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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

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Swine influenza virus (SIV) H3N2 with triple reassorted internal genes (TRIG) has been enzootic in United States since 1998. Transmission of the 2009 pandemic H1N1 (pH1N1) virus to pigs in the United States was followed by reassortment with endemic SIV, resulting in reassorted viruses that include novel H3N2 genotypes (rH3N2p). Between July and December 2011, 12 cases of human infections with swine-lineage H3N2 viruses containing the pandemic matrix (pM) gene [A(H3N2)v] were detected. Whole-genome analysis of H3N2 viruses isolated from pigs from 2009 to 2011 sequenced in this study and other available H3N2 sequences showed six different rH3N2p genotypes present in the U.S. swine population since 2009. The presence of the pM gene was a common feature among all rH3N2p genotypes, but no specific genotype appeared to predominate in the swine population. We compared the pathogenic, transmission, genetic, and antigenic properties of a human A(H3N2)v isolate and two swine H3N2 isolates, H3N2-TRIG and rH3N2p. Our *in vivo* study detected no increased virulence in A(H3N2)v or rH3N2p viruses compared to endemic H3N2-TRIG virus. Antibodies to cluster IV H3N2-TRIG and rH3N2p viruses had reduced cross-reactivity to A(H3N2)v compared to other cluster IV H3N2-TRIG and rH3N2p viruses. Genetic analysis of the hemagglutinin gene indicated that although rH3N2p and A(H3N2)v are related to cluster IV of H3N2-TRIG, some recent rH3N2p isolates appeared to be forming a separate cluster along with the human isolates of A(H3N2)v. Continued monitoring of these H3N2 viruses is necessary to evaluate the evolution and potential loss of population immunity in swine and humans.

Influenza A viruses can infect and cause clinical signs in humans and a wide range of avian and mammalian species. The virus undergoes two major forms of evolution, antigenic drift and shift, which occur through the accumulation of point mutations and genetic reassortment of the multiple gene segments. As a shared disease of animals and humans, transmission of influenza A virus from one species to another may occur (43) and leads to public health concerns about potential transmission events that may lead to the next pandemic.

Swine influenza virus (SIV) is endemic in the pig population worldwide. The evolutionary data for SIV in the United States demonstrates that at least three major human-to-swine influenza virus transmission events have occurred. The emergence of an H3N2 virus with a triple-reassortant internal gene (TRIG) cassette consisting of genes from human (HA, NA, and PB1), avian (PB2 and PA), and swine (NP, M, and NS) viruses (in 1998) and the human-like H1N1 and H1N2 viruses (in 2003 and 2005, respectively) introduced in the U.S. swine population exemplifies the human-to-swine influenza virus transmission (16, 49) that has likewise occurred in other parts of the world. The introduction of the pandemic H1N1 (pH1N1) virus from infected humans to swine in different regions of the world since 2009 is another notable example of influenza A virus spread from humans to pigs (26, 28). At present, North American swine H1 and H3 influenza viruses are grouped into H1- α , - β , - γ , and - δ and H3-I, -II, -III, and -IV clusters based on genetic variation. These major introductory events from human to swine resulted in the establishment of dif-

ferent swine influenza virus H1 and H3 clusters. Novel internal gene combinations resulted from reassortment between the newly introduced viruses and endemic SIVs circulating at the time and contributed to the diverse influenza virus ecology currently recognized in the North American swine population.

Likewise, sporadic reports of humans infected with SIV were previously documented (23). In contrast to the widespread transmission of human influenza viruses demonstrated in pigs, all human cases of influenza caused by nonseasonal swine lineage viruses had limited human-to-human transmission that rarely spread beyond the principal patient (23, 34), with the exception of the 2009 pH1N1. In the United States alone, 41 human cases infected with viruses of swine genetic lineages were detected during 1990 to 2011 (32, 34). From 1990 to 2009, 20 reported cases of human infection with SIV were exclusively of H1 subtype (19 H1N1 and 1 H1N2). Between 2009 and the present, an increasing number of human cases caused by H3N2 viruses containing gene segments similar to those found in SIV have been reported. Seven

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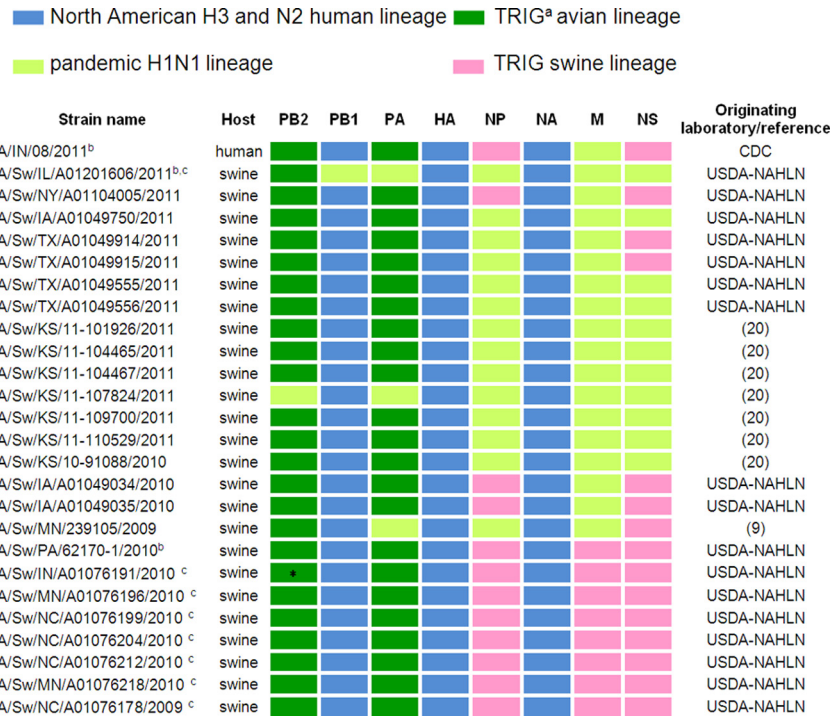


FIG 1 Lineages of each gene segment from different H3N2 genotypes identified through gene sequencing, BLAST, phylogenetic analysis, and previous data. Lists of the originating laboratories and references for the sequences from this study and GenBank database are shown in the far right column. Superscript letters: a, triple reassorted internal gene cassette (TRIG) identified in endemic swine H3N2 virus circulating in North American swine population since 1998; b, virus isolates used as inocula in the swine pathogenesis and transmission study [A/IN/08/2011, A/IN is A(H3N2)v; A/Sw/IL/A01201606/2011, Sw/IL is rH3N2p; and A/Sw/PA/62170-1/2010, Sw/PA is H3N2-TRIG]; c, isolates obtained through USDA-SIV surveillance system that were genetically characterized in this study reported here. *, Partial sequencing. Abbreviations: PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural.

individuals were infected in 2009 and 2010 with a contemporary swine H3N2 subtype with the TRIG cassette (H3N2-TRIG) similar to the endemic H3N2 swine viruses circulating in the North American swine population since 1998 (34). However, 12 recent human influenza cases detected since July 2011 were caused by H3N2-TRIG viruses with the matrix gene derived from the 2009 pH1N1 virus, and this novel virus has been designated as A(H3N2)v (3, 4).

