Virology 553 (2021) 94–101

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Characterization of contemporary 2010.1 H3N2 swine influenza A viruses circulating in United States pigs

Joshua D. Powell [a,1], Eugenio J. Abente [a,1,2], Jennifer Chang [a], Carine K. Souza [a], Daniela S. Rajao [a,3], Tavis K. Anderson [a], Michael A. Zeller [b], Phillip C. Gauger [b], Nicola S. Lewis [c], Amy L. Vincent [a,*]

[a] Virus and Prion Research Unit, National Animal Disease Center, USDA-ARS, Ames, IA, 50010, USA
[b] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA
[c] Department of Pathobiology and Population Sciences, Royal Veterinary College, Hatfield, Hertfordshire, United Kingdom

ARTICLE INFO

Keywords:
Influenza A
H3N2
H3N1
Swine
H3N2v
Antigenic motif

ABSTRACT

In 2012, swine influenza surveillance detected a novel reassorted influenza A virus (IAV) strain containing human-seasonal hemagglutinin (HA) and neuraminidase (NA). Subsequently, these viruses reassorted, maintaining only the human-origin H3, which resulted in a new lineage of viruses that became the most frequently detected H3 clade in US swine (2010.1 HA clade). Here, we assessed the antigenic phenotype, virulence, and transmission characteristics of this virus lineage following its introduction to swine. Relative to 2010.1 viruses from 2012 and 2014, recent 2010.1 contemporary strains from 2015 to 2017 resulted in equivalent macroscopic lung lesions and transmission in pigs. A single mutation at amino acid residue 145 within the previously defined HA antigenic motif was associated with a change of antigenic phenotype, potentially impairing vaccine efficacy. Contemporary 2010.1 viruses circulating in swine since 2012 were significantly different from both pre-2012H3N2 in swine and human-seasonal H3N2 viruses and demonstrated continued evolution within the lineage.

1. Introduction

Influenza A virus (IAV) has a segmented, negative-sense RNA genome and causes respiratory disease in many hosts, including humans and pigs. In humans, IAV has caused four pandemics since the early 1900's (Smith et al., 2009); a hallmark of each emergence is the introduction of a novel hemagglutinin (HA) from animal hosts into the human population. While influenza pandemic events in humans have been documented for more than a century, only more recently is it recognized that human seasonal IAV frequently spillover into the swine population (Nelson et al., 2015; Nelson and Vincent, 2015). H3N2 spillovers were detected in the United States in the late 1990s and again in the 2010s. Cluster IV (C-IV) (H3.1990.4 clade viruses in a global swine H3 nomenclature system) (Anderson et al., 2020), was introduced into pigs from humans in the 1990s (Webby et al., 2000; Olsen et al., 2006) and evolved into multiple phylogenetically distinct clades (C-IV-A through C-IV-F (Anderson et al., 2020; Kitikoon et al., 2013):). The second lineage, (H3.2010.1 clade viruses in global swine H3 nomenclature), were genetically similar to human seasonal H3N2 strains from the 2010–2011 season (Anderson et al., 2020; Rajao et al., 2015). The 2010.1 viruses were first detected in 2012 with human seasonal H3 and N2, and internal genes derived from the 2009 H1N1 pandemic (H1N1pdm09) lineage (P (Rajao et al., 2015)). These H3 viruses (Anderson et al., 2020) reassorted with endemic swine viruses, contributing the HA gene to at least 4 genome constellations, and this H3 lineage continues to circulate in pigs (Zeller et al., 2018). From 2012 to present, the 2010.1 viruses spread and became the numerically dominant H3 lineage in swine (Zeller et al., 2018). The H3 genes of both lineages were typically paired with neuraminidase (NA) genes also of human origin, either introduced in approximately 1998 or 2002 and

* Corresponding author.
E-mail address: amy.vincent@usda.gov (A.L. Vincent).
[1] These authors contributed equally to this work.
[2] Current address: U.S. Naval Medical Research Unit No.6, Venezuela Ave, Block 36, Callao 2, Lima, Peru.
[3] Current address: Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA, 30602 USA.

https://doi.org/10.1016/j.virol.2020.11.006
Received 31 July 2020; Received in revised form 13 November 2020; Accepted 17 November 2020
Available online 21 November 2020

named as such (Nelson et al., 2011). The internal genes of these lineages were derived either from the triple-reassortant H3N2 virus detected in swine populations in the 1990s (TRIG/T (Zhou et al., 1999)) or from the H1N1pdm09 lineage (Garten et al., 2009).

Presently, two major lineages of H3N2 co-circulate in US swine, C-IV-A and 2010.1, and are the targets of vaccine efforts in swine populations. The effects of C-IV HA diversity and genome constellations on transmission and pathology phenotypes has been investigated. While the earlier generations of the 2010.1 viruses have been antigenically characterized and studied in pigs (Rajao et al., 2015), recent changes in antigenicity and how changes in internal gene constellation patterns impacts virulence and transmission has not been evaluated. Understanding how recent shift and drift alters the biology of 2010.1 viruses is important for ensuring vaccine efficacy (Vincent et al., 2017). Further, as 2010.1 swine-to-human cases (termed variant H3N2, or H3N2v) have been detected (Bowman et al., 2017; Duwell et al., 2018), determining whether recent evolution has affected transmission and antigenic phenotype of the virus in swine can inform public health and pandemic preparedness efforts.

Studies linking IAV genetic variation to antigenic phenotype in human seasonal H3 viruses identified seven amino acids (H3 numbering: 145, 155, 156, 158, 159, 189, 193) in the HA globular head that largely determined antigenic phenotype (Koel et al., 2013; Burke and Smith, 2014). In swine H3N2, six of the positions (145, 155, 156, 158, 159, and 189) were also implicated as having a disproportionate impact on antigenic phenotype. These sites were termed the "antigenic motif" and are located adjacent to the receptor binding site within H3 antigenic A and B sites (highlighted in blue, Fig. 1A) (Burke and Smith, 2014; Bolton et al., 2018; Abente et al., 2016). Amino acid 145 is of particular interest within the motif as it is an antibody recognition determinant and influences receptor binding (Koel et al., 2013; Smith et al., 2004; Li et al., 2013). Though the 145 site has limited diversity in wild type viruses, an increasing number of 2010.1H3N2 strains have demonstrated variation at this position, and the effect of mutations at this position on antigenic diversity or transmission in swine has not been characterized.

In this study, we analyzed 2010.1H3 viruses collected from 2012 to 2017 in swine to assess genetic and antigenic evolution, and the potential role of the antigenic motif in determining drift of this lineage. Additionally, the role of a single mutation at position 145 in driving the antigenic phenotype and virus cross-reactivity was investigated with field-sourced swine viruses against seasonal human vaccine antisera raised in pigs. Lastly, we compared different HA and genome constellations detected in IAV from swine respiratory submissions to examine potential pathogenesis and transmission markers. Collectively, these studies provide insight into the impact the 2010.1H3 viruses have in U.S. pigs and how these changes impact antigenic distance from human seasonal H3N2, as a possible risk if variant viruses spill back into people from pigs.

