition, 27.7% of samples tested positive for antibodies to the swine H1 virus, Sw/IND. The rate of seropositivity against the human H3 virus, A/WUH, was 8.0%, while 7.6% of the samples tested positive for antibodies against the avian H1 virus, Dk/ALB (Table 3). When examined temporally, seropositivity followed a similar pattern to that of virus isolation, with peak rates of seropositivity against all 3 reference viruses occurring between November and January. However, it is

Table 3. Results of hemagglutination-inhibition assays of swine sera against classical swine H1, avian H1 and human H3 influenza viruses

Reference virus	% of positive[a] samples
A/Swine/Indiana/1726/88 (classical swine H1N1)	27.7% (657/2,375)
A/Duck/Alberta/35/76 (avian H1N1)	7.6% (180/2,375)
A/Wuhan/359/95 (human H3N2)	8.0% (190/2,375)

[a]Number of positive samples (a positive sample is one reacting in HI assay at a titer = 1:40)

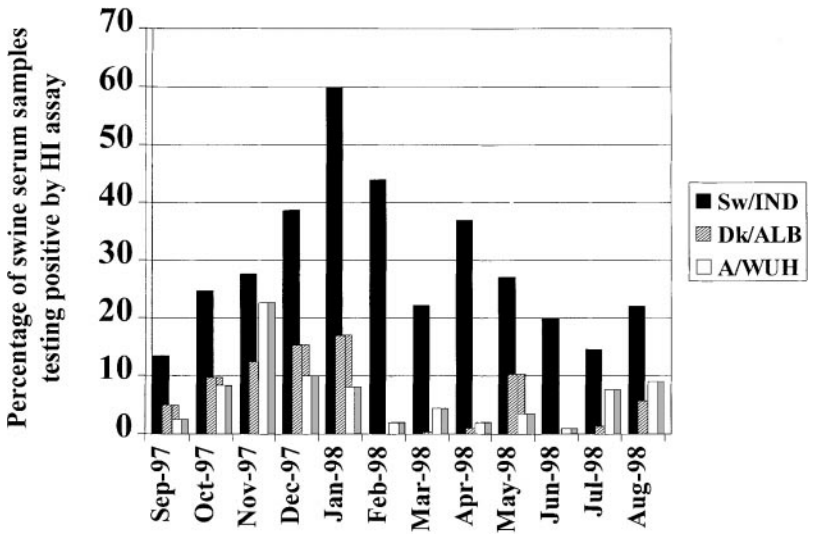

Fig. 4. Temporal distribution of seropositive serum samples by month. Approximately 200 swine serum samples were collected each month and tested by HI assay [56] for the presence of antibodies recognizing 3 reference viruses: Sw/IND (classical swine H1N1 virus); Dk/ALB (avian H1N1 virus); and, A/WUH (human H3N2 virus). The percentage of samples that tested positive (reacting in HI assay at a titer = 1:40) is plotted on the Y-axis against the month of collection on the X-axis

important to note that serum samples were positive for HI antibodies against each of the 3 reference viruses during every month of the year (Fig. 4).

Discussion

Influenza in pigs poses public health concerns because of the zoonotic nature of swine influenza viruses [19, 20, 22, 31, 41, 57, 63, 70, 75, 81, 82], as well as the potential for pigs to serve as hosts for the adaptation of avian viruses to mammals [12] and for reassortment of mammalian and avian influenza viruses [64, 65, 80]. Data concerning the nature of influenza viruses circulating among pigs may, therefore, provide important sentinel information in surveillance for novel strains of influenza viruses in the human population. Additionally, regular and continual antigenic and genetic characterization of swine influenza viruses will help the veterinary community determine when it is necessary to update swine influenza virus vaccines with contemporary antigenic variant strains. Effective vaccination of pigs against influenza virus infection will reduce both morbidity among pigs and the potential for pigs to serve as a reservoir of influenza viruses for humans.