We compared here the pathogenic, genetic, and antigenic properties of H3N2 viruses isolated from U.S. human and swine that inherited genes from TRIG and 2009 pH1N1 genetic lineages. We refer to these swine viruses as H3N2-TRIG or rH3N2p and to the viruses from humans as A(H3N2)v. We report the whole-genome sequence analysis of eight swine H3N2 viruses isolated from pigs from 2009 to 2011, including isolates with the TRIG cassette (H3N2-TRIG) similar to viruses circulating and established in the North American swine population prior to the introduction of pH1N1 and several reassorted H3N2/pH1N1 viruses (rH3N2p) isolated after the pH1N1 virus became established in swine. To evaluate the behavior of a human isolate of A(H3N2)v in swine, we compared A(H3N2)v in a pathogenesis and transmission model in pigs with two swine H3N2 isolates containing different constellations of internal genes derived from the TRIG (H3N2-TRIG) or the 2009 pandemic H1N1 (rH3N2p) viruses.

MATERIALS AND METHODS

Viruses. Swine isolates were obtained from the National Veterinary Services Laboratories (NVSL) through the U.S. Department of Agriculture (USDA)-

National Animal Health Laboratory Network (NAHLN) Swine Influenza Surveillance System. Eight H3N2 swine isolates—A/Swine/Illinois/A01201606/2011, A/Swine/Indiana/A01076191/2010, A/Swine/Minnesota/A01076196/2010, A/Swine/North Carolina/A01076199/2010, A/Swine/North Carolina/A01076204/2010, A/Swine/North Carolina/A01076212/2010, A/Swine/Minnesota/A01076218/2010, and A/Swine/North Carolina/A01076178/2009—were selected for full genome sequencing (Fig. 1). Viral RNA was extracted from Madin-Darby canine kidney (MDCK) cell grown virus with a MagMAX 5X viral RNA isolation kit (Ambion, TX) according to the manufacturer's recommendations.

Three H3N2 viruses (A/Indiana/08/2011 [A/IN], A/Swine/Illinois/A01201606/2011 [Sw/IL], and A/Swine/Pennsylvania/62170-1/2010 [Sw/PA]) were selected for pathogenesis and transmission investigation in the swine host. The human isolate A/IN was provided from the Centers for Disease Control and Prevention (CDC). This A(H3N2)v virus has the matrix gene (pM) from the 2009 pandemic H1N1 virus and was isolated from a human case in Indiana in July 2011 (4). The swine viruses, Sw/PA and Sw/IL, were isolated from outbreaks of respiratory disease in pigs. Sw/PA represents the contemporary swine cluster IV H3N2 viruses (H3N2-TRIG) that endemically circulate in the North American pig population. The H3N2-TRIG was isolated from a 22-week-old pig with clinical signs of coughing, fever, and lethargy and with mild to moderate cranioventral bronchopneumonia at necropsy. Sw/IL virus is a reassortant H3N2 (rH3N2p) virus with an endemic swine HA (cluster IV), NA, and TRIG PB2 and a 2009 pandemic M, NP, NS, PA, and PB1 gene combination. Detailed epidemiological data for Sw/IL are unknown since the USDA-NAHLN system is an anonymous surveillance stream. Viruses were propagated in MDCK cells, harvested, clarified, and stored at -80°C prior to inoculating pigs.

Genetic analysis. Sequences included in the analysis were generated from the present study or obtained from the National Center for Biotechnology Information (NCBI) influenza resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/>). In addition, sequence analysis was performed on viruses that were shed from two principal and two contact pigs of each experimental group (described below) to evaluate nucleotide changes after pig passage. Gene sequencing of Sw/IL was performed by conventional sequencing. All genes were amplified by reverse transcription-PCR (RT-PCR) in their entirety using previously described primer sets (12) with SuperScript III One-Step Reverse Transcriptase and Platinum *Taq* HiFi (Invitrogen, Carlsbad, CA). The fragments were purified by using a QIAquick gel extraction kit (Qiagen, Inc.) prior to labeling using BigDye terminator chemistries (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions and using an ABI 3100 genetic analyzer (Applied Biosystems). The other seven selected swine isolates were deep sequenced using a genome analyzer (Illumina, Inc., San Diego, CA). Samples for sequencing were prepared as described previously (9). In brief, RT-PCR was performed with Uni-12 and Uni-13 primers and gene-specific primers (12) to amplify all eight segments in one reaction with Invitrogen SuperScript III One-Step reverse transcriptase and Platinum *Taq* HiFi. Double-stranded DNA was sonicated in a Covaris AFA (Covaris, Woburn, MA). The 3' overhangs were removed, a poly(A) tail was added, and adapters were ligated to the DNA fragments using E6050L, E6053L, and E6056L kits (New England Biolabs [NEB], Ipswich, MA). The products were then purified by gel electrophoresis using E-Gel size select 2% agarose precast gels (Invitrogen). Index sequences were added to the DNA samples by Phusion DNA polymerase (NEB) before they were loaded on the Illumina sequencer.

Sequencing reads of 150 bp were dynamically trimmed with the Dynamic Trim.pl algorithm from the Solexa QA tools (8). The trimmed reads were further filtered to remove reads that were shorter than 20 bp. Replicated reads that were overrepresented at the ends of some viral genome segments (PB2, PB1, and PA) in some samples were reduced by removing replicated sequences among the reads of a sample (11). A comprehensive, nonredundant database of influenza virus whole-genome sequences was created by extracting all of the whole-genome influenza virus sequences (81,898 sequences accessed on 12 September 2011) in the NCBI influenza virus resource sequence database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/>). The sequences were filtered so that the local database contained only one unique representative of every sequence. The reads from each sample were compared via BLAST to the aforementioned database and subsequently binned according to the influenza virus genome segment that provided the top-scoring blastn match. The prepared reads were assembled using Whole Genome Shotgun and EST Sequence Assembler, MIRA 3.4.0 (German Cancer Research Centre, Heidelberg, Germany). Manual editing for certain sequence was performed with GAP4 from the Staden package (2). Manually edited sequences were exported back to MIRA for recalling of the consensus sequence prior to the final collection of the assembled sequences.

Assembled sequences were aligned by the CLUSTAL W program, and phylogenetic analysis was performed by using Molecular Evolutionary Genetics Analysis software (MEGA, version 4.0) (36). The evolutionary history was inferred using the neighbor-joining method with a bootstrap value from 1,000 replicates indicated next to the branches (31). GenBank accession numbers of sequences obtained from the present study are listed in Table S1 in the supplemental material.

