Novel reassortant human-like H3N2 and H3N1 influenza A viruses detected in pigs are virulent and antigenically distinct from endemic viruses

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Running title: Novel Human-Like H3 Influenza A Viruses in Pigs

Abstract word count: 248; Text word count: 5277

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Abstract

Human-like swine H3 influenza A viruses (IAV) were detected by the USDA surveillance system. We characterized two novel swine human-like H3N2 and H3N1 viruses with HA genes similar to human seasonal H3 strains and the internal genes closely related to 2009 H1N1 pandemic viruses. The H3N2 NA was of the contemporary human N2 lineage, while the H3N1 NA was of the classical swine N1 lineage. Both viruses were antigenically distant from swine H3 viruses that circulate in the U.S. and from swine vaccine strains, and also showed antigenic drift from human seasonal H3N2. Their pathogenicity and transmission in pigs were compared to a human H3N2 with common HA ancestry. Both swine human-like H3 viruses efficiently infected pigs and transmitted to indirect contacts, whereas the human H3N2 was much less efficient. To evaluate the role of genes from the swine isolates on their pathogenesis, reverse genetics-generated reassortants between the swine human-like H3N1 and the seasonal human H3N2 were tested in pigs. Gene segment contribution to virulence was complex with the swine HA and internal genes showing effect *in vivo*. The experimental infections indicate that these novel H3 viruses are virulent and can sustain onward transmission in pigs, and the naturally occurring mutations in the HA were associated with antigenic divergence from H3 IAV from human and swine. Consequently, these viruses could have a significant impact on the swine industry if they cause more widespread outbreaks, and the potential risk of these emerging swine IAV to humans should be considered.

Importance

Pigs are important hosts in the evolution of influenza A viruses (IAV). Human-to-swine transmissions of IAV have resulted in the circulation of reassortant viruses containing human-origin genes in pigs, greatly contributing to the diversity of IAV in swine worldwide. New human-like H3N2 and H3N1 viruses that contain a mix of human and swine gene segments were recently detected by the USDA surveillance system. The human-like viruses efficiently infected pigs and resulted in onward airborne transmission, likely due to multiple changes identified between human and swine H3 viruses. The human-like swine viruses are distinct from contemporary U.S. H3 swine viruses and from the strains used in swine vaccines, which could have a significant impact on the swine industry due to lack of population immunity. Additionally, public health experts should consider appropriate risk assessment for these emerging swine H3N1 for the human population.

Introduction

Swine have a key role in the ecology of influenza A viruses (IAV), and thus represent a risk for future introductions of swine viruses into the human population. Similar to subtypes that circulate in humans, endemic swine IAV are of the H1N1, H3N2, and H1N2 subtypes (1), whereas other subtypes are only sporadically detected in swine as a result of interspecies transmission, such as avian-like H3N1 (2) and H2N3 (3), or equine-like H3N8 (4). The porcine respiratory tract contains both human IAV-preferred sialic acid α2,6-galactose (SAα2,6-Gal) and avian IAV-preferred sialic acid SAα2,3-Gal linked receptors (5), providing an underlying biologic basis for swine as intermediary hosts in the evolution of influenza viruses. Unlike the relatively uncommon event of a swine lineage virus becoming established in the human population, human seasonal virus transmission events to swine have repeatedly led to new genetic lineages of novel viruses that became endemic in various pig populations around the globe (6). Human-origin surface genes have been maintained at a much higher frequency than the internal genes of the seeding virus once it enters a pig population (6), which suggests that barriers exists for the sustained circulation and efficient adaptation of wholly human viruses in swine. A notable human-to-swine event occurred in the late 1990's when a triple reassortant internal gene (TRIG) constellation became established among North American swine (7, 8), containing swine (M, NP, and NS), avian (PB2 and PA), and human (PB1) influenza virus genes. This constellation of internal genes reassorted with different combinations of surface genes, and as a consequence, the dynamics of influenza infection in North American pigs changed drastically. Additionally, more than 49 independent human-to-swine spillover events of the 2009 pandemic H1N1 (H1N1pdm09) have occurred

81 globally since it was introduced into the human population (9). These incursions led to
82 multiple reassortment events between the H1N1pdm09 and endemic swine IAVs (1, 10),
83 creating unique swine IAV configurations and increasing the observed genetic diversity.
84 The H1N1pdm09 highlights the pandemic risk of novel viruses generated through the
85 exchange between human and swine lineages (11). Furthermore, antigenic drift in viral
86 surface glycoproteins contributes to the evolution of swine IAV (12), resulting in the co-
87 circulation of many antigenically distinct viruses in pigs (1, 13).
88 Novel H3N2 and H3N1 viruses with contemporary human seasonal H3 genes were
89 identified through the United States Department of Agriculture (USDA) IAV swine
90 surveillance system. Even though H3N1 viruses have been detected in U.S. swine
91 previously, they are rare (2, 14). The novel H3N1 viruses reported in this manuscript
92 have a unique combination of surface genes from contemporary human seasonal H3N2
93 HA and classical swine H1N1 (cH1N1) NA with internal genes derived from
94 H1N1pdm09, and hence are distinct from current swine H3 viruses circulating in the U.S.
95 as well as human seasonal H3 circulating globally. To assess the impact of these novel
96 H3 viruses, *in vitro* genetic and antigenic characterization along with *in vivo* phenotypic
97 characterization was conducted. We demonstrated that these novel human-like IAV are
98 virulent in swine and pose a significant threat to the swine population due to an expected
99 lack of population immunity. To further understand the role of gene segments on the
100 striking pathogenesis and transmissibility of these viruses compared to a human seasonal
101 H3N2, we constructed reassortants between the swine human-like H3N1 and the human
102 H3N2 by reverse genetics and compared the pathogenesis *in vivo*. Our results suggest that

103 the HA and internal gene constellation were essential for efficient infection and
104 transmission of the novel human-like H3N1 viruses.

105 **Materials and Methods**

106 **Ethics statement.** All animals were housed in biosafety level 2 (BSL2)-containment and
107 cared for in compliance with the Animal Care and Use Committee of the National
108 Animal Disease Center.

109 **Viruses and cell lines.** The swine isolates A/Swine/Missouri/A01476459/2012 (H3N2;
110 Sw/MO/12) and A/Swine/Missouri/A01410819/2014 (H3N1; Sw/MO/14) were obtained
111 from the IAV swine surveillance system repository held at the USDA National
112 Veterinary Service Laboratories in conjunction with the USDA-National Animal Health
113 Laboratory Network (NAHLN). The H3N2 virus was isolated from a breeding herd
114 during the winter of 2012 and the H3N1 was isolated during the winter of 2013 from an
115 epidemiologically linked location. The human H3N2 isolate A/Victoria/361/2011
116 (A/VIC/11, kindly provided by Dr. Richard Webby, St. Jude Children's Research
117 Hospital) was genetically similar to the HA of both swine isolates and the NA of
118 Sw/MO/12 and was included as a control. Viruses were propagated in Madin-Darby
119 canine kidney (MDCK) cells.