2. Materials and methods

2.1. H3 2010.1 data set analysis

Virus sequences for influenza A virus isolates deposited from the United States Department of Agriculture (USDA) Influenza A Virus in Swine Surveillance System (denoted by a nine digit alphanumeric barcode beginning with A0 in the strain name), were obtained from the National Center of Biotechnology Information (NCBI) Influenza Virus Resource (IVR) (Bao et al., 2008) and the Influenza Research Database (IRD) (Zhang et al., 2017) on March 2, 2020. Sequences were classified

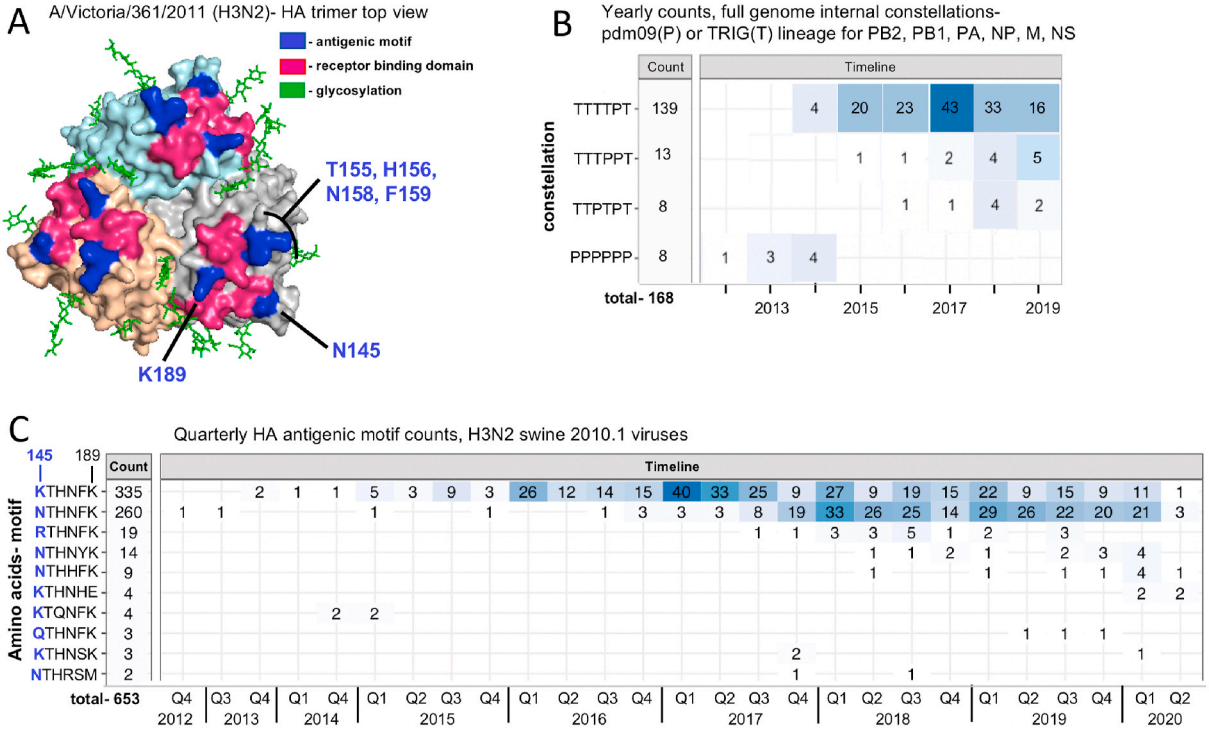

Fig. 1. The antigenic motif and internal genome constellations of 2010.1 swine viruses. A. The HA of A/Victoria/361/2011 adapted from PDB crystal structure: 4O5I (Gao et al., 2017). From above, the monomers of the trimer are shaded in light blue, light tan, and grey. The antigenic motif is shown in blue corresponding to residues 145, 155, 156, 158, 159 and 189. The receptor binding domain is shown in magenta. **B.** Internal gene constellation patterns for 2010.1 viruses from 168 whole genome sequences from 2012 to 2020 that were detected two or more times, where T = triple reassortant internal gene (TRIG) and P═H1N1pdm09 lineages in the order of gene segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), 7 (M), and 8 (NS). **C.** Counts for the top 10 motifs from 2012 to 2019 representing 653 viruses. Counts of whole genome constellations and antigenic motif in HA from strains were collated from the Influenza Research Database (IRD) (Zhang et al., 2017) https://www.fludb.org/ or Influenza Virus Database (Bao et al., 2008) https://www.ncbi.nlm.nih.gov/genomes/FLU/ accessed on 03/02/2020. 2020 Q2 results represent a partial total count with additional sequences expected and missing quarters indicate no H3.2010.1 detections.

into phylogenetic clades using the octoFLU pipeline (Chang et al., 2019) with 673 of 2084H3 swine isolates classified as H3.2010.1. Strains identified as containing the 2010.1H3 were further categorized by the remaining 7 genes. Internal gene constellation patterns were assigned by triple reassortant internal gene (TRIG) or H1N1pdm09 (P) lineages. The antigenic motif (HA positions 145, 155, 156, 158, 159, 189) were summarized by 3 month quarters using Tidyverse packages (Wickham et al., 2019) in the v3.6.0 R programming environment (R Core Team, 2018). The relative proportion of 2010.1 detections compared to total H3 detections in United States pigs was determined using ISU *FLU*ture (Zeller et al., 2018) available at https://influenza.cvm.iastate.edu.

2.2. Viruses

Viruses used for animal studies and antigenic cartography analysis were propagated in Madin–Darby Canine Kidney (MDCK) cells. Four 2010.1 isolates that represent the recent diversity (from 2015-present) of the clade made available through the USDA Influenza A Virus in Swine Surveillance System were studied in pigs for virulence and transmission characteristics and compared to prior findings with 2012 and 2014 2010.1 isolates (Rajao et al., 2015) (described in Table 1). A/swine/Missouri/A01840724/2015 (MO/15) and A/swine/-Missouri/A01671485/2016 (MO/16) were selected as representative 2010.1 viruses for testing in pigs as both contained more recent gene constellations detected in 2010.1 viruses in the United States. Both MO/15 and MO/16 had internal genes derived from the TRIG (T) cassette that has been circulating in North American pigs since the late 1990s (Zhou et al., 1999; Vincent et al., 2008) and the H1N1pdm09 (P). MO/15, MO/16, A/swine/South Dakota/A01678473/2016 (SD/16) and A/swine/Ohio/A01354299/2017 (OH/17) contained TTTTPT constellations, but MO/16 differed by the N2 subclade; MO/16 contained an N2-02A gene, whereas the remaining 3 contained N2–02B genes. SD/16 and OH/17 were selected and tested in pigs as they were >99% similar within the HA but differed at position 145 within the antigenic motif. OH/17 was also of interest as the gene constellation (TTTTPT), NA and HA antigenic motif (N145) were similar to identified H3N2 human variant cases (H3N2v) that occurred in 2016 and 2017 (Bowman et al., 2017; Duwell et al., 2018).