In this paper, we provide antigenic and genetic information on a series of recent H1N1 swine influenza viruses, as well as serologic evidence of human, avian and swine influenza virus infections among pigs in the north-central United States in 1997–1998. The 26 influenza viruses isolated from pigs at slaughter were all H1N1 viruses. This is consistent with previous virologic and serologic

data [16, 31] indicating that H1N1 viruses have been the predominant influenza viruses among pigs in the United States for many years. Antigenic differences in the HA proteins of these viruses compared to the classical swine H1 influenza reference strain, Sw/IND, were detected by Mab analysis. In fact, none of the viruses isolated during this study matched the Mab profile of Sw/IND (Table 2). Currently, however, this level of antigenic drift is unlikely to impact the efficacy of the swine influenza virus vaccine that is available in the United States, since all of the viruses reacted with post-vaccinal pig sera to the same titer as the reference swine H1 virus, Sw/IND.

The HA genes of 11 of the viruses in this study, representing each of the 7 Mab-defined antigenic patterns, were sequenced. The nucleotide and deduced amino acid sequences were compared to reference swine, human and avian H1 influenza viruses to determine their percents identity, and the nucleotide sequences were subjected to phylogenetic analysis (Fig. 2). These results indicate that the HA genes of the viruses isolated during this study are clearly from the classical swine H1 influenza virus lineage (Fig. 2). Additionally, the HA gene sequences were analyzed for mutations in potential glycosylation sites. Only a single glycosylation change was identified: mutations in isolates 125 and 136 at amino acid 306 (S>N) alter the motif (N∼P-S/T∼P) for glycosylation at amino acid 304. Finally, the HA sequences were examined for mutations in specific amino acids previously defined as comprising the antigenic sites on the H1 HA [15, 45, 48, 53, 59, 83]. By doing so, the possible genetic bases for some of the antigenic variability observed in the HI assays could be determined. For example, mutation at amino acid 138 can explain the reduced reactivity of isolates 125 and 136 with Mab 7B1b (antigenic site Sa), mutation at amino acid 156 can explain the reduced reactivity of isolates 458 and 464 with Mab 2-15F1 (antigenic site Ca) and mutation at amino acid 142 can explain the lack of reactivity of isolate 164 with Mab 1-6B2 (antigenic site Sa). However, there are no mutations in or topographically near previously defined antigenic sites to explain the other altered Mab reactivity patterns noted. Sequencing of additional H1 isolates in the future and comparison to the sequences reported here may help to further define the amino acid residues that directly or indirectly contribute to each of these Mab epitopes.

The NP of influenza viruses has been suggested to be an important determinant of virus host range [64, 72, 74]. We sequenced the NP genes of our isolates to assess the possibility that these viruses are reassortants with human or avian internal protein genes. However, both pairwise sequence comparisons and phylogenetic analyses (Fig. 3) indicate that the NP genes of our isolates are, like the HA genes, derived from classical swine influenza viruses.

Several aspects of our serologic findings (Table 3 and Fig. 4) deserve comment. Given a 27.7% seropositivity rate against Sw/IND, it is clear that classical swine H1 influenza viruses continue to circulate widely within the pig population of the north-central United States. It is likely that the vast majority of these seropositive pigs had antibodies because of previous infection rather than vaccination. Vaccination of pigs against H1 influenza virus infection is practiced in the

United States, with approximately 9 million doses of vaccine sold nationwide in 1998 (J. McMillen, pers. comm.). However, only about 40% of this vaccine is used in slaughter pigs, the remainder being used in breeding animals (R. Sibbel, pers. comm.). In 1997 [76] and 1998 [77], 92 and 101 million pigs, respectively, went to slaughter in the United States. Therefore, if animals received the recommended 2 doses/animal, and assuming relatively uniform vaccine usage throughout the country, at most only 1.8–2.0% of slaughtered animals would be expected to be seropositive because of vaccination.

