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Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States

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Abstract

Swine influenza viruses (SIV) of the hemagglutinin subtype 1 (H1) isolated from the United States (U.S.) have not been well-characterized in the natural host. An increase in the rate of mutation and reassortment has occurred in SIV isolates from the U.S. since 1998, including viruses belonging to the H1 subtype. Two independent animal studies were done to evaluate and compare the pathogenesis of 10 SIV isolates dating from 1930 to currently circulating isolates. In addition, the hemagglutinin and neuraminidase genes of each isolate were sequenced for genetic comparison, and serological cross-reactivity was evaluated using all sera and virus combinations in hemagglutination inhibition and serum neutralization assays. Statistically significant differences in percentage of pneumonia and virus titers in the lung were detected between isolates, with modern isolates tending to produce more severe disease, have more virus shedding and higher viral titers. However, nasal shedding and virus titers in the lung were not always correlated with one another or lung lesions. Serologically, the classic historical H1N1 viruses tended to have better cross-reaction between historical sera and antigens, with moderate to good cross-reactivity with modern viral antigens. However, the modern sera were less reactive with historical viruses. Modern viruses tended to have less consistent cross-reactivity within the modern group. Overall, H1 isolates collected over the last 75 years from the U.S. pig population exhibit considerable variability in pathogenicity. There appears to be an increase in genetic and antigenic diversity coincident with the emergence of the swine triple reassortant H3N2 in 1998.

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1. Introduction

Influenza in swine is an acute respiratory disease caused by influenza A viruses within the *Orthomyxoviridae* family, first isolated and identified in North America in 1930 (Shope, 1931). Orthomyxoviruses have negative stranded RNA genomes that are segmented, allowing for reassortment and production of novel viruses. There are 2 major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), important for host range, antigenicity, pathogenesis, and diagnostic detection. The tracheal epithelium in pigs expresses the receptors for avian viruses and human influenza viruses, suggesting the pig as a mixing vessel for the emergence of new isolates with human pandemic potential (Ito et al., 1998; Scholtissek et al., 1993). This underscores the importance of limiting the introduction of new influenza viruses to the swine population and monitoring for newly emerging viruses.

For nearly 70 years, SIV in North America was relatively stable with only one predominant circulating subtype, known as the classical swine H1N1 (cH1N1) (Easterday and Van Reeth, 1999). However in 1998, H3N2 isolates with human, avian, and swine genes were identified in multiple swine populations across the U.S. (Zhou et al., 1999, 2000), and reassortants between the classic H1N1 and the newly introduced H3N2 viruses rapidly appeared. The reassortments produced H1 swine viruses with the HA and NA from the classic swine virus and the internal genes from the H3N2 viruses (rH1N1) or the HA from the classic swine virus and the NA and internal genes from the H3N2 viruses (H1N2) (Gramer and Rossow, 2004; Janke et al., 2004; Karasin et al., 2002; Webby et al., 2004). Subsequently, an increase in the rate of genetic change in North American swine influenza isolates appears to have occurred in both H3 and H1 virus subtypes. Reassortant H1 viruses are reported to be infecting and causing disease in herds that have been routinely vaccinated with commercial vaccines containing cH1N1 despite the observation that the HA gene from the H1N2 and rH1N1 are swine in origin. Moreover, these newly emerged swine viruses have not been well-characterized or compared with classic H1N1 isolates in the pig, nor have serologic comparisons been reported for the three circulating H1 viruses: types cH1N1, H1N2, and rH1N1.

Two independent animal studies were done to evaluate and compare the pathogenesis of 10 swine influenza isolates dating from 1930 to currently active isolates. All isolates evaluated were previously identified as having the type 1 hemagglutinin protein, based on serotyping, genotyping, or sequencing. Four-week old pigs were challenged intra-tracheally with each of the 10 isolates and evaluated for clinical signs, nasal shedding, percentage of lung lesions, and viral titers in the lung. In addition, two pigs per isolate were hyper-immunized and sera collected for use in hemagglutinin inhibition (HI) and serum neutralization (SN) assays. The HA and NA genes from each isolate were sequenced and compared phylogenetically. This study demonstrates considerable variation in shedding, replication, and severity of pneumonia among all isolates, even those considered to be cH1N1. The genetic analysis demonstrated an increase in diversity since 1998. Despite substantial drift in the classical H1N1 isolates in the HA and NA genes based on nucleic acid sequence, this did not appear to interfere with serologic cross-reactivity. As expected from the genetic changes in modern isolates, cross-reactivity in the HI and SN assays have become less predictable since 1998, with sera from modern H1 isolates (isolated after 1998) cross-reacting poorly with historical cH1N1 isolates (isolated before 1998) as well as modern isolates cross-reacting less predictably with other modern H1 isolates.

