JVI Accepts, published online ahead of print on 12 February 2014
J. Virol. doi:10.1128/JVI.03805-13

Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine

Nicola S. Lewis,[a] Tavis K. Anderson,[b,*] Pravina Kitikoon,[b,¶] Eugene Skepner,[a] David F. Burke,[a] Amy L. Vincent[b, #]

Department of Zoology, University of Cambridge, Cambridge, UK[a]; Virus and Prion Research Unit, National Animal Disease Center, USDA-ARS, Ames, IA, USA[b]

[#]Address correspondence to Amy L. Vincent, amy.vincent@ars.usda.gov

[¶]Present address: Merck Animal Health, De Soto, KS, USA

*Present address: Department of Biology, Georgia Southern University, Statesboro, GA, USA

Running Head: Evolution of swine influenza A (H3N2) virus

Abstract = 234 words; Main text = 5814 words.

ABSTRACT

Swine influenza A virus is an endemic and economically important pathogen in pigs with the potential to infect other host species. The hemagglutinin (HA) protein is the primary target of protective immune responses and the major component in swine influenza A vaccines. However, as a result of antigenic drift, vaccine strains must be regularly updated to reflect currently circulating strains. Characterizing the cross-reactivity between strains in pigs and seasonal influenza strains in humans is also important in assessing the relative risk of interspecies transmission of viruses from one host population to the other. Hemagglutination inhibition (HI) assay data for swine and human H3N2 viruses were used with antigenic cartography to quantify the antigenic differences among H3N2 viruses isolated from pigs in the USA from 1998-2013 and the relative cross-reactivity between these viruses and current human seasonal influenza A strains. Two primary antigenic clusters were found circulating in the pig population, but with enough diversity within and between the clusters to suggest updates in vaccine strains are needed. We identified single amino acid substitutions likely responsible for antigenic differences between the two primary antigenic clusters and between each antigenic cluster and outliers. The antigenic distance between current seasonal influenza H3 strains in humans and those endemic in swine suggests that population immunity may not prevent the introduction of human viruses into pigs and possibly vice-versa, reinforcing the need to monitor and prepare for potential incursions.

Importance

Influenza A virus (IAV) is an important pathogen in pigs and humans. The hemagglutinin (HA) protein is the primary target of protective immune responses and the major target of vaccines. However, vaccine strains must be updated to reflect current strains. Characterizing the differences between seasonal IAV in humans and swine IAV is important in assessing the relative risk of interspecies transmission of viruses. We found two primary antigenic clusters of H3N2 in the U.S. pig population with enough diversity to suggest updates in swine vaccine strains. We identified changes in the HA protein that are likely responsible for these differences that may be useful in

predicting when vaccines need to be updated. The difference between human H3N2 and those in swine is enough that population immunity is unlikely to prevent new introductions of human IAV into pigs or vice-versa, reinforcing the need to monitor and prepare for potential introductions.

INTRODUCTION

Influenza A viruses (IAV) have negative sense RNA genomes consisting of 8 segments. To date, the influenza A subtype is comprised of combinations of 17 hemagglutinin (HA) and 11 neuraminidase (NA) surface glycoproteins (1-7). Waterfowl are the natural reservoir of most IAV subtypes and in these species, infections are generally non-pathogenic. In certain instances, these viruses can cause substantial morbidity and mortality following transmission to other species (e.g., (8-10)). However, only H1N1, H1N2, and H3N2 subtypes are endemic in swine populations globally (11) and virulence is variable depending on properties of the virus, environment, and particularly the host and population immunity.

Swine influenza was first recognized as a respiratory disease that coincided with the human Spanish flu pandemic in 1918. The classical swine A(H1N1) viruses were derived from the 1918 human pandemic virus and remained endemic in the swine population with little evidence of antigenic drift for approximately 80 years. In 1998, a novel virus emerged in North American pigs containing what has become known as the triple-reassortant internal gene (TRIG) cassette, with genetic components from classical swine H1N1 (NP, M, NS), human seasonal H3N2 influenza (PB1, HA, NA) and North American avian influenza (PB2, PA) viruses. The HA genes from the triple reassortant H3N2 were the contribution of 3 separate phylogenetically distinct human seasonal virus introductions, termed Clusters I, II, and III (12), with the cluster III H3 evolving into a separate Cluster IV (13). These TRIG-viruses subsequently reassorted with the classical H1N1 swine viruses resulting in distinct H1N1 or H1N2 subtype lineages (14-16). The H1N1 and H1N2 subtypes then evolved in pigs to form the contemporary α, β and γ clusters (17). Then in 2005, H1N1 and H1N2 influenza viruses with the HA and/or NA derived from seasonal human influenza A viruses circulating in 2002 emerged in pigs and spread across the U.S. in swine herds. Currently, H1N1, H1N2 and H3N2 subtypes of IAV are endemic in pigs in North America (12, 18). The marked genetic heterogeneity of HA's circulating in North American pigs have potential antigenic consequences in terms of diagnostic test efficacy, use of vaccine as a means of control, and

assessing the relative risk of further introductions of human seasonal influenza viruses into the pig population.

Introduction of endemic swine IAV into humans continues to occur, with the most recently notable pandemic H1N1 virus (H1N1pdm09) that emerged in the human population in North America in 2009 (19). However, in the summers of 2011-2013 there were multiple infections of H3N2v in people attending agricultural fairs in a number of states in the U.S. (20, 21) with nearly 350 cases of H3N2v now detected in humans (http://www.cdc.gov/flu/swineflu/variant-cases-us.htm). A factor for the increased frequency of H3N2v detections is the relative lack of human population immunity against variants of IAV that have continued to circulate independently in swine, with the ever-present potential for these variants to evolve antigenically, perhaps away from their respective human seasonal precursor viruses and the strains used in contemporary human seasonal vaccines. A substantial proportion of adolescents and young adults were shown to have cross-reactive antibodies against H3N2v; however, children and older adults lacked such protective antibodies (22, 23). The current human seasonal vaccines containing H3N2 do not appear to protect against H3N2v (22, 24). Since the vast majority of cases of H3N2v have been in children with close contact and long periods of exposure time at agricultural fairs, all of these factors point to a unique set of circumstances that collectively increased the odds for H3N2v in these spillover events (25). The unique circumstances do not diminish the epidemic or pandemic risk of H3N2v to humans if these viruses gained the ability to efficiently transmit from human to human, allowing the virus further opportunity to mutate and adapt to the human host. Thus swine IAV not only cost the swine industry in terms of animal health and production (8), but also pose a potential risk to human health. Insights into patterns of swine IAV genetic and antigenic diversity are critical to identify risks to human and swine populations for interspecies transmission and provide criteria for updating influenza diagnostics and vaccine composition.

To accurately assess the risk to pigs from introduction of human seasonal IAV and vice versa requires an understanding of the population dynamics, evolution, and function of circulating

swine IAV. Quantitative analyses of key factors that contribute to zoonotic risk, namely the relative antigenic cross-reactivity of currently circulating human and swine IAV strains, will allow for improved methods of control by optimizing vaccination in swine. Here, we quantified the antigenic and genetic evolution of swine H3N2 influenza A viruses circulating in pigs from 1998-2013 across the U.S. with a focus on contemporary strains and we related the cross-reactivity of these viruses to currently circulating human seasonal H3 influenza viruses used as vaccine strains, demonstrating substantial antigenic differences between contemporary swine H3N2 circulating in the U.S. and those included in human vaccines. Integrating the antigenic data with HA1 domain sequences, we examined the genetic basis for antigenic differences among circulating swine H3N2 isolates, and identified amino acid substitutions that may lead to immune escape and vaccine failure in pigs.

