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

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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 Septem-
ber 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
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, LaJolla, 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'-AGCAAAAGCAGGGAAAATAA-3') and 1747–1771/reverse (5'-CAAGGGTGTTTTTCTCATGTCTC-3'). The NP genes were amplified using primers specific for nucleotides 1-21/forward (5'-GCAGGGTAGATAATCACTCAC-3') and 1533–1557/reverse (5'-CAAGGGTATTTTTCTTAATTGTG-3') (for isolates 125, 136, 163, 164, 166, 168, 235) or the SZANP (5'-CTCGAGAGCAAAAGCAGGGT-3') and SZANP (5'-AGTAGAAACAGGGTATTTTTTC-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.           | [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 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 Z46434 | [47] |
| A/Swine/England/195852/92b 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/2/81b M22579 | [24] |
| A/Swine/Ontario/2/81 M63767  | [26] |
| A/Swine/Hong Kong/126/82b 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/839/89b M63772 | [26] |
| A/Swine/England/195852/92b 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]  |

* n.a. Not available – This sequence has not been deposited in GenBank
  † 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           | 12,800–51,200 | 102,400–204,800 | 204,800 |          |
| A/Swine/Wisconsin/303,1005/98              |            |          |           |          |
| 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), than the HAs of a recent human H1 virus, A/Bayern/7/95 (74% nucleotide identity and 71–72% amino acid identity),
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

| 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 seropos-
itivity that we observed in 1997–1998 reflects infection of pigs with these reasor-
tant viruses or infection with wholly human H3 viruses that are likely to have en-
tered 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.

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