Title: Genetic and antigenic characterization of 2008 H1 swine influenza viruses from the United States - **NPB #08-031**

Investigator: Amy L. Vincent

Institution: USDA, ARS, Midwest Area

Co-Investigators: Sabrina Swenson, Marie R. Gramer, and Kelly M. Lager

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Industry Summary

Influenza A viruses can infect swine, humans, dogs, cats, horses, marine mammals, and many avian species. Influenza A viruses from all host species are classified into subtypes based on the hemagglutinin (HA) and neuraminidase (NA) genes, for example H3N2 or H1N1. Along with the HA and NA genes, influenza A viruses contain 6 additional genes necessary for virus infection and replication. Prior to the introduction of the 2009 pandemic H1N1 virus from human to pigs, four distinct clusters of the HA gene from H1 swine influenza viruses (SIV) co-circulated along with H3N2 viruses in the U.S. Viruses from the classical H1N1 SIV lineage evolved over time to form α-, β-, and γ-clusters based on the genetic makeup of the HA gene. SIV with HA genes most similar to human seasonal H1 viruses emerged in pigs in 2003 to form the δ-cluster. All four HA cluster gene types can be found with neuraminidase genes of either the N1 or N2 subtype. Limited sequence information was available regarding the 6 genes that make up the triple reassortant internal gene (TRIG) cassette in contemporary H1 SIV. In addition, information regarding the antigenic relatedness of the H1 viruses necessary for vaccine development and diagnostic reagent updates were in need due to the dynamic and variable

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For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

nature of H1 SIV. We characterized 12 H1 isolates from 2008 by sequencing and phylogenetic analysis of all eight gene segments and by serologic cross-reactivity in the hemagglutination inhibition (HI) assay. This study provides information on the level of diversity of each gene segment in 2008 U.S. isolates as well as information necessary to make informed vaccine strain selection and diagnostic reagent updates. This data also revealed that the 2008 viruses characterized from U.S. swine were genetically distinct from the 2009 human pandemic H1N1.

Contact: Amy L. Vincent, DVM PhD; 2823 Virus and Prion Diseases Research Unit, USDA-ARS, National Animal Disease Center, 1920 Dayton Avenue, Ames, IA 50010 USA: email: amy.vincent@ars.usda.gov, phone: 515-337-7557, fax: 515-337-7428

Scientific Abstract

Prior to the introduction of the 2009 pandemic H1N1 virus from humans into pigs, four phylogenetic clusters of the hemagglutinin (HA) gene from H1 swine influenza viruses (SIV) could be found in U.S. SIV. Viruses from the classical H1N1 SIV lineage evolved over time to form α-, β-, and γ-clusters. SIV with HA genes most similar to human seasonal H1 viruses emerged in 2003 to form the δ-cluster. All four HA cluster gene types can be found with neuraminidase genes of either N1 or N2 subtype. Limited sequence information was available regarding the 6 genes that make up the triple reassortant internal gene (TRIG) cassette in contemporary H1 SIV. In addition, information regarding the antigenic relatedness of the H1 viruses and diagnostic reagent updates was lacking due to the dynamic and variable nature of H1 SIV. We characterized 12 H1 isolates from 2008 by sequencing and phylogenetic analysis of all eight gene segments and by serologic cross-reactivity in the hemagglutination inhibition (HI) assay. Based on genetic analysis, each of the four previously described phylogenetic clusters of H1 SIV were represented in the 2008 panel. Additionally, it was demonstrated that the δ-cluster HA diverged into sub-clusters δ-1 and δ-2. Serologic cross-reactivity paired with antigenic cartography demonstrated that the phylogenetic clusters are also divergent antigenically. The genes composing the TRIG were all North American in lineage. Genetic diversity was demonstrated in all gene segments, but most notably in the HA gene. Gene segments from the 2009 pandemic H1N1 formed clusters separate from North American swine lineage viruses, suggesting this virus was not present in U.S. pigs prior to 2009. The HA variability has important implications for diagnostic testing as well as vaccine development.

Introduction

Influenza in swine is an acute respiratory disease caused by influenza A viruses of the family *Orthomyxoviridae*. Orthomyxoviruses have negative sense single 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), that define subtypes and are important for host range, antigenicity, pathogenesis, and diagnostic detection. Other than sporadic transmission to humans (1), classical swine influenza A viruses of the H1N1 subtype were historically distinct from avian and other mammalian influenza viruses based on host specificity, serologic type, and/or genotype. A novel H3N2 virus appeared in the U.S. swine population around 1998 and quickly became endemic. The H3N2 viruses were demonstrated to have acquired HA, NA, and PB1 genes of human virus origin; PA and PB2 genes of avian virus origin; and the remaining internal genes, NP, M, and NS, of swine virus origin, thus giving rise to the triple reassortant designation (2). The human lineage PB1, avian lineage PB2 and PA and swine lineage NP, M, and NS found in contemporary swine viruses are referred to as the triple reassortant internal gene (TRIG) constellation (3). Since 1998, 3 predominant swine influenza virus (SIV) subtypes have circulated in US swine, H1N1, H1N2, and H3N2. The H1N1 viruses contain the HA and NA from the classical swine H1N1 virus and the TRIG from triple reassortant H3N2 viruses making them reassortant H1N1 (rH1N1); the H1N2 viruses contain the HA from the classical swine virus and the NA and TRIG from the triple reassortant H3N2 viruses (4-5). Since the acquisition of the TRIG cassette, an increase in the rate of genetic change in North American swine influenza isolates appears to have occurred in H1 virus subtypes, and distinct genetic and antigenic clusters have evolved (6). Currently, there are a number of reassortant viruses that have been identified in pigs, including further drift variants of H3N2 (5, 7-9), H1N2 (4, 10), rH1N1 (5) and H3N1 viruses (11-12). The TRIG was shown to have accepted an avian H2 and N3, producing a novel triple reassortant swine H2N3 in 2006 (13). Furthermore, the recently emerged human 2009 pandemic H1N1 has been demonstrated to contain 6 gene segments of the North American triple reassortant lineage with the M and NA from the Eurasian lineage H1N1 (14-15). The events of

