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Molecular epidemiology of swine influenza A viruses in the Southeastern United States, highlights regional differences in circulating strains

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Abstract

Swine influenza A virus (IAV) can cause widespread respiratory disease with high morbidity, low mortality, and have a substantial economic impact to the swine industry. Swine infection may contribute to pandemic IAV given their susceptibility to both avian and human IAVs. Currently, three IAV subtypes (H1N1, H3N2 and H1N2) circulate in swine in North America frequently combining gene segments from avian or human viruses. This study investigated the prevalence of IAV in commercial swine herds. A total of 1,878 oral fluid samples were collected from pigs of all ages from 201 commercial farms located in North Carolina and South Carolina. Sixty-eight oral fluid samples from 35 farms were positive by MP gene PCR with an overall IAV-positivity of 3.6%. On the herd level, the percentage of IAV positivity was 17.4%. Fifty-six viruses were subtyped, while 12 were partly subtyped or not subtyped at all. Using *de novo* assembly, complete sequences were obtained for 59 HA genes. The majority of IAVs subtyped had an H1 HA demonstrating a considerable prevalence over H3 viruses. Furthermore, only six out of eleven HA types were detected which has implications for the selection of vaccines used by swine producers in the region.

Keywords

Influenza A; Swine Influenza; H1N1; H3N2; H1N2; Haemagglutinin; Orthomyxovirus

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Introduction

Influenza A viruses (IAVs) are Orthomyxoviruses and are single-stranded RNA viruses with a segmented genome (Nicholson et al., 2003). The two surface proteins, haemagglutinin (HA) and neuraminidase (NA), facilitate the entry and release of the virus and they are the primary targets recognized by the immune system after infection and vaccination (Mori et al., 2002; Wiley and Skehel, 1987). Together, HA and NA determine IAV subtypes. The genetic and antigenic changes at these two genes result in virus alteration.

IAVs are among the leading respiratory pathogens in humans, typically causing the death of more than 500,000 people annually and substantial hospitalization (CDC). Waterfowl are the natural reservoir of IAVs; however, a wide range of species can be infected by IAVs such as domesticated poultry, humans, and swine (Ozawa and Kawaoka, 2013; Wahlgren, 2011). The zoonotic capability of IAVs and their potential to reassort have raised health concerns about IAVs emerging in animals and humans. For example, the 2009 H1N1 pandemic virus (pdmH1N1) highlighted the importance of swine in the ecology of IAVs and the protection of public health (Vincent et al., 2014). IAV in swine cause one of the most important viral diseases and place a heavy economic burden on the pork industry. In the US, the annual losses due to swine IAV are estimated between \$360 million and \$1 billion (Dykhuys Haden C et al., 2012; Holtkamp and Garcia, 2007). The clinical manifestation of the disease in swine range from asymptomatic to widespread respiratory disease characterized by abdominal breathing, coughing, sneezing, fever, anorexia and lethargy (Van Reeth et al., 2008). Three subtypes of IAVs have been identified in swine worldwide: H1N1, H3N2 and H1N2 (Kyriakis et al., 2011; Lewis et al., 2016; Nelson et al., 2015b; Simon et al., 2014). Until the late 1990s, only H1N1 virus was found in swine in North America. This IAV strain is known as the “classical swine” H1N1, and has directly descended from the 1918–19 pandemic virus. Since 1998, a variety of additional IAVs have been detected resulting from multiple reassortant events (Anderson et al., 2013; Gramer et al., 2007; Vincent et al., 2009; Webby et al., 2000). H1N1, H3N2 and H1N2 viruses with HA and NA of human origin and different combinations of internal genes have been circulating in the swine population. The so-called “triple reassortant cassette” that includes internal protein genes of human, avian and classical swine has become established as the dominant backbone of IAVs in swine with different combinations of HA and NA proteins. With the introduction of the pdmH1N1 virus to swine, additional reassortment has been observed in swine both in North America and Europe (Bowman et al., 2012; Watson et al., 2015). While the pdmH1N1 has not been able to become established in the swine, reassortant viruses that include one or more genes of pandemic origin are frequently isolated. In 2012–13 the introduction of human seasonal H3 gene in viruses with swine-origin NA and internal protein gene backbone was identified (Rajao et al., 2015). Overall, 11 genetically distinct HAs, including six H1 and five H3 IAVs, have been identified in swine in North America (Anderson et al., 2015; Lewis et al., 2014).