2. Materials and methods

2.1. Animals

Four-week old cross-bred pigs free of influenza and porcine reproductive and respiratory syndrome viruses were challenged intra-tracheally with each of the 10 isolates in 2 separate trials. In experiment 1, 68 pigs were randomly assigned to 7 treatment groups. Each experiment 1 challenge group contained 10 pigs and the negative control sham-challenged group contained 8 pigs. In experiment 2, 44 pigs were randomly assigned to 6 treatment groups. Each experiment 2 challenge group contained 8 pigs and the negative control sham-challenge group contained 4 pigs. All pigs were treated with ceftiofur hydrochloride (Pharmacia & Upjohn, Kalamazoo, MI) for 3 consecutive days to reduce

bacterial contaminants prior to the start of the study. Challenge groups were housed in individual isolation rooms and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. All animals were humanely euthanized 5 days post infection (dpi) with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) and exsanguination.

2.2. Virus strains

Isolates included H1N1 and H1N2 subtypes. Isolates included in experiment 1 were laboratory strains of A/Swine/Iowa/15/1930 H1N1 (IA30), A/Swine/MN/1192/2001 H1N2 (MN01), A/Swine/NC36883/2002 rH1N1 (NC02), and field isolates from the Minnesota Veterinary Diagnostic Lab A/Swine/Minnesota/00194/2003 H1N2 (MN03), A/Swine/Kansas/00246/2004 H1N2 (KS04), and A/Swine/Iowa/00239/2004 rH1N1 (IA04). Isolates included in experiment 2 were IA30, IA 1945 H1N1, ASwine/Wisconsin/1/1968 H1N1 (WI68), IA 1973 H1N1, and A/Swine/Minnesota/37866/1999 H1N1 (MN99). The IA45, WI68, and IA73 were cryopreserved and stored at the NADC until the time of this study. Isolates were grown in embryonated chicken eggs, and allantoic fluids were harvested to collect the viruses. To make pig-passaged challenge material, the allantoic fluids were given intra-tracheally at a dose of 2 ml of 1×10^5 50% tissue culture infective dose ($TCID_{50}$)/ml to 2 week old caesarian derived-colostrum deprived pigs while anesthetized with an intramuscular injection of ketamine, xylazine, tiletamine and zolazepam. The infected lungs were lavaged with Mimimal Essential Medium (MEM) to collect bronchioalveolar lavage fluid (BALF) at necropsy on 3 dpi for use in the pathogenesis study. For the pathogenesis studies, pigs from each challenge group received 2 ml of 1×10^5 $TCID_{50}$/ml of pig-passaged virus given intra-tracheally while anesthetized.

2.3. Clinical evaluation and virology

Pigs were observed daily for clinical signs, and rectal temperatures were taken daily from −2 to 5 dpi. nasal swabs were taken on 0, 3, and 5 dpi and placed in 2 ml MEM and frozen at −80 °C until study completion. Each nasal swab sample was subsequently thawed and vortexed for 15 s, centrifuged for 10 min at $640 \times g$ and the supernatant passed through 0.45 μm filters to reduce contaminates. An aliquot of 200 μl of the filtrate was plated onto confluent MDCK cells washed with PBS in 24-well plates. After 1 h incubation at 37 °C, 200 μl serum-free MEM supplemented with 1 μg/ml TPCK trypsin was added. All wells were evaluated for cytopathic effect (CPE) at 48 h and subsequently frozen. Aliquots of 200 μl from the 24-well frozen-thawed plates were transferred onto confluent MDCK cells in 48-well plates and again evaluated for CPE at 48 h post infection.

After euthanasia, each lung was lavaged with 50 ml MEM to obtain BALF. Ten-fold serial dilutions in serum-free MEM supplemented with trypsin were made with each BALF sample and 100 μl plated in triplicate onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post infection. A $TCID_{50}$ was calculated for each sample using the method of Reed and Muench (Reed and Muench, 1938).