MATERIALS AND METHODS

Viruses

Forty-two swine and human influenza A H3N2 viruses were selected as hemagglutinin inhibition (HI) test antigens and/or antigens for swine H3N2-antisera production (Table 1). The swine H3N2 viruses (n = 33) represented twelve U.S. states and major swine production regions and included 1 cluster I H3 from 1998 and 1 cluster II from 1999 as historical references, and 31 cluster IV isolates from 2006-2013 for our contemporary analysis. Viruses isolated from 1998-2009 (n = 6) from outbreaks of respiratory disease in pigs from diagnostic cases were obtained from the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL, kindly provided by Dr. Marie Culhane). The remaining 2010-2013 viruses were obtained from the USDA-National Animal Health Laboratory Network (NAHLN) voluntary swine influenza A virus (IAV) surveillance system repository held at the National Veterinary Service Laboratories (kindly provided by Dr. Sabrina Swenson). Viruses were selected based on the H3 gene phylogeny, representing the maximum number of swine-producing states, and representing each of the clusters IV and IVA-F. The cluster designations were based upon phylogenetic support (nodes with supportive bootstrap values >70) that also met genetic distance criteria of >5% from other clusters. Available virus isolates meeting

this criteria with acceptable growth properties were then randomly selected for study. Human seasonal H3N2 viruses isolated in 1995-2011 (n = 8) and incorporated into the 1996-2013 human influenza vaccines for the Northern hemisphere were obtained from St. Jude Children's Research Hospital (kindly provided by Dr. Richard Webby). One non-seasonal human virus, A/Indiana/08/2011 was provided from the Center for Disease Control and Prevention (CDC) (kindly provided by the late Dr. Alexander Klimov). This virus was isolated from a human case infected with swine IAV reported in July 2011 and is classified as an H3N2 variant (H3N2v) virus. Viruses were propagated in Madin-Darby canine kidney (MDCK) cells, MDCK-London (MDCK-L, Influenza Reagent Resource, VA, USA) cells or embryonated eggs. Harvested cell culture supernatant or allantoic fluid was clarified by centrifugation and virus was concentrated by ultracentrifugation over a 20% sucrose cushion. Virus pellets were resuspended overnight at 4^{o}C in sterile phosphate buffered saline at pH 7.4 and stored at -80°C.

Swine antisera production

Three week-old cross-bred pigs free of IAV and antibody, porcine reproductive and respiratory syndrome virus, porcine circovirus 2 and *Mycoplasma hyopneumoniae* were obtained. For each virus, two pigs were immunized with 128-256 hemagglutinin units (HAU) of ultraviolet (UV) inactivated IAV combined with 20% commercial adjuvant (Emulsigen D; MVP Laboratories, NE, USA) by the intramuscular route. Two or three doses of UV inactivated vaccines were given approximately 2–3 weeks apart. Pigs were bled weekly post-vaccination to test for HI titers against homologous virus. When HI titers to homologous virus reached at least 1:160, pigs were humanely euthanized with pentobarbital sodium (Fatal Plus, Vortech Pharmaceuticals, MI, USA) for blood collection. Sera were collected and stored at -20^{o}C.

Virus antigenic characterization

HI assays using post-vaccination pig antisera were performed to compare the antigenic properties of swine and human IAV viruses. Prior to HI testing, sera were treated with receptor-destroying enzyme (Sigma-Aldrich, MO, USA), heat inactivated at 56^{o}C for 30 min and adsorbed with 50%

turkey red blood cells (RBC) to remove nonspecific inhibitors of hemagglutination. HI assays were performed by testing reference antisera raised against 18 swine and 9 human influenza A H3N2 viruses with 42 H3N2 viruses according to standard techniques. Serial 2-fold dilutions starting at 1:10 were tested for their ability to inhibit the agglutination of 0.5% turkey RBC with 4 HAU of swine and human H3N2 viruses. All HI assays were performed in duplicate. See Table 1 for list of viruses and reference antisera.

Antigenic cartography

The quantitative analyses of the antigenic properties of swine and human influenza A H3N2 viruses were performed using antigenic cartography as previously described for human H3 and swine influenza A H3 and H1 viruses (26-29). Antigenic clusters were defined using a Ward Hierarchical Clustering approach, as K-means clustering was biased by the large number of antigenic outliers in the dataset, using Euclidean distances among strains in the antigenic map implemented in R version 3.0.2 (30). To quantify the relative distances from vaccines to currently circulating viruses we measured the antigenic distance from representative Cluster I and Cluster IV swine vaccine strains and the human strain, A/Victoria/361/2011, to all other swine influenza A H3N2 viruses and plotted these against year of isolation using R version 3.0.2 (30).

Model of the structure of swine influenza A hemagglutinin

A model of the structure of the HA of A/Swine/Illinois/A01241469/2012 was built by using Choral (31) and Andante (32) based upon the crystal structure of H3N2 HA of the A/Aichi/1/68 (PDB code 2viu) and subsequently visualized with PyMOL (33).

Phylogenetic and sequence analyses

Contemporary H3N2 influenza A hemagglutinin (HA) and neuraminidase (NA) sequences representing clade designations described in (34) were compiled with sequences used in the HI-assay (Table S1). Amino acid alignments of the HA1 domain and NA were generated using default settings in MUSCLE v.3.8.31 (35) with subsequent manual correction in Mesquite (36). For each alignment, we inferred the best-known maximum likelihood tree using RAxML v7.3.4 (37) by

initiating 500 independent tree searches from random start trees generated under parsimony methods implemented with the best fit model of evolution determined in ProtTest v.3.2 ((38): data available upon request). Thereafter, we executed 1000 nonparametric bootstraps and the support values obtained were drawn on the best-scoring tree.

To estimate the average rate of nucleotide substitution in the HA1 domain, we constructed a second dataset incorporating all U.S. swine IAV H3N2 HA1 sequences from 1997 to present: nucleotide sequences were downloaded from the Influenza Virus Resource (39) on July 2, 2013 (Table S2: Figure S1). A maximum likelihood tree was inferred using RAxML (v7.4.2; (37)) on the CIPRES Science Gateway (40) employing a general time-reversible (GTR) model of nucleotide substitution with Γ-distributed rate variation among sites. The starting tree was generated under parsimony methods, with the best-scoring tree and statistical support values obtained with the rapid bootstrap algorithm (1,000 replications). Subsequently, we extracted the patristic distance from A/Wuhan/359/95 in the ML tree to each isolate in Cluster IV H3N2 swine IAV clade using program R v.3.0.2 with the APE (41) and GEIGER (42) packages. Linear models of genetic distance (response vector) and time (linear predictor for response) were fitted using the program R v.3.0.2 (30).

HA1 domain deduced amino acid sequence alignments were used to calculate the number of amino acid substitutions between pairs of isolates. We made genetic maps using a similar method to that used for antigenic maps except that the target distances were the number of amino acid substitutions between the amino acid sequences for each antigen in the antigenic map (26).

Analyses of antigenic evolution

Not all substitutions will be responsible for antigenic changes in the HA. An amino acid substitution X to Y at location L is considered a "cluster-difference" substitution between clusters A and B if all (or all but one) isolates in cluster A have amino acid X at location L and all (or all but one) isolates in cluster B have amino acid Y at location L (26, 43). We used this classification and the HA1 domain amino acid alignments above, to determine which amino acids likely defined the

204 difference among swine influenza A H3N2 virus antigenic clusters and outlying variants, and
205 compared these results to the antigenic effects of the cluster-difference substitutions observed for
206 H3N2 influenza A viruses in humans (43).

