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Reassortment between Swine H3N2 and 2009 Pandemic H1N1 in the United States Resulted in Influenza A Viruses with Diverse Genetic Constellations with Variable Virulence in Pigs

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ABSTRACT Repeated spillovers of the H1N1 pandemic virus (H1N1pdm09) from humans to pigs resulted in substantial evolution of influenza A viruses infecting swine, contributing to the genetic and antigenic diversity of influenza A viruses (IAV) currently circulating in swine. The reassortment with endemic swine viruses and maintenance of some of the H1N1pdm09 internal genes resulted in the circulation of different genomic constellations in pigs. Here, we performed a whole-genome phylogenetic analysis of 368 IAV circulating in swine from 2009 to 2016 in the United States. We identified 44 different genotypes, with the most common genotype (32.33%) containing a clade IV-A HA gene, a 2002-lineage NA gene, an M-pdm09 gene, and remaining gene segments of triple reassortant internal gene (TRIG) origin. To understand how different genetic constellations may relate to viral fitness, we compared the pathogenesis and transmission in pigs of six representative genotypes. Although all six genotypes efficiently infected pigs, they resulted in different degrees of pathology and viral shedding. These results highlight the vast H3N2 genetic diversity circulating in U.S. swine after 2009. This diversity has important implications in the control of this disease by the swine industry, as well as a potential risk for public health if swine-adapted viruses with H1N1pdm09 genes have an increased risk to humans, as occurred in the 2011-2012 and 2016 human variant H3N2v cases associated with exhibition swine.

IMPORTANCE People continue to spread the 2009 H1N1 pandemic (H1N1pdm09) IAV to pigs, allowing H1N1pdm09 to reassort with endemic swine IAV. In this study, we determined the 8 gene combinations of swine H3N2 IAV detected from 2009 to 2016. We identified 44 different genotypes of H3N2, the majority of which contained at least one H1N1pdm09 gene segment. We compared six representative genotypes of H3N2 in pigs. All six genotypes efficiently infected pigs, but they resulted in different degrees of lung damage and viral shedding. These results highlight the vast genetic diversity of H3N2 circulating in U.S. swine after 2009, with important implications for the control of IAV for the swine industry. Because H1N1pdm09 is also highly adapted to humans, these swine viruses pose a potential risk to public health if swine-adapted viruses with H1N1pdm09 genes also have an increased risk for human infection.

KEYWORDS H3N2, genotypes, influenza, pandemic, swine

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Respiratory disease caused by influenza A viruses (IAV) has significant health and subsequent economic consequences for the swine industry worldwide, and swine producers struggle to control the virus with traditional methods (1). The acquisition of the triple reassortant internal gene (TRIG) IAV constellation by swine IAV in the late 1990s in the United States led to an increased rate of reassortments with endemic swine surface genes that had not been previously observed (1, 2). Transfer of viruses from humans to pigs added to the reassortment dynamics and contributed surface genes from human seasonal viruses to swine lineage viruses (3). Antigenic drift of the surface genes (4) further expanded the diversity of IAV in North American swine populations. As a consequence, at least 10 different hemagglutinin (HA) genetic clades cocirculate in U.S. swine, H3-IV-A to -F, H1α, H1β, H1γ, H1δ1, H1δ2, and H1pdm (5, 6).

Human-to-swine virus spillover events with sustained onward transmission have been shown to be far more frequent than the opposite route, with gene segments from independent introductions of human seasonal IAV showing sustained onward transmission in swine in at least eight different countries (7). In addition, the 2009 H1N1 pandemic virus (pdm09) was repeatedly introduced into swine populations across the globe after its initial detection in humans (8–11). At least 49 H1N1pdm09 reverse-zoonosis events were initially reported globally between 2009 and 2012 (12), but recent data show that these numbers continue to grow due to repeated contemporary human-to-swine transmission of H1N1pdm09 into swine populations, and it is likely to continue to occur as long as H1N1pdm09 circulates in humans in a form to which pigs are susceptible (13).

Although H1N1pdm09 has been repeatedly introduced into pigs worldwide, neither the H1N1pdm09 surface genes (HA and neuraminidase [NA]) nor the whole virus has persisted in U.S. pig populations, whereas onward transmission has occurred for the internal genes, particularly polymerase acidic (PA), nucleoprotein (NP), and matrix (M) genes (13). Notably, the introduction of H1N1pdm09 into pigs was concurrent with an increase in genetic diversity at the whole-genome and surface gene levels of contemporary swine IAV. Reassortment events between endemic swine and H1N1pdm09 viruses have been reported in many countries (8, 10, 14, 15), and viruses containing different constellations of H1N1pdm09 internal genes are now predominant among IAV circulating in Chinese pigs (16). In the United States, the M gene from H1N1pdm09 quickly replaced the TRIG M gene, but other H1N1pdm09 internal genes were also detected (5, 17). To follow these early reports, we conducted a phylogenetic analysis of IAV circulating in swine from 2009 to 2016 in North America to characterize reassortant constellations that emerged after the introduction of H1N1pdm09 virus into pigs. To evaluate how genetic constellations impact virus phenotype in pigs, we compared the pathogenesis and transmission of six IAV isolates that were reassortants between contemporary swine H3N2 and H1N1pdm09 viruses. Viruses were selected to represent currently predominant U.S. H3 and N2 phylogenetic lineages, as well as representative combinations of circulating H1N1pdm09 internal genes.

RESULTS

An increasing number of H1N1pdm09 internal genes combined with TRIG genes were found in WGS of H3N2. The whole-genome sequence (WGS) data analyzed here demonstrated that 44 different genotypes of swine H3N2 viruses were detected in the U.S. swine population from 2009 to 2016 (Fig. 1). From the 368 unique WGS collected between 2009 and 2016, 256 contained at least one H1N1pdm09 internal gene and varied between 1 and 6 segments from H1N1pdm09. Since 2012, H1N1pdm09 PA, NP, and M genes were most commonly found to replace TRIG lineage genes (Fig. 2). By 2011, the pandemic matrix (M) gene became predominant over the TRIG M gene and overall was found in 70% ($n = 256$) of the virus isolates analyzed here (Fig. 2). Only a few isolates with WGS from 2016 were available from GenBank at the time of the analysis, hence the lower number of sequences presented in Fig. 2 for the most recent year.