Given the need to better understand IAV epidemiology, a surveillance study in swine focusing on producers in North Carolina was undertaken. Swine production in the United States is characterized by a disproportionately high number of sow farms in North Carolina approaching 1 million sows, which annually wean about 22 million piglets (USDA, 2012).

Weaned piglets (age of 21 to 28 days) are subsequently shipped to the Midwest where they are raised until they are consumed at approximately six months of age. When pigs from different sources are mixed, IAVs have the opportunity to reassort, thus this region of the United States has an important role in the epidemiology of IAVs. In this study, we report the results of our active surveillance of IAVs in the swine population, including viral subtypes and HA gene types identified in the summer of 2014. In summary, a high prevalence of H1N1 and H1N2 viruses was observed compared to H3N2 IAVs, while H1 HA antigenic types were absent, thus these findings have important implications for vaccine selection.

Materials and Methods

Sampling

Between June and August 2014, we performed an active surveillance on swine farms in North Carolina and South Carolina. For active surveillance, clinical specimens are taken on a regular basis regardless of their health status. A total of 1,878 oral fluid samples were collected from 201 farms. Oral fluids are sampled by hanging a cotton rope in a swine pen, allowing the animals to chew on it, and then by collecting fluid that accumulate on the rope. Thus, each sample is not from an individual animal, but from multiple swine, which is an advantage of this sampling method (Decorte et al., 2015). While oral samples contain contaminants, such as faeces and feed, they are not the ideal medium for virus isolation, thus this approach has been proven to be efficient for pathogen screening in swine (Goodell et al., 2016).

Influenza A Virus (IAV) screening

RNA was extracted by the RNAzol RT method (Chomczynski et al., 2013). Briefly, 200µl of each clinical specimen was mixed with 500µl of an acid guanidinium thiocyanate-based commercial product (RNAzol® RT, Molecular Research Center, Inc. USA) and 200µl of molecular grade water. Following centrifugation at 12,000g for 15 minutes, 700µl of supernatant were transferred in a new tube and RNA was isolated after washing once with 100% isopropanol and twice with 75% ethanol. The RNA was re-suspended in 20µl molecular grade water.

Initial IAV screening was conducted by real-time reverse transcription PCR (RRT-PCR) selective for the matrix protein (MP) gene. Sequences for the primers and probe were based on the assay described (Spackman et al., 2002) (Richt et al., 2004). For each reaction, 0.5 pmol of each primer and 0.1µM of probe were combined with 6.75µl of OneStep RRT-PCR Master Mix (Taqman® Fast Virus OneStep Master Mix, Thermo Fisher Scientific, USA) and 2µl of each sample containing 200ng of RNA. Molecular grade water was added to reach a total volume of 25µl. RRT-PCR was carried out using the Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara USA). The reverse transcription (RT) step was set at 50°C for 20 minutes, followed by 10 minutes of RT inactivation at 95°C. The PCR cycling protocol included 40 cycles of 95°C for 10 seconds and 50°C for 20 seconds. Ct values <32 were considered positive. RRT-PCR positive samples were subtyped by Multiplex RRT-PCR and further investigated by Next Generation Sequencing to determine the HA type.

IAV subtyping

The multiplex RRT-PCR for swine IAV subtyping was based on the method described by Zhang and Harmon (Zhang and Harmon, 2014). Two separate RRT-PCR reactions were performed one for the HA and the other for the NA type. The VetMAX™-Gold SIV Detection Kit (Life Technologies, USA) was used which included H1H3 and N1N2 primer-probe mixes. In summary, 6µl of OneStep RRT-PCR Master Mix (Taqman® Fast Virus OneStep Master Mix, Thermo Fisher Scientific, USA) and 8µl of sample of RNA were combined with 1µl of H1H3 or N1N2 primer-probe mix and 10µl molecular grade water to reach a volume of 25µl. The thermocycler was set at following cycles: one RT step at 50°C for 20 minutes, 10 minutes of RT inactivation at 95°C and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Ct values between 24 and 30 in the VIC channel were regarded as positive for H1 and N1 and in the FAM channel for H3 and N2.