In vivo pathogenesis and transmission study. A total of 56 3-week-old conventional pigs were obtained from a healthy herd free of SIV, porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae*. Upon arrival, pigs were confirmed seronegative to SIV by Flock Check AI MultiS-Screen diagnostic kit (Idexx Laboratories, ME) as described previously (7). All pigs were treated with ceftiofur crystalline free acid (Pfizer, NY) and enrofloxacin (Bayer HealthCare AG, NJ) to reduce bacterial contaminants prior to the start of the study. Pigs were divided into four groups of eight, housed in individual isolation rooms, and cared for in compliance with the Institutional Animal Care

and Use Committee of the National Animal Disease Center. The groups included sham-infected negative controls and A/IN-infected, Sw/PA-infected, and Sw/IL-infected pigs. Pigs in each assigned group were inoculated intranasally (1 ml) and intratracheally (2 ml) with $10^{6.5}$ 50% tissue culture infective doses (TCID₅₀) per ml. Phosphate-buffered saline (PBS) was given as a sham inoculum in the negative control group. Inoculation of pigs was performed under anesthesia, using an anesthetic cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg), and Telazol (6 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), administered intramuscularly. Two pigs died from causes unrelated to influenza, leaving seven pigs in the negative control and A/IN-infected groups. Eight contact pigs were cointingled with each of the H3N2 virus-infected groups at 2 days postinfection (dpi) to study virus transmission efficiency. The pigs were observed daily for clinical signs of respiratory disease. Principal pigs, including the negative control group, were humanely euthanized with a lethal dose of pentobarbital (Sleep Away; Fort Dodge Animal Health) and necropsied at 5 dpi, and contact pigs were necropsied at 19 days postcontact (dpc).

Virus titer in nasal swabs and lungs. To evaluate virus shedding, Dacron nasal swabs were taken and placed in 2 ml of minimal essential medium (MEM) on dpi -1 (from all pigs) and dpi 1, 3, and 5 from principal pigs and at dpc 1, 3, 5, and 7 from contact pigs. To assess virus titers in the lungs of infected pigs, bronchoalveolar lavage fluid (BALF) was collected from principal pigs by removing whole lungs from pigs, followed by lavage with 50 ml of MEM. All nasal swabs and BALF samples were stored at -80°C until study completion.

Prior to virus titration, nasal swab samples were thawed and vortexed for 15 s and passed through 0.45- μ m-pore-size filters to reduce bacterial contaminants (this step was omitted for BALF samples). The samples were 10-fold serially diluted with serum-free MEM supplemented with TPCK (tolylsulfonil phenylalanyl chloromethyl ketone) trypsin and antibiotics. Each dilution was plated in triplicate (100 μ l per well) onto PBS-washed confluent MDCK cells in 96-well tissue culture plates. At 48 h, the plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry with an anti-influenza virus A nucleoprotein monoclonal antibody as previously described (17). The virus concentration was calculated for each sample according to the method of Reed and Muench (30).

Pathological examination of lungs. At necropsy, lungs were removed and evaluated for the percentage of the lungs affected with well-demarcated, purple-red consolidation associated with SIV infection. The percentage of the surface affected by pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume.

Tissue samples from the distal tracheas and right cardiac lung lobes and/or affected lobes were fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. A single pathologist blinded to the treatment groups scored all tissues. Lung microscopic lesion scores were based on the following four parameters: (i) the percentage of bronchi and/or bronchioles demonstrating epithelial degeneration, necrosis or proliferation (1 = 0 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%); (ii) the percentage of bronchi and/or bronchioles demonstrating intrapulmonary and submucosal inflammation (1 = 0 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%); (iii) the degree of interstitial pneumonia (0 = none; 1 = mild, focal to multifocal; 2 = moderate, locally extensive to multifocal; 3 = moderate, multifocal to coalescing; and 4 = severe, coalescing to diffuse); and (iv) the magnitude of peribronchiolar lymphocytic cuffing (0 = none; 1 = mild, loosely formed lymphocytic cuffs; 2 = moderate, well-formed lymphocytic cuffs; and 3 = severe, thick, well-formed lymphocytic cuffs). A composite microscopic lung lesion score was computed for each individual pig using the sum of the four individual scores. The average group composite score was used for statistical analysis.

Pigs were screened for additional respiratory pathogens by testing BALF for PRRSV, porcine circovirus type 2 (PCV2), and *M. hyopneumoniae* nucleic acid by real-time RT-PCR with VetMax NA and EU

PRRSV, VetMax PCV2 (custom assay), and VetMax *M. hyopneumoniae* kits, respectively (Life Technologies, CA).

Serological analysis. Blood samples were collected only from contact pigs at 12 dpc and at necropsy (19 dpc) to look for seroconversion against challenge virus by using a hemagglutination inhibition (HI) assay. HI cross-reactive antibodies were evaluated between sera from pigs infected with A/IN, Sw/PA, and Sw/IL and reference swine sera against an H3 cluster I isolate, A/Swine/Texas/4199-1/1998 (Sw/TX/98), and a contemporary cluster IV isolate A/Swine/Illinois/02970/2009 (Sw/IL/09). High HI antibodies against Sw/PA and Sw/IL used for HI cross-reactive test were obtained from two contact pigs (per isolate) with HI antibody titers of $\geq 1:160$ at 19 dpc. Two pigs in the A/IN group were vaccinated with A/IN inactivated virus (160 hemagglutination units (HAU) per dose) plus commercial adjuvant (Emulsigen; MVP Laboratories, Inc., Ralston, NE) to boost the titer since the HI titers to homologous virus in the A/IN contact group were lower than for the other two virus groups. These two pigs were humanely euthanized ~ 3 weeks after vaccination for blood collection.

Prior to HI testing, sera were treated with receptor-destroying enzyme (Sigma-Aldrich, 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 with eight HAU of virus antigen and 0.5% turkey RBC according to standard techniques (48). Reciprocal HI titers were \log_2 transformed for analysis and are reported as geometric means. Seroconversion was considered positive when HI titers to homologous virus were $\geq 1:40$.

Statistical analysis. The percent macroscopic pneumonia, microscopic lesion scores, \log_{10} transformations of BALF, and nasal swab virus titers were analyzed using analysis of variance, with a *P* value of < 0.05 considered significant (GraphPad Prism 5.03; GraphPad Software, Inc., CA).

RESULTS

Sequence analysis of H3N2 viruses. Figure 1 and Fig. S1 to S7 in the supplemental material demonstrate the gene lineage identified by basic local alignment search tool (BLAST) (1) and phylogenetic analysis of each isolate included in the present study, including all rH3N2p reassortant viruses that are available in GenBank (last accessed, December 2011). The last eight isolates in Fig. 1 represent the gene constellation of contemporary swine H3N2 viruses with triple-reassortment internal gene (H3N2-TRIG) cassettes that have been endemic in the North American swine population since 1998.