Our finding of 7.6% and 8.0% seropositivity rates against avian H1 and human H3 viruses, respectively, indicates that pigs in the north-central United States were exposed to these types of viruses in 1997–1998 to a substantially greater degree than was documented in 1976–1977 [31] and 1988–1989 [16]. The fact that seropositivity to these viruses was detected throughout the year (Fig. 4) and that seropositive samples were obtained from both the slaughterhouse and State Laboratory sample populations (data not shown) indicates that these overall levels of seropositivity cannot be explained by a large-scale outbreak on a single farm. In addition, several factors strongly indicate that seropositivity against the avian and human influenza viruses reflects actual infection of pigs with these virus types, and not simply cross-reactivity in the HI assays with classical swine viruses. First of all, although some serum samples had antibodies against either Dk/ALB or A/WUH and Sw/IND, suggestive of dual infections during a pig's lifetime, many samples reacted only with the avian or human viruses. Specifically, 12% of the sera with antibodies to Dk/ALB and 65% of the samples with antibodies to A/WUH tested negative for antibodies to Sw/IND. Furthermore, these sera that tested positive to Dk/ALB or A/WUH and not to Sw/IND also failed to react in HI assays with representative viruses from each of the groups (Table 2) of recent antigenic variant swine H1 viruses [data not shown]. And conversely, sera from pigs that had been experimentally infected with Sw/IND during a previous experiment [42] were tested and completely lacked cross-reactivity in H1 assays with Dk/ALB and A/WUH. Specifically, swine sera with HI titers of 1:128 to 1:512 against Sw/IND had no detectable reactivity ($<$1:8) with either Dk/ALB or A/WUH.

The finding that 8.0% of the pigs in our study population tested positive serologically to human H3 influenza virus during late 1997 and 1998 is of particular significance. H3-subtype influenza viruses have been detected regularly among pigs in Asia and Europe [12, 13, 49, 51, 54], but infection of pigs in the United States with this subtype has been quite rare in the past. Only 1.4% and 1.1% of pigs in the United States had antibodies to H3 influenza viruses in studies conducted in 1976–1977 [31] and 1988–1989 [16], respectively, and only a single H3 isolate had been reported from pigs in the United States prior to 1998 [31]. However, this pattern changed dramatically in 1998. Although we did not isolate any H3 viruses from our slaughterhouse samples, we did isolate H3N2 viruses from pigs on farms in Nebraska, Iowa and Minnesota beginning in March, 1998 [38], and Zhou and colleagues characterized additional viruses from North Carolina, Texas, Iowa and Minnesota [84]. Genetic analyses of these viruses indicated that their HA and

neuraminidase genes were of human influenza virus origin, while the internal genes were either all of swine virus origin or were a mixture of swine and avian virus genes [38, 84]. We cannot determine from our data whether the H3 seropositivity that we observed in 1997–1998 reflects infection of pigs with these reassortant viruses or infection with wholly human H3 viruses that are likely to have entered the swine population prior to development of the reassortant viruses. Given the current presence of multiple subtypes of influenza viruses among American pigs, the potential exists for the emergence of additional reassortant viruses in this population in the future. Therefore, regular and frequent surveillance of swine influenza viruses should continue as part of an overall approach to the prevention of swine influenza epizootics and human influenza pandemics.

Acknowledgements

The authors thank Drs. Bernard Easterday, Virginia Hinshaw, Yoshihiro Kawaoka, and Diane Larsen from the University of Wisconsin and Dr. Nancy Cox from the CDC for reviewing the manuscript and for many helpful suggestions. We also thank Mr. Kyle Whited and Farmland Foods for generously allowing us to come into their facility every 2 weeks for sample collection, Dr. Robert Dellers and Mr. Jeffrey Gifford of the Wisconsin Animal Health Laboratory, Madison, WI for providing serum samples, and Dr. Lynn Cooper from the CDC for providing helpful NP primer suggestions.

This research was supported by USDA Agricultural Experiment Station Grant WIS04080 (CWO), the University of Wisconsin Graduate School (CWO) and a Merial Summer Student Research Fellowship (SC).