120 **Reverse engineered viruses.** The two wild type viruses with phenotypes at the opposite
121 ends of the spectrum were chosen to generate reassortants to test the contribution of
122 genes or combination of genes. Eight viruses were generated by reverse genetics (rg)
123 using an 8-plasmid system as previously described (15) in the bidirectional plasmid
124 vector pDP2002, and their genetic constellations are described in Table 1. Gene

125 combinations were verified by full-length sequencing and viruses were propagated in
126 MDCK cells.
127 **Genetic analysis.** Three genes (HA, NA, and M) of the swine isolates were initially
128 sequenced and submitted to GenBank by the submitting NAHLN (National Animal
129 Health Laboratory Network) veterinary diagnostic lab. Following the identification of the
130 human-origin HA gene, 9 swine isolates were subjected to whole genome next-generation
131 sequencing using the Ion 316 v2 chip and Ion PGM 200 v2 Sequencing Kit (Life
132 Technologies, Carlsbad, CA) as previously described (16). The HA genes from viruses
133 recovered from primary and indirect contact pigs in the *in vivo* studies were sequenced
134 directly from clinical material by conventional sequencing using BigDye® Terminator
135 v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as per manufacturer's
136 instructions using previously described primers (17).
137 Additional representative sequences of North American swine and human viruses were
138 downloaded from GenBank and GISAID. Specifically, using BLASTn (18) we identified
139 15 human isolates from the 2010-11 influenza season with high HA gene sequence
140 identity, and also included randomly selected human isolates from each influenza season
141 from 2008 to 2013. More recent swine human-like H3N1 and H3N2 that were
142 subsequently identified by the USDA surveillance system were also included in the
143 analysis (Table S1 and S2). Sequences were aligned for each of the eight genomic
144 segments using default settings in MUSCLE v.3.8.31 (19), with subsequent manual
145 correction. For each alignment, we inferred the best-known maximum likelihood (ML)
146 tree using RAxML v7.4.2 (20) using the rapid bootstrap algorithm and a general time-
147 reversible (GTR) model of nucleotide substitution with Γ-distributed rate variation

among sites. Statistical support for individual branches was estimated by bootstrap analysis, with the number of bootstrap replicates determined automatically using an extended majority-rule consensus tree criterion (21). The deduced HA1 domain amino acid sequences were aligned and used to identify amino acid differences between the human and the swine viruses.

Animal experiment 1. Fifty 3-week-old crossbred healthy pigs were obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). Prior to the start of the study pigs were treated with ceftiofur crystalline free acid and tulathromycin (Zoetis Animal Health, Florham Park, NJ) to reduce bacterial contaminants and were shown to be seronegative to IAV antibodies. Pigs were divided into four groups: non-challenged (NC; n=5), challenged with A/VIC/11 H3N2 (n=10), with Sw/MO/12 H3N2 (n=10) and with Sw/MO/14 H3N1 (n=10).

Challenged pigs were simultaneously inoculated intranasally (1 ml) and intratracheally (2 ml) with 10^5 50% tissue culture infective dose ($TCID_{50}$) per ml of each assigned virus. 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 Telazol (6 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA). Five contact pigs were placed in separated raised decks in the same room as each inoculated group at 2 days post infection (dpi) to evaluate indirect contact transmission. Nasal swabs (FLOQSwabs™, Copan Diagnostics, Murrieta, CA) were collected at 0, 1, 3, and 5 dpi for primary pigs and from 0 to 5, 7, and 9 days post contact (dpc) for indirect contacts as previously described (22). Two pigs died from causes unrelated to IAV infection, leaving 8 pigs in the A/VIC/11 group. Primary pigs were humanely euthanized with a lethal dose of pentobarbital (Fatal

Plus, Vortech Pharmaceuticals, Dearborn, MI) and necropsied at 5 dpi, when bronchoalveolar lavage fluid (BALF) and tissue samples from the distal trachea and right cardiac or affected lung lobe were collected. Indirect contact pigs were humanely euthanized at 15 dpc for collection of serum to evaluate sero-conversion.

Animal experiment 2. To test the role of the surface genes and internal gene backbones observed *in vivo* with the wild-type Sw/MO/14 H3N1, the reassortant viruses generated above were used in a second pathogenesis study. Eighty-five 3-week-old crossbred healthy pigs obtained from the same source as the previous experiment were used. Groups of 10 pigs were infected with each of the reverse genetics-generated viruses using the same methodology as described above, and five indirect contact pigs were introduced at 2 dpi as described above. Nasal swab samples were collected for primary and indirect contact pigs and necropsies were performed following the same procedures in Experiment 1.

Virus titers in nasal swabs and lungs. Filtered nasal swab (NS) samples were plated for virus isolation onto confluent MDCK, as previously described (22). Ten-fold serial dilutions in serum-free Opti-MEM (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics were prepared for each BALF and virus isolation-positive NS. Each dilution was plated in triplicate onto phosphate-buffered saline (PBS)-washed confluent MDCK cells in 96-well plates. At 48 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry as previously described (23). $TCID_{50}$/ml virus titers were calculated for each sample according to the method of Reed and Muench (24).

194 **Pathological examination of lungs.** At necropsy, lungs were removed and evaluated for
195 the percentage of the lung affected with purple-red consolidation typical of IAV
196 infection. The percentage of the surface affected by pneumonia for the entire lung was
197 calculated based on weighted proportions of each lobe to the total lung volume (25).
198 Tissue samples from trachea and lung were fixed in 10% buffered formalin and were
199 routinely processed and stained with hematoxylin and eosin. Microscopic lesions were
200 evaluated by a veterinary pathologist blinded to treatment groups and scored according to
201 previously described parameters (26). IAV-specific antigen was detected in trachea and
202 lung tissues using immunohistochemistry (IHC) and scored as previously described (26).
203 Individual scores were summed the average group composite scores were used for
204 statistical analysis.
205 **Serology and antigenic cartography.** Two 7-week-old seronegative naïve pigs were
206 used for Sw/MO/14 H3N1 antisera production. Pigs were immunized intramuscularly
207 with 2 doses 2 weeks apart of Sw/MO/14 antigen inactivated by ultraviolet (UV)
208 irradiation. The antigen was used at 128 HA units per 50 µl in PBS with a commercial
209 oil-in-water adjuvant (Emulsigen D, MVP Laboratories, Inc., Ralston, NE) at a 1:5 ratio.
210 Pigs were humanely euthanized as described above for blood collection. Prior to HI, sera
211 were treated with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO), heat
212 inactivated at 56°C for 30 min, and adsorbed with 50% turkey red blood cells (RBC) to
213 remove nonspecific hemagglutinin inhibitors and natural serum agglutinins. HI assays
214 from the experimentally challenged and contact pigs were performed with either
215 A/VIC/11, Sw/MO/12 or Sw/MO/14 as antigens and 0.5% turkey RBCs using standard

techniques (27). Reciprocal titers were divided by 10, $\log_2$ transformed and reported as the geometric mean.

Two-way HI assays were performed as described above, using a panel of reference swine and human H3N2 viruses as HI antigens, including Sw/MO/12, Sw/MO/14 and A/VIC/11, against a reference swine antisera panel (Table S3) (28). The reference panel represents H3 viruses historically or currently circulating in pigs in the U.S., along with recent and historic representatives of human vaccine strains. The HI assay data and antigenic cartography were used to quantify the antigenic inter-relationships between Sw/MO/12, Sw/MO/14 and other H3 isolates, as previously described (12, 29).

Statistical analysis. The percent 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 ≤ 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.