spring 2009 provide compelling evidence that the TRIG has an enhanced ability to pick up novel surface glycoprotein gene segments and these novel viruses can have pandemic potential.

The H3N2, rH1N1, and H1N2 viruses have become endemic and co-circulate in most major swine producing regions of the U.S. and Canada. Additionally, introduction of H1 viruses with the HA gene of human virus origin (hu-like H1) that are genetically and antigenically distinct from the classical swine H1 lineage were reported in pigs in Canada (16). The viruses identified in Canadian pigs were human lineage in entirety or double (human-swine) reassortants. Since 2005, hu-like H1N1 and H1N2 viruses have emerged in swine herds across the U.S. (17). The whole genome sequence analysis of 4 hu-like H1 viruses isolated from U.S. swine, 2 isolated in 2005 and 2 isolated in 2007 demonstrated these viruses had acquired the TRIG (18).

Antigenic changes in the influenza virus are routinely measured using the hemagglutination inhibition (HI) assay (19). Influenza viruses agglutinate red blood cells, and the HI assay tests the ability of antisera raised against reference viruses to inhibit such agglutination. Antigenic differences between circulating strains and vaccine strains can be inferred by comparing their HI titers against a panel of antisera raised to different viruses. Antigenic cartography enables reliable quantitative interpretation of HI assay data, increasing the resolution at which antigenic differences can be determined and facilitates the visualization and interpretation of antigenic data (20). Antigenic cartography has been used to describe the antigenic evolution of influenza A (H3N2) virus, since its appearance in humans in 1968 until 2003, as a two-dimensional (2D) antigenic map (20), and the antigenic evolution of influenza A (H3N2) viruses in swine as a three-dimensional (3D) antigenic map (21) and to investigate the global circulation of seasonal influenza A (H3N2) viruses (22). Antigenic cartography is now an integral part of the World Health Organization human influenza virus vaccine strain selection process and is used to assist OFFLU (the Joint OIE/FAO worldwide scientific network for the control of animal influenza) in avian influenza virus vaccine strain selection. The equine influenza A (H3N8) virus antigenic maps have been formally adopted for use in equine influenza vaccine strain selection at the annual meeting of OIE Experts on Control of Equine Influenza (23).

Objectives:

The objective of this proposal was to characterize 2008 isolates of H1 subtype swine influenza virus (SIV) based on 1) nucleotide sequencing and phylogenetic analysis of the hemagglutinin (HA) and neuraminidase (NA) genes and 2) serologic cross-reactivity in the hemagglutination inhibition (HI) assay and antigenic mapping. Twelve field isolates representing each of the genetic clusters of subtype H1 SIV from 2008 were received from the Minnesota Veterinary Diagnostic Laboratory for use in the study.

Materials and Methods

Swine influenza viruses were isolated from outbreaks of respiratory disease in pigs from routine diagnostic cases submitted to the MVDL. Viruses isolated in 2008 from the U.S. and Canada were selected from a database of 375 viral RNA sequences, each 900 nucleotides long, of the H1 type HA1 gene. The sequences were stored in a group database [Los Alamos National Laboratory, Influenza Sequence Database (http://www.flu.lanl.gov/)] and phylogenetic analyses were performed based on sequence alignment by the Clustal W method using the MegAlign program from the Lasergene package (DNAStar software package (Madison, WI). From the phylogenetic analyses of the HA1 region of the HA gene, 12 viruses were selected for inclusion in the study to represent each of the H1 clusters of SIV (Table 1).

Table I. 2008 MVDL H1 SIV sequence data set.

Pig Location		α-Cluster	β-Cluster	γ-Cluster	δ-Cluster	**Total**
USA	IA	0	11	6	14	**31**
	IL	3	6	26	8	**43**
	IN	0	0	1	0	**1**
	KS	1	4	2	4	**11**
	KY	0	5	0	0	**5**
	MI	0	0	1	1	**2**
	MN	6	58	16	48	**128**
	MO	6	13	7	3	**29**
	NC	0	2	30	18	**50**
	NE	0	7	1	2	**10**
	OH	0	0	11	5	**16**
	OK	0	5	0	16	**21**
	SD	1	0	2	1	**4**
	TX	0	2	1	7	**10**
	WI	0	3	5	1	**9**
CANADA	MB	4	0	0	0	**4**
	SK	1	0	0	0	**1**
TOTAL		**22**	**116**	**109**	**128**	**375**