Data from GenBank and whole-genome analyses from the viruses included here indicate that at least six different genotypes of swine H3N2 viruses can be found in the swine population as a result of H3N2-TRIG/pH1N1 (rH3N2p) reassortment. The majority of the novel rH3N2p viruses were isolated 1 year after the appearance of pH1N1 in 2009. The number of gene segments derived from pH1N1 differed from one to five segments. However, all 17 analyzed swine reassortant viruses representing six different rH3N2p genotypes contained the pM gene. The genotype that includes pandemic M, NP, and NS with the remaining gene segments of H3N2-TRIG was detected most frequently (9 isolates) from three states: Iowa (one isolate), Kansas (six isolates), and Texas (two isolates). It should be noted that the gene combinations of all of the A(H3N2)v isolates from the recent human cases reported since July 2011 were of the H3N2-pM virus genotype, with only the pM gene inherited from 2009 H1N1 viruses. Of 273 swine H3N2 matrix gene sequences available in GenBank (last accessed, December 2011), 125 sequences were North American swine isolates, and 17 of these isolates (14.4%) contained the pM gene. These swine H3N2-pM (rH3N2p) viruses were identified in

six different states including Iowa, Illinois, New York, Kansas, Texas, and Minnesota, whereas the A(H3N2)v-infected human cases were reported in Indiana, Pennsylvania, Maine, Iowa, and West Virginia (3, 4). The pM and TRIG-M genes differ by approximately 12 and 15% at the nucleotide and protein (i.e., M1 protein) levels, respectively. Analysis of the host restricted region on the extracellular domain of the matrix 2 protein (M2e) (21) indicates 100% homology between the pM genes detected in the swine and human isolates. However, four amino acid differences were detected in the M2e host-restricted region of swine H3N2-pM virus compared to swine H3N2-TRIG virus (see Table S2 in the supplemental material).

Phylogenetic analysis of hemagglutinin (HA) genes (Fig. 2) demonstrated that all viruses examined in this analysis were most closely related to the North American swine H3 cluster IV. The amino acid sequence of the HA1 region of the three isolates (A/IN, Sw/PA, and Sw/IL) used in the pathogenesis and transmission study in pigs were further analyzed (Fig. 3). The original viral inocula and virus recovered from two principal and two contact pigs of each experimental group were sequenced to investigate whether amino acid changes occurred after animal passage, with a particular focus on A/IN since this virus was isolated from a human. Differences were detected only in the amino acid sequence of the HA1 region of the Sw/PA virus between the original virus and viruses passed through the principal and contact pigs. Amino acid sequences at the major antigenic sites (A, B, C, and D) were also compared to a prototypic strain of H3 cluster I (A/Swine/Texas/4199-1/1998) commonly used as a reference virus and to a cluster IV isolate (A/Swine/Ontario/33853/2005) to assess changes in the antigenic sites (19, 35, 45). Pairwise amino acid identities of the HA1 protein between the three H3N2 isolates and cluster I and IV prototype viruses is 85.4 to 89.4% and 95.8 to 97.4%, respectively. Compared to the cluster I prototype virus, isolates in cluster IV had eight amino acid changes at the same positions in the four major antigenic sites (A to D). Within these eight amino acid changes, seven were similar among all isolates, while one was unique to the Sw/PA virus, which represents the contemporary swine H3N2-TRIG viruses. Sw/IL, the reassortant H3N2 virus that contained five genes from pH1N1 virus (rH3N2p), demonstrated three additional amino acid changes at the major antigenic sites, A (two) and D (one). Compared to the cluster IV prototype, four and five amino acid changes were observed at the major antigenic sites A, B, and C of Sw/PA and A/IN, respectively. Six amino acid changes were observed at all four major antigenic sites of Sw/IL. Two amino acid changes, H155Y and D158N, were found at the major antigenic site B in all three isolates examined here. Both A/IN and Sw/PA isolates displayed amino acids D190, V226, and S228 at the receptor binding sites (RBS), indicating a preference toward binding a receptor with a sialic acid (SA) attached to galactose via a SA α 2,6 linkage. Sw/IL displayed the same amino acids at positions 190 and 226 as A/IN but contained an S228G change. Deduced amino acid sequences of the HA1 region were analyzed for potential N-linked glycosylation sites using the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Five putative N-glycosylation sites were predicted for all viruses analyzed at positions 22, 38, 63, 126, and 285, whereas all isolates but A/Sw/Texas/4199-1/1998 had also a predicted site at position 246. Sw/PA virus isolated from principal pigs and Sw/IL from inoculum and both principal and contact pigs had one fewer predicted glycosylation site commonly found

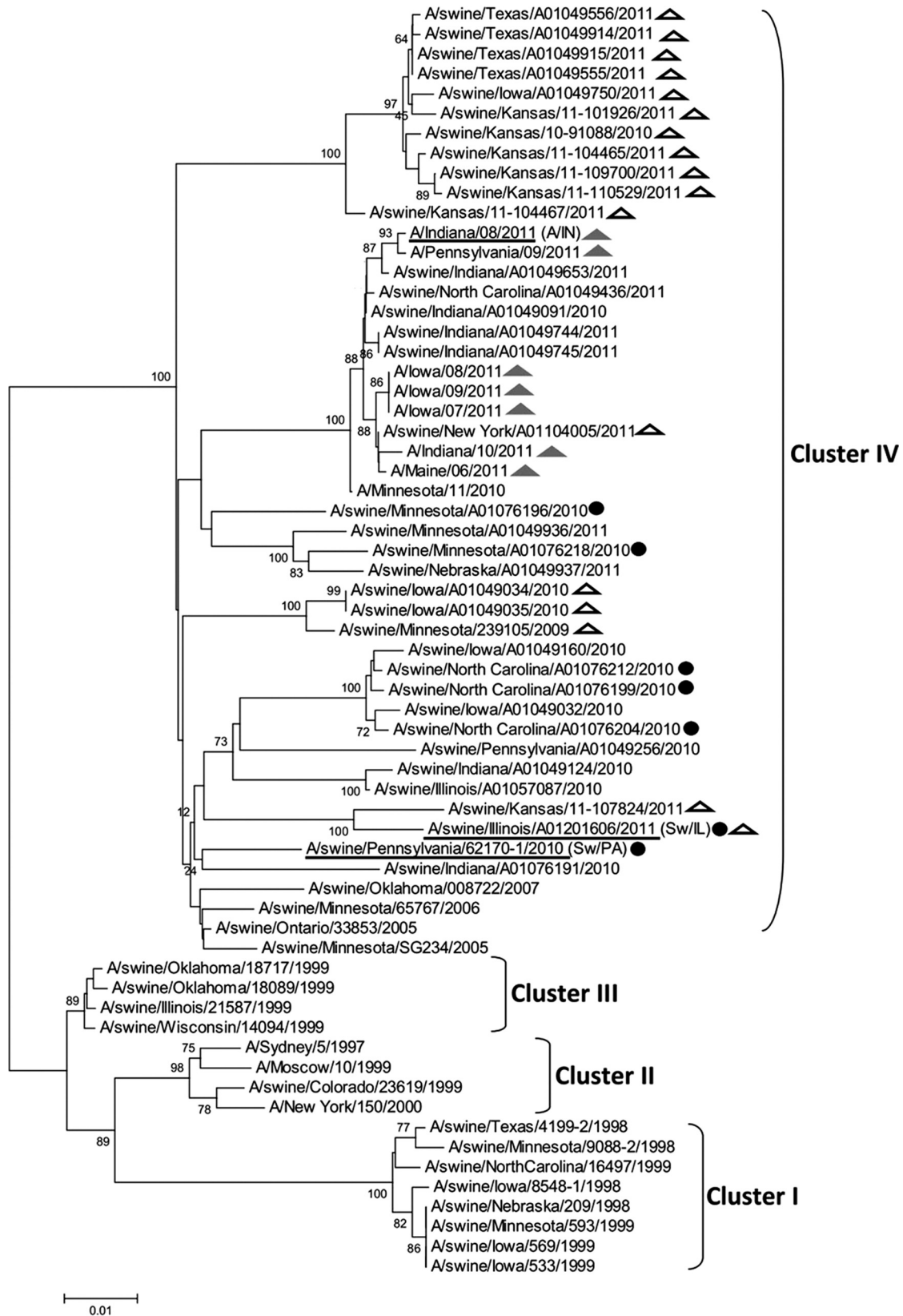


FIG 2 Phylogenetic tree of HA gene generated using the MEGA 4.0 program applying the neighbor-joining algorithm. Tree topology was supported by bootstrap analysis with 1,000 replicates. The isolates used for pathogenesis and transmission study are underlined. The black dots indicate isolates sequenced in the present study. The triangles indicate reassortant H3N2 viruses containing the pandemic matrix (pM) gene. Open or gray color triangles indicate swine (rH3N2p) or human [A(H3N2)v] isolates, respectively.