Results

Genetic characterization of the novel H3 viruses. Phylogenetic analysis of the HA genes of the human-like H3N2 and H3N1 isolates Sw/MO/12 or Sw/MO/14 used in our study, and other human-like H3N1 and H3N2 swine viruses identified in GenBank, demonstrated that they were most closely related to human seasonal H3N2 strains from 2010-2011 (Fig. 1; Fig. S7), and they did not cluster with the contemporary circulating swine H3 genetic clusters (30). The HA genes of the recent swine human-like H3 clustered together in the phylogeny with human seasonal H3 from 2010-11, suggesting these swine isolates were of similar ancestry, and that the Sw/MO/12 isolate most likely

239 evolved from a human-seasonal virus that circulated between the 2010-2011 seasons. The
240 NA phylogeny indicated the NA gene of the initially identified human-like H3N2 swine
241 virus (Sw/MO/12) was closely related to human N2 genes that circulated in 2010-2011,
242 similar to the HA phylogeny (Fig. 2A; Fig. S8A). However, the NA of the more
243 contemporary human-like H3 viruses were closely related to N1 of cH1N1 viruses or the
244 N2 of the swine 2002 N2 lineage (Fig. 2B, Fig. S8B). The internal genes of five human-
245 like H3 viruses (the first H3N2 and four H3N1) were all closely related to H1N1pdm09
246 viruses, and more recent human-like swine H3N2 had a combination of internal genes of
247 the TRIG lineage with the M gene of H1N109pdm lineage (Fig. S1-6). The viruses
248 recovered from two primary and two contact pigs of each infected group (when
249 recoverable) were sequenced and compared to the original inoculum to investigate
250 whether amino acid changes occurred after animal passage, and no differences were
251 found.
252 **Pathogenesis of the swine and human H3 viruses in pigs.** The A/VIC/11 did not cause
253 significant macroscopic or microscopic lesions when compared to non-infected pigs
254 (Table 2). Pigs challenged with the swine viruses (Sw/MO/12 and Sw/MO/14) had
255 significantly higher percentages of the lungs affected with cranioventral consolidation
256 when compared to A/VIC/11 (Table 2), with Sw/MO/14 infected pigs showing the
257 highest percentage of lesions.
258 Microscopic lung lesions in the Sw/MO/14 group consisted of moderate to severe,
259 lobular and patchy to locally extensive interstitial pneumonia and moderately dense
260 peribronchiolar cuffs that extended into the adjacent interstitium. Locally extensive
261 alveolar lumina were expanded by large numbers of neutrophils and macrophages

admixed with mild edema. Multifocal bronchi and bronchioles demonstrated moderate to severe epithelial attenuation and necrosis with infiltrates of neutrophils and occasional macrophages in the airway lumen. Pigs challenged with Sw/MO/12 showed less airway impairment compared with the Sw/MO/14 group, but consistent with uncomplicated influenza virus infection. In contrast, pigs challenged with A/VIC/11 exhibited minimal, patchy interstitial pneumonia and mild and loosely formed peribronchiolar cuffs. Trachea epithelial attenuation or necrosis was mild to moderate in three of ten pigs challenged with Sw/MO/14, although all pigs demonstrated moderate tracheitis, which was also observed in the Sw/MO/12 group. Mild tracheitis was observed in only a few of the A/VIC/11 H3N2 challenged pigs.

Lung and trachea pathology observed for the A/VIC/11rg was consistent with the wild-type strain in Experiment 1; however, pathology for the Sw/MO/14rg was milder than that observed in Experiment 1 for wild-type Sw/MO/14 (Tables 1 and 2), although still relatively high compared to the remaining rg-viruses. The Sw/MO/14 and A/VIC/11 rg-reassortant viruses did not cause significant macroscopic lung lesions when compared to non-infected pigs, with the exception of VIC11-NA (7 genes of Sw/MO/14), with a trend for increased macroscopic lung lesions and significant microscopic lung scores.

IAV-specific antigen staining was detected by IHC in Sw/MO/12 and Sw/MO/14 challenged groups, with average IHC scores in the lungs of 2.0 ± 0.2 and 5.3 ± 0.4 respectively, and average scores in the trachea of 2.5 ± 0.4 and 2.6 ± 0.2 respectively. Immunoreactive IAV signals were not observed in any of the A/VIC/11 challenged pigs.

In Experiment 2 with reassortant viruses, IAV antigen was detected in the lungs and trachea of pigs challenged with Sw/MO/14rg (scores of 2.15 ± 0.3 and 1.7 ± 0.3

285 respectively) and VIC11-NA (scores of 2.4 ± 0.4 and 1.7 ± 0.5, respectively), and in the
286 trachea of pigs challenged with VIC11-HA (score of 0.6 ± 0.4), consistent with virus
287 titers described below.
288 **Infection and transmission of the human-like swine H3 viruses.** The back-titration of
289 the inoculum of A/VIC/11, Sw/MO/12 and Sw/MO/14 were $10^{4.5}$, $10^{4.5}$ and $10^{4.0}$,
290 respectively. IAV was not isolated from BALF or NS of non-challenged (NC) control
291 pigs. Virus was detected in the BALF of all pigs challenged with Sw/MO/12 and
292 Sw/MO/14, with Sw/MO/14 showing the highest average virus titers (Table 2). In
293 contrast, BALF of only two pigs inoculated with A/VIC/11 were virus positive at 5 dpi,
294 and the group mean titer was not significantly different from the non-infected group.
295 The back-titrations of the inoculum used in Experiment 2 ranged from $10^{4.25}$ to $10^{5.0}$.
296 Both rg-generated parental viruses resulted in viral titers in BALF similar to the titers
297 observed for the wild-type viruses in Experiment 1 (Table 3). Although the rg-reassortant
298 viruses did not result in significant lung pathology, significant mean viral titers in the
299 lungs were detected in an increased number of pigs in the two groups containing the HA
300 of Sw/MO/14 on the A/VIC/11 backbone (MO14-HA/NA and MO14-HA; Table 3).
301 The magnitude and kinetics of virus shedding in nasal secretions was considerably
302 different between the human and the swine H3 viruses in Experiment 1. Only two
303 primary pigs shed low titers of A/VIC/11 during the study period (Fig. 3). Pigs infected
304 with Sw/MO/12 started shedding at 1 dpi and all were shedding by 3 dpi. All pigs
305 challenged with Sw/MO/14 shed virus from 1 dpi until the day of necropsy, with titers
306 similar to the Sw/MO/12 pigs at 3 and 5 dpi (Fig. 3).

None of the indirect contact pigs shed A/VIC/11 at any time point. In contrast, pigs in indirect contact with both groups of swine H3-infected pigs shed virus starting at 4 dpc, with similar average titers. One pig in the Sw/MO/14 contact group was still shedding at 9dpc. By 15 dpc, all Sw/MO/12- and Sw/MO/14-contact pigs had seroconverted to homologous virus (average HI titers of 422.2 ± 13.2 and 2228.6 ± 12.9 respectively), confirming exposure to the challenge virus. None of the A/VIC/11-contact pigs seroconverted.

Pigs infected with both parental rg-generated viruses in Experiment 2 showed similar nasal shedding patterns as pigs infected with the wild-type viruses in Experiment 1 (Fig. 4), consistent with what was observed for viral replication in the lungs. Despite detectable virus titers in lungs for MO14-HA/NA and MO14-HA, all reassortant viruses that contained A/VIC/11 internal genes or NA alone resulted in significant loss in nasal viral shedding compared to Sw/MO/14rg (Fig. 4). In contrast, the HA of A/VIC/11 with the Sw/MO/14 backbone (VIC11-HA/NA and VIC11-HA) demonstrated the opposite pattern, with significant virus titers in nasal swabs (Fig. 4) despite limited replication in the lung (Table 3). Apart from the shedding patterns observed in primary infected pigs in Experiment 2, only Sw/MO/14rg resulted in airborne transmission to indirect contacts, with similar titers to the wild-type Sw/MO/14 (data not shown).

Antigenic analysis of the novel H3N1. The antigenic distances between the human-like H3 viruses (Sw/MO/12 and Sw/MO/14) and human and swine H3N2 reference viruses are shown in Fig. 5 (tabulated cross-HI titers are shown in Table S4 in the supplemental material), with the antigens color-coded according to Lewis et al. (28). The human-like swine H3 viruses did not cluster with either of the two major antigenic clusters recently

identified for contemporary swine H3 viruses descended from the historic cluster III, or with prototypic antigens representing historic swine H3 clusters I and II (Fig. 5A). The novel human-like H3N1 and H3N2 were positioned at least 5 antigenic units away from other contemporary influenza viruses endemic in swine (Fig. 5B). Sw/MO/12 was located 1.4 antigenic units away from Sw/MO/14. The human seasonal H3 representative, A/VIC/11, was 1.9 and 3.1 antigenic units away from Sw/MO/12 and Sw/MO/14, respectively (Fig. 5B).