207 **RESULTS**

208 ***Swine H3N2 viruses are antigenically diverse***

209 Cross HI titers were tabulated (Table S3) and used for antigenic cartography analyses. One strain,
210 A/swine/Minnesota/01146/2006, showed the broadest cross-reactivity against the swine H3N2
211 antisera tested and may serve as a suitable contemporary reference strain. However, among the
212 swine and human influenza A H3N2 viruses, HI cross-reactivity was highly variable and these
213 antigenic relationships are shown in the 3D antigenic map in Figure 1A, with each antigen colored
214 according to the antigenic cluster to which it belonged. The swine viruses circulating between
215 2006-2013 formed two major antigenic groups, the cyan and the red antigenic clusters. Other more
216 antigenically diverse strains arising between 2010-13 were also observed, classified as outliers in
217 the cluster analyses, and identified with unique color-coding. A/Wuhan/359/1995 and the cluster I
218 prototype swine influenza A H3N2 virus are shown in light blue, and A/Sydney/5/1997,
219 A/Moscow/10/1999 and the cluster II prototype swine influenza A H3N2 viruses are shown in light
220 pink. Light grey spheres are human H3N2 isolates from 2002 -2011 and the large grey sphere is
221 A/Victoria/361/2011.

222 ***Genetic evolution of U.S. swine H3 between 1995 and 2013***

223 The ML phylogenetic tree (Figure 2A) shows that the genetic evolution of the swine influenza A
224 (H3N2) viruses consisted of 5 contemporary clades evolved from Cluster IV. In agreement with the
225 criteria previously suggested, a 5-7% average pairwise nucleotide distance threshold (18) continued
226 to define the new putative clusters of contemporary swine H3. Thus, clusters A, B, C, E and F were
227 identified as newly formed genetic clusters, as evidenced by the pairwise criteria as well as onward
228 transmission into 2013 and continued genetic evolution. Figure 2C shows the genetic map made

from pairwise differences among strains and again demonstrates the HA clade evolution of currently circulating strains.

There was a lack of concordance between the topology of the HA and NA gene phylogenies (Figure 2A and 3). The NA phylogeny reveals that the majority of our contemporary isolates have an N2 gene derived from a 2002 human origin N2 lineage. However, there were no H3 cluster predilections for the possession of the 1998 or 2002 lineage N2 genes with the exception of Cluster IV-F. This is demonstrated by isolates classified with specific Cluster IV lineages using the HA gene, but being scattered incongruously throughout NA gene lineage.

To evaluate the amount of variation accrued over time in the swine H3 genes, the distance from A/Wuhan/359/95 to each isolate in Cluster IV was plotted as a function of time (Figure 4). The regression line had a slope of 0.006 (x-intercept = -11.58 ± 0.27 S.E.: Adjusted R^2 = 0.75: p-value < 0.0001): the slope gives the rate of evolution of nucleotide substitutions per year. There was an apparent increase in diversity since the emergence and reassortment of the H1N1pdm09 viruses in the U.S. swine population since 2009; however, limited sampling prior to 2009 may have biased our inference. The solid regression line for the three years prior to 2009 had a slope of 0.003 (x-intercept = -7.84 ± 1.31 S.E.: Adjusted R^2 = 0.30: p-value < 0.0001) whereas the hatched regression line for 2010-present had a slope of 0.005 (x-intercept = -10.40± 0.68 S.E.: Adjusted R^2 = 0.31: p-value < 0.0001). Though the regression lines had significantly different intercepts (ANCOVA: p-value < 0.0001), the difference in rates of evolutionary change was suggestive but not statistically significant (ANCOVA: p-value = 0.12). Retrospective sampling of viruses isolated prior to 2009 or alternate phylogenetic techniques are required to tease apart these dynamics.

Predictability of antigenic cluster by phylogenetic cluster

Since vaccine strain selection or choice of currently available vaccine for swine in the US relies primarily on the genetic similarity at the nucleotide level between vaccine strains and the outbreak strain, we investigated whether the antigenic phenotype could be predicted from the genetic cluster of a particular isolate. When we colored the ML phylogenetic tree (Figure 2B) and genetic map

(Figure 2D) according to antigenic cluster to which each strain belonged we found that the antigenic cluster was not predicted from the phylogenetic clade alone, or pairwise comparison of amino acid sequences, particularly for isolates in Clusters IV A, B, D and F. For example, red antigenic cluster isolates were located genetically in the newly formed Clusters IV-A, -B and -D. Cyan-colored antigenic cluster isolates were located genetically in Clade IV and Clade IV-F. The isolate A/swine/Pennsylvania/A01076777/2010 was a genetic outlier not clustered in one of the newly emerged phylogenetic branches, yet mapped antigenically with the red cluster.

Genetic basis for antigenic differences among currently circulating swine influenza A (H3) viruses

To investigate the molecular basis of the antigenic clusters, we aligned the amino acid sequences used in this study (Figure S2), grouped and color-coded based on the antigenic cluster, and marked with the cluster-defining amino acid substitutions relative to the earliest Cluster I H3 cluster in pigs from a Wuhan 95-like human seasonal influenza A H3 virus introduction. A subsequent seasonal human influenza A H3 introduction into pigs from a Sydney 97-like virus led to the swine Cluster II viruses and differs from the Wuhan-like Cluster I strains at amino acid positions 156 and 158 for all antigens.

Focusing on the currently circulating strains in North American pigs, we found 2 main antigenic clusters and 10 different antigenic variants mapping a significant antigenic distance away from the two primary clusters (red cluster in Figure 1B and cyan cluster in 1C). The cyan cluster consisted of strains isolated from 2004-2012, and contains strains from the same genetic cluster as the putative Cluster IV vaccine strain. The red cluster consisted of strains isolated from 2010-2013 and included the H3N2v strain A/Indiana/08/2011 representing the human agricultural fair outbreaks of 2011-12. The two spheres colored in gold represent the isolate A/swine/Minnesota/A01125993/2012, which differed from the red cluster at position 145 and 159 (2 A/swine/Minnesota/A01125993/2012 isolates with different passage history were analysed here and thus the data were not combined). One or both of these 2 amino acid substitutions (N145K and

Y159N) likely results in the 6 antigenic unit distance from the red cluster. The strain colored in light green isolated in 2013 (A/swine/Wyoming/A01444562/2013) differed from the red cluster at only position 145 and was 5.5 antigenic units away from the red cluster. The strain colored in blue A/Swine/Michigan/A01203498/2012 differed from the majority of the red cluster strains at positions 145 and 155 and was positioned ~5 antigenic units away. A/swine/North Carolina/A01432566/2013 (dark green) differed from the red cluster at positions 145, 156, and 189 and was 7.5 antigenic units away. The two strains colored in purple (A/swine/Nebraska/A01271549/2012 and A/swine/Iowa/A01432500/2013) differed from the red cluster at two amino acid positions (N145K, K189R/S) and were 8 and 9.4 antigenic units away. They differed from each other only at position 189 and are ~1 antigenic unit apart. Thus, 189R was likely antigenically equivalent to 189S with little effect on antigenicity in this background. A/swine/Nebraska/A01241171/2012 (dark pink) differed from the red cluster at positions 145 and 189 and was also ~9 antigenic units away.

The cyan cluster differed from the red cluster at amino acid positions 155 and 189. The strains colored in brown (A/swine/Indiana/A01202866/2011 and A/swine/Michigan/A01432375/2013), representative of strains that were isolated from both pigs and turkeys, differed from the cyan cluster at amino acid positions 155, 156, 158, 159 and 189 and were 4.4-4.9 antigenic units away. The orange strain (A/swine/Iowa/A01203196/2012) differed from the cyan cluster at positions 145, 156 and 189 and was 4.4 antigenic units away. Thus, despite ongoing genetic evolution at the nucleotide and amino acid level across the entire length of the HA, as few as one or two amino acid substitutions in the HA1 domain were sufficient to change the antigenic properties of the swine influenza A (H3N2) viruses sufficiently to move them between the red or cyan clusters or to define a new antigenic cluster or outlier. The amino acids that distinguished clusters when mapped onto the HA trimer (Figure 1D) were found to be close to the receptor binding site.