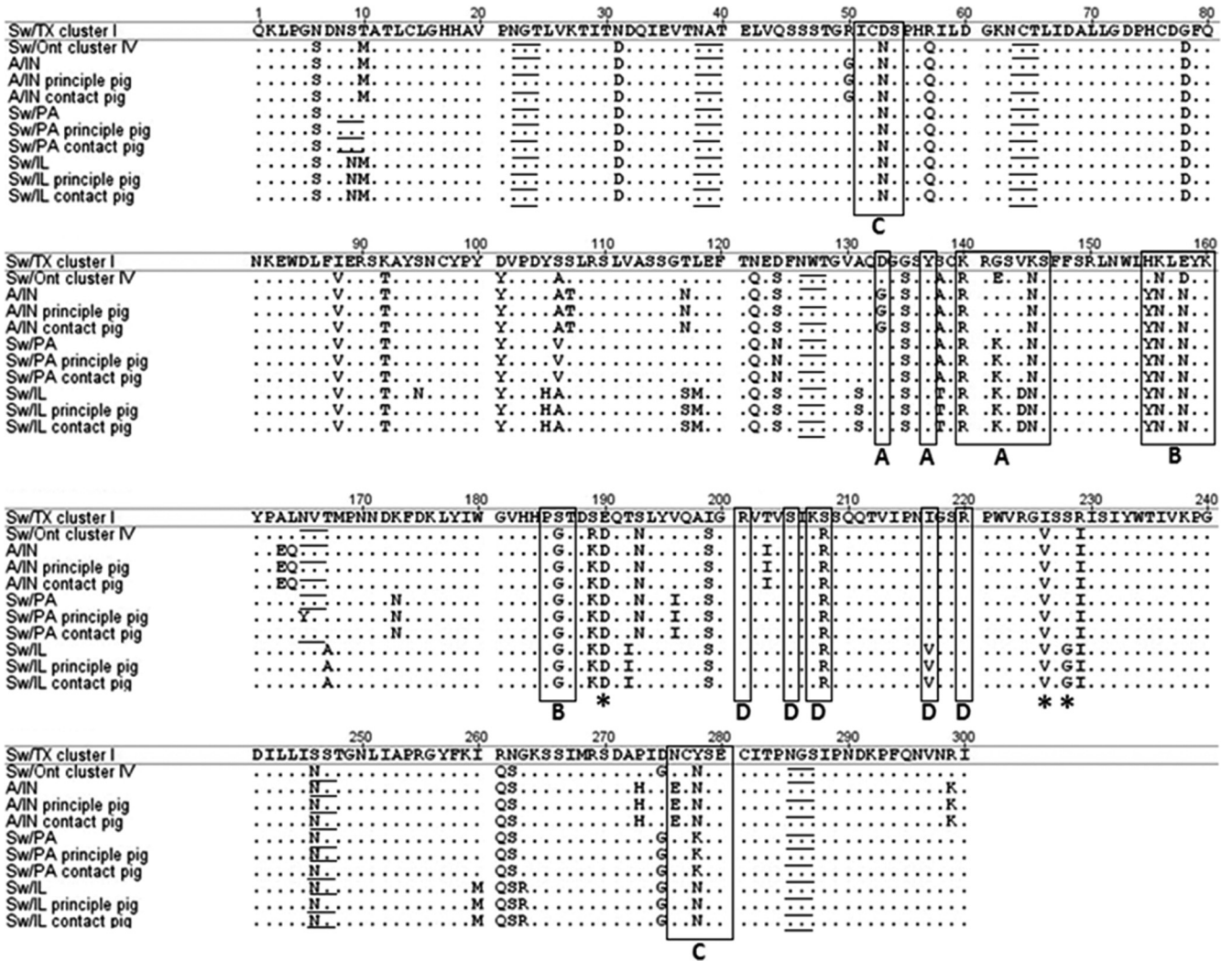


FIG 3 Alignment of amino acid sequences of the HA1 protein of H3N2 viruses from viral inocula used for experimental infection in pigs and from nasal shedding of two principal and contact pigs per group. Sequences were compared to H3N2, A/Sw/TX/4199-1/1998 (Sw/TX), an H3 cluster I prototype (42). Sw/Ont is A/Sw/Ontario/33853/2005, an H3 cluster IV prototype and representative of older seasonal swine H3N2-TRIG (25). A/IN is A/IN/08/2011, a swine A(H3N2)v virus isolated from a human case in July 2011. Sw/PA is A/Sw/PA/62170-1/2010, a representative of the contemporary swine H3N2-TRIG virus in the North American swine population. Sw/IL is A/Sw/IL/A01201606/2011, another swine reassortant H3N2 (rH3N2p) with pandemic M, NP, NS, PB1, and PA genes. Dots represent consensus sequences compared to reference strain (Sw/TX). Amino acids in the open boxes represent antigenic sites of H3 (47). *, Receptor binding sites (RBS) identified previously. Letters in open boxes represents substitutions. Predicted putative N-glycosylation sites are underlined.

in other isolates (position 165). The Sw/PA, however, had a predicted glycosylation site at amino acid position 8 which was not found in the other two isolates.

Pathogenesis of A(H3N2)v, rH3N2p, and H3N2-TRIG virus infections in pigs. In general, all infected pigs demonstrated mild clinical illness similar to that caused by contemporary SIV. Pigs infected with the human isolate A/IN had significantly lower macroscopic lesions than pigs infected with the H3N2-TRIG virus, Sw/PA (Table 1 and see Fig. S8 in the supplemental material). Pigs infected with Sw/IL displayed lung lesions intermediate in severity between the Sw/PA and A/IN groups.