Human-like H3 genes from swine contained many mutations. To investigate a possible molecular basis for antigenic properties and pathogenesis observed with the human-like swine H3 viruses studied here, the deduced HA1 amino acid sequences were compared against a panel of reference H3 strains. The human-like Sw/MO/14 H3 gene differed in 25 amino acids in comparison to the human vaccine strain with similar evolutionary history (A/VIC/11; Fig. S9); eight of these mutations were located in the previously recognized antigenic sites (A to E) (31, 32) (Fig. S9). The human-like Sw/MO/12 differed in 18 positions from A/VIC/11, three in the antigenic sites, and in 16 positions from the 2014 H3N1 (Fig. S9). Positions 140 and 145, which differed in Sw/MO/14 from A/VIC/11 and the other swine H3 strains, might be key in determining the relative antigenic map position among these strains. Putative N-linked glycosylation sites were predicted using the Net NGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Substitutions predicted to result in the loss of putative *N*-glycosylation sites were detected at four amino acid sites observed in Sw/MO/14 H3N1 and at two positions for the Sw/MO/12 H3N2 in comparison to A/VIC/11.

Discussion

Despite a certain level of host specificity, many interspecies transmission events of influenza A viruses have been documented (33). In that context, pigs are an important natural host for IAV and are closely associated with the ecology and evolution of IAV (33). Notably, human IAV can infect swine and establish new lineages of endemic viruses (6, 9). The continuous spillover of human viruses into pig populations followed by reassortment and evolution has resulted in the circulation of swine IAV containing human-origin segments in North America, such as the TRIG H3N2 viruses and the human seasonal H1-related viruses known as the delta-cluster swine viruses (1, 8, 34). In our study, swine human-like H3 viruses newly identified through the USDA surveillance system caused significant lung pathology in infected pigs and resulted in airborne transmission. This is consistent with evidence from recent diagnostic investigations that demonstrate the virus has spread to a second U.S. state to a location without known epidemiologic links to the index case in Missouri. However, submissions to the USDA IAV surveillance system are voluntary and anonymous, including viruses described in this report. Therefore, details regarding the clinical disease on some of the source farms and potential epidemiologic links between the outbreaks were not always available. Both the human-like viruses were antigenically distinct from swine H3 viruses currently circulating in the U.S. and antigenic drift from human seasonal H3N2 vaccine strains was also apparent.

Globally, endemic strains of IAV in pigs are of three main subtypes: H1N1, H1N2, and H3N2 (1, 33). Nevertheless, H3N1 viruses resulting from the reassortment between swine viruses (14, 35, 36) or from interspecies transmission and reassortment (2, 37, 38) have

been detected previously. The HA of the newly emerging H3N2 and H3N1 viruses we describe are most genetically similar to recent human seasonal H3N2 strains from 2010-2011, suggesting these viruses evolved from a relatively recent spillover event of a human virus into pigs. These human-like viruses have been detected in multiple reassorted genome constellations, containing human H3, either human N2, classical swine N1, or swine 2002 N2, and internal genes from H1N1pdm09 or TRIG with H1N1pdm09 M genes. Recently, Nelson et al. (6) showed that relatively frequent human-to-swine transmission occurred since 1965 in at least 8 countries, often with the replacement of the human IAV internal genes with swine-origin genes, suggesting reassortment and swine adaptation are important for sustained onward transmission. The human-like H3N2 detected first appears to be a precursor to the H3N1 viruses, differing from the H3N1 primarily by mutations in the HA gene and in the subtype of the NA gene. The N1 gene of the H3N1 human-like viruses is of the classical N1 lineage that circulates at a relatively similar frequency as N2 in pigs. Two lineages of N2 co-circulate in swine in the U.S., one of a human seasonal N2 lineage from approximately 1998 and the other a more recent human seasonal N2 lineage from approximately 2002 (1). Sw/MO/12-like H3N2 viruses containing human-origin NA were not detected by the USDA system since 2012, yet the H3N1 was repeatedly detected in 2013-2014, suggesting the N1 replaced the human-origin N2, although a direct evolutionary link to an N1 source virus could not be made. However, the most recent evaluation of the surveillance data revealed that human-like H3 viruses with swine N2 of the 2002 lineage are now being detected as a third generation reassortant from a putative human seasonal

398 precursor. These findings underscore that these novel viruses continue to evolve and
399 adapt to the swine host.
400 The internal gene constellation also appears to be important in the evolution of these
401 human-like viruses in swine. Reassortants containing surface genes from endemic viruses
402 and the TRIG constellation with the H1N1pdm09 M gene have become predominant in
403 North American swine IAV (1, 39), and other H1N1pdm09 internal genes are
404 increasingly being detected through the USDA surveillance system (39). The novel field
405 isolates studied here contained all internal genes from H1N1pdm09, leading to a
406 speculation that they may be associated with the fitness of these viruses in the swine host.
407 Indeed, pairing the A/VIC/11 HA or HA and NA with the H1N1pdm09-lineage internal
408 genes from the Sw/MO/14 virus resulted in significantly higher nasal shedding compared
409 to the whole human virus. More recent isolates detected in the surveillance system
410 contain TRIG plus pandemic M gene constellations, but were detected after these studies
411 were initiated and will be the subject of future studies.
412 Our results demonstrated that the human-like viruses efficiently infected pigs, caused
413 moderate to severe pneumonia and resulted in airborne transmission to indirect contacts.
414 In contrast, the prototypic human A/VIC/2011 H3N2 virus did not cause significant
415 pathology and failed to transmit to indirect contacts. Unaltered wild type human IAV
416 were shown to cause mild respiratory disease and lung pathology in comparison to swine-
417 adapted virus previously (40). Conversely, the H1N1pdm09, a swine-origin human
418 seasonal virus, causes typical influenza-like clinical signs and shedding in pigs (41, 42),
419 suggesting IAV has the potential to be fully adapted to humans and swine. Individual
420 gene segments or mutations within gene segments as well as combinations of genes

contribute to viral fitness; for example, an ideal balance between surface genes HA and NA is necessary to result in effective influenza infection (43). Our results suggest that the Sw/MO/14 HA alone conferred the ability to replicate in the lungs regardless of the NA or internal genes paired with it. However, the HA combined with the other genes (NA and/or internal genes) were critical for ability to replicate in nasal epithelium and transmit to indirect contacts. While the HA from Sw/MO/14 contributed to replication in the lower respiratory tract, the virus containing the HA of A/VIC/11 replicated in the upper respiratory tract when paired with the H1N1pdm09-lineage internal genes of the Sw/MO/14. These findings indicate that the Sw/MO/14 HA played a critical role in the adaptation of these novel viruses to swine, but the combination and balance between viral genes was also essential.