2.4. Pathologic examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of SIV. The percent of the surface affected with pneumonia was visually estimated for each lung lobe, and then a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume (Halbur et al., 1995). Tissue samples from the trachea and right cardiac lung lobe were taken and fixed in formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Lung sections were given a score from 0 to 4 to reflect the severity of bronchial epithelial changes based on previously published methods (Richt et al., 2003). The lung sections were scored according to the following criteria: 0 = normal airways with medium-to-tall columnar epithelial cells with ciliated apical borders and basal pseudostratified nuclei; 1 = mostly normal airways with few isolated airways displaying minor loss of cilia and/or slight proliferation; 2 = localized cluster of affected airways, often within one or two lobules, with affected airways displaying proliferation, epithelial cell disorganization and irregular

luminal borders; 3 = low number of airways affected similar to criteria 2, but affected airways are located throughout most of the section; 4 = many airways of all sizes affected, often severely, with marked epithelial cell disruption, disorganization, necrotic cells visible in lumen, and marked proliferation. A single pathologist scored all slides and was blinded to the treatment groups.

2.5. Subtype RT-PCR and sequencing

Viral RNA was extracted from allantoic or MDCK cell fluids with the QIAamp viral RNA Mini Kit (Qiagen Inc., Valencia, CA). To verify the subtype of each isolate, a subtyping RT-PCR was done using previously reported methods (Choi et al., 2002a,b). For sequencing, the HA and NA genes were amplified by RT-PCR in their entirety and the fragments were purifed by QIAquick® Gel Extraction Kit (Qiagen, Inc.) and sequenced for genetic comparison. Products were quantitated using the Pico Green assay for dsDNA (Invitrogen Corporation, Carlsbad, CA). The appropriate quantity of dsDNA PCR product was labeled in both directions using Big Dye terminator chemistries (Applied Biosystems Inc., Foster City, CA) according to manufacturer's instructions. The labeled products were sequenced using an ABI 3100 genetic analyzer (Applied Biosystems Inc.) using the primary PCR primers and a series of internal forward and reverse primers. Primer sequences are available upon request. The sequences were analyzed using SeqMan and phylogenetic trees produced by Clustal W alignment using MegAlign of the LaserGene software package (DNAStar, Inc., Madison, WI). Swine influenza virus sequences from the U.S. published on GenBank were included in the multiple alignment and are identified by their accession numbers.

2.6. Serologic assays

To develop high HI antibodies, 2 pigs per isolate were vaccinated with two doses of 10^6 TCID$_{50}$/ml UV-inactivated virus plus commercial adjuvant (Emulsigen, MVP Laboratories, Inc., Ralston, NE), with the second dose approximately 2–3 weeks following the priming dose. This was followed 3–4 weeks post vaccination with a live intra-tracheal challenge as

described above. Serum was collected 2 weeks post challenge. The serum samples were evaluated using a commercial H1N1 ELISA for presence of antibody according to the manufacturer's protocol (HerdCheck, IDEXX Laboratories, Inc., Westbrook, ME). For use in the HI assay, sera were heat inactivated at 56 °C and treated to remove non-specific agglutinators with a 20% suspension of Kaolin (Sigma Aldrich, St. Louis, MO) followed by adsorption with 0.5% turkey red blood cells (RBC). The HI assays were done with turkey RBC using all sera and virus combinations using standard techniques (Palmer et al., 1975). The SN assays were done with virus isolates grown in MDCK cells and standardized to concentrations of 100 TCID$_{50}$/reaction. The virus was incubated with an equal volume of 2-fold serial dilutions of serum at 37 °C for 1 h. All media used for virus and serum dilutions was serum-free MEM supplemented with trypsin. The virus/sera mixtures were then transferred to 96-well plates containing confluent monolayers of MDCK cells washed with PBS and evaluated for CPE between 48 and 72 h post infection. Reciprocal titers for HI and SN assays were log$_2$ transformed for statistical analysis and reported as geometric means.

2.7. Statistical analysis

Macroscopic pneumonia scores, virus titers, average rectal temperatures, microscopic pneumonia scores, and log$_2$ transformations of HI and SN reciprocal titers were analyzed using analysis of variance (ANOVA) and daily rectal temperatures were analyzed with multivariate analysis of variance (MANOVA) (JMP, SAS institute, Cary, NC) with a p-value ≤0.05 considered significant. Response variables shown to have a significant effect by challenge group were subjected to pair-wise comparisons using the Student's t-test.