2.3. Antigenic characterization

Representative 2010.1 strains with the following antigenic motifs (145 in bold, 155–156, 158–159, 189 HA positions) are described in Table 1: MO/12 **N**THNFK, MO/14 **K**THNSK, OH/16 **K**THNFK, OH/17 **N**THNFK were used in hemagglutinin inhibition (HI) assays (Kitikoon et al., 2014) for antigenic characterization. A panel of monovalent anti-sera against human seasonal vaccine strains and MO/14, as representative of 2010.1 lineage (described in Table S1), were raised in swine following a previously described protocol (Rajao et al., 2018). HI data was used to determine the antigenic relationships between contemporary 2010.1 swine IAV and recent human seasonal IAV vaccine strains (1995–2014) using antigenic cartography three-dimensional maps (Lewis et al., 2014). Antigenic distances between antigens were calculated in antigenic units (AU), in which 1 AU is equivalent to a 2-fold difference in HI cross-reactivity. The antigenic distances generated in the 3-D map between the H3N2 antigens were plotted in Graph Pad Prism Version 7.03, San Diego, CA.

2.4. Animal study design

All pigs were cared for in compliance with the Institutional Animal Care and Use Committee of the USDA National Animal Disease Center. Pigs were treated with ceftiofur crystalline free acid and tulathromycin (Zoetis Animal Health, Florham Park, NJ) and were seronegative to IAV antibodies by a commercial ELISA kit (Swine Influenza Virus Ab Test, IDEXX, Westbrook, ME) prior to the start of the study. At 4–5 weeks of age (0 days post-infection; dpi), 3 ml of 1×10^5 TCID50/ml of either MO/12, MO/14, MO/15, MO/16, SD/16 or OH/17 challenge virus (described in Table 1) were delivered to pigs intratracheally (2 ml) and intranasally (1 ml). Pigs were challenged under anesthesia, using an intramuscular injection of ketamine (8 mg/kg of body weight; Phoenix, St. Joseph, MO), xylazine (4 mg/kg; Lloyd Inc., Shenandoah, IA), and Telazol (6 mg/kg; Zoetis Animal Health, Florham Park, NJ) cocktail. The number of challenged pigs for each strain is described in Table 1. Nasal swabs were collected on 0, 3, and 5 days post infection (dpi) and used for virus isolation as previously described (Vincent et al., 2012). At day two, five contact pigs for each respective group were placed in a separate raised deck in the same containment room to assess transmission dynamics, with pigs swabbed at 1, 2, 3, 5 and 7 days post contact (dpc). All pigs were humanely euthanized at either 5 dpi or 16 dpc with a lethal dose of pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Bronchoalveolar lavage fluids (BALF) from directly inoculated pigs were collected in minimal essential media (MEM) for analysis.

2.5. Pathological examination and virus detection

At necropsy, the percent of lung surface affected with pneumonia was calculated as previously described (Gauger et al., 2011, 2012; Halbur et al., 1995). Lung microscopic lesions were scored according to previously described parameters (Gauger et al., 2012) and individual composite scores for each pig were computed, with a maximum composite score of 22. Virus isolation-positive nasal swabs and BALF were titrated in MDCK cells as previously described (Vincent et al., 2012; Gauger et al., 2014) and $TCID_{50}$/ml virus titers were calculated for each sample by the Reed and Muench method (Reed and Muench, 1938).

Table 1
Swine H3 viruses characterized in animals and in antigenic cartography.

Abbreviation	Animals challenged	Strain	Internal genes[d]	NA lineage	Antigenic motif [e]
MO/12 (Gen. 1)[a,b]	n = 10[c]	A/swine/Missouri/A01476459/2012	PPPPPP	N2-hu	NTHNFK
MO/14 (Gen. 2)[a,b]	n = 20[c]	A/swine/Missouri/A01410819/2014	PPPPPP	N1-Sw	KTHNSK
MO/15 (Gen. 3)[a]	n = 5	A/swine/Missouri/A01840724/2015	TTTTPT	N2–02B-Sw	KTHNFK
OH/16 (Gen. 3)[b]	N.D.[f]	A/swine/Ohio/16TOSU4788/2016	TTTTPT	N2-02A-Sw	KTHNFK
MO/16 (Gen. 3)[a]	n = 10	A/swine/Missouri/A01671485/2016	TTTTPT	N2-02A-Sw	KTHNFK
SD/16 (Gen. 3)[a]	n = 10	A/swine/South Dakota/A01678473/2016	TTTTPT	N2–02B-Sw	KTHNFK
OH/17 (Gen. 3)[a,b]	n = 9	A/swine/Ohio/A01354299/2017	TTTTPT	N2–02B-Sw	NTHNFK

[a] Animal study viruses to assess IAV phenotype and transmission.
[b] Viruses used as test antigens in antigenic cartography.
[c] Includes a subset of animals previously reported in Rajao et al. [8].
[d] P = H1N1pdm09, T = TRIG, lineage and internal gene constellation order is PB2, PB1, PA, NP, M and NS.
[e] Antigenic motif amino acids defined by HA positions 145, 155, 156, 158, 159, 189.
[f] N.D. = Not done.

2.6. Statistical analysis

Animal data were analyzed by analysis of variance (ANOVA), with $P \leq 0.05$ considered significant (Prism software; GraphPad, La Jolla, CA) and variables with significant effects by treatment group were subjected to pairwise mean comparisons using the Tukey-Kramer test. Means include all pigs within a group, including those with no detectable virus (value = 0).

3. Results

3.1. Genomic evolution within the 2010.1 swine H3 viruses

From 2012 to 2018, there were at least three major reassortment events involving the HA of 2010.1 viruses (Rajao et al., 2015). The first generation viruses contained an HA and NA derived from human seasonal H3 viruses with internal genes derived from the H1N1pdm09 lineage (gene constellation: PPPPPP). The second generation of viruses were H3N1 subtype, acquiring an NA N1 gene from the classical swine lineage, with a PPPPPP internal gene constellation. The third generation of viruses contained the 2010.1 HA, a 2002 lineage swine N2, and internal genes from the TRIG lineage except the H1N1pdm09 lineage matrix gene (gene constellation: TTTTPT). Out of the 172 2010.1 H3 whole genomes available from USDA surveillance, the PPPPPP constellation was not detected since late 2014 when viruses with the TTTTPT constellations were initially detected (Fig. 1B). From 2016 to present, over 80% of genome sequences were the TTTTPT constellation, although constellations with PA or NP segments of the H1N1pdm09 lineage (TTTPPT and TTPTPT) were detected in lower numbers (n = 21).