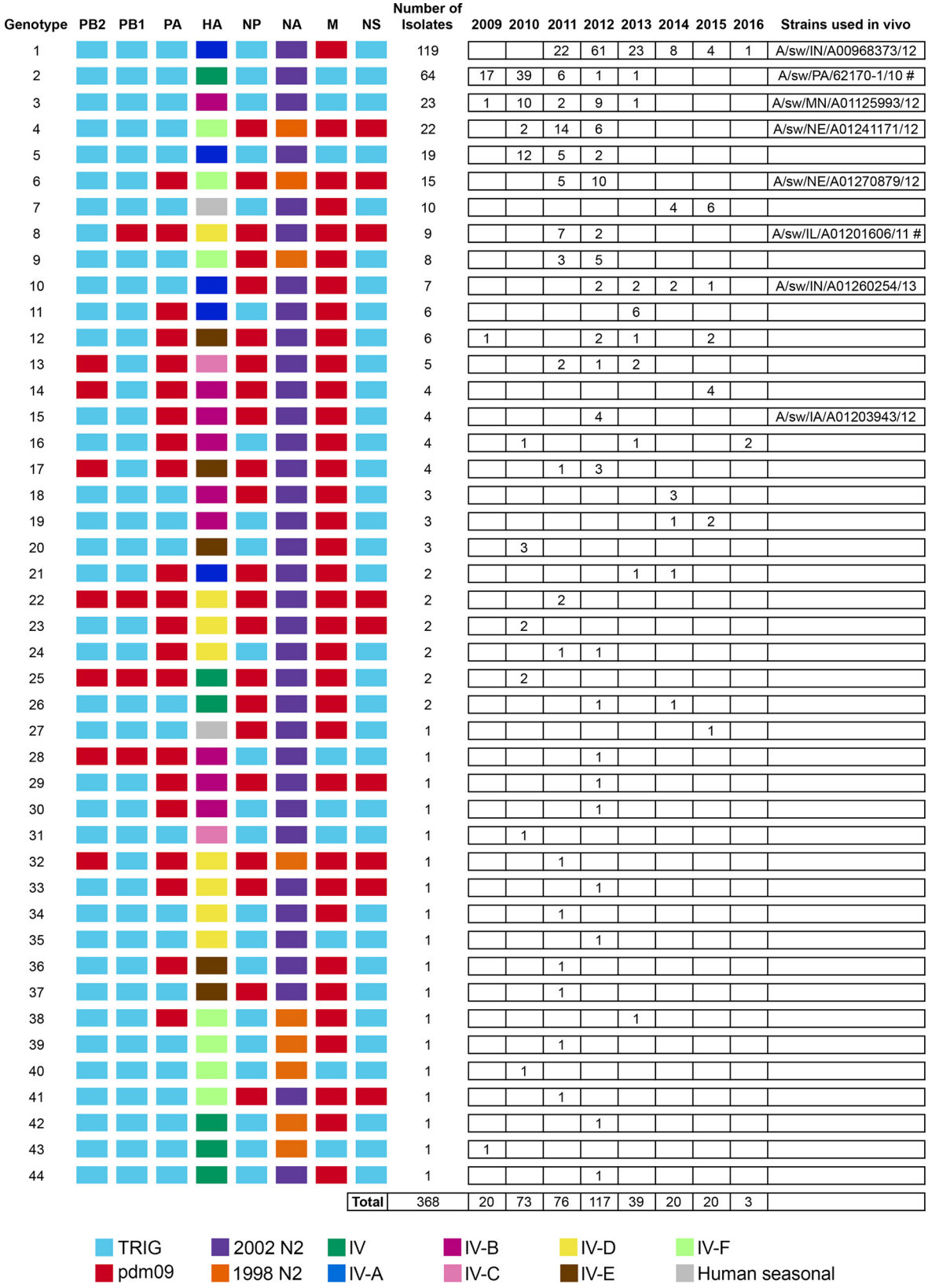

FIG 1 Genotypes of H3N2 whole-genome sequence isolates from United States swine from 2009 to 2016. The origin of each gene segment is color coded according to the key. Abbreviations: PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural; TRIG, triple reassortant internal gene constellation lineage; H1N1pdm09, pandemic 2009 lineage; I, II, and IV-A to -F, HA genetic clades; SIV, swine influenza virus. #, viruses evaluated *in vivo* by Kitikoon et al. (23).

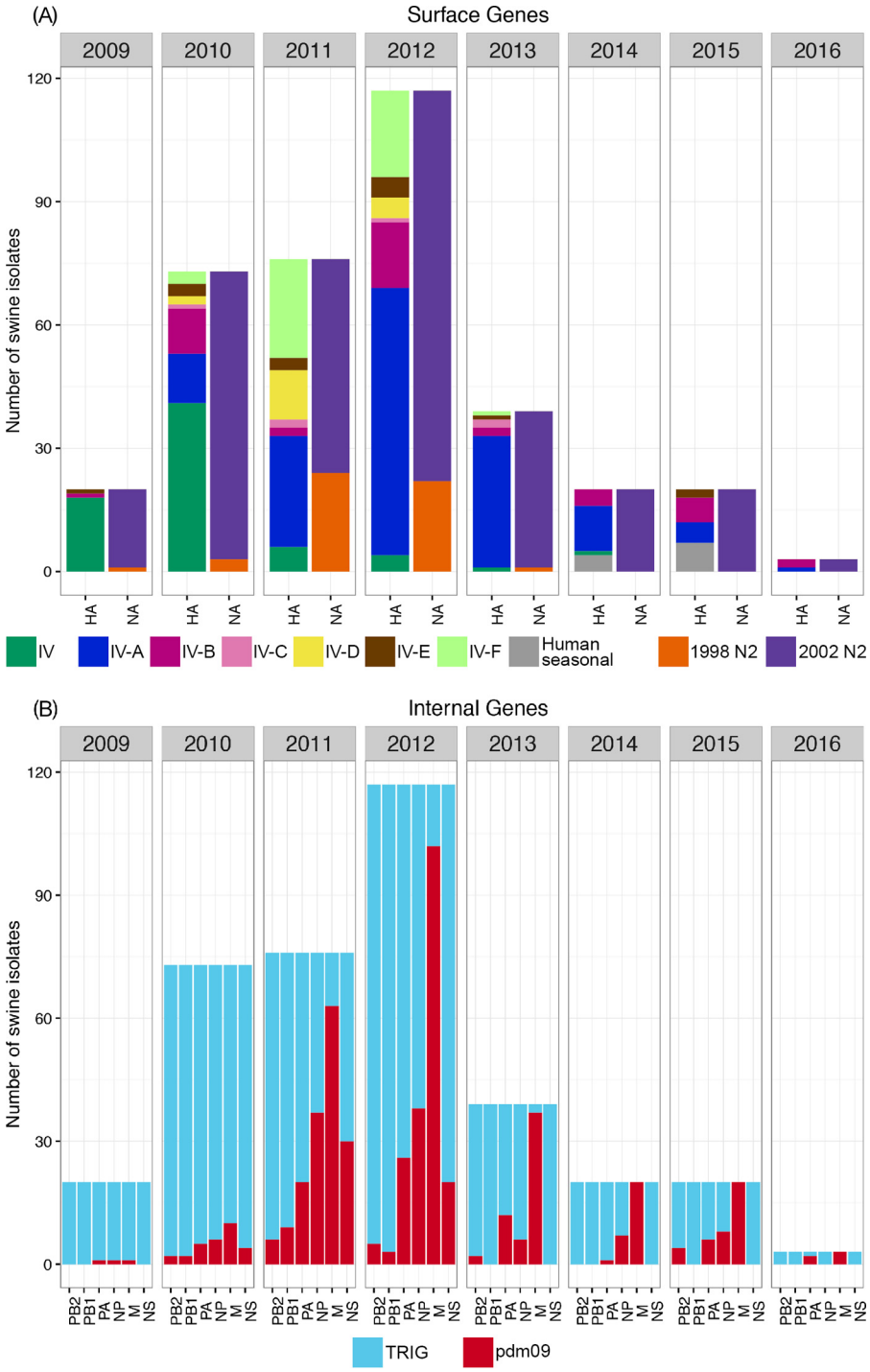

FIG 2 Relative frequency of surface (A) and internal (B) genes of H3N2 swine influenza viruses in the United States from 2009 to 2016. The number of swine isolates by lineage available as whole-genome sequences through the USDA Surveillance System are shown by year.

The most frequently detected genotype ($n = 119$) contained a clade IV-A HA gene, a 2002-lineage NA gene, a pandemic M gene segment, and the remaining gene segments of TRIG origin (Fig. 1). The genotype containing all TRIG internal genes, a clade IV HA gene, and a 2002-lineage NA gene was the next most frequent (64 isolates).