Group mean composite microscopic lung lesion scores demonstrated a trend similar to that of the group mean percent macroscopic lung consolidation (Table 1). All influenza virus-inoculated groups demonstrated significantly higher ($P \leq 0.05$) mean composite lung lesion scores compared to the negative control

TABLE 1 Mean macroscopic lung consolidation, mean composite microscopic lung lesion scores, and mean BALF virus titers in control and A/IN-, Sw/PA-, and Sw/IL-inoculated groups at 5 dpi

Group	Mean \pm SEM ^a		
	Macroscopic pneumonia (%)	Microscopic lesion score (0–15)	Log ₁₀ virus titer (TCID ₅₀) in BALF ^b
Control (sham inoculated)	0.0 \pm 0.0 ^C	0.2 \pm 0.1 ^C	0.0 \pm 0.0 ^C (0/8)
A/IN [A(H3N2)v]	1.8 \pm 0.5 ^{B,C}	5.0 \pm 0.8 ^B	2.0 \pm 0.6 ^B (6/7)
Sw/PA (H3N2-TRIG)	7.6 \pm 1.9 ^A	7.7 \pm 0.9 ^A	3.0 \pm 0.5 ^A (6/7)
Sw/IL (rH3N2p)	5.9 \pm 1.2 ^{A,B}	5.6 \pm 0.4 ^{A,B}	0.2 \pm 0.2 ^C (1/8)

^a Values within a column not connected by the same superscript capital letter are significantly different ($P \leq 0.05$). BALF, bronchoalveolar lavage fluid.

^b The number of virus-positive pigs/the total number of pigs tested is indicated in parentheses.

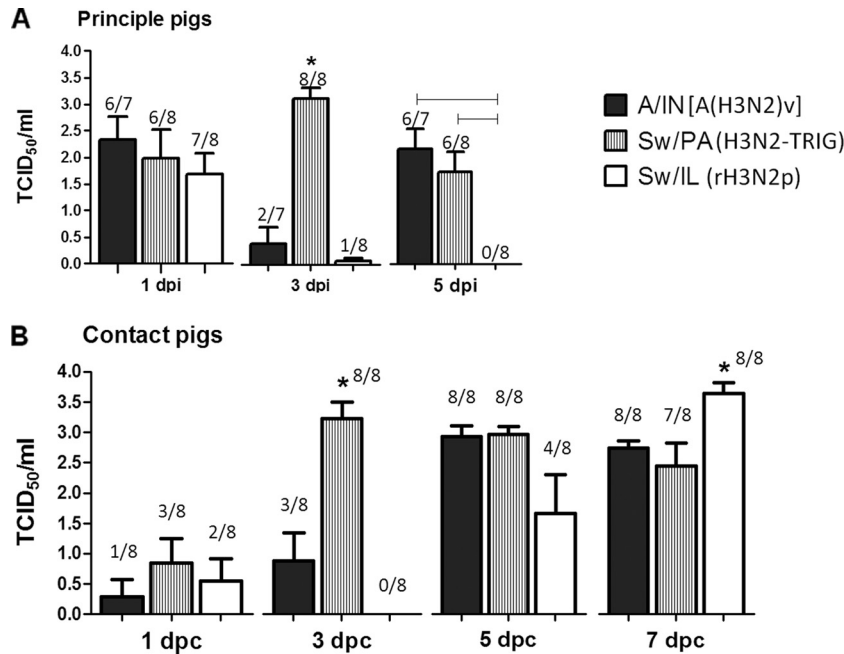


FIG 4 Mean TCID₅₀ log₁₀ virus titers ± the standard error of the mean from nasal swabs of principal pigs at 1, 3, and 5 dpi (A) and contact pigs at 1, 3, 5 and 7 dpc (B). The number of virus positive pigs is indicated at the top of each column. Significant differences ($P \leq 0.05$) between groups were compared within the same collection dates.

pigs. The group mean Sw/PA (H3N2-TRIG) microscopic lung lesion scores were significantly higher ($P \leq 0.05$) than the A/IN A(H3N2)v lesion scores. However, significant differences were not observed between the Sw/IL (rH3N2p)-inoculated group and the Sw/PA- or A/IN-inoculated groups.

The amount of virus detected in the BALF of Sw/PA-infected pigs at the time of necropsy was significantly higher compared to all other groups (Table 1). Six of seven pigs in the A/IN group were virus positive at necropsy, with a significantly higher BALF titer compared to the Sw/IL-infected pigs. In contrast, only one of eight pigs in the Sw/IL group was virus positive in BALF at 5 dpi. Back-titrations of each inoculum were $10^{6.5}$, $10^{6.3}$, and $10^{6.3}$ for A/IN, Sw/PA, and Sw/IL, respectively.

Virus shedding and transmission of A(H3N2)v, rH3N2p, and H3N2-TRIG virus infection in pigs. All three H3N2 virus isolates replicated in pigs with different efficiencies (Fig. 4A). The Sw/PA replicated more efficiently than the other isolates, with the highest titer detected in nasal swabs at 3 dpi. A/IN-infected pigs shed virus inconsistently and demonstrated a titer equivalent to that for the Sw/PA group at 5 dpi. Pigs infected with Sw/IL appeared to shed the lowest amount of virus with the shortest duration.

All three isolates were transmitted from principal to contact pigs. The transmission patterns were similar between the isolates, but a delay in the kinetics was observed between the contact groups (Fig. 4B). Sw/PA (H3N2-TRIG) transmitted most efficiently at 3 dpc, as contact pigs shed virus with a significantly higher titer compared to other groups. Transmission of A/IN [A(H3N2)v] and Sw/IL (rH3N2p) to contact pigs was slightly delayed compared to the Sw/PA group. However, at 7 dpc most pigs in all of the contact groups were still shedding virus.

Seroconversion in contact pigs and HI cross-reactivity test between H3N2 viruses. Prior to contact with principal infected

pigs, all contact pigs were influenza A virus seronegative by enzyme-linked immunosorbent assay and had no HI titers to homologous challenge virus. By 12 dpc, all pigs had seroconverted to homologous virus, indicating exposure to the challenge virus. Antigenic characterization was evaluated by HI cross-reactivity between sera and virus antigens used for experimental infection and previously circulating isolates (Table 2). All homologous antisera and antigen combinations displayed high mean reciprocal HI titers (≥ 320). Sera from pigs immunized with Sw/TX/98, a prototypic H3N2 reference isolate grouped in phylogenetic cluster I of swine H3 HA, tended to have low to moderate cross-reactivity

TABLE 2 Hemagglutinin inhibition cross-reactivity between swine sera and H3N2 virus antigen combinations

Antiserum	HI cross-reactivity for virus antigen ^a :				
	Sw/TX/98 ^b (C1, 1998)	Sw/IL/09 (C4, 2009)	Sw/PA ^c (C4, 2010)	Sw/IL ^d (C4, 2011)	A/IN ^e (C4, 2011)
Sw/TX/98	2,560	160	160	40	80
Sw/IL/09	30	320	120	100	15
Sw/PA	0	120	480	960	320
Sw/IL	0	20	240	960	60
A/IN	5	320	1280	960	1,280

^a The isolates used for swine antiserum production are identified in the far left column, with the geometric mean reciprocal titer of two serum samples per isolate given in each row. Virus isolates used as test antigens are identified in the top row. The hemagglutinin gene cluster (C) and year the virus was isolated are indicated in parentheses in the subheadings. Homologous serum/antigen combinations are shaded gray. HI, hemagglutinin inhibition.

^b Result from one reference serum sample.

^c Swine H3N2 virus with triple-reassortant internal gene (TRIG) cassette, H3N2-TRIG.

^d Reassortant H3N2 virus with TRIG-HA, -NA, and -PB2 and pandemic M, NP, NS, PA, and PB1 gene combinations (rH3N2p).