3.2. Evolution of the HA in 2010.1 swine H3 viruses

The amino acid sequences of 2010.1H3 genes were analyzed for antigenic motif patterns. The 2010.1 swine viruses contain an H3 with a common human seasonal H3N2 ancestor similar to the human vaccine strain A/Victoria/361/2011 (H3N2) (Rajao et al., 2015). The top ten unique antigenic motifs with at least two or more detections in the HA of 2010.1 swine viruses are shown in Fig. 1C. At first detection, the antigenic motif of the swine strain, A/Missouri/A01476459/2012 (MO/12), was NTHNFK. From 2012 to 2015, only three 2010.1 strains had an asparagine (N) at position 145 (shown in blue, Fig. 1A,C). As the 2010.1H3 clade increased in detection frequency in USDA IAV swine surveillance data, 2010.1H3 genes with a lysine (K) at position 145 became commonly detected, then N145 increased in detection in mid-2016. Since 2018, 2010.1H3 containing either N or K at position 145 were frequently detected. In total, over 95% of 2010.1 viruses had either a lysine or asparagine at position 145, with KTHNFK or NTHNFK detected as the dominant antigenic motifs. In the last two years, additional diversification at position 145 was evident as arginine (R) and glutamine (Q) were also detected, albeit in low numbers. Mutation within the six amino acid antigenic motif was most prominent at position 145, and to a lesser extent at positions 159 and 189. There was minimal variation evident at positions 155 or 156.

3.3. Antigenic diversity of human-like swine H3 viruses to human seasonal strains

Due to the risk of zoonosis evidenced by H3N2v infections in agricultural fairs, we assessed whether contemporary 2010.1H3 swine

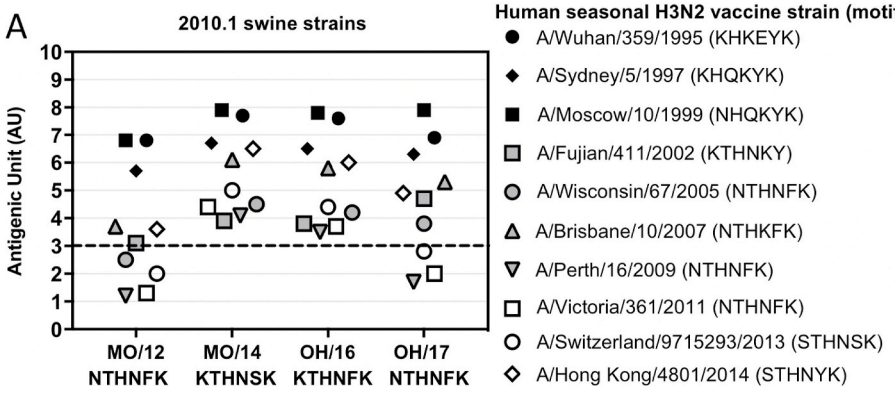

Fig. 2. Similarity of hemagglutinin proteins between 2010.1 swine and human seasonal H3. A. Antigenic distance between 2010.1 swine viruses and human seasonal H3N2 vaccine strains extracted from antigenic cartography. Pairwise hemagglutination inhibition (HI) titers were converted to a 3-dimensional map and antigenic distances between strains were estimated. One antigenic unit distance corresponds to a 2-fold difference in titer in assays and three antigenic units (dashed line) is equivalent to an 8-fold difference and a significant loss in HI cross-reactivity. **B.** Percent amino acid similarity for the swine and human vaccine strains tested for the full length mature HA. Although MO/12 and OH/17 have identical antigenic motifs, the HA are only 96% identical, the NA are of different lineages, and possess different internal gene constellations.

B Hemagglutinin amino acid % identity

MO/12	MO/14	OH/16	OH/17	Human seasonal H3N2 vaccine strain
91.5	91.0	91.0	89.9	A/Wuhan/359/1995
92.1	91.7	91.3	90.3	A/Sydney/5/1997
92.1	91.7	91.3	90.3	A/Moscow/10/1999
95.1	94.5	94.2	93.1	A/Fujian/411/2002
95.2	94.0	93.8	93.5	A/Wisconsin/67/2005
96.5	95.0	94.9	94.2	A/Brisbane/10/2007
96.6	95.2	95.2	94.5	A/Perth/16/2009
96.1	95.2	95.2	94.5	A/Victoria/361/2011
94.9	94.7	94.0	93.5	A/Switzerland/9715293/2013
94.7	94.2	94.0	93.1	A/Hong Kong/4801/2014
				2010.1 swine strains
100.0	96.8	96.3	95.6	A/swine/Missouri/A01476459/2012 (MO/12)
96.8	**100.0**	98.4	97.4	A/swine/Missouri/A01410819/2014 (MO/14)
96.3	98.4	**100.0**	98.9	A/swine/Ohio/16TOSU4788/2016 (OH/16)
95.6	97.4	98.9	**100.0**	A/swine/Ohio/A01354299/2017 (OH/17)

viruses had antigenically drifted from human IAV seasonal vaccines used over three decades (1995–2014). The impact of three different antigenic motifs with differences at position 145 and 159 in swine 2010.1H3 was assessed by HI assays and antigenic cartography (Fig. 2). A/swine/Ohio/16TOSU4788/2016 (OH/16) was representative of MO/15, MO/16 and SD/16, as they shared a common antigenic motif (Table 1) and the antigenic site B regions were identical. The more recent virus A/Ohio/A01354299/2017 (OH/17) had an identical antigenic motif as the early strain A/Missouri/A01476459/2012 (MO/12), but these two viruses shared less than 96% amino acid similarity within the HA (Fig. 2B), with differences in the globular HA1 head at residues 131, 132, 138, 144, 166.

Antigenic cartography analysis revealed that the first detected 2010.1 swine virus, MO/12, retained antigenic similarity to human seasonal IAV vaccine strains that were isolated between 2009 and 2011 (approximately 1 AU). MO/12 had highest sequence similarity to the 2007–2011 human vaccine strains with greater than 96% identity (Fig. 2B). Subsequent evolution in swine (represented by MO/14 and OH/16) resulted in viruses that were significantly different from all human vaccine strains tested (greater than 3 AU). A more recent