Antigenic distance from swine influenza A vaccine strains

To consider the effect this observed antigenic diversity might have on vaccine strain efficacy, we measured the antigenic distance from genetic representatives of putative vaccine strains (the actual strain identity being proprietary information) to currently circulating swine H3N2 viruses. Current vaccine strains in fully licensed swine IAV products are either genetic Cluster I (Figure 2A, orange viruses) or Cluster IV (Figure 2A, red viruses). The most recent vaccine representative from Cluster IV was isolated in 2005. When we measured the antigenic distances from a serum raised to either a Cluster I (Figure 5A) or a Cluster IV (Figure 5B) strain to other currently circulating influenza A H3N2 strains in pigs, we found that all currently circulating strains were greater than 2 antigenic units from the Cluster I vaccine serum, and most strains were over 4 antigenic units away. Within the panel of Cluster IV viruses, we found that some strains were within 2 antigenic units of the putative vaccine serum, but the majority of isolates were greater than 3 antigenic units from the vaccine strain. Therefore, vaccination with Clade I or Clade IV vaccine strains are unlikely to prevent virus infection and/or shedding (e.g., (44)). We also found that the distances from the two putative swine vaccine sera to human seasonal H3N2 strains were over 4 antigenic units in viruses isolated since 1995, and a seasonal strain isolated from humans in 2011 (A/Victoria/361/2011) was 6 antigenic units from the Cluster IV serum and 8 antigenic units from the Cluster I serum. Such antigenic distances suggest that future incursions of a current human seasonal H3N2 strain into pigs are unlikely to be mitigated by immunity from either the Cluster I or Cluster IV vaccines currently in use in pigs.

Antigenic distance to human seasonal vaccine strains

We also quantified the antigenic distance between currently circulating swine strains and A/Victoria/361/2011, the most recent human seasonal vaccine strain representative (Figure 5C). We found that all currently circulating swine strains were over 4 antigenic units away from the most recent representative human H3N2, and some as many as 8 antigenic units away, thus future incursions of current swine strains into humans may not be mitigated by immunity to the current human seasonal vaccines.

DISCUSSION

Here, we quantified the antigenic diversity among currently circulating swine and human H3 influenza A viruses using HI assay data and antigenic cartography. The swine H3N2 viruses demonstrated antigenic diversity in the cross-HI assays. In the antigenic maps we saw a clustered antigenic evolution, similar to that shown for the H1 viruses (α, β, γ, δ-1, and δ-2: (28)) with a marked antigenic distance among and between two broad antigenic clusters, demonstrating the substantial antigenic diversity in the milieu of genetically evolving H3N2 viruses circulating in U.S. pigs. Although a previous study utilizing different methodology with a ferret antiserum panel against 8 swine H3N2 viruses identified two antigenic clusters of swine H3N2 from 2006-2012 primarily from one U.S. state (20), our study with a serum panel of 18 swine H3N2 covering 12 states and major hog producing regions and generated in the natural host demonstrated greater overall antigenic diversity and a greater number of outliers. In addition, our study included representatives from each of the newly emerging phylogenetically defined clusters, contributing significantly to the amount of antigenic diversity we observed.

The genetic evolution of both the HA and the NA of H3N2 viruses in pigs was visualized in the ML phylogenetic trees and was consistent with previous analyses (18, 34). However we observed that there was a relative mismatch between the phylogenetic topology of HA and NA, where the NA gene segment was not necessarily consistent with that of the HA gene segment, with the exception of the 2012 and 2013 H3N2v and the Clade IV-F viruses, where there was good correlation between relative tree topology of HA and NA. This suggests that co-evolution between HA and NA pairs may not have an important role in contemporary H3N2 virus fitness, and the inconsistent tree topologies likely arose from frequent reassortment or other ecological or immunological pressures (45).

The rate of change in ML likelihood distances of the HA gene in U.S. swine H3N2 of 0.006 per year was similar to an estimate of 0.0047 from previously published work on evolution of swine H3N2 viruses in European pigs and 0.006 in human seasonal H3N2 from 1982-2002, reported in

359 the same study (27). However, the overall genetic evolutionary rate in U.S. swine H3N2 from 1997-
360 2013 was weighted by the increased evolution from 2010-2013, following the introduction and
361 subsequent reassortment with the H1N1pdm09. Mutation rates of circulating H3 HA genes also
362 appear to differ between animal hosts. For example, the rate of change of ML-likelihood distances
363 of H3 subtype viruses in horses was 0.002, less than half the rate observed in pigs. This is likely
364 due to a number of factors, including virus, individual host and population factors.

365 The rapid genetic evolution seen with our swine H3 data prompted our investigation into
366 quantifying the impact on antigenic diversity in contemporary U.S. swine H3N2. We found
367 clustered antigenic evolution of H3N2 viruses in pigs from the USA, similar to that previously
368 quantified in European pigs. In Europe, H3N2 viruses continued to circulate and evolve in pigs
369 following the H3 pandemic of 1968 in humans and subsequent introduction into the pig population.
370 Despite similar rates of genetic change, European swine H3N2 viruses evolved 6 times more slowly
371 antigenically than human influenza H3N2 viruses over a similar time period (27). Although H3N2
372 viruses were introduced into North American pigs around 1997 and have continued to circulate,
373 new antigenic variants arose that are antigenically distinct from ancestrally related H3N2 viruses
374 circulating in humans. In addition, we observed far greater antigenic diversity of H3N2 viruses
375 circulating in U.S. pigs in a much shorter time period when compared with viruses circulating in
376 European pigs. Coupled with the antigenic diversity, we observed co-circulation of different
377 antigenic clusters within the pig population, rather than replacement, as seen in human influenza H3
378 evolution. Despite relatively similar genetic evolution rates in North American and European swine
379 H3N2, as well as human seasonal H3N2, the within-host antigenic evolution in pigs in the U.S.
380 does not parallel the antigenic evolutionary patterns of H3N2 viruses in European pigs, people or
381 horses, likely because of host population and virus factors that are currently undetermined.

382 Surprisingly, the substitutions that resulted in marked antigenic differences were attributed
383 in most cases to one or two amino acid changes in the HA-1 domain, located at 6 amino acid
384 positions (145, 155, 156, 158, 159 and 189), strikingly similar to the 7 key amino acid changes

recently identified in human antigenic switches from 1968 to 2003: 145, 155, 156, 158, 159, 189, and 193 (43). Furthermore, similarities were also observed between the H3 HA evolution seen in humans, pigs and horses (see Table S4 for direct comparison of observed substitutions in three host species). Although the precise amino acid substitutions differed or were present in different combinations in a particular host species, similar amino acid positions were associated with antigenic cluster-defining substitutions in all three hosts and all were located close to the receptor-binding site. These observations were consistent whether natural host sera were used to characterize the antigenic properties of the viruses as was the case in this study, or when ferret sera were used as a small animal model to characterize H3 viruses circulating in either horses or humans. Of particular note is position 145, which caused the difference between A/Beijing/1992 and A/Wuhan/1995 viruses in humans (43). Here, we observed that A/swine/Minnesota/A01125993/2012 had substitutions at position 145 (N145K) and position 159 (Y159N) associated with an antigenic change of 6 antigenic units away from the red cluster. This is remarkably at the same linear amino acid position as the S145N/R substitution associated with the difference between the first and the second antigenic clusters that emerged in European pigs (27). We also found that positions 155 and 189 defined the antigenic difference between the red (155Y: 189K) and the cyan (155H: 189R) swine antigenic clusters, whereas Feng, *et. al.* (20) reported only the R189K as defining the two antigenic clusters of U.S. swine H3N2 in that study. The role of the R189K substitution has been explored in swine (46), and this position 189 has been consistently identified as cluster defining in other species as well. In the evolution of equine H3, the European-like cluster was defined from the American-like cluster by the amino acid substitution K189N, -D, -Q, or -E (47). Position 189 was also key in the human influenza A H3 antigenic evolution from A/England/1972 to A/Victoria/1975 and in combination with positions 155 and 159 in the evolution from A/Bangkok/1979 to A/Sichuan/1987 (43). Although position 189 seems to be more consistently identified among different species and in different studies, it is clear that it is not the sole position responsible for cluster-transition substitutions in human (43) and now swine H3N2.