Among the H3 phylogenetic subclades, the less frequently detected clades IV-C, IV-D, and IV-E were paired more frequently with H1N1pdm09 internal genes, with the majority containing H1N1pdm09 PA, NP, and M (Fig. 3). Most IV-D strains also contained

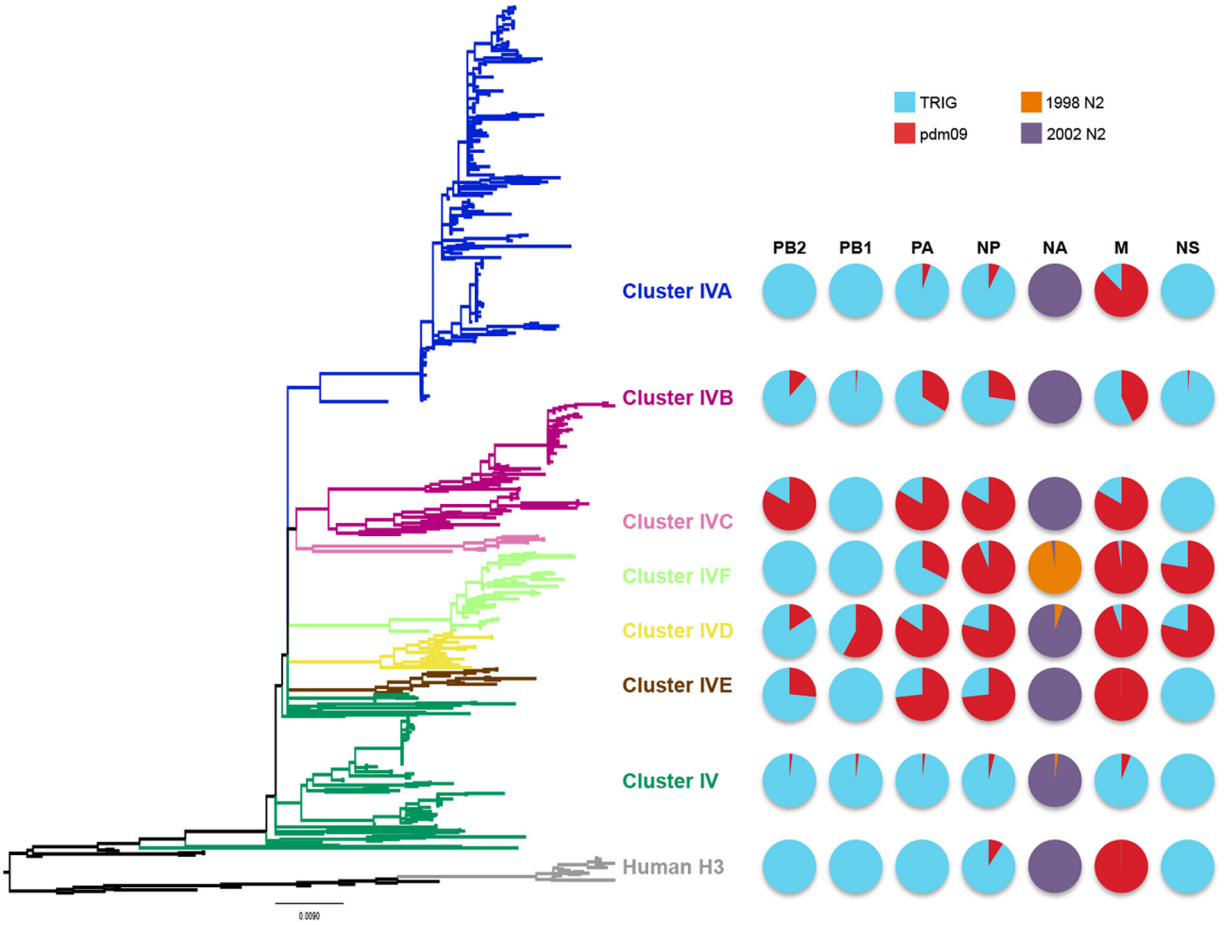

FIG 3 Pictorial representation of whole-genome H3N2 viruses from 2009 to 2016. The phylogenetic tree shows the HA gene, while the composition of the other 7 genes is shown using pie charts.

PB1 and NS from H1N1pdm09, and most IV-C strains contained PB2 from H1N1pdm09 (Fig. 3). Most clade IV-F isolates contained H1N1pdm09 NP, M, and NS genes, while the two most prevalent clades, IV-A and IV-B, show most internal genes of TRIG lineage (Fig. 3). Remarkably, all HA clades predominantly paired with the 2002 N2 lineage, except for clade IV-F (Fig. 3). The virus isolates selected for the *in vivo* pathogenesis study belonged to the most prevalent HA clades and represented the different genotypes of swine H3N2 circulating between 2009 and 2016.

Swine H3N2 with different genome constellations efficiently infected pigs but resulted in different degrees of pathology. All pigs infected with H3N2 viruses demonstrated mild clinical signs, including mild lethargy and anorexia, consistent with experimental IAV infection. Pigs infected with the genotype 15 virus showed the highest percentages of lung lesions, significantly higher than those of pigs infected with the genotype 6 virus (Table 1). Infection with the genotype 10 virus resulted in a percentage of lesions similar to that of genotype 15, although it was not significantly different from the other groups. Overall, microscopic lung lesion scores followed the pattern of macroscopic lesions, with the exception of the group infected with genotype 1 (Table 1).

In contrast to the pattern observed for lung pathology, substantial mean virus titers were found in the BALF for all groups, and virus was detected in the lungs of all pigs in each group (Table 1). However, there were subtle differences in mean titers; pigs infected with genotype 4 had significantly higher titers than pigs infected with genotype 15. Pigs infected with the genotype 3 virus had lower BALF virus titers than all other groups except the genotype 15 group (Table 1).

TABLE 1 Characteristics of infection with different swine IAV genotypes[a]

Group	Macroscopic pneumonia (%)	Microscopic lung scores (0-22)	Microscopic trachea scores (0-8)	BALF virus titer[b] (log_{10} $TCID_{50}$)
NC	0.5 ± 0.3^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a} (0/7)
Genotype 1	$3.8 \pm 1.0^{a,b}$	$6.3 \pm 1.0^{b,d}$	2.7 ± 0.5^{b}	$5.9 \pm 0.2^{b,d}$ (9/9)
Genotype 3	$3.3 \pm 0.7^{a,b}$	$3.0 \pm 0.6^{a,c}$	$3.7 \pm 0.5^{b,c}$	5.1 ± 0.1^{c} (10/10)
Genotype 4	$5.2 \pm 1.1^{a,b}$	$6.4 \pm 0.8^{b,d}$	4.9 ± 0.4^{c}	6.4 ± 0.2^{d} (10/10)
Genotype 6	2.4 ± 0.9^{a}	$3.4 \pm 0.4^{c,d}$	$3.3 \pm 0.6^{b,c}$	$5.8 \pm 0.2^{b,d}$ (10/10)
Genotype 10	6.2 ± 1.2^{b}	$5.4 \pm 0.6^{b,c}$	$3.6 \pm 0.5^{b,c}$	$5.9 \pm 0.1^{b,d}$ (10/10)
Genotype 15	7.8 ± 1.7^{b}	7.0 ± 0.9^{b}	$4.3 \pm 0.5^{b,c}$	$5.7 \pm 0.2^{b,c}$ (10/10)

[a]Shown are the mean (±SEM) percentage of macroscopic lung consolidation, microscopic lung and trachea composite scores, and virus titers in bronchoalveolar lavage fluid (BALF) of pigs infected with A/Swine/Indiana/A00968373/2012 (genotype 1), A/Swine/Minnesota/A01125993/2012 (genotype 3), A/Swine/Nebraska/A01241171/2012 (genotype 4), A/Swine/Nebraska/A01270879/2012 (genotype 6), A/Swine/Indiana/A01260254/2013 (genotype 10), A/Swine/Iowa/A01203943/2012 (genotype 15) at 5 dpi. Lowercase superscript letters within a column indicate significantly different values ($P \leq 0.05$).
[b]The number of virus-positive pigs/total number of pigs tested is indicated in parentheses.