Viruses were grown in Madin-Darby Canine Kidney (MDCK) cells in culture. Inactivated viruses for immunization were prepared at approximately 128 HA units per 50 µL (or maximum HA titer) with inactivation by ultraviolet irradiation and addition of a commercial adjuvant (Emulsigen D, MVP Labs) at a v:v ratio of 4:1 virus to adjuvant. Virus isolates contained in the inactivated vaccines were: A/SW/NC/02023/2008 H1N1, A/SW/OH/02026/2008 H1N1, A/SW/MO/02060/2008 H1N1, A/SW/IA/02096/2008 H1N1, A/SW/KY/02086/2008 H1N1, A/SW/MN/02011/2008 H1N2, A/SW/MN/02093/2008 H1N1, A/SW/MN/02053/2008 H1N1, A/SW/NE/02013/2008 H1N1, A/SW/NC/02084/2008 H1N1, A/SW/TX/01976/2008 H1N2, and A/SW/IA/02039/2008 H1N2 (Table I). Similarly prepared immune sera

from additional studies conducted at USDA NADC (6, 18, 24) were included in the antigenic mapping and described in

Table II. Selected 2008 H1 SIV and previous NADC H1 SIV isolates used for generation of anti-sera.

2008 H1 SIV	Cluster	Subtype	NADC H1 SIV	Cluster	Subtype
A/SW/MN/02053/2008	α	H1N1	A/SW/MN/37866/1999	α	H1N1
A/SW/MN/02093/2008	α	H1N1	A/SW/IA/1973	α	H1N1
A/SW/IA/02096/2008	β	H1N1	A/SW/WI/1/1968	α	H1N1
A/SW/KY/02086/2008	β	H1N1	A/SW/IA/1945	α	H1N1
A/SW/NE/02013/2008	β	H1N1	A/SW/IA/15/1930	α	H1N1
A/SW/NC/02084/2008	β	H1N1	A/SW/IA/00239/2004	β	H1N1
A/SW/NC/02023/2008	γ	H1N1	A/SW/NC/36883/2002	β	H1N1
A/SW/OH/02026/2008	γ	H1N1	A/SW/OH/511445/2007	γ	H1N1
A/SW/MO/02060/2008	γ	H1N1	A/SW/KS/00246/2004	γ	H1N2
A/SW/TX/01976/2008	δ1	H1N2	A/SW/MN/00194/2003	γ	H1N2
A/SW/IA/02039/2008	δ1	H1N2	A/SW/MN/1192/2001	γ	H1N2
A/SW/MN/02011/2008	δ1	H1N2	A/SW/MN/07002083/2007	δ2	H1N1
			A/CA/04/2009	2009p	H1N1
			A/Mexico/4108/2009	2009p	H1N1

Genetic characterization

The 12 selected viruses were grown at low passage number in MDCK cell culture, concentrated, and semi-purified on a sucrose cushion followed by library preparation for *de novo* pyrosequencing on a Genome Sequencer FLX system following the manufacturer's recommendations with modifications. Briefly, viral RNA was extracted, fragmented, reverse transcribed, and ligated to oligonucleotide adaptors containing multiplex identifier (MID) labels. Pools containing MID-labeled viral cDNA were used to prepare sequencing beads via Roche's GS-FLX standard chemistry emulsion-based PCR. Each pool contained viral cDNA from three to five different viruses. Prepared beads were loaded into the small 16 regions on a GS-FLX standard chemistry pico-titer plate according to manufacturer's recommendations and sequenced using the GS-FLX LR 70 standard chemistry. Sequencing reads were compared to an influenza database created from >85,000 influenza sequences extracted from GenBank in December, 2008. Using the BLAST results, sequencing reads were filtered, influenza specific reads extracted, and extracted reads assembled with the Roche GS De Novo Assembler (Newbler) version 2.0 application. Gene segments with large gaps were closed by traditional primer based sequencing using an ABI 3100 genetic analyzer. The sequence contigs were analyzed using SeqMan (DNASTAR). Sequence alignment for each individual gene segment and phylogenetic analyses were conducted using MEGA version 4.

Antigenic characterization

Four-week-old cross-bred pigs were obtained from a herd free of both influenza virus and porcine reproductive and respiratory syndrome virus (PRRSV) infections. Animals were group-housed in individual isolation rooms and cared for in compliance with the NADC Institutional Animal Care and Use Committee. All pigs were administered ceftiofur crystalline-free acid at the beginning of the acclimation period. Prior to vaccination, influenza virus was not detected in nasal swab samples and anti-influenza antibodies were not detected by the serum HI assay. Thus, all animals were demonstrated to be free of influenza virus. Two pigs per virus were immunized with inactivated virus combined with commercial adjuvant by an intramuscular route. Two doses of vaccine were given approximately 2-3 weeks apart; pigs with HI titers <1:80 after the second dose were given a

third dose of vaccine prior to final blood collection. At the end of the vaccination period, pigs were humanely euthanized with pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) approximately 2 weeks after the final vaccination for blood collection.

Sera were heat inactivated at 56°C and treated to remove non-specific agglutinators with a 20% suspension of kaolin followed by adsorption with 0.5% turkey red blood cells (RBC). The HI assays were performed with turkey RBC's with standard techniques (WHO Manual on Animal Influenza Diagnosis and Surveillance, http://www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf). The reciprocal of the ratio between heterologous and homologous HI titers for individual serum samples were calculated to indicate the fold-change over homologous reactions.