References

1. Altmuller A, Kunerl M, Muller K, Hinshaw VS, Fitch WM, Scholtissek C (1991) Genetic relatedness of the nucleoprotein (NP) of recent swine, turkey, and human influenza A virus isolates. Virus Res 22: 79–87
2. Austin FJ, Kawaoka Y, Webster RG (1990) Molecular analysis of the haemagglutinin gene of an avian H1N1 influenza virus J Gen Virol 71: 2 471–2 474
3. Bikour MH, Frost EH, Deslandes S, Talbot B, Elazhary Y (1995) Persistence of a 1930 swine influenza A (H1N1) virus in Quebec. J Gen Virol 76: 2 539–2 547
4. Both GW, Shi CH, Kilbourne ED (1983) Hemagglutinin of swine influenza virus: a single amino acid change pleiotropically affects viral antigenicity and replication. Proc Natl Acad Sci USA 80: 6 996–7 000
5. Briedis DJ, Tobin MB (1984) Complete nucleotide sequence of the influenza B/Lee/40 virus genome RNA segment 5 encoding the nucleoprotein and comparison with the B/Singapore/222/79 nucleoprotein. Virology 133: 448–455
6. Brown IH, Chakraverty P, Harris PA, Alexander DJ (1995) Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. Vet Rec 136: 328–329
7. Brown IH, Done SH, Spencer YI, Cooley WA, Harris PA, Alexander DJ (1993) Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet Rec 132: 598–602
8. Brown IH, Harris PA, McCauley JW, Alexander DJ (1998) Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in emergence of an H1N2 virus of novel genotype. J Gen Virol 79: 2 947–2 955

9. Brown IH, Hill ML, Harris PA, Alexander DJ, McCauley JW (1997) Genetic characterisation of an influenza A virus of unusual subtype (H1N7) isolated from pigs in England. Arch Virol 142: 1 045–1 050
10. Brown IH, Ludwig S, Olsen CW, Hannoun C, Scholtissek C, Hinshaw VS, Harris PA, McCauley JW, Strong I, Alexander DJ (1997) Antigenic and genetic analyses of H1N1 influenza A viruses from European pigs. J Gen Virol 78: 553–562
11. Buckler-White AJ, Murphy BR (1986) Nucleotide sequence analysis of the nucleoprotein gene of an avian and a human influenza virus strain identifies two classes of nucleoproteins. Virology 155: 345–355
12. Campitelli L, Donatelli I, Foni E, Castrucci MR, Fabiani C, Kawaoka Y, Krauss S, Webster RG (1997) Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology 232: 310–318
13. Castrucci MR, Campitelli L, Ruggieri A, Barigazzi G, Sidoli L, Daniels R, Oxford JS, Donatelli I (1994) Antigenic and sequence analysis of H3 influenza virus haemagglutinins from pigs in Italy. J Gen Virol 75: 371–379
14. Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG (1993) Genetic reassortment between avian and human influenza viruses in Italian pigs. Virology 193: 503–506
15. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W (1982) The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31: 417–427
16. Chambers TM, Hinshaw VS, Kawaoka Y, Easterday BC, Webster RG (1991) Influenza viral infection of swine in the United States 1988–1989. Arch Virol 116: 261–265
17. Claas ECJ, Kawaoka Y, de Jong JC, Masurel N, Webster RG (1994) Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology 204: 453–457
18. Concannon P, Cummings IW, Salser WA (1984) Nucleotide sequence of the influenza virus A/USSR/90/77 hemagglutinin gene. J Virol 49: 276–278
19. Dasco CC, Couch RB, Six HR, Young JF, Quarles JM, Kasel JA (1984) Sporadic occurrence of zoonotic swine influenza virus infections. J Clin Microbiol 20: 833–835
20. de Jong JC, Paccaud MF, de Ronde-Verloop FM, Huffels NH, Verwei C, Weijers TF, Bangma PJ, van Kregten E, Kerckhaert JAM, Wicki F, Wunderli W (1988) Isolation of swine-like influenza A (H1N1) viruses from men in Switzerland and the Netherlands. Annu Inst Pasteur/Virol 139: 429–437
21. Dea S, Bilodeau R, Sauvageau R, Montpetit C, Martineau GP (1992) Antigenic variant of swine influenza virus causing proliferative and necrotizing pneumonia in pigs. J Vet Diagn Invest 4: 380–392
22. Eason RJ, Sage MD (1980) Deaths from influenza A, subtype H1N1, during the 1979 Auckland epidemic. N Zeal Med J 91: 129–131
23. Easterday BC, Hinshaw VS (1992) Swine influenza. In: Leman AD, Straw BE, Mengeling WL, D'Allaire SD, Taylor DJ (eds) Diseases of swine. Iowa State Press, Ames, pp 349–357
24. Gammelin M, Altmüller A, Reinhardt U, Mandler J, Harley VR, Hudson PJ, Fitch WM, Scholtissek C (1990) Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol 7: 194–200
25. Gammelin M, Mandler J, Scholtissek C (1989) Two subtypes of nucleoproteins (NP) of influenza A viruses. Virology 170: 71–80
26. Gorman OT, Bean WJ, Kawaoka Y, Donatelli I, Guo Y, Webster RG (1991) Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 65: 3 704–3 714