3. Results

3.1. Clinical evaluation and virology

All pigs were negative prior to the start of the experiment for H1N1 and H3N2 antibodies by HI assay. Clinically, the uncomplicated influenza infections

were relatively mild for all the isolates evaluated in this study with almost no detectable coughing or anorexia. All challenged pigs had elevated rectal temperatures compared to the sham-inoculated pigs at some point during the clinical evaluation period, with 3 dpi being the peak febrile day for most challenge groups. Although elevated over the sham control group, the MN99 and KS04 group means never reached the minimum value for fever ($\geq$40 °C), calculated as two standard deviations above the mean of pre-challenge and sham temperatures. The IA04 group continued to have fevers from 3 dpi until necropsy, whereas most other group temperatures had decreased to within normal ranges. In addition, there were statistically significant differences between challenge groups for mean rectal temperature over the trial period (Table 1).

Significant differences were detected between challenge groups for virus titration in BALF from experiments 1 and 2 (Table 1). Least squares means and standard errors are given for virus titration in BALF. The percentages of nasal swabs found to be positive by virus isolation are reported in Table 1. The IA30, IA45 and IA73 isolates appeared to have less nasal shedding. With the exception of IA73 and NC02, modern isolates (present after 1998) appeared to replicate more efficiently in the host than historical isolates; however, viral loads in the lung did not always correlate with the ability to isolate virus from nasal swabs.

Fig. 1. Macroscopic and microscopic lung lesions for 10 H1 subtype SIV isolates. (A) Mean percentage of lung surface with macroscopic pneumonic lesions on 5 dpi. (B) Mean microscopic lung lesion scores (0–4) on 5 dpi. Error bars represent S.E.

3.2. Pathologic examination of lungs

The lungs were removed *in toto* from each pig and examined for macroscopic evidence of influenza pneumonia. All isolates induced some level of lung pathology, although the percentage varied, sometimes dramatically, between isolates. There was a statistically significant difference in percentage of macroscopic pneumonia between isolates, as is summarized

Table 1
Summary of mean rectal temperatures, percentage of nasal shedding (N.S.) on 3 and 5 dpi, and virus titers in BALF for 10 H1 subtype SIV isolates

SIV isolate	Temperature (°C) 1–5 dpi	% Pos N.S. 3 dpi	% Pos N.S. 5 dpi	Virus titer[a] ($\log_{10}$)
Sham[b]	39.3[D]	0.0	0.0	0.0 ± 0.2[F]
IA30 H1N1[b]	39.8[A,B]	5.6	27.8	4.9 ± 0.2[E]
IA45 H1N1	39.9[A,B]	0.0	0.0	5.8 ± 0.2[B,C,D]
WI68 H1N1	40.1[A]	100.0	100.0	5.3 ± 0.2[D,E]
IA73 H1N1	40.1[A]	37.5	62.5	6.5 ± 0.2[A,B]
MN99 H1N1	39.7[A,B,C,D]	100.0	100.0	5.8 ± 0.2[C,D]
MN01 H1N2	39.4[B,C,D]	100.0	100.0	6.6 ± 0.2[A]
NC02 rH1N1	39.7[A,B,C,D]	88.9	100.0	5.4 ± 0.2[D,E]
MN03 H1N2	39.7[A,B,C]	100.0	100.0	6.3 ± 0.2[A,B,C]
KS04 H1N2	39.4[C,D]	100.0	100.0	6.5 ± 0.2[A]
IA04 rH1N1	39.9[A]	100.0	100.0	6.5 ± 0.2[A]

Treatment group means with different superscript letters (A–F) statistically different at $p < 0.05$.
[a] Mean ± S.E.
[b] Values for sham and IA30 are from experiment 1 and 2 combined.
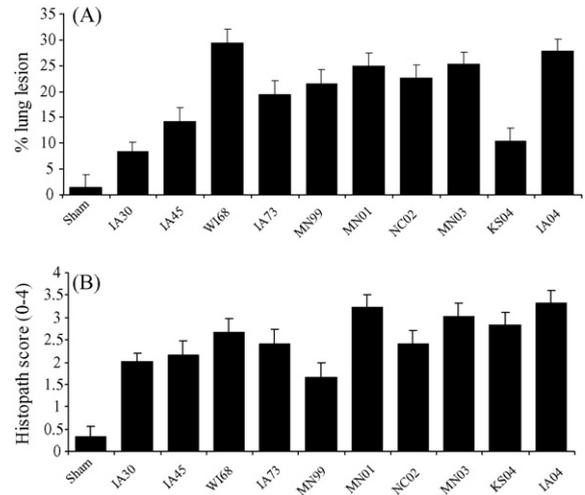
in Fig. 1A. Histopathologically, the extent of damage to the bronchiolar epithelium also varied between isolates (Fig. 1B). Modern viruses isolated since 1999 with reassortant genomes tended to produce more severe macroscopic and microscopic lesions, with the exception of WI68 and KS04. In experiment 1, several of the negative control pigs had small foci of consolidation indistinguishable from influenza pneumonia. These pigs remained seronegative for SIV, were negative for virus isolation, and tested PCR positive for *Bordetella bronchiseptica*, which can cause mild bronchopneumonia. The microscopic lung lesion scores for the sham pigs also showed very mild to no changes in bronchiolar architecture and the sham group means for macroscopic and microscopic evaluation were significantly lower than all challenge groups.