The quantitative analyses of the antigenic properties of swine influenza A (H1) viruses were performed using antigenic cartography, previously used for human and swine influenza A (H3N2) viruses (20-22) and swine origin A (H1N1) influenza virus in humans (15). In an antigenic map, the distance between antiserum point S and antigen point A corresponds to the difference between the $\log_2$ of the maximum titer observed for antiserum S against any antigen and the $\log_2$ of the titer for antiserum S against antigen A. Therefore, each titer in an HI table can be thought of as specifying a target distance for the points in an antigenic map. Modified multidimensional scaling methods are then used to arrange the antigen and antiserum points in an antigenic map to best satisfy all the target distances specified by the HI data. The result is a map in which the distance between points best represents antigenic distance as measured by the HI assay. Because antisera are tested against multiple antigens, and antigens tested against multiple antisera, many measurements are used to determine the position of the antigen and antiserum points in an antigenic map, thus potentially increasing the accuracy of point placement beyond that of individual HI measurements.

Results

Genetic Characterization

The pyrosequencing technology was successfully applied to multiplexed *de novo* sequencing of up to five viruses per region by use of oligo adaptors containing MID. Because of the large number of non-influenza virus sequence reads in preliminary sequencing efforts (background RNA from culture source, ie. MDCK cells) a two-step sequential sucrose semi-purification was performed in the final sequencing run. Additionally, a quantitative real-time RT-PCR for the matrix gene (25) was utilized to quantitate influenza virus-specific RNA prior to cDNA synthesis, and real-time PCR for the same matrix gene target was used to semi-quantitate viral cDNA just prior to sequencing. The average percentage of the twelve viral genomes sequenced by the Genome Sequencer was 79% of approximately 13,500 bases. In most cases, large contigs were assembled by the Newbler software for each gene segment for each virus. Comparison of the large *de novo* contigs by BLAST with known influenza sequences allowed lineage identification for all genes. Sanger sequencing using targeted oligo primers was utilized to walk across gaps or clean up sequences. No large contigs were produced for the NS gene of A/SW/OH/02026/2008.

Each of the eight gene segments were individually analyzed for phylogenetic relationships. The phylogenetic trees for the surface glycoproteins HA and NA are shown in Figure 1 A and B. The twelve selected H1 SIV represented each of the 4 previously described HA phylogenetic clusters. In addition, the HA from the δ-cluster viruses were shown to have diverged into two distinct sub-clusters, δ-1 and δ-2. All of the NA genes from the α, β, and γ-cluster viruses were N1 subtype. The 3 δ-1 viruses characterized in this study were N2 subtype. Variation was demonstrated in the 6 genes that make up the TRIG, but no HA cluster-specific patterns were detected among the TRIG genes (Figure 2 A-F). Importantly, no genes from the 2008 U.S. H1 viruses clustered with the genes of the 2009 pandemic H1N1 that emerged in the human population in the spring of 2009. In all of the phylogenetic analyses of each gene segment, the pandemic H1N1 formed a distinct branch from the U.S. swine lineage genes.

Antigenic Characterization

All immunized pigs seroconverted to the respective virus used in the vaccine preparation. Serologic cross-reactivity in the HI assay is summarized in Table III and the fold-reduction between homologous and heterologous serum and virus pairs is described in Table IV. Viruses representing the phylogenetic clusters evolved from the cH1N1 swine-lineage (α, β, and γ) demonstrated moderate to strong cross-reactivity within a cluster, especially within recent β- and γ-cluster viruses. However, cross-reactivity between clusters was more variable, ranging from no cross-reactivity to strong cross-reactivity. The lack of cross-reactivity was most dramatically demonstrated between the cH1N1 swine-lineage clusters and the δ-cluster. Based on the phylogenetic anaysis, all of the 2008 viruses were grouped as δ-1 whereas the A/sw/MN/07002083/2007 virus is in the δ-2 cluster; however, limited HI cross-reactivity was demonstrated within the δ-cluster overall. Because of this, the antigenic mapping of the δ-cluster viruses and anti-sera was less than optimal with only two viruses being successfully mapped.

Anti-sera generated in a subsequent experiment against two isolates of human 2009 pandemic H1N1 were additionally tested against select 2007-8 swine H1 viruses and results reported in Table V A and B. Reductions in cross-reactivity were evident; nonetheless, moderate cross-reactivity was demonstrated between the 2009 anti-sera and the α- and β-cluster swine isolates tested. Cross-reactivity between SIV antisera and 2009 viruses have been reported in a separate publication (26).

Discussion

In 2008, five genetic and antigenic clusters of H1 SIV co-circulated in the U.S. pig population with limited HI cross-reactivity between them (α-, β-, γ-, δ-1, and δ-2). Genetic diversity was demonstrated in all gene segments, but most notably in the HA gene. The antigenic diversity was consistent with the HA genetic diversity as measured by HI cross-reactivity and antigenic mapping. Antigenic cross-reactivity was limited between H1 clusters and supports the practice of combining several H1 viruses with H3N2 viruses in multivalent vaccines in most swine producing regions. This study demonstrates the antigenic diversity in swine influenza viruses in the U.S. both within a given year and over a period of years. The co-circulation of distinct

antigenic clusters that continue to drift emphasize the need for increased surveillence incorporated with antigenic analysis for vaccine strain selection. Further study is required to evaluate the usefulness of antigenic mapping for vaccine strain selection through experimental efficacy studies in pigs.