Distinct patterns of viral shedding and transmission were observed between swine H3N2 IAV reassortants. Both clade IV-B viruses were delayed at the start of virus shedding, and there was no virus detected in nasal swabs of any pigs in these groups at 1 day postinfection (dpi) (Fig. 4A). Although pigs in the genotype 15 group exhibited the highest percentage of lung pathology, lower virus titers were consistently detected in nasal swabs until 4 dpi. Although not statistically significant, the group infected with the genotype 6 virus showed a trend for lower viral shedding, but with titers comparable to those of the genotype 15 virus.

All viruses transmitted to aerosol-transmission pigs at some point during the course of exposure, with virus being detected in nasal swabs as early as 1 day postcontact (dpc) in some groups (Fig. 4B). Consistent with the delayed viral shedding in the principal pigs in genotype 3 and genotype 15 groups, virus was not detected in the aerosol-transmission pigs in these groups until 3 and 5 dpc, respectively (Fig. 4B).

H3 clade diversification impacted serologic cross-reactivity. All aerosol-transmission pigs seroconverted by 15 dpc, with homologous hemagglutination inhibition (HI) titers between 80 and 5,120 (data not shown). Cross-reactivity was detected between the H3N2 viruses and antisera against the other challenge viruses within the same HA genetic clades (Fig. 5). In general, clade IV-A viruses (genotypes 1 and 10) were detected by a greater range of reference antisera to different H3 clade viruses, followed by clade IV-B (genotypes 3 and 15) and IV-F (genotypes 4 and 6) (Fig. 5).

DISCUSSION

Human-to-swine transmission of the H1N1pdm09 virus has been reported globally since 2009 (8, 10, 18, 19) and continues to occur in association with H1N1pdm09 activity in humans (13). However, onward transmission of H1N1pdm09 in pigs in the

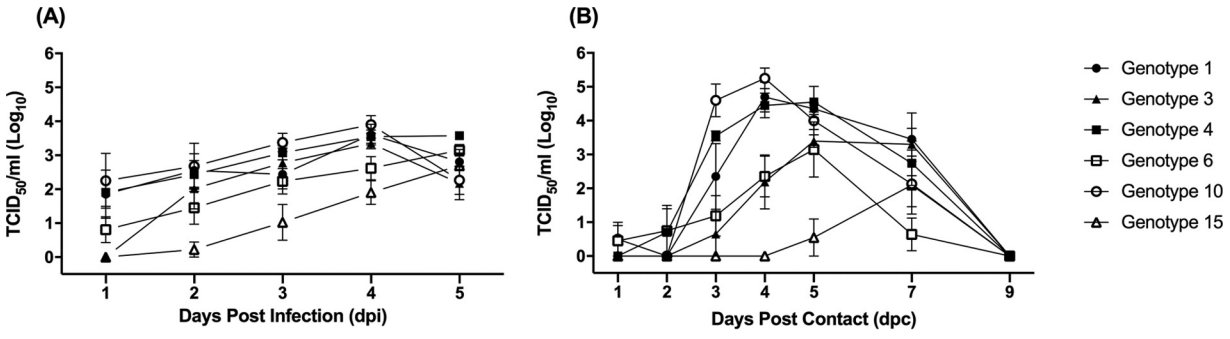

FIG 4 Virus titers (means ± standard errors of the means) in nasal swabs of principal (A) and aerosol (B) transmission in pigs infected with A/Swine/Indiana/A00968373/2012 (genotype 1), A/Swine/Minnesota/A01125993/2012 (genotype 3), A/Swine/Nebraska/A01241171/2012 (genotype 4), A/Swine/Nebraska/A01270879/2012 (genotype 6), A/Swine/Indiana/A01260254/2013 (genotype 10), and A/Swine/Iowa/A01203943/2012 (genotype 15) at 1 to 5 days postinfection (dpi) or 1 to 5, 7, and 9 days postcontact (dpc).

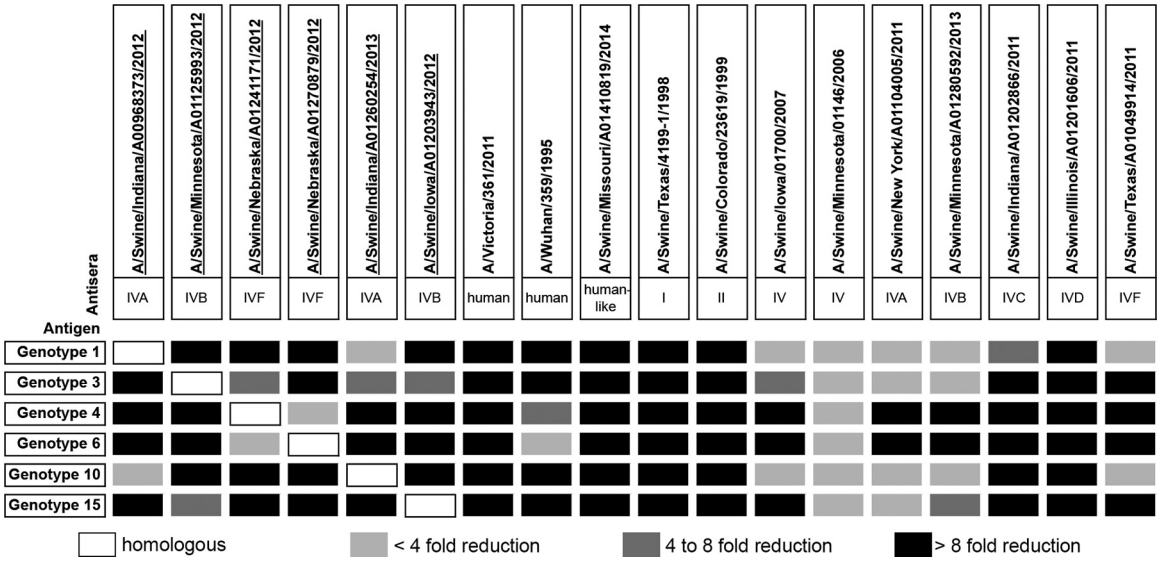

FIG 5 Fold difference of hemagglutination inhibition (HI) titers of the challenge viruses (underlined) using homologous antisera from aerosol-transmission pigs and selected reference antisera. Results are shown as fold reduction in reciprocal HI titers and were calculated compared to the titers of each test antigen against homologous antisera.