411 While the amino acid positions associated with antigenic variability were conserved among host
412 species, the mechanism by which the individual substitutions act in the different host species is not
413 known. The amino acid changes may cause structural differences in the hemagglutinin leading to
414 receptor binding constraints in different hosts, differences in qualitative and quantitative adaptive
415 immune recognition, or a combination of the above.

416 In the context of the swine humoral response represented by our serum panel and circulating
417 swine H3N2 viruses, contemporary human seasonal H3N2 were shown to have substantial
418 antigenic distance from the contemporary swine H3N2 although these lineages share a common
419 ancestor from the mid-1990s. We showed that between 4 and 8 antigenic units separated the human
420 seasonal vaccine strain representative A/Victoria/361/2011 from all currently circulating strains in
421 pigs. This indicates that despite a potentially high level of immunity against swine H3N2 in the pig
422 population in the U.S., a future incursion of human seasonal H3N2 is possible if the event produced
423 a virus fit for pig-to-pig transmission. The increasing antigenic distance of the A/Victoria/361/2011
424 H3N2 and other previous human seasonal vaccine strains to the contemporary swine H3N2 also
425 suggests the youngest of the human population may become increasingly susceptible to incursions
426 of swine H3N2 due to lack of cross-reacting immunity. Indeed, a dramatic number of H3N2v
427 infections in humans, primarily children, in the USA were detected in 2011-2013 and studies with
428 human sera demonstrated a lack of cross-reacting HI antibodies in children and the elderly (22, 23).
429 Further study of human sera tested against a panel of swine H3N2 representing the antigenic
430 diversity we demonstrate here is required to fully understand the level of human population
431 immunity to H3N2 endemic in the pig population.

432 The marked antigenic diversity seen in H3N2 viruses in pigs since 2010 poses problems in
433 assessing the relative risk of a swine variants emerging in the human population and in using
434 vaccine as an effective means of IAV control in pigs. How differences in host factors alter the
435 relative evolution of viruses in these two hosts is poorly understood, but some factors that might
436 alter the evolutionary pattern of the HA gene within pigs in comparison with H3 in the human

437 population host include differing lifespan and replacement rates of pigs versus humans; more
438 continental separation of pigs compared to humans; and relative spatial separation of sow farms but
439 movement and mixing of weaned pigs to the Midwest from Southeast and Southwest USA and
440 Canada (48). All these factors might lead to different population immunological profiles and thus
441 alter the evolutionary patterns of viruses. This complex immunological profile is exacerbated by a
442 difficulty in updating inactivated vaccines to contain representatives of currently circulating strains
443 and subsequent lack of an ideal vaccine for all situations in pig production.

444 A national surveillance system was established in 2009 by the U.S. Department of
445 Agriculture in response to the 2009 H1N1 pandemic, the growing diversity of swine viruses, and
446 increasing number of detections of zoonotic events in humans (18). The number of isolates with
447 sequence data from this surveillance stream has grown significantly, building the foundation for
448 systematic sequence analyses to pair with antigenic assessment. Phylogenetic analysis of
449 contemporary H3 suggested increasing evolution since the emergence and subsequent reassortment
450 with the H1N1pdm09 (18, 34), and here we demonstrate the resulting antigenic diversity. The
451 USDA surveillance system and analyses such as ours reported here can now begin to be used to
452 inform vaccine strain selection for swine. However, to improve and further facilitate vaccine strain
453 updates, a vaccine strain selection working group established to collectively provide cross-HI and
454 phylogenetic data from various laboratories and sectors together for interpretation and discussion
455 would be beneficial. Changes in regulatory processes to allow rapid replacement of HA and NA
456 onto approved IAV backbones or platforms would also be extremely useful for improving control
457 measures against influenza A virus in swine. Additionally, platforms not currently available in
458 swine, such as live attenuated influenza vaccines or vectored vaccines, have shown great promise in
459 experimental settings for improved heterologous protection and greater efficacy in the face of
460 maternally derived antibodies (44, 49-54).

461 Here, we found that as few as one or two amino acid substitutions resulted in new antigenic
462 clusters and/or outliers. Since these cluster defining amino acid changes were shown to be enough

to result in vaccine failure in other host species, we need to continue to systematically monitor the evolution of swine IAV for vaccine strain updates. Such information is also critical to increase our understanding of what governs the evolutionary mechanisms in different hosts and in improving control measures for influenza A viruses to protect the health and wellbeing of swine, a primary protein food source for humans, as well as the respiratory health of the human population.

ACKNOWLEDGMENTS

We wish to thank Michelle Harland, Gwen Nordholm, and Alicia Janas-Martindale for excellent technical assistance and Jason Huegal, Jason Crabtree and Tyler Standley for animal care and handling assistance. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Funding was provided by USDA-ARS and USDA-APHIS. NSL was funded by USDA-ARS SCA agreement number 58-3625-2-103F, and the EC FP7 award number 259949. USDA is an equal opportunity provider and employer.

REFERENCES

1. **Anon.** 1980. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull. World Health Organ. **58**:585–591.
2. **Fouchier RAM**, **Munster V**, **Wallensten A**, **Bestebroer TM**, **Herfst S**, **Smith D**, **Rimmelzwaan GF**, **Olsen B**, **Osterhaus ADME**. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. **79**:2814–2822.
3. **Hinshaw VS**, **Air GM**, **Gibbs AJ**, **Graves L**, **Prescott B**, **Karunakaran D**. 1982. Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J. Virol. **42**:865–872.
4. **Kawaoka Y**, **Yamnikova S**, **Chambers TM**, **Lvov DK**, **Webster RG**. 1990. Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology **179**:759–767.
5. **Röhm C**, **Zhou N**, **Süss J**, **Mackenzie J**, **Webster RG**. 1996. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology **217**:508–516.
6. **Tong S**, **Li Y**, **Rivailler P**, **Conrardy C**, **Alvarez Castillo DA**, **Chen L-M**, **Recuenco S**, **Ellison JA**, **Davis CT**, **York IA**, **Turmelle AS**, **Moran D**, **Rogers S**, **Shi M**, **Tao Y**, **Weil MR**, **Tang K**, **Rowe LA**, **Sammons S**, **Xu X**, **Frace M**, **Lindblade KA**, **Cox NJ**, **Anderson LJ**, **Rupprecht CE**, **Donis RO**. 2012. A distinct lineage of influenza A virus from bats. Proc. Natl. Acad. Sci. USA **109**:4269–4274.
7. **Tong S**, **Zhu X**, **Li Y**, **Shi M**, **Zhang J**, **Bourgeois M**, **Yang H**, **Chen X**, **Recuenco S**, **Gomez J**, **Chen L-M**, **Johnson A**, **Tao Y**, **Dreyfus C**, **Yu W**, **McBride R**, **Carney PJ**, **Gilbert AT**, **Chang J**, **Guo Z**, **Davis CT**, **Paulson JC**, **Stevens J**, **Rupprecht CE**, **Holmes EC**, **Wilson IA**, **Donis RO**. 2013. New world bats harbor diverse influenza a viruses. PLoS Pathog. **9**:e1003657–e1003657.

8. **Brown IH**. 2000. The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. **74**:29–46.

9. **Myers KP**, **Olsen CW**, **Gray GC**. 2007. Cases of Swine Influenza in Humans: A Review of the Literature. Clin. Infect. Dis. **44**:1084–1088.