Vaccinating pigs against influenza virus is a common practice in the U.S. swine industry, with fully licensed commercial vaccines or autogenous vaccines. Autogenous vaccine usage against influenza virus has continued to increase due to the diversity of viruses circulating in the North American pig population and the inability of the animal biologics industry to change the vaccine composition as rapidly as the viruses are changing. Over half of the doses of SIV vaccine released in 2008 were autogenous (Rick Hill, USDA-APHIS-Center for Veterinary Biologics, personal communication). One of the highest priorities for the recently launched USDA SIV surveillance plan is to enhance monitoring of the evolution of viruses for relevant commercial vaccine strain selection and diagnostic reagent development.

The Genome Sequencer pyrosequencing technology was successfully applied to multiplexed *de novo* sequencing for nearly all segments of the 12 viruses. No large contigs were produced for the NS gene of A/SW/OH/02026/2008 and several segments had significant gaps to close, but the large contigs produced from the multiplexed pyrosequencing allowed identification of the sequences as influenza A as well as virus lineage determination by sequence analysis. The sequences generated from the Genome Sequencer were utilized for primer design for traditional Sanger sequencing to add additional sequence data. Further sequencing must be conducted to complete coding sequences for a number of segments prior to publication of the sequence. All of the α-, β-, and γ-cluster H1 viruses were paired with an N1 subtype. However, the 3 δ-1, and δ-2 cluster H1 viruses were paired with an N2 subtype. Additional testing is necessary to determine if this result extends as a trend among other field isolates of swine H1 influenza virus. In constrast to the HA and NA phylogenetic patterns, no patterns of clustering according to H1 cluster or NA subtype could be extended to the 6 genes composing the TRIG. This suggests that reassortment and drift of the H1 HA is occuring randomly on the TRIG backbone.

The pig has been suggested to be a "mixing vessel" capable of generating reassorted influenza viruses with pandemic potential due to the presence of both avian and mammalian receptors expressed by respiratory tract epithelial cells (27) along with human (28) and japanese quail (29). The 2009 pandemic A/H1N1 underscores the potential risk to the human population of other influenza virus subtypes and genotypes with the SIV TRIG backbone and demonstrates the potential for viruses with genes from swine lineages to emerge and cause a pandemic in the human population. However, gene segments from the 2009 pandemic H1N1 formed clusters separate from U.S. swine lineage viruses, suggesting this virus was not present in U.S. pigs prior to 2009 and likely some time prior to 2008.

The HA variability has important implications for diagnostic testing as well as vaccine development. Increased surveillance and monitoring for SIV in the swine population as well as the pandemic A/H1N1 worldwide are critical to understand the dynamic ecology of influenza A viruses in this susceptible host population. Likewise, development of a vaccine strain selection system through the USDA surveillance plan is critical for controlling SIV and reducing the risk of such reassortment events with the current 2009 A/H1N1 or other emerging viruses in the future.

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Table III. HI titers for individual serum samples*

A.

SERA → / VIRUS ↓	874	875	876	877	532	595	589	590	596	870	871	872	873	878	879	880	881	577	558
A/SW/MN/02053/2008	320	320	160	160	320	320	160	640	320	20	80	40	40	40	40	<10	20	160	80
A/SW/MN/02093/2008	160	320	160	320	320	320	80	2560	160	10	10	<10	20	10	10	<10	20	80	80
A/SW/MN/37866/1999	20	40	<10	40	160	160	320	640	320	<10	<10	<10	40	20	10	<10	10	80	40
A/SW/IA/1973	20	40	20	20	320	5120	1280	1280	1280	10	20	10	80	20	20	<10	40	160	80
A/SW/IA/1945	10	20	<10	20	160	160	1280	320	1280	<10	<10	<10	20	<10	<10	<10	<10	80	<10
A/SW/WI/1/1968	40	80	20	80	320	160	320	640	640	10	20	20	80	20	20	10	20	160	80
A/SW/IA/15/1930	20	20	<10	20	80	320	640	640	640	<10	<10	10	20	20	10	<10	<10	40	20
A/SW/IA/02096/2008	160	40	40	40	160	640	80	1280	640	80	320	80	160	160	80	<10	40	640	640
A/SW/KY/02086/2008	160	40	80	80	640	80	20	640	640	10	20	80	640	80	80	80	20	160	160
A/SW/NE/02013/2008	80	40	20	20	160	160	80	640	640	40	80	40	160	640	160	80	40	320	160
A/SW/NC/02084/2008	160	40	40	20	320	160	160	640	640	40	80	40	160	320	160	160	80	320	320
A/SW/IA/00239/2004	80	40	40	80	320	320	320	640	320	40	40	40	160	160	160	80	40	640	160
A/SW/NC/36883/2002	20	20	40	40	160	320	160	640	320	40	40	40	80	80	40	40	40	320	160
A/SW/NC/02023/2008	20	40	10	20	160	80	80	320	40	<10	<10	<10	10	<10	10	<10	<10	80	20
A/SW/OH/02026/2008	40	40	80	40	320	320	160	320	320	10	40	80	80	40	40	<10	40	160	1280
A/SW/MO/02060/2008	20	40	10	20	160	160	80	160	<10	<10	<10	<10	<10	<10	<10	<10	<10	40	<10
A/SW/OH/511445/2007	80	320	160	160	640	320	320	2560	320	80	160	40	160	160	160	80	80	320	160
A/SW/KS/00246/2004	80	160	40	80	320	160	80	320	80	<10	<10	<10	20	10	40	<10	20	80	40
A/SW/MN/00194/2003	40	40	10	10	80	20	20	40	20	20	10	10	10	20	10	<10	<10	20	10
A/SW/MN/1192/2001	40	40	10	10	320	80	160	80	<10	10	10	<10	20	10	10	<10	10	80	<10
A/SW/TX/01976/2008	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/02039/2008	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/SW/MN/02011/2008	<10	<10	<10	<10	20	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
A/SW/MN/07002083/2007	<10	<10	<10	<10	<10	<10	<10	40	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

B.