United States is limited to its internal gene segments, particularly the PA, NP, and M genes (13–15, 20). Reassortants with the M-pdm09 gene and remaining endemic TRIG genes are predominant in U.S. swine (5), contributing to the genetic diversity of swine IAV and to the origin of an H3N2 variant (H3N2v) that infected more than 300 people in 2011 to 2012 in the United States (21). Very recently, 18 detections of H3N2v in August 2016 were reported by the CDC that were associated with exhibition pigs (22), underscoring the importance of monitoring IAV in swine and the potential impact on humans. Many different IAV genotype patterns containing H1N1pdm09 internal genes were initially reported in U.S. pigs (14, 17, 23, 24), yet H3N2 reassortants with additional H1N1pdm09-TRIG internal gene constellations continued to emerge. Here, we performed whole-genome characterization of 368 swine H3N2 IAV isolates circulating in the United States since 2009 and identified at least 44 different genotypes. We then evaluated the pathogenesis and transmission phenotype of six viruses representative of these genotypes in pigs. Our results show that although all six genotypes efficiently infected pigs, they resulted in different degrees of pathology and viral shedding.

Prior to the introduction of H1N1pdm09 from humans into pigs in the United States in 2009, reassortment among endemic IAV, including H3, N2, and the six internal gene segments, was frequent (25), but the TRIG cassette was maintained and the clade IV HA gene was relatively evolutionarily stable. However, the introduction of H1N1pdm09 was concurrent with an expansion of genetic and antigenic diversity of the HA gene (26). In the first 3 years after H1N1pdm09 emergence, 11 distinct genomic patterns of H3N2 viruses were observed circulating in U.S. swine through the USDA surveillance system, although no single genotype predominated (17). Furthermore, it was observed that the well-established H3 clade IV diversified into six different clades, IV-A to IV-F (17). Importantly, the hundreds of human cases of H3N2v in 2011 to 2012 were associated with one of the newly emerged genotypes containing the H3 IV-A and the M-pdm09 gene on the TRIG backbone (21).

From the 44 different H3N2 genotypes that circulated in the United States from 2009 to 2016, the predominant genetic constellation (genotype 1) was found in 32.33% of the isolates. The next two most frequent genotypes (genotype 2, 17.39%; genotype 3, 6.25%) contained all internal genes of the TRIG lineage but were last detected in 2013. These all-TRIG genotypes likely represent low-prevalence isolates or isolates no longer in circulation in the United States, since the M gene of H1N1pdm09 has almost exclusively been reported in swine IAV based on all available M gene sequences in

GenBank, and all available WGS analyzed here from 2014 to 2016 contained M-pdm (5). Complex IAV genetic patterns were also observed among European swine since H1N1pdm09 emergence, with 19 different H1 but only 4 H3 genotypes identified (27). Interestingly, reassortments involving the H1N1pdm09 and European swine virus internal genes were rare, with only 1% detected (27), whereas reassortants between H1N1pdm09 and TRIG internal genes represented more than 70% of the isolates in the United States. A possible explanation is that the TRIG lineage is more permissive to reassortment with H1N1pdm09 and incorporation of internal genes. Reassortants with H1N1pdm09-TRIG internal genes were also more frequently detected in China, and the reassortants that circulated the longest had at least five H1N1pdm09-origin genes (16). These Chinese H1N1pdm09 reassortants became predominant with time, and the authors speculated that the H1N1pdm09 internal genes replace the Eurasian and TRIG internal genes in China (16). In our analysis, although there was an increased detection of H1N1pdm09 internal genes early after introduction, as of 2013 only the PA, NP, and M genes were extensively sustained. Similar to what was observed in both Europe and China, the HA genetic diversity was the major contributor to the overall genetic diversity constellations in the United States. However, the H3 gene diversity of North American viruses is much more extensive than reported from other regions (16, 27).

We then compared the pathogenesis and transmission properties of six contemporary H3N2 swine viruses of the most prevalent HA subclades from 2009 to 2016, IV-A, IV-B, and IV-F, with different internal gene constellations. Clinical signs were similar in all challenged groups and typical of influenza infection in pigs. Although all viruses infected pigs and efficiently replicated in the lungs, the resulting lung viral titers and lung pathology differed between virus groups. However, there was no correlation between lesions and viral titers in the lungs. For example, infection with genotype 6 virus resulted in BALF virus titers comparable to those of the other viruses but with less lung pathology. A similar inconsistency between low viral replication in the lungs and a high percentage of lung lesions was observed in a previous study testing viruses from genotype 2 and genotype 8; nevertheless, both genotypes resulted in efficient infection and transmission in pigs (23). Many factors are involved with influenza pathogenesis, such as infectivity, virulence, and replication efficiency, including the balance between the HA binding and NA cleavage activities (28) or 627K or 627E/701N amino acids in the PA gene (29). Recently, different H3N2 reassortants containing either three (NP, M, and NS) or five (PA, PB2, NP, M, and NS) genes from H1N1pdm09 were shown to infect pigs at different efficiencies, likely a result of the combination of genes rather than specific segments (30). In that study, the presence of a human seasonal lineage N2 or increased polymerase activity were suggested to be associated with increased viral transmissibility rather than the H1N1pdm09 internal genes (30). Similarly, the two viruses in our study with the highest and lowest percentages of pneumonia contained PA, NP, and M of pdm09 lineage, underscoring that combinations of additional gene segments likely played a role in the different pathology observed here.

The kinetics of virus shedding also differed among the infected groups. The genotype 15 H3N2 virus (containing PA-, NP-, and M-pdm09) displayed delayed viral shedding compared to the other groups, which subsequently delayed transmission to naive pigs. The M-pdm09 gene was shown to increase the aerosol transmission of poorly transmissible IAV in a guinea pig model, and it also increased the transmissibility of viruses with the swine TRIG backbone (31). Overall, among the genotypes containing M-pdm09, there was 98.8 to 100% amino acid sequence homology for this segment. The M segment of the genotype 1 virus contained three amino acid differences (M1 L74P and A227T and M2 V27A) compared to the M genes of all other genotypes with M-pdm09. Additionally, genotype 15 (M1 L234I) and genotype 4 (M1 D232N) contained a single mutation each compared to the remaining genotypes with M-pdm09. These amino acid positions have not been implicated in transmissibility, so a specific role for these mutations remains unknown. Since the M gene of genotype 15, the group with the lowest viral titers and delayed transmission, had only one amino acid difference compared to genotypes 6 and 10 and two amino acid differences compared to

genotype 4, our results suggest that transmission efficiency, like replication in the lungs, is a multigenic characteristic and may also be host species dependent. Furthermore, the M-pdm09 segment alone combined with the TRIG backbone resulted in a loss in transmissibility in pigs, but transmission was restored when M-pdm09 and NA-pdm09 were combined (32). Although M-pdm09 was shown to increase transmissibility in a PR8 backbone, NA-pdm09 also contributed to the transmission of the PR8 reassortant virus in the guinea pig model (33). Changes in the M segment were also correlated with increased neuraminidase activity, and the authors speculated that these changes were mediated by changes to virus morphology. In contrast to the reverse-engineered reassortant studies, viruses used in this study were wild-type field isolates, with amino acid differences between genes even from the same lineage, and therefore further studies are necessary to confirm the role of individual genes in the replication and transmission of reassortant swine H3N2 viruses.

We also compared the serological response and cross-reactivity between the six viruses used in this study against a panel of reference antisera made against human and swine IAV. The two viruses from clade IV-A were broadly detected by monovalent antisera to heterologous viruses, particularly antisera raised against more contemporary viruses. Based on amino acid sequences, these viruses contain the antigenic motif (145N, 155Y, 156N, 158N, 159Y, and 189K) that predicts them to be in the red antigenic cluster of swine H3 IAV according to Lewis et al. (26). Conversely, the two viruses of the IV-F genetic clade had 145K, 155H, 156K, 158N, 159Y, and 189R/S in antigenic motif positions, which could explain their lack of recognition by the reference panel shown here. As previously observed by Lewis et al. (26), the phylogenetic clade to which an H3 virus belongs does not predict its antigenic relationships, particularly for clades IV-A, -B, and -F tested here, hence the differences in cross-reactivity observed in this study between antisera raised against viruses of the same H3 clade.