Human influenza viruses have been shown to replicate more efficiently at 33-34°C due to amino acid 627K in the PB2 gene (44, 45). In contrast, the baseline body temperature of pigs ranges between 38.5-39.5ºC, and thus may restrict replication like observed for the human A/VIC/11 virus backbone in the pig's respiratory tract. However, H1N1pdm09 virus has been shown to efficiently replicate in both the upper and lower respiratory tracts of pigs (42), and this internal gene backbone likely contributed to the increased replication of the reassortants with the A/VIC/11 HA in the upper respiratory tract. The ability of the H1N1pdm09 to replicate in the lower respiratory tract and thus result in lung pathology has been associated, among other factors, with lower number of glycosylations in the HA and reduced surfactant protein D (SP-D)-mediated clearance (46). The two wild-type human-like swine H3 viruses described here had fewer predicted

443 *N*-glycosylation sites in the HA protein when compared to the putative human IAV
444 ancestor, which might have contributed to their increased pathogenicity in pigs.
445 Additionally, the presence of carbohydrates on the HA might alter the antigenicity of
446 IAV (47), and the reduction observed in the Sw/MO viruses may have impacted the
447 cross-reactivity to the H3 reference antisera panel, in addition to other potential
448 antigenic-impacting amino acid substitutions. Substitutions in as few as seven amino acid
449 positions were shown to be largely responsible for the antigenic evolution of H3N2
450 viruses circulating in humans for 35 years (48). In addition, positions 145 and 159 near
451 the receptor-binding site, among others, are likely responsible for antigenic changes in
452 H3N2 swine virus evolution (28). Amino acid substitutions in these two positions as well
453 as others detected in the human-like swine H3 likely contributed to the low cross-
454 reactivity observed here between the human-like Sw/MO viruses and the swine endemic
455 IAV. However, the magnitude of the effect of each of these individual substitutions is
456 unclear at the current time. Commercially available swine IAV vaccines in the U.S.
457 contain swine strains from phylogenetic clusters I and/or IV in their composition. The
458 human-like H3N2 and H3N1 showed little HI cross-reactivity with current and historical
459 swine H3N2 and, therefore, immune response elicited by the commercial swine vaccines
460 are highly unlikely to result in cross-protection against these novel H3 viruses.
461 Though new subtypes or genotypes of IAV are sporadically detected in pigs, the
462 properties required for a virus to efficiently transmit and become established in pig
463 populations are still largely unknown and likely contextual with the whole genome. The
464 recurring bidirectional exchange between swine and human influenza A viruses has
465 contributed much to the diversity of viruses circulating in pigs currently, and the frequent

incursions of human seasonal viruses to swine have greatly influenced the dynamics of IAV evolution in swine. We demonstrated that wild type field isolates of the human-origin H3N2 and H3N1 swine viruses efficiently infected pigs and resulted in onward transmission. However, the adaptation of human viruses to swine appears to be complex as the HA gene as well as the internal gene constellation played important but variable roles in infectivity, replication, transmission, and pathogenicity in swine, with different phenotypes in the upper compared to lower respiratory tract. Importantly, the novel human-like viruses were antigenically divergent from all U.S. swine viruses included in our contemporary H3N2 serum panel and from the strains used in commercially available swine vaccines, therefore pigs likely have limited immune protection against these novel human-like viruses. Hence, effective surveillance and close monitoring of the evolution of these human-origin viruses in pigs are critical for vaccine preparedness and to improve preventive measures in the swine industry.

Acknowledgements

We gratefully acknowledge pork producers, swine veterinarians, and laboratories for participating in the USDA Influenza Virus Surveillance System for swine. The authors thank Michelle Harland and Gwen Nordholm for assistance with laboratory techniques, and Jason Huegel, Ty Standley, and Jason Crabtree for assistance with animal studies. We thank Dr Susan Brockmeier for assisting with bacterial screening and Kerrie Franzen for whole genome sequencing. Funding was provided from USDA-ARS and USDA-APHIS. D.S. Rajao was a CNPq-Brazil scholarship recipient. T.K. Anderson and E.J. Abente were supported in part by an appointment to the ARS-USDA Research Participation Program administered by the Oak Ridge Institute for Science and Education

(ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and USDA. ORISE is managed by ORAU under DOE contract number DE-AC05-06OR23100.

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672 **Tables**

673 **Table 1.** List of viruses generated by reverse genetics (rg) using A/Victoria/361/2011
674 (A/VIC/11) and A/Swine/Missouri/ A01410819/2014 (Sw/MO/14) used as challenge
675 viruses in Experiment 2.

Virus	HA gene origin	NA gene origin	Internal genes origin
Sw/MO/14rg	Sw/MO/14	Sw/MO/14	Sw/MO/14
VIC11-HA/NA	A/VIC/11	A/VIC/11	Sw/MO/14
VIC11-HA	A/VIC/11	Sw/MO/14	Sw/MO/14
VIC11-NA	Sw/MO/14	A/VIC/11	Sw/MO/14
A/VIC/11rg	A/VIC/11	A/VIC/11	A/VIC/11
MO14-HA/NA	Sw/MO/14	Sw/MO/14	A/VIC/11
MO14-HA	Sw/MO/14	A/VIC/11	A/VIC/11
MO14-NA	A/VIC/11	Sw/MO/14	A/VIC/11

676 HA= hemagglutinin; NA= neuraminidase

677

678 **Table 2.** Macroscopic pneumonia, lung and trachea microscopic pathology, and lung
679 virus titers obtained in pigs challenged with wild-type A/Victoria/361/2011 (A/VIC/11),
680 A/Swine/Missouri/A01476459/2012 (Sw/MO/12), or
681 A/Swine/Missouri/A01410819/2014 (Sw/MO/14), and non-challenged controls (NC).
682 Results shown as means ± standard error of the means.

Group	Macroscopic pneumonia (%)	Microscopic pneumonia score (0-22)	Microscopic tracheitis score (1-8)	Log_{10} virus titer ($TCID_{50}$) in BALF
NC	$0.0 \pm 0.0^{a,x}$	0.2 ± 0.1^{a}	0.1 ± 0.1^{a}	0.0 ± 0.0^{a} (0/5)[y]
A/VIC/11	0.0 ± 0.0^{a}	0.8 ± 0.2^{a}	0.8 ± 0.2^{a}	0.6 ± 0.4^{a} (2/8)
Sw/MO/12	4.2 ± 1.0^{b}	4.9 ± 0.5^{b}	2.8 ± 0.5^{b}	3.6 ± 0.2^{b} (10/10)
Sw/MO/14	12.0 ± 0.8^{c}	9.4 ± 0.6^{c}	2.1 ± 0.3^{b}	5.1 ± 0.2^{c} (10/10)

683 [x]Different lower case letters within the same column indicate significant differences
684 ($p \leq 0.05$).
685 [y]The number of virus-positive pigs/total number of pigs tested is indicated in parentheses.
686

687 **Table 3.** Macroscopic pneumonia, lung and trachea microscopic pathology, and lung
688 virus titers in pigs challenged with reverse genetics-generated A/VIC/11rg, Sw/MO/14rg,
689 VIC11-HA/NA, VIC11-HA, VIC11-NA, MO14-HA/NA, MO14-HA, and MO14-NA,
690 and non-challenged controls (NC). Results shown as means ± standard error of the
691 means.

Group	Macroscopic pneumonia (%)	Microscopic pneumonia score (0-22)	Microscopic tracheitis score (1-8)	Log_{10} virus titer ($TCID_{50}$) in BALF
NC	$0.3 \pm 0.2^{a,x}$	0.1 ± 0.1^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a} $(0/5)^{y}$
Sw/MO/14rg	6.3 ± 1.9^{b}	6.4 ± 1.0^{b}	1.1 ± 0.4^{b}	4.1 ± 0.3^{b} (10/10)
VIC11-HA/NA	0.5 ± 0.4^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.0 ± 0.0^{a} (0/10)
VIC11-HA	0.0 ± 0.0^{a}	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}	0.0 ± 0.0^{a} (0/10)
VIC11-NA	2.7 ± 0.7^{a}	2.3 ± 0.7^{b}	0.2 ± 0.1^{a}	$3.6 \pm 0.4^{b,c}$ (9/10)
A/VIC/11rg	0.4 ± 0.2^{a}	0.6 ± 0.2^{a}	0.2 ± 0.2^{a}	0.4 ± 0.3^{a} (2/10)
MO14-HA/NA	1.0 ± 0.3^{a}	0.5 ± 0.2^{a}	0.4 ± 0.2^{a}	1.7 ± 0.4^{c} (7/10)
MO14-HA	1.1 ± 0.5^{a}	0.9 ± 0.2^{a}	0.1 ± 0.1^{a}	2.6 ± 0.4^{c} (9/10)
MO14-NA	1.4 ± 0.6^{a}	0.4 ± 0.1^{a}	0.0 ± 0.0^{a}	0.4 ± 0.2^{a} (2/10)

692 [x]Different lower case letters within the same column indicate significant differences
693 ($p \leq 0.05$).
694 [y]The number of virus-positive pigs/total number of pigs tested is indicated in parentheses.