SERA →	864	865	866	867	868	869	821	581	560	592	882	883	884	885	886	887	328
VIRUS ↓																	
A/SW/MN/02053/2008	40	40	20	640	1280	640	320	320	40	80	<10	<10	<10	<10	<10	<10	<10
A/SW/MN/02093/2008	80	160	40	2560	1280	1280	1280	640	40	160	<10	<10	<10	<10	<10	<10	<10
A/SW/MN/37866/1999	80	80	10	1280	640	640	320	80	20	80	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/1973	40	80	<10	640	640	320	320	160	<10	1280	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/1945	40	40	<10	1280	640	640	160	80	<10	40	<10	<10	<10	<10	<10	<10	<10
A/SW/WI/1/1968	<10	40	10	1280	1280	1280	320	80	20	80	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/15/1930	<10	20	<10	640	320	160	80	20	<10	40	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/02096/2008	40	80	20	320	640	160	320	320	20	160	<10	<10	<10	<10	<10	<10	<10
A/SW/KY/02086/2008	40	40	<10	320	160	80	160	80	20	1280	<10	<10	<10	<10	<10	<10	<10
A/SW/NE/02013/2008	20	80	10	640	640	160	160	40	<10	80	<10	<10	<10	<10	<10	<10	<10
A/SW/NC/02084/2008	40	80	10	1280	640	320	320	160	20	320	<10	<10	<10	<10	<10	<10	<10
A/SW/IA/00239/2004	160	80	80	640	640	640	160	160	40	320	<10	<10	<10	<10	<10	<10	<10
A/SW/NC/36883/2002	40	40	20	320	640	320	80	40	40	20	<10	<10	<10	<10	<10	<10	<10
A/SW/NC/02023/2008	320	320	40	640	640	640	640	320	80	80	<10	<10	<10	<10	<10	<10	<10
A/SW/OH/02026/2008	160	640	80	1280	640	640	640	640	640	320	<10	<10	<10	<10	<10	<10	10
A/SW/MO/02060/2008	80	320	40	1280	1280	2560	640	160	40	40	<10	<10	<10	<10	<10	<10	<10
A/SW/OH/511445/2007	320	1280	160	2560	2560	1280	2560	1280	320	320	80	80	20	20	320	80	<10
A/SW/KS/00246/2004	160	160	160	640	1280	640	640	640	160	320	40	20	20	80	20	20	<10
A/SW/MN/00194/2003	40	80	40	320	320	320	160	80	640	80	40	20	20	40	40	40	10
A/SW/MN/1192/2001	160	640	40	1280	640	1280	640	320	80	640	10	<10	<10	10	<10	<10	<10
A/SW/TX/01976/2008	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	40	40	<10	<10	<10	<10	10
A/SW/IA/02039/2008	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	10	40	320	40	<10	<10	<10
A/SW/MN/02011/2008	<10	<10	<10	<10	<10	<10	<10	<10	20	40	40	40	40	20	2560	2560	40
A/SW/MN/07002083/2007	<10	10	<10	<10	10	10	<10	<10	<10	<10	160	80	20	<10	<10	<10	640

*Reciprocal HI titers for individual serum samples are reported for 24 different U.S. H1 swine influenza viruses. Tables IIA and IIB were split due to size constraints. Phylogenetic clusters are color coded by α-cluster (classic-like swine; cyan), β-cluster (rH1-like swine; pink), γ-cluster (In/00 or H1N2-like swine; blue), and δ-cluster (human-like; gold). Shaded cells indicate homologous anti-serum and virus and double bar boxes indicate reactions within phylogenetic clusters.

Table IV. Fold-reduction compared to homologous HI titer.*

A.