3.3. Subtype RT-PCR and sequencing

As expected, all isolate subtypes were confirmed by the gel-based subtyping RT-PCR as H1N1 for IA30, IA45, WI68, IA73, MN99, NC02, and IA04 or H1N2 for MN01, MN03, and KS04 (data not shown). The
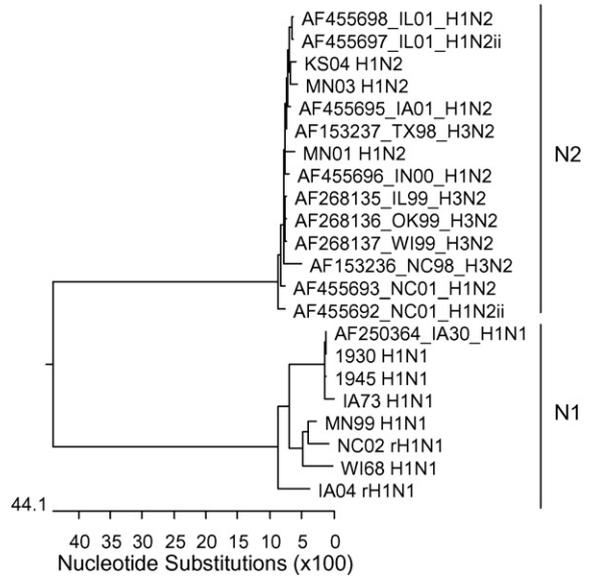

Fig. 3. Phylogenetic tree based on the NA nucleotide sequence for 10 H1 SIV isolates and other SIV sequences available from GenBank. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. The two broad clusters of related viruses, N1 and N2, are indicated by the bars on the right.
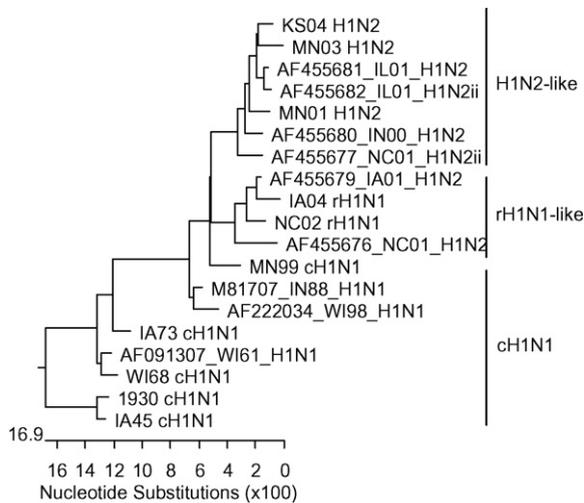
HA and NA genes were sequenced and analyzed for genetic relatedness (Figs. 2 and 3, respectively). The HA1 region of the HA gene sequence was used for phylogenetic analysis. Based on genetic sequence analysis, the 10 isolates each appeared to be unique with greater than 3% divergence between either the HA or NA genes. Analyses of the HA genes showed H1 viruses clustered into three distinct genetic groups as previously described (Webby et al., 2004): cH1N1, H1N2-like, and rH1N1-like groups. There was considerable variation within groups at the nucleotide and amino acid level, most notably in the cH1N1 group, with 18% difference between the IA30 and MN99 cH1N1 viruses. The HA nucleotide divergence between historical cH1N1 and modern reassortant isolates ranged from 11 to 21% difference. There were 2 groups present according to NA gene analyses, N1 and N2, with greater nucleotide divergence between N1 subtypes. The NA nucleotide divergence between the historical N1 and modern N1 ranged from 8 to 16% difference.

Fig. 2. Phylogenetic tree based on the HA1 nucleotide sequence for 10 H1 SIV isolates and other SIV sequences available from GenBank. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. The three broad clusters of related viruses, cH1N1, rH1N1-like, and H1N2-like are indicated by the bars on the right.