Figure Legends

Fig. 1. Phylogenetic analysis of HA genes of the swine human-like H3 viruses. Maximum likelihood phylogeny of the HA of 20 human-like H3 swine viruses and 155 H3N2 viruses collected from humans and swine in the United States. Branch color reflects evolutionary history and is indicated in the inset: swine human-like H3 in purple; human seasonal H3 in gray; swine cluster I H3 in brown; swine cluster II H3 in blue; swine cluster IV H3 in orange; and human reference H3 vaccine strain in red. Numbers above or below branches indicate bootstrap support (%): bootstrap values ≤50% are not shown. The tree is midpoint rooted for clarity and all branch lengths are drawn to scale: scale bar indicates nucleotide substitutions per site. A phylogeny with taxon names indicating viral isolate, prefaced by GenBank or GISAID EpiFlu accession identifier, is presented in the supplementary material.

Fig. 2. Phylogenetic analysis of NA genes of the swine human-like H3 viruses. Maximum likelihood phylogeny of the NA of 20 human-like H3 viruses and 155 representative viruses collected from humans, swine, and turkeys in the United States; (A) N2 influenza A virus isolates; and (B) N1 influenza A virus isolates. Numbers above or below branches indicate bootstrap support (%): bootstrap values ≤50% are not shown. H3N2 NA sublineages are colored: (A) the 1998 swine-lineage in magenta, the 2002 swine-lineage in green, and human seasonal lineage in gray; and (B) the H1N1pdm09 lineage in red and the classical swine lineage in cyan. The novel human-like H3 viruses described in this study are colored purple. The trees are midpoint rooted for clarity and all branch lengths are drawn to scale: scale bar indicates nucleotide substitutions per site.

717 Phylogenies with taxon names indicating viral isolate, prefaced by GenBank or GISAID
718 EpiFlu accession identifier, are presented in the supplementary material.
719 **Fig. 3.** Nasal viral shedding observed in the *in vivo* Experiment 1. Virus titers in nasal
720 swabs of (A) primary pigs at 1, 3, and 5 days post infection (dpi) with
721 A/Swine/Missouri/A01476459/2012 (Sw/MO/12), A/Swine/Missouri/A01410819/2014
722 (Sw/MO/14) or A/Victoria/361/2011 (A/VIC/11) and of (B) their respective indirect
723 contact pigs at 4, 5, 7, and 9 days post contact (dpc). Results shown as means and
724 standard error of the means. Numbers of infected pigs/total number of pigs are indicated
725 in parentheses. Different lowercase letters between groups within the same sampling day
726 indicate significant differences ($p \leq 0.05$).
727 **Fig. 4.** Nasal viral shedding observed in the *in vivo* Experiment 2 with reassortant
728 viruses. Virus titers in nasal swabs of primary pigs at 1, 3, and 5 days post infection (dpi)
729 with reverse genetics generated parental viruses (A) A/Swine/Missouri/A01410819/2014
730 (Sw/MO/14rg) or (B) A/Victoria/361/2011 (A/VIC/11rg), and reassortant viruses with
731 surface genes exchanged on the parental backbones (A: VIC11-HA/NA, VIC11-HA and
732 VIC11-NA in the Sw/MO/14rg backbone; B: MO14-HA/NA, MO14-HA and MO14-NA
733 in the A/VIC/11rg backbone). Results shown as means and standard error of the means.
734 Numbers of infected pigs/total number of pigs are indicated in parentheses. Different
735 lowercase letters within the same sampling day indicate significant differences ($p \leq 0.05$).
736 Levels of lung replication indicated for comparison: crosses illustrate approximated log
737 viral titers.
738 **Fig. 5.** Antigenic relationships between the swine human-like H3 viruses and a panel of
739 reference H3N2 viruses. (A) 3D antigenic map of swine and human H3 influenza viruses.

740 (B) Graph illustrating the antigenic distances between the human-like swine H3 viruses
741 (Sw/MO/12 in the first panel and Sw/MO/14 in the second panel) and all viruses
742 represented in the 3D map. The viruses used in this study, Sw/MO/12 H3N2, Sw/MO/14
743 H3N1, and A/VIC/11, are represented by green, purple and gray larger spheres/circles,
744 respectively. Swine and human isolates are colored according to Lewis et al. (28):
745 A/Wuhan/359/1995 and the cluster I prototype swine H3N2 are shown in light blue;
746 A/Sydney/5/1997, A/Moscow/10/1999, and the cluster II prototype swine H3N2 are
747 shown in light pink; swine H3 antigenic clusters are shown in red and cyan, and outliers
748 as multicolor; and human vaccine strains are shown in gray. The scale bar represents one
749 antigenic unit distance, corresponding to a 2-fold dilution of antiserum in the HI assay.
750

Fig. 1. Phylogenetic analysis of HA genes of the swine human-like H3 viruses. Maximum likelihood phylogeny of the HA of 20 human-like H3 swine viruses and 155 H3N2 viruses collected from humans and swine in the United States. Branch color reflects evolutionary history and is indicated in the inset: swine human-like H3 in purple; human seasonal H3 in gray; swine cluster I H3 in brown; swine cluster II H3 in blue; swine cluster IV H3 in orange; and human reference H3 vaccine strain in red. Numbers above or below branches indicate bootstrap support (%): bootstrap values ≤50% are not shown. The tree is midpoint rooted for clarity and all branch lengths are drawn to scale: scale bar indicates nucleotide substitutions per site. A phylogeny with taxon names indicating viral isolate, prefaced by GenBank or GISAID EpiFlu accession identifier, is presented in the supplementary material.