^e A(H3N2)v virus with pandemic matrix (pM) isolated from a human case.

with the contemporary H3N2 antigens. Likewise, sera from pigs infected with the contemporary H3N2 isolates had minimal to no cross-reactivity to the historical Sw/TX/98 virus but tended to have higher cross-reactivity among the modern isolates. The A/IN virus demonstrated significant loss in HI cross-reactivity with the majority of the swine H3N2 antisera tested.

DISCUSSION

Multiple-reassortment events have taken place in swine since the H3N2 virus with TRIG emerged in the North American swine population in 1998 (24, 39, 41). These H3N2-TRIG viruses consist of HA, NA, and PB1 genes of human virus lineage, the M, NP, and NS genes of swine virus lineage, and the PA and PB2 genes of avian virus lineage. The H3N2-TRIG virus spread widely in swine and reassorted with the classical swine H1N1 (cH1N1) virus, generating a new subtype, H1N2, and reassortant H1N1 (rH1N1) viruses, both containing the six internal genes of the TRIG cassette. Subsequently, two reverse zoonotic events in 2003 and 2005 introduced H1N2 and H1N1 viruses with human virus lineage HA and NA genes into the SIV gene pool (16, 38). These H1 viruses reassorted with endemic SIV circulating in swine at that time, but retained the TRIG cassette (see review in reference 39). The emergence of the pandemic H1N1 (pH1N1) virus in 2009 in the human population, followed by further reverse zoonotic events, introduced yet another H1N1 virus with a new gene constellation into the North American swine population (44). The pH1N1 virus had five gene segments (PB2, PB1, PA, NP, and NS) genetically related to the TRIG cassette, as well as an HA genetically related to the γ -H1 cluster found in the North American swine lineage viruses, while the M and NA genes were from Eurasian lineage (avian-like) swine influenza viruses (10). Reports of pH1N1 reassortment with endemic H1 SIV resulting in different SIV genotypes detected in swine have been documented around the world (9, 13, 18, 22, 29). In contrast to the increasing genetic diversity and evolution detected in the H1 subtype in recent years, swine H3N2 viruses have not evolved further into additional distinct genotypes beyond the HA cluster IV. Since the introduction of the H3N2-TRIG in 1998 until 2009, all descendant H3N2 viruses have demonstrated the human-like surface HA and NA genes with a similar TRIG cassette, suggesting that this combination of gene segments favored optimal adaptation to and transmission between swine hosts. A previous publication showed that nine H3N2 viruses obtained from Minnesota in 2006-2007 maintained the same human (HA, NA, and PB1), avian (PB2 and PA), and swine (NP, M, and NS) virus genes that were demonstrated in earlier strains (19), confirming limited reassortment events had taken place at the time. However, the appearance of pH1N1 in swine led to reassortment between pH1N1 and endemic H3N2-TRIG viruses in pigs.

A recent study identified nine reassortant pH1N1/endemic swine H1 and H3 influenza viruses collected during an active surveillance project conducted in 2009-2010 (9). Seven genotypes were detected among the three subtypes: H1N1 (one isolate), H3N2 (one isolate), and H1N2 (five isolates). All genotypes consistently contained the pM gene. The single reassortant H3N2 virus was collected in 2009 from pigs in Minnesota and contained pandemic M, NP, and PA genes. Sequence data from the USDA-NAHLN Swine Influenza Surveillance System in the present study, including recently published data (20) (Fig. 1), revealed five different genotypes of reassortant pH1N1/swine H3N2 viruses that differ from the 2009 isolate reported by Ducatez et al. (9).

Reassortant H3N2 viruses collected from all studies mentioned here and publicly available data in GenBank were found from six different states. The number of gene segments in the reassortant H3N2 viruses that were derived from pH1N1 differed from one to five segments. Phylogenetic analysis of all gene segments indicated that reassortment possibly occurred as multiple events since no individual gene cluster can be rooted to a single virus. Similar to what was reported previously, our analysis of the published sequences and viruses submitted through the USDA-NAHLN Swine Influenza Surveillance System did not find a single genotype that was predominant in the swine population. However, the presence of the pM gene was consistent among all of the H3N2 reassortants detected in this and previous studies (9, 20). These data suggest that pM from the pH1N1/Eurasian swine lineage may be necessary for the genetic fitness of subsequent rH3N2p reassortants in the swine host.

Swine H3N2-TRIG and H1 subtypes have thus far caused only sporadic human cases despite their wide circulation in the swine population for over a decade. In the United States, 41 swine-origin influenza cases in humans have been reported since 1990 to the present with limited human-to-human transmission (3, 4, 10, 32, 34). However, the pandemic H1N1 2009 virus, which had the ability to infect humans through aerosol transmission, spread rapidly in the human population around the world and was able to spill back into pigs and spread widely among naive pig populations. Although genetic evolution was apparent in all gene segments, the major genetic differences between pH1N1 and swine γ cluster H1N1-TRIG viruses are the NA and M genes. Since July 2011, there have been reports of 12 cases of human infection with A(H3N2)v, which are viruses with seven genes from the swine-lineage H3N2-TRIG and the pM gene. These 12 cases were identified in five states: Indiana ($n = 2$), Iowa ($n = 3$), Maine ($n = 2$), Pennsylvania ($n = 3$), and West Virginia ($n = 2$) (3, 4, 5). All of these cases demonstrated influenza-like clinical signs, and three of the infected people required hospitalization. Eleven of the twelve cases were children. Three cases in Iowa, as well as two cases in West Virginia, had no documented history of direct or indirect swine exposure, suggesting limited human-to-human transmission of A(H3N2)v viruses (3, 5). The number of individuals infected with A(H3N2)v virus observed in 2011 was a concern and thus prompted the experimental pathogenesis and transmission pig study reported here to determine the efficiency of this virus in spreading in the swine population.

We obtained an A(H3N2)v (A/IN) virus isolated from the first human case in July 2011 and compared its pathogenesis and transmission efficiency to a swine H3N2-TRIG (Sw/PA), as well as to a reassortant swine H3N2 virus with pandemic M, NS, NP, PA, and PB1 genes (Sw/IL) in the natural swine host. It was unknown whether the presence of genes from the 2009 pandemic H1N1 viruses in these reassortant H3N2p viruses (rH3N2p) would lead to a different phenotypic behavior compared to H3N2-TRIG-infected pigs. Our results demonstrated the ability of all three tested H3N2 viruses to infect, cause pneumonia and histopathologic lung lesions, and transmit to contact pigs. Clinical signs in all groups were typical of SIV-associated disease reported previously by our and other groups with no increase in clinical illness or pathogenesis detected in any A(H3N2)v or rH3N2p virus-infected pigs. In fact, the degree of macroscopic and microscopic lung lesions and the virus titers in nasal swabs and BALF were lower in both of the reassortant virus-infected groups than in

the H3N2-TRIG-infected group. It appeared that the novel A(H3N2)v virus isolated from a human was the least pathogenic, and the swine rH3N2p virus (with pandemic M, NS, NP, PA, and PB1 genes) replicated to the lowest titers of the three viruses. Overall, all three viruses maintained the ability to transmit from the inoculated to contact pigs (indicated by seroconversion). The kinetics of virus shedding in the A(H3N2)v contact pigs were delayed compared to the H3N2-TRIG group; however, it was intermediate to the kinetics of the rH3N2p virus. Considering that the surface glycoproteins, HA and NA, are relatively similar, the differences in shedding may be due to different gene constellations found in the three viruses. Future studies utilizing reverse genetics to swap individual genes of the reassortant viruses are needed to investigate the role of the pM or other genes in pathogenesis and transmission *in vivo*.