10. **Shinde V**, **Bridges CB**, **Uyeki TM**, **Shu B**, **Balish A**, **Xu X**, **Lindstrom S**, **Gubareva LV**, **Deyde V**, **Garten RJ**, **Harris M**, **Gerber S**, **Vagasky S**, **Smith F**, **Pascoe N**, **Martin K**, **Dufficy D**, **Ritger K**, **Conover C**, **Quinlisk P**, **Klimov A**, **Bresee JS**, **Finelli L**. 2009. Triple-Reassortant Swine Influenza A (H1) in Humans in the United States, 2005–2009. N. Engl. J. Med. **360**:2616–2625.

11. **Vincent A**, **Awada L**, **Brown I**, **Chen H**, **Claes F**, **Dauphin G**, **Donis R**, **Culhane M**, **Hamilton K**, **Lewis N**, **Mumford E**, **Nguyen T**, **Parchariyanon S**, **Pasick J**, **Pavade G**, **Pereda A**, **Peiris M**, **Saito T**, **Swenson S**, **Van Reeth K**, **Webby R**, **Wong F**, **Ciacci-Zanella J**. 2013. Review of Influenza A Virus in Swine Worldwide: A Call for Increased Surveillance and Research. Zoonoses Public Health. doi: 10.1111/zph.12049.

12. **Webby RJR**, **Swenson SLS**, **Krauss SLS**, **Gerrish PJP**, **Goyal SMS**, **Webster RGR**. 2000. Evolution of swine H3N2 influenza viruses in the United States. J. Virol. **74**:8243–8251.

13. **Olsen CW**, **Karasin AI**, **Carman S**, **Li Y**, **Bastien N**, **Ojkic D**, **Alves D**, **Charbonneau G**, **Henning BM**, **Low DE**, **Burton L**, **Broukhanski G**. 2006. Triple reassortant H3N2 influenza A viruses, Canada, 2005. Emerging Infect. Dis. **12**:1132–1135.

14. **Karasin AI**, **Landgraf J**, **Swenson S**, **Erickson G**, **Goyal S**, **Woodruff M**, **Scherba G**, **Anderson G**, **Olsen CW**. 2002. Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. J. Clin. Microbiol. **40**:1073–1079.

15. **Webby RJ**, **Rossow K**, **Erickson G**, **Sims Y**, **Webster R**. 2004. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res. **103**:67–73.

16. **Vincent AL**, **Ma W**, **Lager KM**, **Janke BH**, **Richt JA**. 2008. Swine influenza viruses a North American perspective. Adv. Virus Res. **72**:127–154.

17. **Vincent AL**, **Ma W**, **Lager KM**, **Gramer MR**, **Richt JA**, **Janke BH**. 2009. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. Virus Genes **39**:176–185.

18. **Anderson TK**, **Nelson MI**, **Kitikoon P**, **Swenson SL**, **Korslund JA**, **Vincent AL**. 2013. Population dynamics of cocirculating swine influenza A viruses in the United States from 2009 to 2012. Influenza Other Respi. Viruses **7**:42–51.

19. **Garten RJ**, **Davis CT**, **Russell CA**, **Shu B**, **Lindstrom S**, **Balish A**, **Sessions WM**, **Xu X**, **Skepner E**, **Deyde V**, **Okomo-Adhiambo M**, **Gubareva L**, **Barnes J**, **Smith CB**, **Emery SL**, **Hillman MJ**, **Rivailler P**, **Smagala J**, **de Graaf M**, **Burke DF**, **Fouchier RAM**, **Pappas C**, **Alpuche-Aranda CM**, **López-Gatell H**, **Olivera H**, **López I**, **Myers CA**, **Faix D**, **Blair PJ**, **Yu C**, **Keene KM**, **Dotson PD**, **Boxrud D**, **Sambol AR**, **Abid SH**, **St George K**, **Bannerman T**, **Moore AL**, **Stringer DJ**, **Blevins P**, **Demmler-Harrison GJ**, **Ginsberg M**, **Kriner P**, **Waterman S**, **Smole S**, **Guevara HF**, **Belongia EA**, **Clark PA**, **Beatrice ST**, **Donis R**, **Katz J**, **Finelli L**, **Bridges CB**, **Shaw M**, **Jernigan DB**, **Uyeki TM**, **Smith DJ**, **Klimov AI**, **Cox NJ**. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325**:197–201.

20. **Feng Z**, **Gomez J**, **Bowman AS**, **Ye J**, **Long L-P**, **Nelson SW**, **Yang J**, **Martin B**, **Jia K**, **Nolting JM**, **Cunningham F**, **Cardona C**, **Zhang J**, **Yoon K-J**, **Slemons RD**, **Wan X-F**. 2013. Antigenic characterization of H3N2 influenza A viruses from Ohio agricultural fairs. J. Virol. **87**:7655–7667.

21. **Lindstrom S**, **Garten R**, **Balish A**, **Shu B**, **Emery S**, **Berman L**, **Barnes N**, **Sleeman K**, **Gubareva L**, **Villanueva J**, **Klimov A**. 2012. Human infections with novel reassortant influenza A(H3N2)v viruses, United States, 2011. Emerging Infect. Dis. **18**:834–837.

22. **Skowronski DM**, **Janjua NZ**, **De Serres G**, **Purych D**, **Gilca V**, **Scheifele DW**, **Dionne M**,

555 **Sabaiduc S**, **Gardy JL**, **Li G**, **Bastien N**, **Petric M**, **Boivin G**, **Li Y**. 2012. Cross-reactive
556 and vaccine-induced antibody to an emerging swine-origin variant of influenza A virus
557 subtype H3N2 (H3N2v). J. Infect. Dis. **206**:1852–1861.
558 23. **Centers for Disease Control and Prevention (CDC)**. 2012. Prevention and control of
559 influenza with vaccines: recommendations of the Advisory Committee on Immunization
560 Practices (ACIP)--United States, 2012-13 influenza season. MMWR Morb. Mortal. Wkly.
561 Rep. Morbidity and Mortality Weekly Report.
562 24. **Houser KV**, **Pearce MB**, **Katz JM**, **Tumpey TM**. 2013. Impact of Prior Seasonal H3N2
563 Influenza Vaccination or Infection on Protection and Transmission of Emerging Variants of
564 Influenza A(H3N2)v Virus in Ferrets. J. Virol. **87**:13480–13489.
565 25. **Bowman AS**, **Sreevatsan S**, **Killian ML**, **Page SL**, **Nelson SW**, **Nolting JM**, **Cardona C**,
566 **Slemons RD**. 2012. Molecular evidence for interspecies transmission of H3N2pM/H3N2v
567 influenza A viruses at an Ohio agricultural fair, July 2012. Emerg. Microbes Infect. **1**:e33.
568 26. **Smith DJ**, **Lapedes AS**, **de Jong JC**, **Bestebroer TM**, **Rimmelzwaan GF**, **Osterhaus**
569 **ADME**, **Fouchier RAM**. 2004. Mapping the antigenic and genetic evolution of influenza
570 virus. Science **305**:371–376.
571 27. **De Jong JC**, **Smith DJ**, **Lapedes AS**, **Donatelli I**, **Campitelli L**, **Barigazzi G**, **Van Reeth**
572 **K**, **Jones TC**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Fouchier RAM**. 2007. Antigenic and
573 genetic evolution of swine influenza A (H3N2) viruses in Europe. J. Virol. **81**: 4315–4322.
574 28. **Lorusso A**, **Vincent AL**, **Harland ML**, **Alt D**, **Bayles DO**, **Swenson SL**, **Gramer MR**,
575 **Russell CA**, **Smith DJ**, **Lager KM**, **Lewis NS**. 2011. Genetic and antigenic characterization
576 of H1 influenza viruses from United States swine from 2008. J. Gen. Virol. **92**:919–930.
577 29. **Nfon CK**, **Berhane Y**, **Hisanaga T**, **Zhang S**, **Handel K**, **Kehler H**, **Labrecque O**, **Lewis**
578 **NS**, **Vincent AL**, **Copps J**, **Alexandersen S**, **Pasick J**. 2011. Characterization of H1N1
579 Swine Influenza Viruses Circulating in Canadian Pigs in 2009. J. Virol. **85**:8667–8679.
580 30. **Core Team R**. 2013. R: A Language and Environment for Statistical Computing, 3rd ed. R