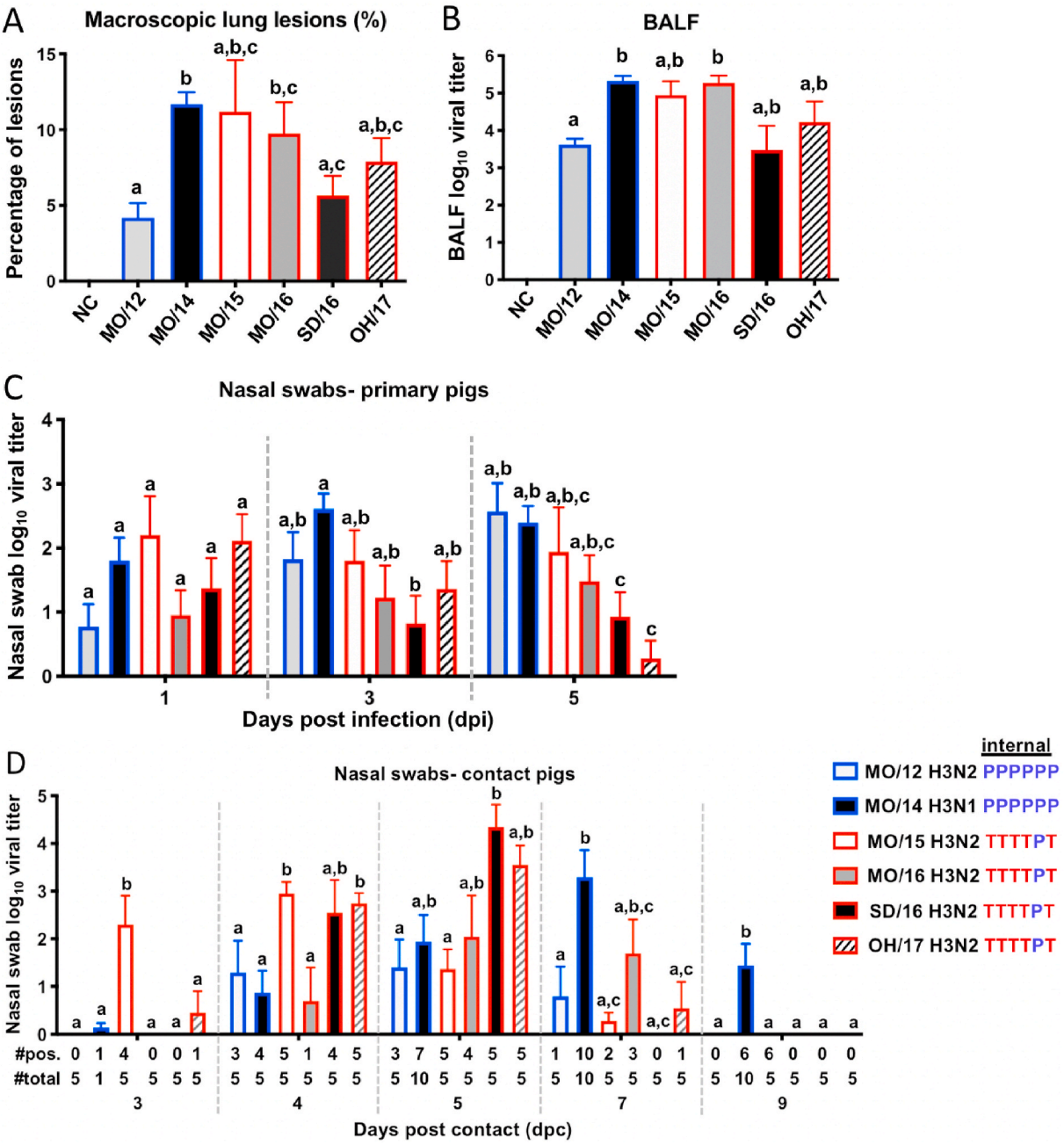

Fig. 3. Infection and transmission of human IAV A/Victoria/361/2011 and six 2010.1 swine H3 viruses in pigs. A. Percentage of macroscopic lung lesions were evaluated at 5 days post infection (dpi). **B.** Viral titers were measured in bronchoalveolar lavage fluid (BALF) at 5 dpi. **C-D.** Nasal swab samples were collected every other day with contact pigs placed in a separate pen in the same room at day 2. The number of contact pigs in which virus was recovered and total number of contact pigs are noted below the Fig. 3D axis. Data presented are group means±standard error of the means for all pigs in each respective group. Treatment group means with statistically significant differences ($P \leq 0.05$) are identified by different lowercase letters above the group mean bars (a, b, c, etc.). NC, not challenged-negative control. Internal gene constellations are indicated in the figure key, with P (pandemic in blue) and T (TRIG in red) internal genes in the order of PB2, PB1, PA, NP, M and NS.

representative strain, OH/17 had greater antigenic similarity to the 2009 and 2011 seasonal vaccine strains compared to MO/14 and OH/16 (less than 3 AU). OH/17 and MO/12 contained the antigenic motif NTHNFK, while OH/16 contained the motif KTHNFK, demonstrating the importance of position 145. All of the contemporary swine 2010.1 viruses tested were significantly distant to the A/Hong Kong/481/2014 human seasonal H3 vaccine component (Fig. 2A). Importantly, the continued drift of 2010.1H3 strains in swine with either an N or K at 145 resulted in significant loss of HI cross-reactivity to recent human seasonal vaccines (Rajao et al., 2015).

3.4. Infection and transmission of contemporary 2010.1 swine H3 viruses in pigs

Pigs were challenged with MO/12, MO/14, MO/15, MO/16, SD/16, or OH/17. The percentage of lung lesions at 5 days post infection (dpi) was assessed as a measure of relative virulence (Fig. 3A). Viral titers (5 dpi) from BALF (Fig. 3B) as well as nasal swab titers from primary challenged pigs (Fig. 3C) and indirect contact pigs placed in the room two days after initial infection were also assessed (Fig. 3D). As shown previously (Rajao et al., 2015), MO/14 induced significantly higher percentages of pneumonia than MO/12 (Fig. 3A) (11.7% vs. 4.2% lung lesion score) and had significantly higher BALF titers (5.3 log10 vs. 3.6 log10) (Fig. 3B), but similar peak nasal titers in inoculated pigs (Fig. 3C) (2.4 log10 on dpi 3 vs. 2.6 log10 on dpi 5). The third generation viruses of the 2010.1 lineage with the TTTTPT internal gene constellation (Fig. 3, highlighted in red) demonstrated equivalent virulence, with pneumonia lesions below 10% on average (Fig. 3A, right). Histopathological examination of lung sections for influenza-like microscopic lesions were all comparable. Out of a maximum score of 22, MO/12 group average score was 4.9 ± 0.5 compared to 9.4 ± 0.6 for MO/14, as reported previously (Rajao et al., 2015). More recent generation viruses had scores between MO/12 and MO/14 with 6.9 ± 2.7, 5.9 ± 2.6, and 2.8 ± 2.1 for MO/16, SD/16 and OH/17, respectively. The primary inoculated pigs in the SD/16 and OH/17 groups had lower nasal titers but higher nasal titers in the indirect contact pigs than earlier generations. The number of shedding contact pigs and titers differed between virus groups. Infectious virus was detected in all contact pigs in all groups by 5 dpc with the exception of MO/12 and MO/14, but the kinetics differed between virus groups. MO/12 demonstrated reduced transmission and titers overall, and MO/14 showed slower kinetics over the course of the experiment. Finally, OH/17 and SD/16, which encode HA's that differ from each other by only four amino acids (56, 138, 145, 209), including the K145N difference in the antigenic motif, were not significantly different to one another in percentage of lung lesions (Fig. 3A), BALF titers (Fig. 3B), or in transmission characteristics as assessed by peak viral titer in primary inoculated (Fig. 3C) and contact animals (Fig. 3D). Although group means of the 6 virus groups showed subtle differences in the parameters measured, all of the 2010.1H3 viruses representing different antigenic motifs and genotypes induced viral pneumonia typical of influenza, replicated in the upper and lower respiratory tract to typical titers, and efficiently transmitted by aerosol to contact pigs.