Repeated spillover of H1N1pdm09 from humans to pigs has resulted in substantial changes in the evolution of swine influenza viruses in recent years, increasing the number of possible gene segment combinations and clearly contributing to the genetic and antigenic diversity of IAV in this species. Although neither the H1N1pdm09 virus in its entirety nor its surface genes have been maintained in pigs in the United States, the internal genes are amenable to reassortment with endemic swine viruses, with some gene combinations becoming established and predominant in pigs. Here, we highlight the outstanding diversity of H3N2 genomic constellations that circulated in U.S. swine in the 7 years following the introduction of H1N1pdm09. This diversity has important implications for the swine industry for controlling this rapidly changing pathogen. Notably, the adaptation to swine of viruses containing different combinations of human-adapted genes emphasizes the potential risk for the reintroduction of these viruses to humans, which could have important health consequences, as seen in recent zoonotic events and detections of H3N2v in 2011 to 2012 and more recently in the summer of 2016.

MATERIALS AND METHODS

Genetic analysis. Whole-genome sequences were obtained for the six swine isolates used for the pathogenesis study. Briefly, next-generation sequencing was done using the Ion 316 v2 chip and Ion PGM 200 v2 sequencing kit (Life Technologies, Carlsbad, CA) as previously described (34) or the Illumina MiSeq using the MiSeq reagent kit v2 (500 cycle; Illumina Inc., San Diego, CA), generating two 250-bp paired-end reads. All sequence data are available in GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/), and accession numbers for viruses used in the pathogenesis study are provided in Table 2.

An additional 553 U.S. swine H3N2 viruses with whole-genome sequences (WGS) were obtained from GenBank (as of 2 June 2016). Nucleotide sequences were aligned for each of the eight gene segments using default settings in MUSCLE v.3.7, available via the CIPRES portal (35), and then translated to amino acid sequences with subsequent manual correction. To remove identical whole-genome sequences from the data set, amino acid sequences of the 8 gene segments for individual viruses were concatenated and duplicates were removed. The same process was used to eliminate duplicate whole-genome sequences of multiple virus representatives of a single outbreak event collected on the same date and from the same location, leaving 368 WGS.

Trees were generated using MrBayes v3.2.2 (36) using the CIPRES portal (35). For each gene segment, three MrBayes runs (by using different start seeds) of two independent, parallel Markov chain Monte

TABLE 2 GenBank accession numbers of the swine H3N2 viruses used in this study

Strain name	Genotype	Accession no.							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/Sw/IN/A00968373/2012	1	JX534979	JX534980	JX534981	JX534982	JX534983	JX534984	JX534985	JX534986
A/Sw/MN/A01125993/2012	3	KX851891	KX851892	KX838371	JX422257	KX851893	JX422256	JX422255	KX851894
A/Sw/NE/A01241171/2012	4	JX128298	JX128299	JX128300	JX422575	JX128301	JX422574	JX422573	JX128302
A/Sw/NE/A01270879/2012	6	KF026735	KF026734	KF026736	KC139741	KF026738	KC139740	KC139739	KF026737
A/Sw/IN/A01260254/2013	10	KF386768	KF386769	KF386770	KF179107	KF386771	KF179108	KF179109	KF386772
A/Sw/IA/A01203943/2012	15	KC435042	KC435043	KC435044	KC134399	KC435045	KC134398	KC134397	KC435046

Carlo (MCMC) analyses, consisting of four MCMC chains, were run for five million generations, with subsampling every 100th generation. Convergence of the MCMC chains was determined by assessing the stationarity of molecular evolution parameters by effective sample sizes (ESS) of >200 in Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). The consensus trees were visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree). For the purpose of genotype classification, each gene segment was assigned to one of the following lineages: TRIG, H1N1pdm09, human seasonal, H3 clade IV, H3 clade IV-A, H3 clade IV-B, H3 clade IV-C, H3 clade IV-D, H3 clade IV-E, H3 clade IV-F, N2 1998, and N2 2002. Genotypes were defined when the phylogenies of the eight gene segments resulted in a unique gene combination for one or more isolates.

Viruses. Six swine H3N2 viruses were selected for *in vivo* study to represent prevalent genotypes containing the HA of the most prevalent H3 gene clades from 2009 to 2016. Genotypes were specifically selected to represent combinations of gene segments between H1N1pdm09 and TRIG internal genes not yet tested in previous experiments. The isolates were obtained from clinical cases of respiratory disease in pigs and maintained in the repository held at the National Veterinary Services Laboratories (NVSL) through the U.S. Department of Agriculture (USDA) IAV swine surveillance system in conjunction with the USDA-National Animal Health Laboratory Network (NAHLN). The selected viruses were A/swine/Indiana/A00968373/2012 (genotype 1, clade IV-A), A/swine/Minnesota/A01125993/2012 (genotype 3, clade IV-B), A/swine/Nebraska/A01241171/2012 (genotype 4, clade IV-F), A/swine/Nebraska/A01270879/2012 (genotype 6, clade IV-F), A/swine/Indiana/A01260254/2013 (genotype 10, clade IV-A), and A/swine/Iowa/A01203943/2012 (genotype 15, clade IV-B). Detailed epidemiological data for the viruses are unknown, as they were anonymous submissions to the USDA-NAHLN surveillance system. Viruses were propagated in Madin-Darby canine kidney (MDCK) cells. The pathogenesis of a virus belonging to genotype 2, A/Swine/Pennsylvania/62170-1/2010, was tested previously by our group in a similar study design (23).

***In vivo* pathogenesis and transmission study.** Three-week-old crossbred healthy pigs were obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). Upon arrival, pigs were treated with ceftiofur crystalline free acid (Zoetis Animal Health, Florham Park, NJ) and enrofloxacin (Bayer HealthCare AG, Whippany, NJ) to reduce bacterial contaminants and were shown to be seronegative to IAV antibodies by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Flock Check AI MultiS-Screen kit; IDEXX, Westbrook, ME). Pigs were divided into six groups of 10 according to challenge virus as described above and one control group of 8, housed in biosafety level 2 (BSL2) containment, and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center.

Each group of challenged pigs was inoculated intratracheally (2 ml) and intranasally (1 ml) with 1×10^5 50% tissue culture infective doses ($TCID_{50}$) per ml of one of the assigned viruses. Inoculation was performed under anesthesia, using an intramuscular injection of a cocktail of ketamine (8 mg/kg of body weight), xylazine (4 mg/kg), and tiletamine HCL and zolazepam HCL (Telazol; 6 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA). At 2 days postinfection (dpi), five naive pigs were placed in a separated raised deck in the same room as each inoculated group to evaluate indirect or aerosol transmission. This route is initially aerosol exposure but may also include direct contact with pigs in this group that become infected by aerosol first and then serve as direct-contact sources for the remaining pigs in the deck.

Two pigs died from causes unrelated to IAV infection, leaving 7 pigs in the nonchallenged group (NC) and 9 pigs in the genotype 1 groups. Principal pigs were humanely euthanized at 5 dpi with a lethal dose of pentobarbital (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI). Bronchoalveolar lavage fluid (BALF) and tissue samples from the distal trachea and right cardiac or affected lung lobe were collected. Aerosol-transmission pigs were bled and humanely euthanized at 15 days postcontact (dpc).