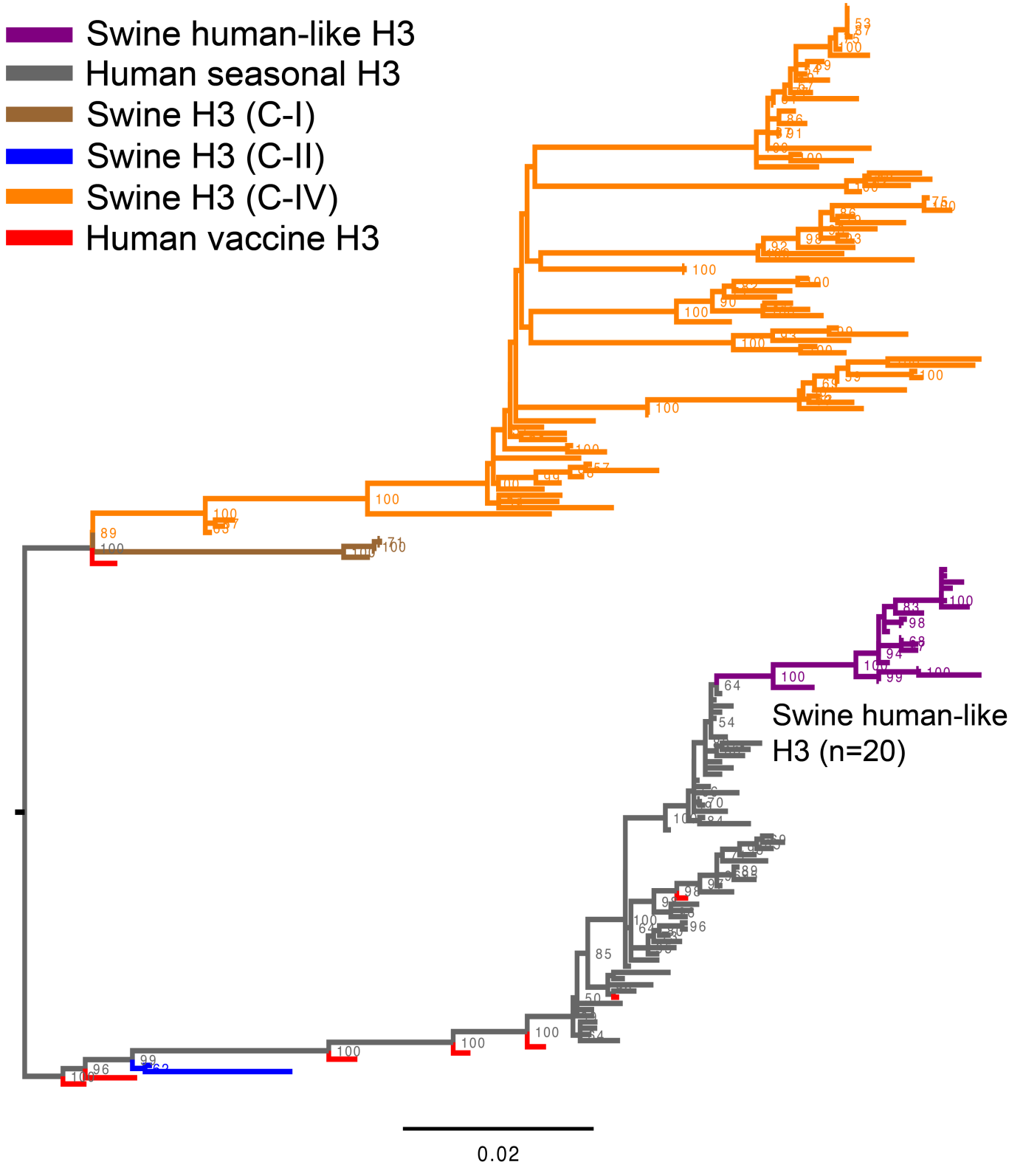

Fig. 2. Phylogenetic analysis of NA genes of the swine human-like H3 viruses. Maximum likelihood phylogeny of the NA of 20 human-like H3 viruses and 155 representative viruses collected from humans, swine, and turkeys in the United States; (A) N2 influenza A virus isolates; and (B) N1 influenza A virus isolates. Numbers above or below branches indicate bootstrap support (%): bootstrap values ≤50% are not shown. H3N2 NA sublineages are colored: (A) the 1998 swine-lineage in magenta, the 2002 swine-lineage in green, and human seasonal lineage in gray; and (B) the H1N1pdm09 lineage in red and the classical swine lineage in cyan. The novel human-like H3 viruses described in this study are colored purple. The trees are midpoint rooted for clarity and all branch lengths are drawn to scale: scale bar indicates nucleotide substitutions per site. Phylogenies with taxon names indicating viral isolate, prefaced by GenBank or GISAID EpiFlu accession identifier, are presented in the supplementary material.

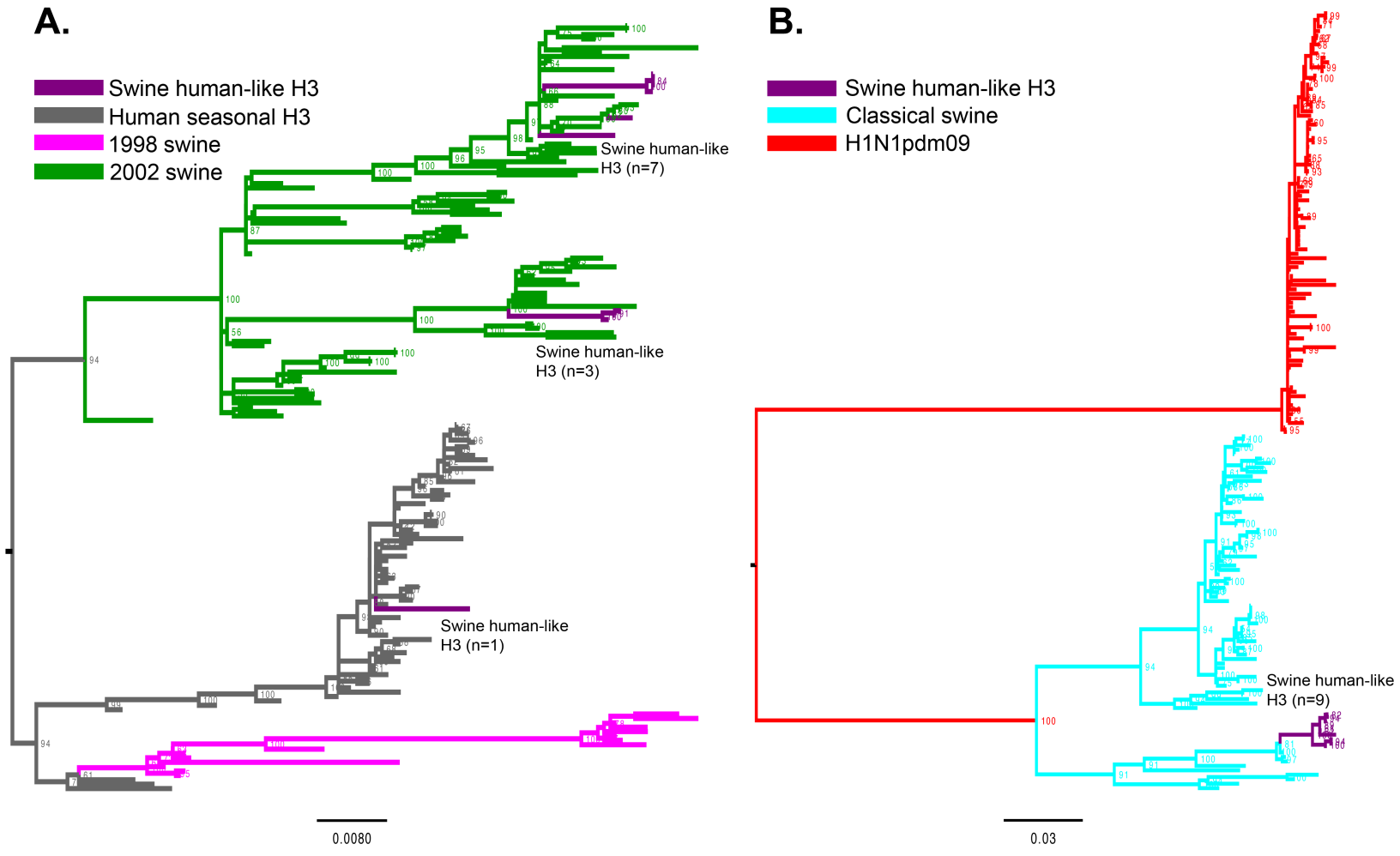

Fig. 3. Nasal viral shedding observed in the *in vivo* Experiment 1. Virus titers in nasal swabs of (A) primary pigs at 1, 3, and 5 days post infection (dpi) with A/Swine/Missouri/A01476459/2012 (Sw/MO/12), A/Swine/Missouri/A01410819/2014 (Sw/MO/14) or A/Victoria/361/2011 (A/VIC/11) and of (B) their respective indirect contact pigs at 4, 5, 7, and 9 days post contact (dpc). Results shown as means and standard error of the means. Numbers of infected pigs/total number of pigs are indicated in parentheses. Different lowercase letters between groups within the same sampling day indicate significant differences ($p \leq 0.05$).