27. Gorman OT, Bean WJ, Kawaoka Y, Webster RG (1990) Evolution of the nucleoprotein gene of influenza A virus. J Virol 64: 1 487–1 497
28. Gourreau JM, Kaiser C, Valette M, Douglas AR, Labie J, Aymard M (1994) Isolation of two H1N2 influenza viruses from swine in France. Arch Virol 135: 365–382
29. Groschup MH, Brun A, Haas B (1993) Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. J Vet Med 40: 681–689
30. Guan Y, Shortridge KF, Krauss S, Li PH, Kawaoka Y, Webster RG (1996) Emergence of avian H1N1 influenza viruses in pigs in China. J Virol 70: 8 041–8 046
31. Hinshaw VS, Bean, WJ, Webster RG, Easterday BC (1978) The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. Virology 84: 51–62
32. Hinshaw VS, Webster RG, Bean WJ, Downie J, Senne DA (1983) Swine influenza viruses in turkeys – a potential source of virus for humans? Science 220: 206–208
33. Hinshaw VS, Webster RG, Turner B (1978) Novel influenza A viruses isolated from Candian feral ducks: including strains antigenically related to swine influenza (Hsw1N1) viruses. J Gen Virol 41: 115–127
34. Influenza Virus Branch, CDC (1999) Unpublished data
35. Inkster M, Hinshaw VS, Schulze IT (1993) The hemagglutinins of duck and human H1 influenza viruses differ in sequence conservation and in glycosylation. J Virol 67: 7 436–7 373
36. Ito T, Couceiro JNSS, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72: 7 367–7 373
37. Kanegae Y, Sugita S, Shortridge KF, Yoshioka Y, Nerome K (1994) Origin and evolutionary pathways of the H1 hemagglutinin gene of avian, swine and human influenza viruses: cocirculation of two distinct lineages of swine virus. Arch Virol 134: 17–28
38. Karasin AI, Schutten MM, Cooper LA, Smith CB, Subbarao K, Anderson GA, Carman S, Olsen CW (2000) Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. Virus Res (in press)
39. Katsuda K, Shirahata T, Kida H, Goto H (1995) Antigenic and genetic analyses of the hemagglutinin of influenza viruses isolated from pigs in 1993. J Vet Med Sci 57: 1 023–1 027
40. Kay RM, Done SH, Paton DJ (1994) Effect of sequential porcine reproductive and respiratory syndrome and swine influenza on the growth and performance of finishing pigs. Vet Rec 135: 199–204
41. Kimura K, Adlakha A, Simon PM (1998) Fatal case of swine influenza virus in an immunocompetent host. Mayo Clin Proc 73: 243–245
42. Larsen DL, Karasin AI, Zuckermann F, Olsen CW (2000) Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. Vet Microbiol (in press)
43. Larsen DL, Karasin AI, Olsen CW (2000) Immunization of pigs against influenza virus infection by DNA vaccine priming followed by killed-virus vaccine boosting. Vaccine (submitted)
44. Lindstrom SE, Hiromoto Y, Nerome R, Omoe K, Sugita S, Yamazaki Y, Takahashi T, Nerome K (1998) Phylogenetic analysis of the entire genome of influenza A (H3N2) viruses from Japan: evidence for genetic reassortment of the six internal genes. J Virol 72: 8 021–8 031