3.5. Proportion of 2010.1 detections vs. all swine H3 detections

The 2010.1 lineage H3N2 became frequently detected IAV in swine after late 2015. The 2010.1H3 gene was frequently in veterinary diagnostic laboratory data from ISU *FLU*ture, which aggregates USDA surveillance and private sequence data (Zeller et al., 2018). There was a total of 509 detections for 2010.1 lineage IAV documented from 2018 to 2020, compared to 315 detections for IAV with clade 1990.4H3 (Fig. 4).

4. Discussion

The current 2010.1 viruses have an HA gene derived from human seasonal H3 that circulated in humans in 2010–11, an NA gene that has circulated in swine since 2002, and most often have an internal gene constellation consisting of TRIG genes and a 2009H1N1 pandemic matrix gene (TTTTPT). During the period described here, there were no detections of viruses with human-seasonal H3N2 internal genes in swine. The first 2010.1 virus detected in swine (MO/12) had reassorted and acquired pandemic internal genes, suggesting that there are barriers for circulation of wholly human H3N2 viruses in pigs. This was further

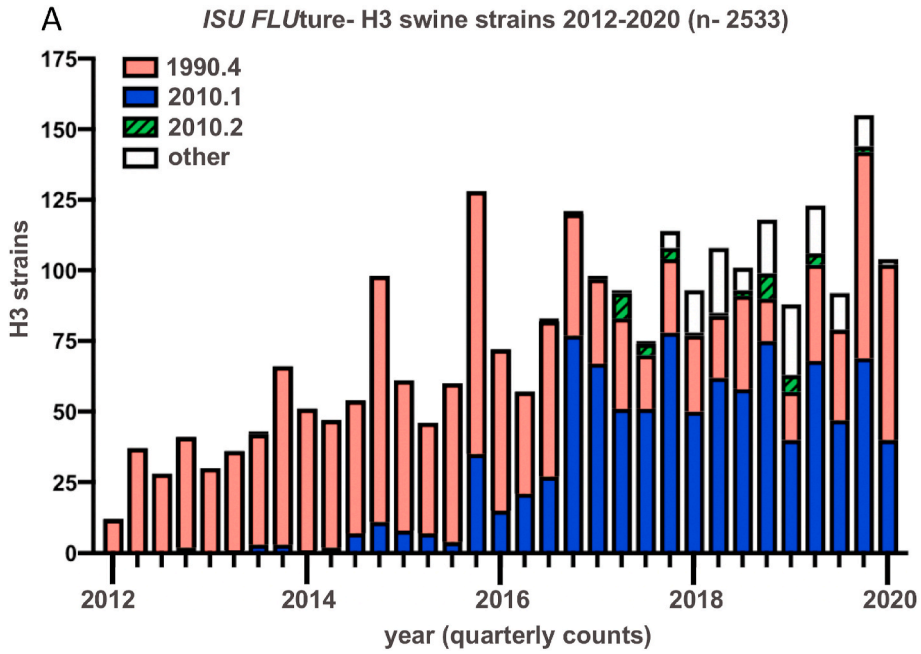

Fig. 4. United States H3 swine viruses collated by H3 clade from the *ISU FLU*ture database. Blue- 2010.1; Red- 1990.4 (Cluster-IV); Green- 2010.2 viruses detected during 2017–2019; White-other H3 (Cluster I or human-seasonal) HA detection events. Counts reflect accessed data on 05/20/2020 on https://influenza.cvm.iastate.edu.

supported by the findings in Rajao et al. (2015) that showed that the 2010–2011 human seasonal A/Victoria/361/2011 virus failed to transmit in pigs. Following human-to-swine spillover, adaptation through multiple rounds of reassortment with endemic swine IAV resulted in multiple 2010.1 virus genotypes, followed by mutation in the HA, and our results showed that they maintained similar virulence and transmission phenotypes. Specifically, transmission kinetics and peak titers of 2010.1H3 viruses collected in 2016 and 2017 were not different compared to swine 2010.1 from 2012 and 2014; further there was no significant difference in virulence over time except for the first generation virus.

The amino acid changes in each of the eight viral segments of 2010.1 viruses required for human viruses to become pig-adapted have not been identified. Rajao et al. (2015) demonstrated that a reverse engineered virus with the HA of A/Victoria/361/2011 combined with seven segments of the swine backbone of MO/14 was detected in nasal swabs at increased levels compared to the wholly-human A/Victoria/361/2011, but replication in the lower lung was minimal compared to the MO/14 virus and it did not transmit to respiratory contacts. MO/14 and A/Vic/11 HA share a common ancestor, but differed by 25 amino acids, nine of which were within five antigenically critical H3 sites (sites A to E) (Gerhard and Webster, 1978). The amino acid changes that were detected soon after the introduction of a human HA may have been associated with adaptation, changes in receptor-binding specificity, and sustained circulation of these 2010.1 viruses in pigs. However, immune pressure from existing H3 immunity in pigs induced from C-IV lineage viruses cannot be ruled out as possible pressure on the amino acid positions in the antigenic sites. Additionally, amino acid changes affecting antigenicity may also affect receptor-binding, with N145K shown to influence binding (Li et al., 2013). We were unable to demonstrate that genome constellations among the three generations of 2010.1 had an impact on transmission since detection of Generation 1 and 2 viruses in surveillance were extremely limited; however, after the human H3 reassorted with endemic swine viruses, transmission was sufficient for the lineage to be maintained. The first detection in 2012 contained genes from human seasonal and H1N1pdm09 viruses (human-origin HA and NA, with six internal pdm09-related segments (MO/12)), albeit the pdm09 internal genes were derived from swine IAV prior to 2009. However, the PPPPPP constellation is not frequently detected in US endemic swine IAV without H1N1pdm09 HA and NA. Shortly thereafter, two other reassortment episodes resulted in a virus with the 2010.1 human-like H3 that acquired a swine-adapted N2 (2002 lineage) and TRIG-dominant internal genes with H1N1pdm09 M (TTTTPT, represented by MO/15, MO/16, SD/16), an internal gene constellation that is frequently found in contemporary circulating swine IAV (Rajao et al., 2015; Gao et al., 2017). Nevertheless, the transmission and pathogenesis study in this paper demonstrated that all three generations of 2010.1 viruses replicated and transmitted in pigs with no significant increase observed for more recent viruses.

The continued evolution of 2010.1 viruses, both in their internal gene constellations and antigenic motif, should be closely monitored. Further antigenic drift of HA amino acids in the HA1 head domain increases the risk to the human population if zoonotic transmission occurs at the swine-human interface, as immunity due to vaccination or exposure in humans may not be protective. While the K145N mutation found in swine 2010.1H3N2 since 2017 demonstrated increased the antigenic similarity to the 2009–2013 human seasonal vaccine strains, the current generation of 2010.1 swine viruses containing either 145K or 145N were >3AU from the more recent A/Hong Kong/4801/2014 vaccine strain, indicating further antigenic evolution and risk to humans may occur in the future. These antigenic changes are also likely to impact swine vaccine effectiveness, and vaccination is still presently the most frequently utilized intervention strategy to prevent infection and spread of IAV in swine and limit spillover of these viruses into humans.