SERA → / VIRUS ↓	874	875	876	877	532	595	589	590	596	870	871	872	873	878	879	880	881	577	558
A/SW/MN/02053/2008			1	2	0.5	16	8	1	2	4	4	2	16	16	4	>16	4	4	2
A/SW/MN/02093/2008	2	1			0.5	16	16	0.25	4	8	32	>8	32	64	16	>16	4	8	2
A/SW/MN/37866/1999	16	8	>16	8		32	4	1	2	>8	>32	>8	16	32	16	>16	8	8	4
A/SW/IA/1973	16	8	8	16	0.5		1	0.5	0.5	8	16	8	8	32	8	>16	2	4	2
A/SW/IA/1945	32	16	>16	16	1	32		2	0.5	>8	>32	>8	32	>64	>16	>16	>8	8	>16
A/SW/WI/1/1968	8	4	8	4	0.5	32	4		1	8	16	4	8	32	8	16	4	4	2
A/SW/IA/15/1930	16	16	>16	16	2	16	2	1		>8	>32	8	32	32	16	>16	>8	16	8
A/SW/IA/02096/2008	2	8	4	8	1	8	16	0.5	1			1	4	4	2	>16	2	1	0.25
A/SW/KY/02086/2008	2	8	2	4	0.25	64	64	1	1	8	16			8	2	2	4	4	1
A/SW/NE/02013/2008	4	8	8	16	1	32	16	1	1	2	4	2	4			2	2	2	1
A/SW/NC/02084/2008	2	8	4	16	0.5	32	8	1	1	2	4	2	4	2	1			2	0.5
A/SW/IA/00239/2004	4	8	4	4	0.5	16	4	1	2	2	8	2	4	4	1	2	2		1
A/SW/NC/36883/2002	16	16	4	8	1	16	8	1	2	2	8	2	8	8	4	4	2	2	
A/SW/NC/02023/2008	16	8	16	16	1	64	16	2	16	>8	>32	>8	64	>64	16	>16	>8	8	8
A/SW/OH/02026/2008	8	8	2	8	0.5	16	8	2	2	8	8	1	8	16	4	>16	2	4	0.125
A/SW/MO/02060/2008	16	8	16	16	1	32	16	4	>64	>8	>32	>8	>64	>64	>16	>16	>8	16	>16
A/SW/OH/511445/2007	4	1	1	2	0.25	16	4	0.25	2	1	2	2	4	4	1	2	1	2	1
A/SW/KS/00246/2004	4	2	4	4	0.5	32	16	2	8	>8	>32	>8	32	64	4	>16	4	8	4
A/SW/MN/00194/2003	8	8	16	32	2	256	64	16	32	4	32	8	64	32	16	>16	>8	32	16
A/SW/MN/1192/2001	8	8	16	32	0.5	64	8	8	>64	8	32	>8	32	64	16	>16	8	8	>16
A/SW/TX/01976/2008	>16	>16	>16	>32	>16	>512	>128	>64	>64	>8	>32	>8	>64	>64	>16	>16	>8	>64	>16
A/SW/IA/02039/2008	>16	>16	>16	>32	>16	>512	>128	>64	>64	>8	>32	>8	>64	>64	>16	>16	>8	>64	>16
A/SW/MN/02011/2008	>16	>16	>16	>32	8	>512	>128	>64	>64	>8	>32	>8	>64	>64	>16	>16	>8	>64	>16
A/SW/MN/07002083/2007	>16	>16	>16	>32	>16	>512	>128	16	>64	>8	>32	>8	>64	>64	>16	>16	>8	>64	>16

B.

SERA → / VIRUS ↓	864	865	866	867	868	869	821	581	560	592	882	883	884	885	886	887	328
A/SW/MN/02053/2008	8	8	4	2	1	4	8	2	16	8	>4	>4	>32	>4	>256	>256	>64
A/SW/MN/02093/2008	4	2	2	0.5	1	2	2	1	16	4	>4	>4	>32	>4	>256	>256	>64
A/SW/MN/37866/1999	4	4	8	1	2	4	8	8	32	8	>4	>4	>32	>4	>256	>256	>64
A/SW/IA/1973	8	4	>8	2	2	8	8	4	>64	0.5	>4	>4	>32	>4	>256	>256	>64
A/SW/IA/1945	8	8	>8	1	2	4	16	8	>64	16	>4	>4	>32	>4	>256	>256	>64
A/SW/WI/1/1968	>32	8	8	1	1	2	8	8	32	8	>4	>4	>32	>4	>256	>256	>64
A/SW/IA/15/1930	>32	16	>8	2	4	16	32	32	>64	16	>4	>4	>32	>4	>256	>256	>64
A/SW/IA/02096/2008	8	4	4	4	2	16	8	2	32	4	>4	>4	>32	>4	>256	>256	>64
A/SW/KY/02086/2008	8	8	>8	4	8	32	16	8	32	0.5	>4	>4	>32	>4	>256	>256	>64
A/SW/NE/02013/2008	16	4	8	2	2	16	16	16	>64	8	>4	>4	>32	>4	>256	>256	>64
A/SW/NC/02084/2008	8	4	8	1	2	8	8	4	32	2	>4	>4	>32	>4	>256	>256	>64
A/SW/IA/00239/2004	2	4	1	2	2	4	16	4	16	2	>4	>4	>32	>4	>256	>256	>64
A/SW/NC/36883/2002	8	8	4	4	2	8	32	16	16	32	>4	>4	>32	>4	>256	>256	>64
A/SW/NC/02023/2008			2	2	2	4	4	2	8	8	>4	>4	>32	>4	>256	>256	>64
A/SW/OH/02026/2008	2	0.5			2	4	4	1	1	2	>4	>4	>32	>4	>256	>256	64
A/SW/MO/02060/2008	4	1	2	1			4	4	16	16	>4	>4	>32	>4	>256	>256	>64
A/SW/OH/511445/2007	1	0.25	0.5	0.5	0.5	2		0.5	2	2	0.5	0.5	16	2	8	32	>64
A/SW/KS/00246/2004	2	2	0.5	2	1	4	4		4	2	1	2	16	0.5	128	128	>64
A/SW/MN/00194/2003	8	4	2	4	4	8	16	8		8	1	2	16	1	64	64	64
A/SW/MN/1192/2001	2	0.5	2	1	2	2	4	2	8		4	>4	>32	4	>256	>256	>64
A/SW/TX/01976/2008	>32	>32	>8	>128	>128	>256	>256	>64	>64	>64			>32	>4	>256	>256	64
A/SW/IA/02039/2008	>32	>32	>8	>128	>128	>256	>256	>64	>64	>64	4	1			>256	>256	>64
A/SW/MN/02011/2008	>32	>32	>8	>128	>128	>256	>256	>64	32	16	1	1	8	2			16
A/SW/MN/07002083/2007	>32	32	>8	>128	128	256	>256	>64	>64	>64	0.25	0.5	16	>4	>256	>256	

*The reciprocal of the ratio between heterologous and homologous HI titers for individual serum samples are reported for 24 different U.S. H1 swine influenza viruses. Tables IIA and IIB were split due to size constraints. Phylogenetic clusters are color coded by α-cluster (classic-like swine; cyan), β-cluster (rH1-like swine; pink), γ-cluster (In/00 or H1N2-like swine; blue), and δ-cluster (human-like; gold). Solid cells indicate homologous anti-serum reactions and virus and double bar boxes indicate reactions within phylogenetic clusters.