581 Foundation for Statistical Computing, Vienna, Austria.
582 31. **Montalvao RW**, **Smith RE**, **Lovell SC**, **Blundell TL**. 2005. CHORAL: a differential
583 geometry approach to the prediction of the cores of protein structures. Bioinformatics
584 **21**:3719–3725.
585 32. **Smith RE**, **Lovell SC**, **Burke DF**, **Montalvao RW**, **Blundell TL**. 2007. Andante: reducing
586 side-chain rotamer search space during comparative modeling using environment-specific
587 substitution probabilities. Bioinformatics **23**:1099–1105.
588 33. **Schrodinger,, LLC**. 2010. The PyMOL Molecular Graphics System, Version 1.3r1.
589 34. **Kitikoon P**, **Nelson MI**, **Killian ML**, **Anderson TK**, **Koster L**, **Culhane MR**, **Vincent AL**.
590 2013. Genotype patterns of contemporary reassorted H3N2 virus in US swine. J. Gen. Virol.
591 **94**:1236–1241.
592 35. **Edgar RC**. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
593 throughput. Nucleic Acids Res. **32**:1792–1797.
594 36. **Maddison WP**, **Maddison DR**. 2011. Mesquite: a modular system for evolutionary analysis,
595 2nd ed. http://mesquiteproject.org.
596 37. **Stamatakis A**. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses
597 with thousands of taxa and mixed models. Bioinformatics **22**:2688–2690.
598 38. **Darriba D**, **Taboada GL**, **Doallo R**, **Posada D**. 2011. ProtTest 3: fast selection of best-fit
599 models of protein evolution. Bioinformatics **27**:1164–1165.
600 39. **Bao Y**, **Bolotov P**, **Dernovoy D**, **Kiryutin B**, **Zaslavsky L**, **Tatusova T**, **Ostell J**, **Lipman**
601 **D**. 2008. The influenza virus resource at the National Center for Biotechnology Information.
602 J. Virol. **82**:596–601.
603 40. **Miller MA**, **Pfeiffer W**, **Schwartz T**. 2010. Creating the CIPRES Science Gateway for
604 Inference of Large Phylogenetic Trees 1–7.
605 41. **Paradis E**, **Claude J**, **Strimmer K**. 2004. APE: Analyses of Phylogenetics and Evolution in
606 R language. Bioinformatics **20**:289–290.

607 42. **Harmon LJ**, **Weir JT**, **Brock CD**, **Glor RE**, **Challenger W**. 2008. GEIGER: investigating
608 evolutionary radiations. Bioinformatics **24**:129–131.
609 43. **Koel BF**, **Burke DF**, **Bestebroer TM**, **van der Vliet S**, **Zondag GCM**, **Vervaet G**,
610 **Skepner E**, **Lewis NS**, **Spronken MIJ**, **Russell CA**, **Eropkin MY**, **Hurt AC**, **Barr IG**, **De**
611 **Jong JC**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Fouchier RAM**, **Smith DJ**. 2013.
612 Substitutions Near the Receptor Binding Site Determine Major Antigenic Change During
613 Influenza Virus Evolution. Science **342**:976–979.
614 44. **Loving CL**, **Lager KM**, **Vincent AL**, **Brockmeier SL**, **Gauger PC**, **Anderson TK**,
615 **Kitikoon P**, **Perez DR**, **Kehrli ME**. 2013. Efficacy in Pigs of Inactivated and Live
616 Attenuated Influenza Virus Vaccines against Infection and Transmission of an Emerging
617 H3N2 Similar to the 2011-2012 H3N2v. J. Virol. **87**:9895–9903.
618 45. **Nelson MI**, **Detmer SE**, **Wentworth DE**, **Tan Y**, **Schwartzbard A**, **Halpin RA**, **Stockwell**
619 **TB**, **Lin X**, **Vincent AL**, **Gramer MR**, **Holmes EC**. 2012. Genomic reassortment of
620 influenza A virus in North American swine, 1998-2011. J. Gen. Virol. **93**:2584–2589.
621 46. **Ye J**, **Xu Y**, **Harris J**, **Sun H**, **Bowman AS**, **Cunningham F**, **Cardona C**, **Yoon KJ**,
622 **Slemons RD**, **Wan X-F**. 2013. Mutation from arginine to lysine at the position 189 of
623 hemagglutinin contributes to the antigenic drift in H3N2 swine in. Virology **446**:225–229.
624 47. **Lewis NS**, **Daly JM**, **Russell CA**, **Horton DL**, **Skepner E**, **Bryant NA**, **Burke DF**, **Rash**
625 **AS**, **Wood JLN**, **Chambers TM**, **Fouchier RAM**, **Mumford JA**, **Elton DM**, **Smith DJ**.
626 2011. Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to
627 2007. J. Virol. **85**:12742–12749.
628 48. **Nelson MI**, **Lemey P**, **Tan Y**, **Vincent A**, **Lam TT-Y**, **Detmer S**, **Viboud C**, **Suchard MA**,
629 **Rambaut A**, **Holmes EC**, **Gramer M**. 2011. Spatial Dynamics of Human-Origin H1
630 Influenza A Virus in North American Swine. PLoS Pathog. **7**:e1002077.
631 49. **Pena L**, **Vincent AL**, **Loving CL**, **Henningson JN**, **Lager KM**, **Lorusso A**, **Perez DR**.
632 2012. Restored PB1-F2 in the 2009 Pandemic H1N1 Influenza Virus Has Minimal Effects in

633 Swine. J. Virol. **86**:5523–5532.
634 50. **Gauger PC**, **Vincent AL**, **Loving CL**, **Lager KM**, **Janke BH**, **Kehrli Jrr ME**, **Roth JA**.
635 2011. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like
636 (d-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus.
637 Vaccine **29**:2712–2719.
638 51. **Khurana S**, **Loving CL**, **Manischewitz J**, **King LR**, **Gauger PC**, **Henningson J**, **Vincent**
639 **AL**, **Golding H**. 2013. Vaccine-induced anti-HA2 antibodies promote virus fusion and
640 enhance influenza virus respiratory disease. Sci. Transl. Med. **5**:200ra114.
641 52. **Vincent AL**, **Lager KM**, **Janke BH**, **Gramer MR**, **Richt JA**. 2008. Failure of protection
642 and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an
643 inactivated classical swine H1N1 vaccine. Vet. Microbiol. **126**:310–323.
644 53. **Salmon H**, **Berri M**, **Gerdts V**, **Meurens F**. 2009. Humoral and cellular factors of maternal
645 immunity in swine. Dev. Comp. Immunol. **33**:10–10.
646 54. **Polewicz MM**, **Gracia AA**, **Buchanan RR**, **Strom SS**, **Halperin SAS**, **Potter AAA**,
647 **Babiuk LAL**, **Gerdts VV**. 2011. Influence of maternal antibodies on active pertussis toxoid
648 immunization of neonatal mice and piglets. Vaccine **29**:7718–7726.
649