Virus titration. Nasal swabs (FLOQSwabs; Copan Diagnostics, Murrieta, CA) were collected daily from 0 to 5 dpi for primary pigs and from 0 to 5, 7, and 9 dpc for aerosol-transmission pigs in 2 ml minimum essential medium (MEM). For virus isolation, filtered nasal swab (NS) samples were plated in 24-well plates onto confluent MDCK cells washed twice with phosphate-buffered saline (PBS), as previously described (37). For virus titration, BALF and virus isolation-positive NS were 10-fold serially diluted in serum-free Opti-MEM (Gibco, Life Technologies, Carlsbad, CA) supplemented with 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics and plated in 96-well plates; plates were stained at 48 h as previously described (38). Virus titers were calculated as $TCID_{50}$ per milliliter for each sample (39).

Pathology. At necropsy, lungs were removed and the percentage of the surface affected by pneumonia was calculated based on weighted proportions of each lobe to the total lung volume (40). Trachea and lung tissue samples fixed in 10% buffered formalin were routinely processed and stained

with hematoxylin and eosin. Microscopic lesions were evaluated by a veterinary pathologist blinded to treatment groups and scored according to previously described parameters (41). IAV-specific antigen was detected in trachea and lung tissues using immunohistochemistry (IHC), and detection was scored as previously described (41). Individual scoring parameters were combined into a composite score for each individual pig by tissue and staining type and analyzed as average group composite scores.

Cytokine assays. Cytokine levels in cell-free BALF samples were determined using a multiplex ELISA according to the manufacturer's recommendations (SearchLight; Aushon Biosystems, Billerica, MA). Cytokine concentrations were reported as the means of duplicate samples for statistical analysis.

Serologic assays. Serum samples were obtained from aerosol-transmission pigs at 15 dpc to test for seroconversion by HI assay. Prior to HI, sera were treated with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO), heat inactivated at 56°C for 30 min, and adsorbed with 50% turkey red blood cells (RBC) to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins. HI assays were performed using standard techniques (42). Cross-reactive HI was performed with a reference swine anti-H3N2 antiserum panel using the challenge viruses as antigens. Reciprocal titers were divided by 10, $\log_2$ transformed, and reported as the geometric mean.

Statistical analysis. The percentage of macroscopic lesions, microscopic lesion scores, and $\log_{10}$-transformed BALF and NS virus titers were analyzed using analysis of variance, with a P value of ≤ 0.05 considered significant (GraphPad Prism 6; GraphPad Software, La Jolla, CA). Response variables shown to have significant effects by treatment group were subjected to pairwise mean comparisons using the Tukey-Kramer test.

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REFERENCES

1. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. 2008. Swine influenza viruses: a North American perspective. Adv Virus Res 72:127–154. https://doi.org/10.1016/S0065-3527(08)00403-X.
2. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73:8851–8856.
3. Nelson MI, Vincent AL. 2015. Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface. Trends Microbiol 23:142–153. https://doi.org/10.1016/j.tim.2014.12.002.
4. de Jong JC, van Nieuwstadt AP, Kimman TG, Loeffen WL, Bestebroer TM, Bijlsma K, Verweij C, Osterhaus AD, Class EC. 1999. Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. Vaccine 17:1321–1328. https://doi.org/10.1016/S0264-410X(98)00392-2.
5. 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 Respir Viruses 7:42–51. https://doi.org/10.1111/irv.12193.
6. Lorusso A, Vincent AL, Gramer ME, Lager KM, Ciacci-Zanella JR. 2013. Contemporary epidemiology of North American lineage triple reassortant influenza A viruses in pigs, p 113–132. *In* Richt JA, Webby RJ (ed), Swine influenza. Springer, Berlin, Germany. https://doi.org/10.1007/82_2011_196.
7. Nelson MI, Wentworth DE, Culhane MR, Vincent AL, Viboud C, LaPointe MP, Lin X, Holmes EC, Detmer SE. 2014. Introductions and evolution of human-origin seasonal influenza a viruses in multinational swine populations. J Virol 88:10110–10119. https://doi.org/10.1128/JVI.01080-14.
8. Pasma T, Joseph T. 2010. Pandemic (H1N1) 2009 infection in swine herds, Manitoba, Canada. Emerg Infect Dis 16:706–708. https://doi.org/10.3201/eid1604.091636.
9. Pereda A, Cappuccio J, Quiroga MA, Baumeister E, Insarralde L, Ibar M, Sanguinetti R, Cannilla ML, Franzese D, Escobar Cabrera OE, Craig MI, Rimondi A, Machuca M, Debenedetti RT, Zenobi C, Barral L, Balzano R, Capalbo S, Risso A, Perfumo CJ. 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis 16:304–307. https://doi.org/10.3201/eid1602.091230.
10. Sreta D, Tantawet S, Na Ayudhya SN, Thontiravong A, Wongphatcharachai M, Lapkuntod J, Bunpapong N, Tuanudom R, Suradhat S, Vimolket L, Poovorawan Y, Thanawongnuwech R, Amonsin A, Kitikoon P. 2010. Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg Infect Dis 16:1587–1590. https://doi.org/10.3201/eid1610.100665.