In summary, the contemporary swine 2010.1H3N2 viruses persisted and antigenically evolved in the U.S. pig population. This lineage of H3 viruses also infected more than 50 people in agricultural fairs in the U.S. in 2016 and 2017, and our data suggest that its antigenic drift may leave subsets of the human populations at higher risk from these viruses if human to human transmission occurred. Consequently, vaccination in swine may help prevent spillover into human populations. For swine, there have been significant strides made in the reporting of HA and NA, as well as a subset of whole genome sequences that circulate in swine in the United States due to the coordination and funding efforts of the USDA Influenza A Virus in Swine Surveillance System (Anderson et al., 2013). Additionally, tools such as ISU *FLU*ture provide real time visualization of trends in swine IAV sequence diversity and can be used to detect novel reassortment events (Zeller et al., 2018). As in-field diagnostics and surveillance methods become faster and more informative, along with advancement of convenient swine-influenza related online analysis tools (Zeller et al., 2018; Chang et al., 2019; Rambo--Martin et al., 2020; Anderson et al., 2016), bidirectional transmission events between humans and swine can be more rapidly detected and vaccines may be designed to better match antigenically circulating strains.

Declaration of competing interest

No conflict of interest exists.

Acknowledgments

We thank producers, swine veterinarians, and veterinary diagnostic laboratories for participating in the USDA Influenza A Virus in Swine Surveillance System. The authors thank Michelle Harland and Nicholas Otis for technical assistance with laboratory techniques, Jason Huegel, Justin Miller, Randy Leon, Alyssa Dannen and Keiko Sampson for animal care/necropsy, Zebulun Arendsee for bioinformatics assistance and Bryan Kaplan for critical review. This study was supported by USDA-ARS, USDA-APHIS, and by an NIH-National Institute of Allergy and Infectious Diseases (NIAID) interagency agreement (R21AI098079) associated with Center of Research in Influenza Pathogenesis, an NIAID funded Center of Excellence in Influenza Research and Surveillance (HHSN272201400008C). This research used resources provided by the SCINet project of the USDA Agricultural Research Service, ARS project number 0500-00093-001-00-D. JDP, CKS, and JC were supported in part by an appointment to the USDA-ARS 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 and USDA under contract number DE-AC05-06OR23100. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture, DOE, or ORISE. USDA is an equal opportunity provider and employer.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.11.006.

References

Abente, E.J., Santos, J., Lewis, N.S., Gauger, P.C., Stratton, J., Skepner, E., Anderson, T. K., Rajao, D.S., Perez, D.R., Vincent, A.L., 2016. The molecular determinants of antibody recognition and antigenic drift in the H3 hemagglutinin of swine influenza A virus. J. Virol. 90, 8266–8280.

Anderson, T.K., Nelson, M.I., Kitikoon, P., Swenson, S.L., Korslund, J.A., Vincent, A.L., 2013. Population dynamics of cocirculating swine influenza A viruses in the United States from 2009 to 2012. Influenza Other Respir Viruses 7 (Suppl. 4), 42–51.

Anderson, T.K., Macken, C.A., Lewis, N.S., Scheuermann, R.H., Van Reeth, K., Brown, I. H., Swenson, S.L., Simon, G., Saito, T., Berhane, Y., Ciacci-Zanella, J., Pereda, A., Davis, C.T., Donis, R.O., Webby, R.J., Vincent, A.L., 2016. A phylogeny-based global

nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza A viruses. mSphere 1.
Anderson, T.K., Chang, J., Arendsee, Z.W., Venkatesh, D., Souza, C.K., Kimble, J.B., Lewis, N.S., Davis, C.T., Vincent, A.L., 2020. Swine influenza A viruses and the tangled relationship with humans. Cold Spring Harb Perspect Med. https://doi.org/10.1101/cshperspect.a038737.
Bao, Y., Bolotov, P., Dernovoy, D., Kiryutin, B., Zaslavsky, L., Tatusova, T., Ostell, J., Lipman, D., 2008. The influenza virus resource at the national center for Biotechnology information. J. Virol. 82, 596–601.
Bolton, M.J., Abente, E.J., Venkatesh, D., Stratton, J.A., Zeller, M., Anderson, T.K., Lewis, N.S., Vincent, A.L., 2018. Antigenic Evolution of H3N2 Influenza A Viruses in Swine in the United States from 2012 to 2016. Influenza Other Respir Viruses. https://doi.org/10.1111/irv.12610.
Bowman, A.S., Walia, R.R., Nolting, J.M., Vincent, A.L., Killian, M.L., Zentkovich, M.M., Lorbach, J.N., Lauterbach, S.E., Anderson, T.K., Davis, C.T., Zanders, N., Jones, J., Jang, Y., Lynch, B., Rodriguez, M.R., Blanton, L., Lindstrom, S.E., Wentworth, D.E., Schiltz, J., Averill, J.J., Forshey, T., 2017. Influenza A(H3N2) virus in swine at agricultural fairs and transmission to humans, Michigan and Ohio, USA, 2016. Emerg. Infect. Dis. 23, 1551–1555.
Burke, D.F., Smith, D.J., 2014. A recommended numbering scheme for influenza A HA subtypes. PloS One 9, e112302.
Chang, J., Anderson, T.K., Zeller, M.A., Gauger, P.C., Vincent, A.L., 2019. octoFLU: automated classification for the evolutionary origin of influenza A virus gene sequences detected in U.S. Swine. Microbiol Resour Announc 8.
Duwell, M.M., Blythe, D., Radebaugh, M.W., Kough, E.M., Bachaus, B., Crum, D.A., Perkins Jr., K.A., Blanton, L., Davis, C.T., Jang, Y., Vincent, A., Chang, J., Abney, D. E., Gudmundson, L., Brewster, M.G., Polsky, L., Rose, D.C., Feldman, K.A., 2018. Influenza A(H3N2) variant virus outbreak at three fairs - Maryland, 2017. MMWR Morb. Mortal. Wkly. Rep. 67, 1169–1173.
Gao, S., Anderson, T.K., Walia, R.R., Dorman, K.S., Janas-Martindale, A., Vincent, A.L., 2017. The genomic evolution of H1 influenza A viruses from swine detected in the United States between 2009 and 2016. J. Gen. Virol. 98, 2001–2010.
Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., Lopez-Gatell, H., Olivera, H., Lopez, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson Jr., P.D., Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., et al., 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325, 197–201.
Gauger, P.C., Vincent, A.L., Loving, C.L., Lager, K.M., Janke, B.H., Kehrli Jr., M.E., Roth, J.A., 2011. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. Vaccine 29, 2712–2719.
Gauger, P.C., Vincent, A.L., Loving, C.L., Henningson, J.N., Lager, K.M., Janke, B.H., Kehrli Jr., M.E., Roth, J.A., 2012. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. Vet. Pathol. 49, 900–912.
Gauger, P.C., Vincent, A.L., 2014. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine. In: Spackman, E. (Ed.), Animal Influenza Virus. Springer, New York, NY, pp. 313–324. https://doi.org/10.1007/978-1-4939-0758-8_26.
Gerhard, W., Webster, R.G., 1978. Antigenic drift in influenza A viruses. I. Selection and characterization of antigenic variants of A/PR/8/34 (HON1) influenza virus with monoclonal antibodies. J. Exp. Med. 148, 383–392.
Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. 32, 648–660.
Kitikoon, P., Nelson, M.I., Killian, M.L., Anderson, T.K., Koster, L., Culhane, M.R., Vincent, A.L., 2013. Genotype patterns of contemporary reassorted H3N2 virus in US swine. J. Gen. Virol. 94, 1236–1241.
Kitikoon, P., Gauger, P.C., Vincent, A.L., 2014. Hemagglutinin inhibition assay with swine sera. Methods Mol. Biol. 1161, 295–301.
Koel, B.F., Burke, D.F., Bestebroer, T.M., van der Vliet, S., Zondag, G.C., Vervaet, G., Skepner, E., Lewis, N.S., Spronken, M.I., Russell, C.A., Eropkin, M.Y., Hurt, A.C., Barr, I.G., de Jong, J.C., Rimmelzwaan, G.F., Osterhaus, A.D., Fouchier, R.A., Smith, D.J., 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342, 976–979.
Lewis, N.S., Anderson, T.K., Kitikoon, P., Skepner, E., Burke, D.F., Vincent, A.L., 2014. Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J. Virol. 88, 4752–4763.
Li, Y., Bostick, D.L., Sullivan, C.B., Myers, J.L., Griesemer, S.B., Stgeorge, K., Plotkin, J.B., Hensley, S.E., 2013. Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering. J. Virol. 87, 9904–9910.
Nelson, M.I., Vincent, A.L., 2015. Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface. Trends Microbiol. 23, 142–153.
Nelson, M.I., Lemey, P., Tan, Y., Vincent, A., Lam, T.T., Detmer, S., Viboud, C., Suchard, M.A., Rambaut, A., Holmes, E.C., Gramer, M., 2011. Spatial dynamics of human-origin H1 influenza A virus in North American swine. PLoS Pathog. 7, e1002077.
Nelson, M.I., Stratton, J., Killian, M.L., Janas-Martindale, A., Vincent, A.L., 2015. Continual reintroduction of human pandemic H1N1 influenza A viruses into swine in the United States, 2009 to 2014. J. Virol. 89, 6218–6226.
Olsen, C.W., Karasin, A.I., Carman, S., Li, Y., Bastien, N., Ojkic, D., Alves, D., Charbonneau, G., Henning, B.M., Low, D.E., Burton, L., Broukhanski, G., 2006. Triple reassortant H3N2 influenza A viruses, Canada, 2005. Emerg. Infect. Dis. 12, 1132–1135.
Rajao, D.S., Gauger, P.C., Anderson, T.K., Lewis, N.S., Abente, E.J., Killian, M.L., Perez, D.R., Sutton, T.C., Zhang, J., Vincent, A.L., 2015. Novel reassortant human-like H3N2 and H3N1 influenza A viruses detected in pigs are virulent and antigenically distinct from swine viruses endemic to the United States. J. Virol. 89, 11213–11222.
R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Rajao, D.S., Anderson, T.K., Kitikoon, P., Stratton, J., Lewis, N.S., Vincent, A.L., 2018. Antigenic and genetic evolution of contemporary swine H1 influenza viruses in the United States. Virology 518, 45–54.
Rambo-Martin, B.L., Keller, M.W., Wilson, M.M., Nolting, J.M., Anderson, T.K., Vincent, A.L., Bagal, U.R., Jang, Y., Neuhaus, E.B., Davis, C.T., Bowman, A.S., Wentworth, D.E., Barnes, J.R., 2020. Influenza A virus field surveillance at a swine-human interface. mSphere 5.
Reed, LJ., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 493–497.
Smith, D.J., Lapedes, A.S., de Jong, J.C., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D., Fouchier, R.A., 2004. Mapping the antigenic and genetic evolution of influenza virus. Science 305, 371–376.
Smith, G.J., Bahl, J., Vijaykrishna, D., Zhang, J., Poon, L.L., Chen, H., Webster, R.G., Peiris, J.S., Guan, Y., 2009. Dating the emergence of pandemic influenza viruses. Proc. Natl. Acad. Sci. U. S. A. 106, 11709–11712.
Vincent, A.L., Ma, W., Lager, K.M., Janke, B.H., Richt, J.A., 2008. Swine influenza viruses a North American perspective. Adv. Virus Res. 72, 127–154.
Vincent, A.L., Ma, W., Lager, K.M., Richt, J.A., Janke, B.H., Sandbulte, M.R., Gauger, P. C., Loving, C.L., Webby, R.J., Garcia-Sastre, A., 2012. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease. J. Virol. 86, 10597–10605.
Vincent, A.L., Perez, D.R., Rajao, D., Anderson, T.K., Abente, E.J., Walia, R.R., Lewis, N. S., 2017. Influenza A virus vaccines for swine. Vet. Microbiol. 206, 35–44.
Webby, R.J., Swenson, S.L., Krauss, S.L., Gerrish, P.J., Goyal, S.M., Webster, R.G., 2000. Evolution of swine H3N2 influenza viruses in the United States. J. Virol. 74, 8243–8251.
Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., 2019. Welcome to the Tidyverse. Journal of Open Source Software 4 (43), 1686.
Zeller, M.A., Anderson, T.K., Walia, R.W., Vincent, A.L., Gauger, P.C., 2018. ISU FLUture: a veterinary diagnostic laboratory web-based platform to monitor the temporal genetic patterns of Influenza A virus in swine. BMC Bioinf. 19, 397.
Zhang, Y., Aevermann, B.D., Anderson, T.K., Burke, D.F., Dauphin, G., Gu, Z., He, S., Kumar, S., Larsen, C.N., Lee, A.J., Li, X., Macken, C., Mahaffey, C., Pickett, B.E., Reardon, B., Smith, T., Stewart, L., Suloway, C., Sun, G., Tong, L., Vincent, A.L., Walters, B., Zaremba, S., Zhao, H., Zhou, L., Zmasek, C., Klem, E.B., Scheuermann, R. H., 2017. Influenza Research Database: an integrated bioinformatics resource for influenza virus research. Nucleic Acids Res. 45, D466–D474.
Zhou, N.N., Senne, D.A., Landgraf, J.S., Swenson, S.L., Erickson, G., Rossow, K., Liu, L., Yoon, K., Krauss, S., Webster, R.G., 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J. Virol. 73, 8851–8856.