650
651 **FIGURE LEGENDS**

652 **Figure 1.** 3D antigenic maps of swine influenza A(H3N2) and human influenza A(H3N2) viruses
653 from 1998–2013 and position of key amino acids on the three-dimensional trimeric model of the
654 hemagglutinin protein. The relative positions of isolates (colored spheres) and antisera (open grey
655 cubes) were computed (A) such that the distances between isolates and antisera in the map
656 correspond with the least error to measurements in the HI assay (26). Swine isolate color represents
657 the antigenic cluster to which each isolate belongs and grey spheres represent recent human
658 influenza A (H3N2) viruses. The large grey sphere is A/Victoria/361/2011. The white scale bar
659 represents 1 unit of antigenic distance, corresponding to a twofold dilution of antiserum in the HI
660 assay. Antigenic maps with only swine influenza A(H3N2) viruses showing the antigenic effect of
661 the amino acid substitutions for each antigenic variant that was not located within the red (B) or the
662 cyan (C) antigenic clusters. The arrows radiate from the consensus in each cluster to the outlying
663 antigen and numeric values show the number of antigenic units separating the outlier from the
664 antigens representing the consensus. A trimeric structure of A/Swine/Illinois/A01241469/2012 (red
665 antigenic cluster) was generated to demonstrate the location of the antigenic-determining amino
666 acid positions (D). The receptor binding site was colored wheat. An α2,6 glycan (LSTc) is shown
667 docked in the binding site as sticks. The six amino acid positions associated with antigenic outliers
668 were colored red. Images were produced using PyMOL (33).
669
670 **Figure 2**. Maximum likelihood phylogenies (A and B) and genetic maps (C and D) of
671 representative H3N2 swine influenza A isolates using HA1 domain amino acid sequences. Numbers
672 above or below branches in the phylogenetic trees indicate bootstrap support (%) estimated from
673 1,000 resamplings of the sequence data; bootstrap values ≤ 50% are not shown. H3N2 HA
674 sublineages are indicated by bolded square parantheses (Cluster I, II, II, and IV-A/B/C/D/E/F).
675 Taxon names indicate viral isolate, followed by Genbank or GISAID EpiFlu accession identifiers in
676 parentheses. Branches were colored by genetic cluster (A) and antigenic cluster (B); branches in

light grey were not part of antigenic study. Scale bars in the phylogenies indicate amino acid substitutions per site. Genetic maps were made from pairwise differences among strains and spheres representing virus strains were colored by genetic cluster (C) or antigenic cluster (D). The white scale bars in the genetic maps correspond to 5 amino acid substitutions.

Figure 3. Maximum likelihood phylogeny of neuraminidase (NA) gene amino acid sequences from viruses in the antigenic study and representative H3N2 swine influenza A isolates. Branches were colored by HA genetic cluster; branches in light grey were not part of study. Numbers above or below branches in the phylogenetic trees indicate bootstrap support (%) estimated from 1,000 resamplings of the sequence data; bootstrap values ≤ 50% are not shown. H3N2 NA sublineages are indicated by bolded square parentheses (1998 vs. 2002). Taxon names indicate viral isolate, followed by Genbank or GISAID EpiFlu accession identifiers in parentheses. Scale bar in the phylogeny indicates amino acid substitutions.

Figure 4. Patristic distance from A/Wuhan/359/95 in the maximum likelihood phylogenetic tree presented in Figure S1 to each isolate in Cluster IV H3N2 swine influenza A virus clade plotted as a function of time. The solid line represents the regression for the three years prior to 2009 with a slope of 0.003 (x-intercept = -7.84 ± 1.31 S.E.: Adjusted R^2 = 0.30: p-value < 0.0001) whereas the hatched line represents the regression for the isolates from 2010-present with a slope of 0.005 (x-intercept = -10.40± 0.68 S.E.: Adjusted R^2 = 0.31: p-value < 0.0001).

Figure 5. Antigenic distances from putative Cluster I (A) and Cluster IV (B) swine vaccine sera and antigenic distance from the human seasonal vaccine strain A/Victoria/361/2011 (C) swine sera to circulating strains in pigs by year.

Table 1. Viruses used to raise reference antisera in swine (underlined) and test antigens in the hemagglutination inhibition (HI) assay.

Viruses	H3 Cluster	Virus propagation	H3 Accession
Swine			
A/swine/Texas/4199-2/1998	H3-I	MDCK	CY095675
A/swine/Colorado/23619/1999	H3-II	MDCK	AF268128
A/swine/Minnesota/01146/2006	H3-IV	MDCK	CY099035
A/swine/Iowa/01700/2007	H3-IV	MDCK	CY099027
A/swine/Minnesota/02782/2009	H3-IV	MDCK	CY099103
A/swine/Illinois/02907/2009	H3-IV	MDCK	KF739390
A/swine/Pennsylvania/A01076777/2010	H3-IV	MDCK	JF263535
A/swine/New York/A01104005/2011	H3-IV (A)	MDCK	JN940422
A/swine/Indiana/A00968373/2012	H3-IV (A)	MDCK	JX534982
A/swine/Illinois/A01241469/2012	H3-IV (A)	MDCK	JX422497
A/swine/Michigan/A01259000/2012	H3-IV (A)	MDCK-L	JX442056
A/swine/Wyoming/A01444562/2013	H3-IV (A)	MDCK	KC562197
A/swine/North_Carolina/A01432566/2013	H3-IV (A)	MDCK	KC841842
A/swine/Minnesota/A01300213/2012	H3-IV (B)	MDCK	JX657030
A/swine/Minnesota/A01125993/2012	H3-IV (B)	MDCK	JX422257
A/swine/Minnesota/A01327922/2012	H3-IV (B)	MDCK	JX422521
A/swine/Iowa/A01300195/2012	H3-IV (B)	MDCK	JX657018
A/swine/Minnesota/A01432544/2013	H3-IV (B)	MDCK	KC841830
A/swine/Minnesota/A01280592/2013	H3-IV (B)	MDCK	KC589443
A/swine/Indiana/A01202866/2011	H3-IV (C)	MDCK	JX092535
A/swine/Michigan/A01432375/2013	H3-IV (C)	MDCK	KC534987
A/swine/Illinois/A01201606/2011	H3-IV (D)	MDCK	CY107066
A/swine/Iowa/A01202613/2011	H3-IV (D)	MDCK-L	JX092307
A/swine/Iowa/A01202889/2011	H3-IV (D)	MDCK-L	JX092542
A/swine/Iowa/A01203196/2012	H3-IV (D)	MDCK-L	JQ739697
A/swine/Michigan/A01203498/2012	H3-IV (D)	MDCK	JX163265
A/swine/Iowa/A01049750/2011	H3-IV (F)	MDCK	JN652493
A/swine/Texas/A01049914/2011	H3-IV (F)	MDCK	JN652507
A/swine/Illinois/A01241066/2012	H3-IV (F)	MDCK	JX422557
A/swine/Iowa/A01203121/2012	H3-IV (F)	MDCK-L	JX092555
A/swine/Nebraska/A01241171/2012	H3-IV (F)	MDCK-L	JX422575
A/swine/Nebraska/A01271549/2012	H3-IV (F)	MDCK	KC222305

A/swine/Iowa/A01432500/2013	H3-IV (F)	MDCK	KC755694
Human			
A/Wuhan/359/1995	Vaccine strain (1996-1998)	MDCK-L	AY661190
A/Sydney/5/1997	Vaccine strain (1998-2000)	MDCK-L	CY039079
A/Moscow/10/1999	Vaccine strain (2000-2004)	MDCK-L	AY531035
A/Fujian/411/2002	Vaccine strain (2004-2005)	MDCK-L	EF541397
A/Wisconsin/67/2005	Vaccine strain (2006-2008)	MDCK-L	CY034116
A/Brisbane/10/2007	Vaccine strain (2008-2010)	Egg	CY039087
A/Perth/16/2009	Vaccine strain (2010-2012)	Egg	GQ293081
A/Victoria/361/2011	Vaccine strain (2012-2013)	Egg	KC306165
A/Indiana/08/2011	H3N2v: H3-IV(A)	MDCK	JN638733

704