Table 2
Cross-reactivity between all sera and viral antigen combinations in the HI assay

Virus[a]	IA30	IA45	WI68	IA73	MN99	MN01	NC02	MN03	KS04	IA04
IA30 H1N1	5120.0	2560.0	905.1	640.0	640.0	113.1	640.0	10.0	113.1	905.1
IA45 H1N1	1810.2	1280.0	452.5	452.5	452.5	113.1	320.0	14.1	56.6	640.0
WI68 H1N1	905.1	905.1	1810.2	905.1	2560.0	160.0	320.0	28.3	452.5	640.0
IA73 H1N1	320.0	160.0	226.3	905.1	226.3	40.0	113.1	10.0	56.6	160.0
MN99 H1N1	320.0	640.0	905.1	452.5	1810.2	452.5	452.5	226.3	1280.0	905.1
MN01 H1N2	56.6	56.6	56.6	80.0	160.0	160.0	20.0	56.6	160.0	160.0
NC02 rH1N1	40.0	56.6	113.1	113.1	226.3	40.0	640.0	40.0	80.0	640.0
MN03 H1N2	10.0	56.6	56.6	28.3	160.0	226.3	56.6	640.0	452.5	56.6
KS04 H1N2	56.6	320.0	320.0	160.0	640.0	905.1	80.0	160.0	1280.0	226.3
IA04 rH1N1	40.0	40.0	113.1	40.0	226.3	40.0	320.0	14.1	80.0	1280.0

[a]Isolates used in the vaccine are identified in the far left column, with geometric mean reciprocal titers of 2 sera samples per isolate in rows. Virus isolates used as antigens in the HI assay are identified in the top row. Homologous sera and antigen combinations have bold borders and negative or low positives are shaded gray.

3.4. Serologic assays

Two pigs per isolate were hyper-immunized to collect serum for use in the commercial ELISA, HI and SN assays. Sera from the hyper-immunized pigs were used in HI assays with all possible sera and antigen combinations. The reciprocal titer means for each pair are summarized in Table 2. The homologous sera and antigen combinations have bold borders and negative or low-positive HI titers are shaded gray. All homologous anti-sera and antigen combinations displayed high mean HI titers with the exception of MN01 with a moderate mean reciprocal titer of 160. Sera from pigs primed with historical isolates tended to have better cross-reactions among historical antigens, and moderate to good cross-reactivity with modern antigens. However, the sera from pigs primed with modern isolates were less reactive with historical viruses. Modern isolates also tended to be less consistent in cross-reactivity within the modern isolate group. The MN99 isolate appeared to have the best cross-reactivity overall, both as an anti-serum and as an antigen in the HI assay.

The SN assay was also done with all virus and sera combinations. The SN cross-reactivity tended to be highly correlated with the HI cross-reactivity, although some sera showed slightly higher virus neutralizing activity as compared to hemagglutination inhibiting activity (data not shown). Despite high HI titers to the homologous virus, the two serum samples raised against the MN03 isolate were negative on the commercial ELISA (data not shown). All other samples were positive, although several other serum samples from modern isolates were only marginally above the S/P ratio positive cut-off of 0.4, even in the face of high isolate specific HI titers.

4. Discussion

Swine influenza in the U.S. was relatively stable until 1998, with the only major circulating isolate

being the classic H1N1 that emerged in swine coincidentally with the 1918 human H1N1 pandemic (Easterday and Van Reeth, 1999). The cH1N1 remained highly conserved genetically until the 1990s, with only a limited number of nucleotide changes leading to amino acid substitutions (Luoh et al., 1992; Noble et al., 1993). Antigenically, the virus was also highly conserved based on reactivity with monoclonal antibodies and cross-reactivity in the H.I. assay (Sheerar et al., 1989). However, since the introduction of H3N2 viruses with genes of human and avian origin into pig populations and the resultant reassortment with cH1N1 viruses, the dynamics of clinical disease and prevention of outbreaks has changed dramatically only recently. This is in contrast to European countries where the epidemiology of swine influenza has been changing rapidly for many years (Brown, 2000). The cH1N1 was only sporadically identified in Europe from the 1940s until 1976, when it re-emerged in Italy. By the late 1970s, the cH1N1 had spread rapidly across the continent and into the U.K., becoming endemic in the pig population. In the 1970s, an avian-like H1N1 and a human-like H3N2 virus began circulating in European pigs. The avian-like H1N1 has maintained a solely avian-like genome and gradually became the dominant H1N1 over the cH1N1 in Europe. More recently, the dominant H3N2 viruses isolated from pigs in Europe are avian-human reassortants and appear to have replaced the human-like H3N2 of the 1970s and 1980s (Castrucci et al., 1993). An H1N2 virus related to the circulating human-like swine H3N2 and human-like H1N1 with avian-like internal genes subsequently emerged and also became endemic in Great Britain (Brown et al., 1998) and Europe (Marozin et al., 2002; Van Reeth et al., 2000). It appears that although a different series of introductions and reassortments have occurred in the U.S., the epidemiology of swine influenza in the U.S. is now as complex and dynamic as that in continental Europe and Great Britain.