45. Lubeck MD, Gerhard W (1981) Topological mapping of antigenic sites on the influenza A/PR/8/34 virus hemagglutinin using monoclonal antibodies. Virology 113: 64–72
46. Ludwig S, Haustein A, Kaleta EF, Scholtissek C (1994) Recent influenza A (H1N1) infections of pigs and turkeys in northern Europe. Virology 202: 281–286
47. Ludwig S, Stitz L, Planz O, Van H, Fitch WM, Scholtissek C (1995) European swine virus as a possible source for the next influenza pandemic. Virology 212: 555–561
48. Luoh SM, McGregor MW, Hinshaw VS (1992) Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. J Virol 66: 1 066–1 073
49. Mancini G, Donatelli I, Rozera C, Ruiz GA, Butto S (1985) Antigenic and biochemical analysis of influenza A H3N2 viruses isolated from pigs. Arch Virol 83: 157–167
50. McGregor MW, Bradshaw M, Hinshaw VS (1995) Sequence of the nucleoprotein gene of A/Swine/Indiana/1726/88. Unpublished data
51. Nakajima K, Nakajima S, Shortridge KF, Kendal AP (1982) Further genetic evidence for maintenance of early Hong Kong-like influenza A (H3N2) strains in swine until 1976. Virology 116: 562–572
52. Nerome K, Ishida M, Oya A, Oda K (1982) The possible origin of H1N1 (Hsw1N1) virus in the swine population of Japan and antigenic analysis of the isolates. J Gen Virol 62: 171–175
53. Olsen CW, McGregor MW, Cooley AJ, Schantz B, Hotze B, Hinshaw VS (1993) Antigenic and genetic analysis of a recently isolated H1N1 swine influenza virus. Am J Vet Res 54: 1 630–1 636
54. Ottis K, Sidoli L, Bachman PA, Webster RG, Kaplan MM (1982) Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. Arch Virol 73: 103–108
55. Ouchi A, Nerome K, Kanegae Y, Ishida M, Nerome R, Hayashi K, Hashimoto T, Kaji M, Kaji Y, Inaba Y (1996) Large outbreak of swine influenza in southern Japan caused by reassortant (H1N2) influenza viruses: its epizootic background and characterization of the causative viruses. J Gen Virol 77: 1 751–1 759
56. Palmer DF, Dowdle WR, Coleman MT, Schild GC (1975) Advanced laboratory techniques for influenza diagnosis. United States Department of Health, Education and Welfare Immunology Series
57. Patriarca PA, Kendal AP, Zakowski PC, Cox NJ, Trautman MS, Cherry JD, Auervach DM, McCusker J, Belliveau RR, Kappus KD (1984) Lack of significant person-to-person spread of swine influenza-like virus following fatal infection of an immunocompromised child. Am J Epidemiol 119: 152–158
58. Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA (1981) Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bull World Health Organ 59: 75–78
59. Raymond FL, Caton AJ, Cox NJ, Kendal AP, Brownlee GG (1986) The antigenicity and evolution of influenza H1 haemagglutinin, from 1950–1957 and 1977–1983: two pathways from one gene. Virology 148: 275–287
60. Rekik MR, Arora DJS, Dea S (1994) Genetic variation in swine influenza virus A isolate associated with proliferative and necrotizing pneumonia in pigs. J Clin Microbiol 32: 515–518
61. Robertson JS (1987) Sequence analysis of the haemagglutinin of A/Taiwan/1/86, a new variant of human influenza A (H1N1) virus. J Gen Virol 68: 1 205–1 208
62. Rocha E, Cox NJ, Black RA, Harmon MW, Harrison CJ, Kendal AP (1991) Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. J Virol 65: 2 340–2 350