The Ducatez et al. (9) study that identified the nine reassortant pH1N1/endemic swine influenza viruses mentioned above also assessed virus replication and pathogenicity of the reassortant H3N2-pM, -NP, and -PA in comparison to pH1N1 and endemic H3N2-TRIG viruses using a ferret model. The H3N2 reassortant virus was shown to cause only mild clinical signs in ferrets, suggesting no enhancement of virulence properties had occurred through reassortment. The study did not evaluate transmission efficiency and thus cannot interpret whether pM and/or these particular gene combinations altered the transmission phenotype in ferrets. A recent study in guinea pigs used reverse genetics to separately swap the M or NA genes of pH1N1 virus into PR8, a representative of historic human H1N1 (6). Wild-type PR8 replicated and transmitted poorly in the guinea pig model, but the presence of the pM gene in the virus resulted in an increased transmission rate of 62.5% compared to the wild-type virus. Although the exact mechanism contributing to the increased aerosol transmission in guinea pigs was not determined, the study indicated that the pM gene of Eurasian swine virus lineage contributed a selective advantage for viral transmission in the guinea pig model. This finding correlates with the epidemiology of the emerging reassortant H3N2 viruses where the different genotypes all contain the pM gene. However, although the pM may be required for successful reassortment and/or fitness of new gene combinations, acquisition of pH1N1 genes does not appear to confer an advantage over previously swine-adapted H3N2 in the swine host.

The surface hemagglutinin (HA) glycoprotein of influenza viruses are known to play a major role in influenza virus cross-species transmission since it contains the viral receptor binding sites (RBS). Compatibility between RBS on the HA protein to the corresponding receptor expressed on the cells of the host species is required for successful transmission (14). Human and swine influenza viruses preferentially bind to sialic acid (SA) attached to galactose in an SA α -2,6 linkage on the host epithelial cells. The majority of the upper (nasal passage and trachea) and lower (bronchi and alveoli) respiratory tract of humans and pigs have SA α -2,6 receptors. The receptors preferred by avian influenza viruses, SA α -2,3 can be found on the nonciliated cuboidal bronchiolar cells in the lower respiratory tracts of humans and pigs but are also found in swine trachea (15, 33, 37). As a result, pigs (and humans) can potentially be infected by both mammalian-adapted and avian influenza viruses for generating new reassortant influenza viruses.

Amino acids at positions 190, 226, and 228 of the RBS in the HA1 protein were shown to correlate with receptor specificity. In

human H3 viruses, amino acid positions D190, L1226, and S228 correlated with SA α -2,6 receptors, while in swine H3 viruses those positions are D/A190, V226, and S228 (27, 40, 46). All three isolates—A/IN A(H3N2)v, Sw/PA H3N2-TRIG, and Sw/IL rH3N2p—were able to infect and transmit to contact pigs. The RBS of both A/IN and Sw/PA viruses displayed the sequence of the reported SA α -2,6 receptor preference, while Sw/IL contained an amino acid S228G change. G228 is more typically found in the HA1 protein of avian influenza viruses with a preference for SA α -2,3 receptor (40). This mutation that suggests the a SA α -2,3 receptor preference possibly contributed to the low virus titer in the lungs and a short virus shedding period detected in the principal Sw/IL-infected pigs, as well as the slight delay in the kinetics of virus transmission in the contact pigs. A number of amino acid mutations were also detected in the major antigenic sites A, B, C, and D of the three viruses compared to both the cluster I and IV prototype viruses. The total number of mutations was the lowest in Sw/PA (H3N2-TRIG) and highest in Sw/IL (rH3N2p). These mutations appeared to have affected the antibody binding property, as evidenced by the HI cross-reactivity test where antisera against the historic H3N2 cluster I virus (Sw/TX/98) cross-reacted to Sw/IL 2-fold lower than to Sw/PA. The lack of HI cross-reactivity indicates antigenic drift of the tested viruses from the cluster I virus. HI cross-reactive titers were higher within the cluster IV viruses. Antibodies primed against the swine H3N2-TRIG (Sw/PA) cross-reacted against both A(H3N2)v (A/IN) and rH3N2p (Sw/IL) and an older H3N2 cluster IV. This suggests that the North American swine population should have some level of immunity against the new reassortant pH1N1/H3N2-TRIG viruses if they had been previously exposed or vaccinated with a contemporary cluster IV H3N2 virus. However, the A/IN virus A(H3N2)v had a loss in cross-reactivity against all contemporary and reference virus-antisera, suggesting a potential antigenic drift in this newly emerging phylogenetic node of H3 from the swine-lineage HA. In addition, it was demonstrated that ferret sera primed against H3N2-TRIG viruses isolated from human cases do not cross-react with the seasonal human H3N2 virus incorporated in human influenza vaccines and vice versa (34). This demonstrates that although the H3N2 viruses currently circulating in swine were originally transmitted from humans in the mid-1990s, they are now antigenically distinct from contemporary human H3N2 viruses. Some subsets of the human population may be vulnerable to both TRIG- and reassortant-H3N2 virus infections. It can be speculated that A(H3N2)v viruses in humans may also diverge separately and distinctly from H3N2-TRIG and rH3N2p viruses found in swine if successfully established in the human population, and thus continued monitoring of H3 viruses in both populations is necessary.

In summary, our data demonstrate that at least six different swine rH3N2p genotypes currently circulate in the North American swine population as the result of genetic reassortment between pH1N1 and contemporary swine H3N2-TRIG viruses. However, all genotypes contained some internal gene segments, as well as the surface glycoprotein genes, HA and NA, that are already found in the swine population, and thus far no specific genotype appears to predominate in the swine population based on diagnostic case submissions and surveillance efforts. The results from our pathogenesis and transmission study with two different H3N2 viruses, rH3N2p and H3N2-TRIG isolated from pigs and A(H3N2)v isolated from a human, detected no major biological

differences compared to that observed with the swine H3N2-TRIG virus. Antibodies specific to the H3N2-TRIG virus cross-reacted with both reassortant H3N2 viruses. Genetic analysis of all of the reassortant H3N2-pM viruses with different internal gene combinations were grouped in the same HA gene cluster as the contemporary swine H3N2-TRIG, although the most recent isolates appear to be forming a separate cluster with the human isolates of A(H3N2)v. Despite the fact that some degree of antigenic cross-reactivity is conserved at this point, continued monitoring of this group of H3 viruses is necessary to evaluate population immunity in both swine and humans. Overall, these findings further substantiate the continued genetic instability and evolution of influenza A viruses, that influenza A viruses are readily shared between humans and animals, and the role of pigs in generating reassortant influenza viruses. Thus, it is essential to continue to monitor influenza viruses through whole-genome analysis, antigenic cross-reactivity, and *in vivo* studies to examine the phenotypic nature of viruses with novel and emerging genetic and/or antigenic combinations.

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