In 1998 and 1999, U.S. H3N2 viruses were detected that were double reassortants between the classic swine virus and circulating human viruses as well as triple reassortants between the classic swine virus (NP, M, and NS genes), human-like viruses (PB1, HA, and NA genes), and avian-like viruses (PB2 and PA genes) (Zhou et al., 1999, 2000). The triple reassortant H3N2 subsequently became established in U.S. swine herds,

and reassortants between the triple reassortant and cH1N1 resulted in an H1N2 with the swine HA gene and the remaining genes derived from the H3N2 (Webby et al., 2000). Since their identification, the H1N2 subtype viruses have also become established in U.S. herds (Karasin et al., 2002). This was followed by further reassortment producing H1N1 viruses with the HA and NA from the classic swine virus, but with internal genes from the triple reassortant H3N2 (Gramer and Rossow, 2004; Janke et al., 2004). The PA and PB2 genes of modern isolates that have been characterized are almost exclusively avian-like in origin, similar to that of the H3N2 triple reassortant (Janke, 2004). In addition, the HA genes of most current H1 isolates are genetically more closely related to the reassortant viruses rather than the cH1N1 isolates, forming genetic clusters apart from classic HA gene sequences as demonstrated in this study. With the acquisition of avian-like polymerase genes, the current swine H1 viruses appear to have increased the rate of mutation and therefore the ability to evade established herd immunity. Consequently, the commercially available killed vaccines are at present not able to provide the coverage necessary for the changing milieu of circulating swine influenza viruses.

Although characterization of the genetic, antigenic and pathogenic characteristics of the newly circulating H3N2 viruses in the U.S. has been described (Richt et al., 2003), only genetic or antigenic comparisons of H1 isolates from 2002 or prior has been reported (Choi et al., 2002a,b; Karasin et al., 2002; Luoh et al., 1992; Noble et al., 1993; Webby et al., 2004). These H1 studies also did not include the rH1N1 viruses. No recent reports comparing the clinical and pathologic characteristics as well as the genetic and antigenic properties of modern U.S. rH1N1, modern H1N2, and historical H1N1 were found. The purpose of this study was to compare 10 H1 isolates ranging from 1930 to 2004 using pathogenic, genetic, and antigenic parameters with the goal of identifying any potential trends in the newly emerging viruses. The antigenic study was done to evaluate serum cross-reactivity to potentially address the problem of influenza outbreaks in the face of vaccination or natural immunity.

The H1 swine isolates evaluated in this study were shown to have differences in the clinical and pathologic course of disease as evaluated by rectal

temperatures, nasal shedding, viral titers in the lung, and percentage of macroscopic and microscopic pneumonia. The variability was especially evident within the cH1N1 group of viruses. Although we did not evaluate all gene segments, it is presumed that all cH1N1 viruses contain closely related swine-like internal genes, so the source of the variation remains unexplained. All isolates were passed through pigs once to eliminate variation due to laboratory culture methods. Based on the isolates used in this study, current reassortant H1 isolates in general caused more severe pneumonia, had higher efficiency of nasal shedding, and grew to higher titers in the lung, suggesting the newly emerged viruses have adapted well to the swine host and may have greater transmission potential while inducing more severe disease. The KS04 H1N2 was an exception with relatively milder pneumonia, but high shedding and virus titers. The WI68 cH1N1 and IA73 cH1N1 isolates were also exceptions; with the WI68 isolate having higher percentage pneumonia and viral shedding and the IA73 isolate having moderately high percentage of pneumonia and higher virus titers. There was no strong correlation between any of the clinical and/or pathologic measurements with the exception of the virus titer in the BALF and the severity of microscopic lesions ($r = 0.7$). The *B. bronchiseptica* contamination in experiment 1 appeared to play a minor role in macroscopic or microscopic lung pathology with very mild to no lesions in uninfected SIV controls. Only a limited number of *B. bronchiseptica* PCR positive pigs were found throughout all treatment groups.