63. Rota PA, Rocha EP, Harmon MW, Hinshaw VS, Sheerar MG, Kawaoka Y, Smith TL (1989) Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza, J Clin Microbiol 27: 1 413–1 416
64. Scholtissek C, Burger H, Kistner O, Shortridge K (1985) The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147: 287–294
65. Scholtissek C, Naylor E (1988) Fish farming and influenza pandemics. Nature 331: 215
66. Schultz U, Fitch WM, Ludwig S, Mandler J, Scholtissek C (1991) Evolution of pig influenza virus. Virology 183: 61–73
67. Sheerar MG, Easterday BC, Hinshaw VS (1989) Antigenic conservation of H1N1 swine influenza viruses. J Gen Virol 70: 3 297–3 303
68. Shu LL, Bean WJ, Webster RG (1993) Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1993 to 1990. J Virol 67: 2 723–2 729
69. Shu LL, Lin YP, Wright SM, Shortridge KF, Webster RG (1994) Evidence for interspecies transmission and reassortment of influenza A viruses in pigs in Southern China. Virology 202: 825–833
70. Smith TF, Burgert EO, Dowdle WR, Noble GR, Campbell RJ, Van Scoy RE (1976) Isolation of swine influenza virus from autopsy lung tissue of man. N Engl J Med 294: 708–710
71. Snacken R, Kendal AP, Haaheim LR, Wood JM (1999) The next influenza pandemic: lessons from Hong Kong, 1997. Emerg Infect Dis 5: 195–203
72. Snyder MH, Buckler-White AJ, London WT, Tiernney EL, Murphy BR (1987) The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. J Virol 61: 2 857–2 863
73. Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, Gojobori T, Nerome K, Oya A (1991) Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 32: 16–23
74. Tian S-F, Buckler-White AJ, London WT, Reck LJ, Chanock RM, Murphy BR (1985) Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/Mallard/NY/78 virus and its reassortants in squirrel monkey respiratory tract. J Virol 53: 771–775
75. Top FH, Russell PK (1977) Swine influenza at Fort Dix, NJ. IV. Summary and speculation. J Infect Dis 136: S376–S380
76. United States Department of Agriculture (1998) Pork Facts National Pork Producers Council Publication 4057: 19
77. United States Department of Agriculture (1998) Livestock Slaughter Annual Summary National Agricultural Statistics Service
78. Van Reeth K, Nauwynck H, Pensaert M (1996) Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. Vet Microbiol 48: 325–335
79. Webster RG (1998) Influenza: an emerging disease. Emerg Infect Dis 4: 436–441
80. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152–179
81. Wentworth D, Xian X, Cooley AJ, McGregor MW, Hinshaw VS, Cox N (1994) An influenza A (H1N1) virus closely related to swine influenza responsible for a fatal case of human influenza. J Virol 68: 2 051–2 058
82. Wentworth DE, McGregor MW, Macklin MD, Neumann V, Hinshaw VS (1997)

Transmission of swine influenza virus to humans after exposure to experimentally infected pigs. J Infect Dis 175: 7–15
83. Winter G, Fields S, Brownlee GG (1981) Nucleotide sequence of the haemagglutinin gene of a human influenza virus H1 subtype. Nature 292: 72–75
84. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K-J, Krauss S, Webster RG (1999) Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73: 8 851–8 856
85. Zou S (1997) A practical approach to genetic screening for influenza virus variants. J Clin Microbiol 35: 2 623–2 627

Authors' address: Dr. C. W. Olsen, Assistant Professor of Public Health, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive West, Madison, WI 53706, U.S.A.

Received December 1, 1999