11. Welsh MD, Baird PM, Guelbenzu-Gonzalo MP, Hanna A, Reid SM, Essen S, Russell C, Thomas S, Barrass L, McNeilly F, McKillen J, Todd D, Harkin V, McDowell S, Choudhury B, Irvine RM, Borobia J, Grant J, Brown IH. 2010. Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs. Vet Rec 166:642–645. https://doi.org/10.1136/vr.4851.
12. Nelson MI, Gramer MR, Vincent AL, Holmes EC. 2012. Global transmission of influenza viruses from humans to swine. J Gen Virol 93:2195–2203. https://doi.org/10.1099/vir.0.044974-0.
13. Nelson MI, Stratton J, Killian ML, Janas-Martindale A, Vincent AL. 2015. Continual reintroduction of human pandemic H1N1 influenza A viruses into swine in the United States, 2009 to 2014. J Virol 89:6218–6226. https://doi.org/10.1128/JVI.00459-15.
14. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton JC, Lowe J, Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerg Infect Dis 17:1624–1629. https://doi.org/10.3201/1709.110338.
15. Vijaykrishna D, Poon LL, Zhu HC, Ma SK, Li OT, Cheung CL, Smith GJ, Peiris JS, Guan Y. 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 328:1529. https://doi.org/10.1126/science.1189132.
16. Liang H, Lam TT, Fan X, Chen X, Zeng Y, Zhou J, Duan L, Tse M, Chan CH, Li L, Leung TY, Yip CH, Cheung CL, Zhou B, Smith DK, Poon LL, Peiris M, Guan Y, Zhu H. 2014. Expansion of genotypic diversity and establishment of 2009 H1N1 pandemic-origin internal genes in pigs in China. J Virol 88:10864–10874. https://doi.org/10.1128/JVI.01327-14.
17. Kitikoon P, Nelson MI, Killian ML, Anderson TK, Koster L, Culhane MR, Vincent AL. 2013. Genotype patterns of contemporary reassorted H3N2 virus in US swine. J Gen Virol 94:1236–1241. https://doi.org/10.1099/vir.0.051839-0.
18. Rajao DS, Costa AT, Brasil BS, Del Puerto HL, Oliveira FG, Alves F, Braz GF, Reis JK, Guedes RM, Lobato ZI, Leite RC. 2013. Genetic characterization of influenza virus circulating in Brazilian pigs during 2009 and 2010 reveals a high prevalence of the pandemic H1N1 subtype. Influenza Other Respir Viruses 7:783–790. https://doi.org/10.1111/irv.12072.
19. Hofshagen M, Gjerset B, Er C, Tarpai A, Brun E, Dannevig B, Bruheim T, Fostad IG, Iversen B, Hungnes O, Lium B. 2009. Pandemic influenza A(H1N1)v: human to pig transmission in Norway? Euro Surveill 14:19406.
20. Pereda A, Rimondi A, Cappuccio J, Sanguinetti R, Angel M, Ye J, Sutton T, Dibarbora M, Olivera V, Craig MI, Quiroga M, Machuca M, Ferrero A, Perfumo C, Perez DR. 2011. Evidence of reassortment of pandemic H1N1 influenza virus in swine in Argentina: are we facing the expansion of potential epicenters of influenza emergence? Influenza Other Respir Viruses 5:409–412. https://doi.org/10.1111/j.1750-2659.2011.00246.x.
21. Epperson S, Jhung M, Richards S, Quinlisk P, Ball L, Moll M, Boulton R, Haddy L, Biggerstaff M, Brammer L, Trock S, Burns E, Gomez T, Wong KK, Katz J, Lindstrom S, Klimov A, Bresee JS, Jernigan DB, Cox N, Finelli L. 2013. Human infections with influenza A(H3N2) variant virus in the United States, 2011-2012. Clin Infect Dis 57:S4–S11. https://doi.org/10.1093/cid/cit272.
22. Schicker RS, Rossow J, Eckel S, Fisher N, Bidol S, Tatham L, Matthews-Greer J, Sohner K, Bowman AS, Avrill J, Forshey T, Blanton L, Davis CT, Schiltz J, Skorupski S, Berman L, Jang Y, Bresee JS, Lindstrom S, Trock SC, Wentworth D, Fry AM, de Fijter S, Signs K, DiOrio M, Olsen SJ, Biggerstaff M. 2016. Outbreak of influenza A(H3N2) variant virus infections among persons attending agricultural fairs housing infected swine–Michigan and Ohio, July-August 2016. MMWR Morb Mortal Wkly Rep 65:1157–1160. https://doi.org/10.15585/mmwr.mm6542a1.
23. Kitikoon P, Vincent AL, Gauger PC, Schlink SN, Bayles DO, Gramer MR, Darnell D, Webby RJ, Lager KM, Swenson SL, Klimov A. 2012. Pathogenicity and transmission in pigs of the novel A(H3N2)v influenza virus isolated from humans and characterization of swine H3N2 viruses isolated in 2010-2011. J Virol 86:6804–6814. https://doi.org/10.1128/JVI.00197-12.
24. Liu Q, Ma J, Liu H, Qi W, Anderson J, Henry SC, Hesse RA, Richt JA, Ma W. 2012. Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States. Arch Virol 157:555–562. https://doi.org/10.1007/s00705-011-1203-9.
25. Nelson MI, Detmer SE, Wentworth DE, Tan Y, Schwartzbard A, Halpin RA, Stockwell TB, Lin X, Vincent AL, Gramer MR, Holmes EC. 2012. Genomic reassortment of influenza A virus in North American swine, 1998-2011. J Gen Virol 93:2584–2589. https://doi.org/10.1099/vir.0.045930-0.
26. Lewis NS, Anderson TK, Kitikoon P, Skepner E, Burke DF, Vincent AL. 2014. Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J Virol 88:4752–4763. https://doi.org/10.1128/JVI.03805-13.
27. Watson SJ, Langat P, Reid SM, Lam TTY, Cotten M, Kelly M, Van Reeth K, Qiu Y, Simon G, Bonin E, Foni E, Chiapponi C, Larsen L, Hjulsager C, Markowska-Daniel I, Urbaniak K, Dürrwald R, Schlegel M, Huovilainen A, Davidson I, Dán Á Loeffen W, Edwards S, Bublot M, Vila T, Maldonado J, Valls L, Brown IH, Pybus OG, Kellam P. 2015. Molecular epidemiology and evolution of influenza viruses circulating within European swine between 2009 and 2013. J Virol 89:9920–9931. https://doi.org/10.1128/JVI.00840-15.
28. Wagner R, Matrosovich M, Klenk HD. 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol 12:159–166. https://doi.org/10.1002/rmv.352.
29. Steel J, Lowen AC, Mubareka S, Palese P. 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog 5:e1000252. https://doi.org/10.1371/journal.ppat.1000252.
30. Ma J, Shen H, Liu Q, Bawa B, Qi W, Duff M, Lang Y, Lee J, Yu H, Bai J, Tong G, Hesse RA, Richt JA, Ma W. 2015. Pathogenicity and transmissibility of novel reassortant H3N2 influenza viruses with 2009 pandemic H1N1 genes in pigs. J Virol 89:2831–2841. https://doi.org/10.1128/JVI.03355-14.
31. Chou YY, Albrecht RA, Pica N, Lowen AC, Richt JA, Garcia-Sastre A, Palese P, Hai R. 2011. The M segment of the 2009 new pandemic H1N1 influenza virus is critical for its high transmission efficiency in the guinea pig model. J Virol 85:11235–11241. https://doi.org/10.1128/JVI.05794-11.
32. Ma W, Liu Q, Bawa B, Qiao C, Qi W, Shen H, Chen Y, Ma J, Li X, Webby RJ, Garcia-Sastre A, Richt JA. 2012. The neuraminidase and matrix genes of the 2009 pandemic influenza H1N1 virus cooperate functionally to facilitate efficient replication and transmissibility in pigs. J Gen Virol 93:1261–1268. https://doi.org/10.1099/vir.0.040535-0.
33. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. 2014. The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. J Virol 88:3802–3814. https://doi.org/10.1128/JVI.03607-13.
34. Bowman AS, Sreevatsan S, Killian ML, Page SL, Nelson SW, Nolting JM, Cardona C, Slemons RD. 2012. Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A viruses at an Ohio agricultural fair, July 2012. Emerging Microbes Infect 1:e33. https://doi.org/10.1038/emi.2012.33.
35. Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Abstr Gateway Computing Environ Workshop (GCE). IEEE, New York, NY.
36. Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755. https://doi.org/10.1093/bioinformatics/17.8.754.
37. Vincent AL, Ma W, Lager KM, Richt JA, Janke BH, Sandbulte MR, Gauger PC, Loving CL, Webby RJ, Garcia-Sastre A. 2012. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease. J Virol 86:10597–10605. https://doi.org/10.1128/JVI.01439-12.
38. Gauger PC, Vincent AL. 2014. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine, p 313–324. *In* Spackman E (ed), Animal influenza virus. Springer, New York, NY. https://doi.org/10.1007/978-1-4939-0758-8_26.
39. Reed J, Muench H. 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 27:493–497.
40. Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA. 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 32:648–660. https://doi.org/10.1177/030098589503200606.
41. Gauger PC, Vincent AL, Loving CL, Henningson JN, Lager KM, Janke BH, Kehrli ME, Jr, Roth JA. 2012. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. Vet Pathol 49:900–912. https://doi.org/10.1177/0300985812439724.
42. Kitikoon P, Gauger PC, Vincent AL. 2014. Hemagglutinin inhibition assay with swine sera, p 295–301. *In* Spackman E (ed), Animal influenza virus. Springer, New York, NY. https://doi.org/10.1007/978-1-4939-0758-8_24.