Based on nucleotide sequence of the HA gene, there was considerable variation between all H1 isolates, as well as within the three clusters of H1 isolates. This was most dramatic in the cH1N1 cluster. The cH1N1 viruses have been endemic in U.S. pig populations for over 70 years and the diversity is most likely due to drift mutations accumulating over time. The N1 cluster of viruses also demonstrated greater divergence in the NA gene, similar to the variation shown for the HA gene in cH1N1 swine viruses and is likely due to genetic drift as well. In contrast to the clinical, pathogenic, and genetic variation, the cH1N1 isolates were much more consistent in cross-reactivity in serologic assays. However, there has been a loss of HI cross-reactivity between H1 isolates over time,

most pronounced between antibodies against modern isolates and historical cH1N1 antigens as well as within the modern isolate group overall. Modern isolates in the same phylogenetic grouping tended to cross-react to some level, but not always as strongly as would be predicted based on sequence homology. Nucleic acid similarity was not always a good predictor of cross-reactivity, with KS04 and MN03 as an example. These two isolates were in the same H1N2-like phylogenetic group and were 97% homologous in the HA1 nucleic acid sequence, but the KS04 sera cross-reacted much weaker to the MN03 antigen compared to its reaction to the homologous antigen. In addition, cross-reactivity was not always bi-directional in that sera from virus X may cross-react strongly with virus Y, but sera from virus Y cross-reacted weakly with virus X. The IA30 sera cross-reactivity to modern viral antigens as compared to the IA30 antigen cross-reactivity with modern sera demonstrates the best example of this phenomenon. One isolate appeared to be superior both in producing cross-reacting antibodies as well as cross-reacting with other anti-sera as an antigen. The MN99 sera cross-reacted quite well with all antigens evaluated in this study and the antigen was most consistent in cross-reacting with other anti-sera. Phylogenetically, the MN99 appears to be an intermediate between the historical classic viruses and the new reassortant H1 viruses. This isolate may be useful both as a diagnostic reagent and as an inactivated antigen used in immunizing pigs for maximum cross-protection against the circulating H1 viruses.

Despite high HI and SN titers to homologous antigen, the commercial ELISA was negative for one current isolate and had low S/P ratios for two others. The antibody levels in the sera used here are likely to be elevated due to hyper-immunization of the pigs as compared to sera from naturally infected or vaccinated animals in the field. Thus, the commercial ELISA may have even less sensitivity when used for vaccine compliance or outbreak monitoring. Based on this study, it appears that the HI assay with a panel of cross-reacting H1 and H3 viruses is the better diagnostic test for influenza serology. However, this continues to be a challenge for diagnosticians with the constant evolution of influenza virus isolated from the field.

All hyper-immune sera demonstrated moderately high to very high titers against homologous antigens in

the HI assay, therefore it is unknown how low or moderate titers produced under field conditions would cross-react. It can be presumed that lower titers would reduce cross-reactivity. Additionally, it is not clear if cross-reactivity in the HI or SN assays would be directly correlated to cross-protection from heterologous challenge. This must be addressed in future studies. Although immunity acquired from natural challenge can provide better cross-protection against a heterologous virus challenge (Heinen et al., 2001; Van Reeth et al., 2003, 2004), it has been shown that antibodies induced by inactivated vaccines that do not cross-react in HI assays show a lack of cross-protection in an in vivo challenge. All swine influenza virus vaccines currently available for use in the U.S. must be inactivated, so the development of vaccines with broad cross-protection and sufficient coverage of newly emerging viruses remains a weakness in the ability to control this evolving disease. The H3N2 viruses continue to circulate and evolve in the swine population as well, adding another layer of complexity to the development of vaccines and control strategies.

Finally, it is evident based on sequence homology that viruses of human origin have spilled over into the U.S. swine population and continue to do so (Gramer, 2006). There is also serologic evidence that swine farm personnel and swine veterinarians have been exposed to cH1N1 and H1N2 swine viruses, although there have been relatively few documented cases of swine-to-human influenza based on virus isolation (Myers et al., 2006; Olsen et al., 2002). Continued surveillance for virus and antibody from swine workers and pigs in their care is necessary to monitor the change in circulating viruses and emergence of novel isolates that may evade immunity and/or diagnostic testing. With the current evolution of swine viruses, the zoonotic potential, and the lack of effective vaccines to control currently circulating viruses, it is increasingly important to continue investing resources in preventing further introduction of human and avian viruses into swine herds as well as the development of broader acting vaccines.

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