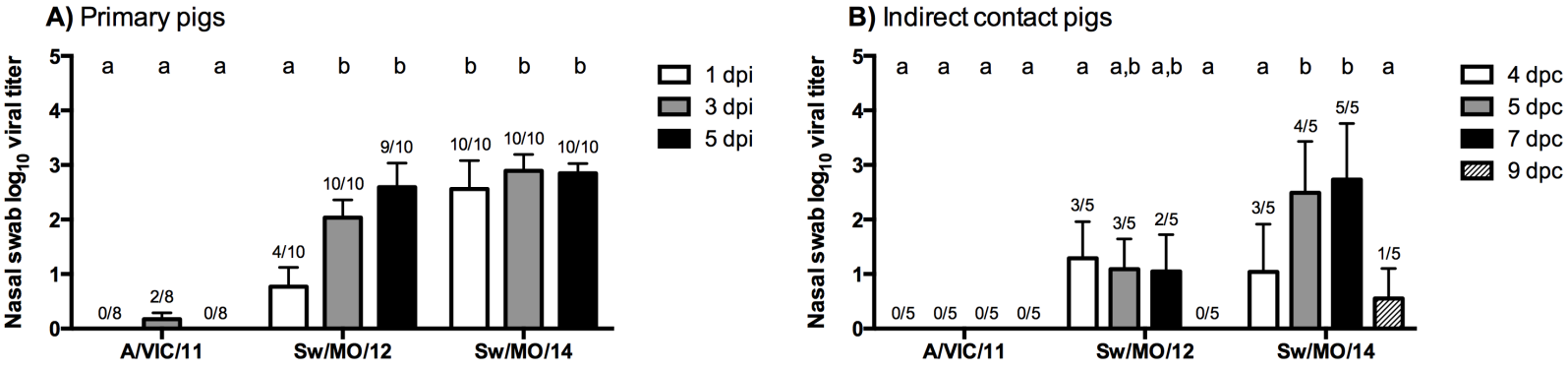

Fig. 4. Nasal viral shedding observed in the *in vivo* Experiment 2 with reassortant viruses. Virus titers in nasal swabs of primary pigs at 1, 3, and 5 days post infection (dpi) with reverse genetics generated parental viruses (A) A/Swine/Missouri/A01410819/2014 (Sw/MO/14rg) or (B) A/Victoria/361/2011 (A/VIC/11rg), and reassortant viruses with surface genes exchanged on the parental backbones (A: VIC11-HA/NA, VIC11-HA and VIC11-NA in the Sw/MO/14rg backbone; B: MO14-HA/NA, MO14-HA and MO14-NA in the A/VIC/11rg backbone). Results shown as means and standard error of the means. Numbers of infected pigs/total number of pigs are indicated in parentheses. Different lowercase letters within the same sampling day indicate significant differences ($p \leq 0.05$). Levels of lung replication indicated for comparison: crosses illustrate approximated log viral titers.

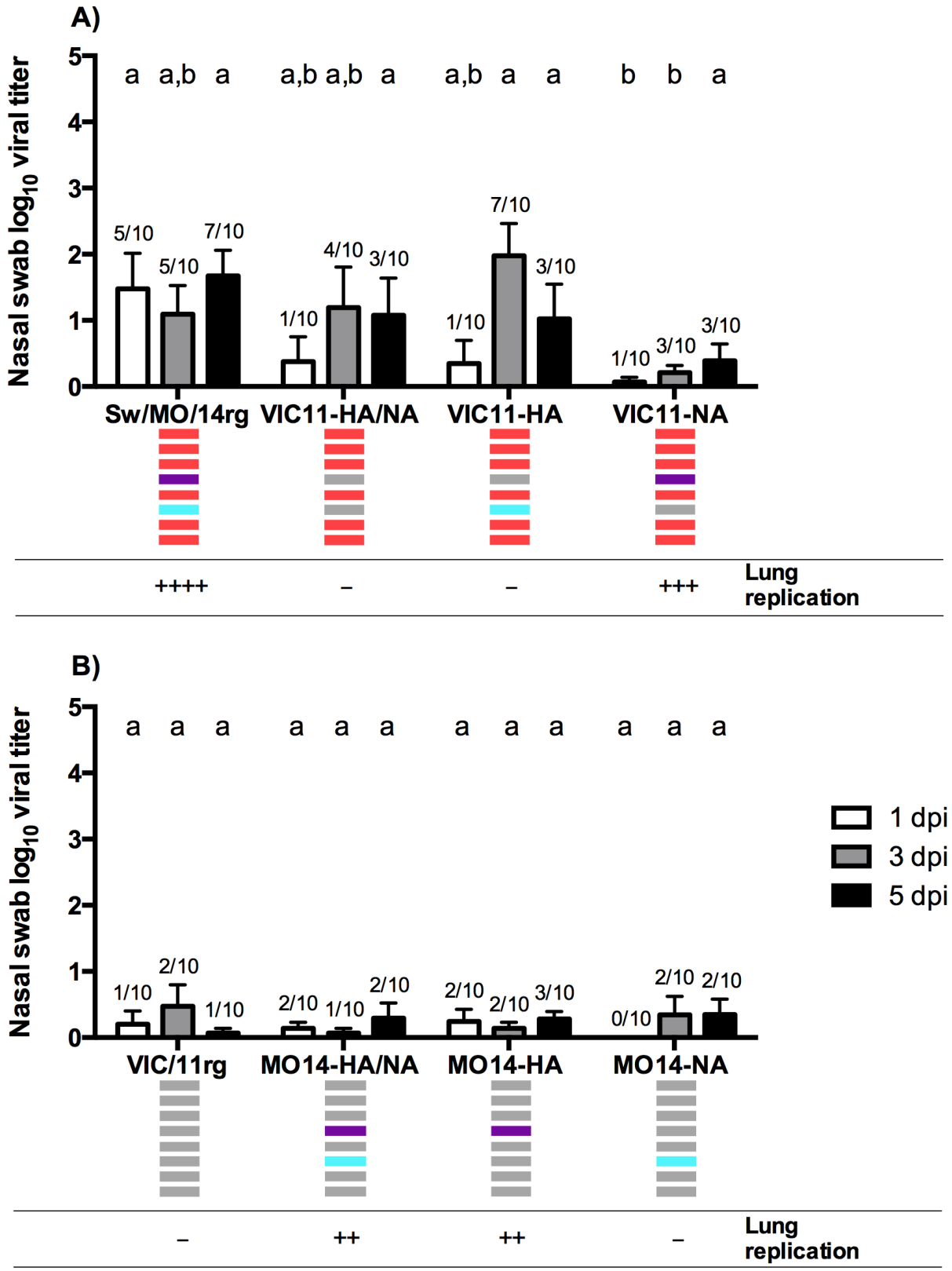

Fig. 5. Antigenic relationships between the swine human-like H3 viruses and a panel of reference H3N2 viruses. (A) 3D antigenic map of swine and human H3 influenza viruses. (B) Graph illustrating the antigenic distances between the human-like swine H3 viruses (Sw/MO/12 in the first panel and Sw/MO/14 in the second panel) and all viruses represented in the 3D map. The viruses used in this study, Sw/MO/12 H3N2, Sw/MO/14 H3N1, and A/VIC/11, are represented by green, purple and gray larger spheres/circles, respectively. Swine and human isolates are colored according to Lewis et al. (28): A/Wuhan/359/1995 and the cluster I prototype swine H3N2 are shown in light blue; A/Sydney/5/1997, A/Moscow/10/1999, and the cluster II prototype swine H3N2 are shown in light pink; swine H3 antigenic clusters are shown in red and cyan, and outliers as multicolor; and human vaccine strains are shown in gray. The scale bar represents one antigenic unit distance, corresponding to a 2-fold dilution of antiserum in the HI assay.