Table V

A. HI titers for individual serum samples*

SERA →	960	962	963	966
VIRUS ↓				
A/CA/04/09	640	640	320	320
A/Mexico/4108/09	640	640	640	1280
A/Sw/MN/02053/08	10	40	20	20
A/Sw/MN/2093/08	160	160	40	40
A/Sw/IA/02096/08	80	80	80	40
A/Sw/KY/02086/08	80	160	160	80
A/Sw/NE/02013/08	40	20	80	20
A/Sw/NC/02084/08	80	160	80	80
A/Sw/NC/02023/08	160	160	160	160
A/Sw/OH/02026/08	80	80	80	80
A/Sw/MO/02060/08	80	80	80	40
A/Sw/0H/511445/07	80	80	80	80

*Reciprocal HI titers for individual serum samples against human isolates of 2009 pandemic H1N1 are reported for 2007-8 U.S. H1 swine influenza viruses representing the swine-lineage α-, β-, and γ-clusters. Phylogenetic clusters are color coded by α-cluster (classic-like swine; cyan), β-cluster (rH1-like swine; pink), γ-cluster (In/00 or H1N2-like swine; blue), δ-cluster (human-like; gold), A(H1N1) pdm (red). Shaded cells indicate homologous anti-serum and virus and double bar boxes indicate phylogenetic clusters.

B. Fold-reduction compared to homologous HI titer*

SERA →	960	962	963	966
VIRUS ↓				
A/CA/04/09			2	2
A/Mexico/4108/09	1	1		
A/Sw/MN/02053/08	64	16	32	32
A/Sw/MN/2093/08	4	4	16	16
A/Sw/IA/02096/08	8	8	8	16
A/Sw/KY/02086/08	8	4	4	8
A/Sw/NE/02013/08	16	32	8	32
A/Sw/NC/02084/08	8	4	8	8
A/Sw/NC/02023/08	4	4	4	4
A/Sw/OH/02026/08	8	8	8	8
A/Sw/MO/02060/08	8	8	8	16
A/Sw/0H/511445/07	8	8	8	8

*The reciprocal of the ratio between heterologous and homologous HI titers for individual serum samples are reported against anti-sera from human isolates of 2009 pandemic H1N1 are reported for 2007-8 U.S. H1 swine influenza viruses representing the swine-lineage α-, β-, and γ-clusters. Phylogenetic clusters are color coded by α-cluster (classic-like swine; cyan), β-cluster (rH1-like swine; pink), γ-cluster (In/00 or H1N2-like swine; blue), and δ-cluster (human-like; gold). Solid cells indicate homologous anti-serum and virus reactions.

Figure 1. Phylogenetic trees for each of the HA and NA gene segments based on nucleotide sequences from the 12 U.S. isolates (indicated by closed circles) and other sequences available from GenBank. A). HA phylogenetic tree with four clusters of related viruses, H1α (cH1N1), H1β (rH1N1-like), H1γ (H1N2-like), and H1δ (hu-like H1) as indicated by the bars on the right of the tree. The H1δ cluster can be further split into δ-1 and δ-2 sub-clusters. B). NA phylogenetic tree with N1 and N2 subtypes and lineages indicated by bars on the right. Phylogenetic analyses were conducted in MEGA4. The reference viruses used in the analysis are abbreviated with their state and year of origin, subtype preceded by host abbreviation (sw=swine; hu=human; du= duck; and tu=turkey), and GeneBank accession number.

Figure 2. Phylogenetic trees for the 6 internal gene segments based on nucleotide sequences from the 12 U.S. H1 isolates (indicated by closed circles) and other sequences available from GenBank: A) PB2; B) PB1; C) PA; D) NP; E) M; F) NS. The triple reassortant internal gene (TRIG) constellation is indicated by the bar to the right and other lineages are indicated by group labels of compressed branches. Phylogenetic analyses were conducted in MEGA4. The reference viruses used in the analysis are abbreviated with their state and year of origin, subtype preceded by host abbreviation (sw=swine; hu=human; du= duck; ch=chicken; and tu=turkey), and GeneBank accession number.

Figure 3. Antigenic map of swine influenza A (H1) viruses. The relative positions of strains (colored dots) and swine hyperimmune antisera (open squares) were computed such that the distances between strains and antisera in the map with the least error represent the corresponding HI measurements (Smith et al, 2004). Strain color indicates α-cluster (classic-like swine; cyan), β-cluster (rH1-like swine; pink), γ-cluster (In/00 or H1N2-like swine; blue), δ-cluster (human-like; gold), A(H1N1) pdm (red). Each grid square represents 1 unit of antigenic distance, corresponding to a twofold dilution of antiserum in the HI assay.