Next Generation Sequencing (NGS)

The MiSeq Platform (Illumina, USA) was used for Next Generation Sequencing (NGS) and as described by Rutvisuttinunt and coworkers (Rutvisuttinunt et al., 2013). For sample preparation, cDNA synthesis was performed using Superscript III RT (Life Technologies, USA) combined with Uni-12 primer (AGCAAAGCAGG), set at the thermocycler at 65°C for 5 minutes and then placed on ice for at least 1 minute. RNaseOUT (Life Technologies, USA) and Superscript III RT were then added to the reaction and incubated at 50°C for 60 minutes, then inactivated at 70°C for 15 minutes. Influenza genome amplification was performed by PCR, combining the cDNA with Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, USA) and using an influenza-specific universal set of primers at a concentration of 0.1µM per primer (Hoffmann et al., 2001). Prior to library preparation, the Agilent 2200 TapeStation (Agilent Technologies, USA) was used for quantification cDNA. AMPure XP beads were used for DNA purification at the end of every step. For the library preparation we used the Nextera DNA Library Preparation kit (Illumina, USA) and followed the manufacturer's protocol. Finally, samples were pooled together and loaded into the cartridge of the Illumina MiSeq Reagent Kit V2. Sequencing was performed as multiplex double read libraries for 400 cycles.

The sequencing adaptor and unpaired ends were removed by using the Trimmomatic package (Bolger et al., 2014). To ensure the high quality of reads, a score of 30 was used as the filter threshold. Assembly was performed in the Iterative Virus Assembler, which has shown outperformance relative to existing virus de novo assemblers (Hunt et al., 2015). Assembled sequences were then subject to HA classification in a local BLAST search, which background reference is listed in Table 1. The best BLAST hit was used to classify HA.

Results

Of the 1,878 oral fluid samples examined, 68 (3.6%) were positive by the MP gene RRT-PCR. These samples came from 35 of 201 farms, which indicates 17.4% (35/201) IAV prevalence in the swine herds. Table 2 shows the subtypes of swine IAVs identified by multiplex RRT-PCR. Of the 68 positive samples, 56 were subtyped, while 6 samples were

partially subtyped and the remainder were not able to be genotyped. The major IAV subtype identified was H1N1 (35.3%), followed by H1N2 (23.5%) and H3N2 (16.2%). No H3N1 virus was identified in our study. Interestingly, 5 samples showed a mixed infection with two or more subtypes of IAV, which may create potential for reassortment events. Sequencing rendered 59 full-length HA sequences as shown in Figure 1, which demonstrates their HA classification by North American lineages. Of the 46 H1 HAs, 37 were classified as classical gamma H1, 5 as human-origin delta 1 H1, 2 as human-origin delta 2, and 2 as pandemic H1 HAs. All 13 identified H3 HAs belong to cluster IV H3. No classical alpha or beta H1 HAs or cluster I, II, III and novel human-like H3 HAs were identified. Overall, 3.6% of individual samples were IAV-positive samples, while IAVs were circulating in 17.4% of the farms. While clinical manifestation of swine IAV was been documented from October to June, IAV may be present year-round. The percentage of positive samples, while it may appear low, is comparable with other IAV active surveillance studies conducted in swine in the Midwest (Corzo et al., 2013).

Discussion

An important issue addressed by this study was the practical application of oral fluids as a sampling method for surveillance of IAV. While IAVs were detected and subtyped, no virus was isolated from RRT-PCR positive samples in cell culture. This may in part be attributed to how samples were collected for porcine respiratory and reproductive syndrome (PRRS) virus monitoring that was routinely done on the farms where it was stored at 4°C for at least three weeks before tested for IAV. Also, oral fluid samples contain environmental contaminants, like urine, faeces and feed, but also saliva, which may inactivate virus. However, studies have indicated that IAV can be isolated in most cases (>75%) when processing of oral fluid samples is initiated within 48 hours of collection. However, performing NGS for whole genome sequencing directly from oral fluid samples may be problematic as the isolated material contains RNA from swine cells, bacteria, fungi and other viruses.

The subtyping data showed a high prevalence of H1 over H3 IAVs. Specifically, 47 of 60 samples that were fully or partially subtyped were identified as containing H1 IAV. However, only 16 samples contained an H3 IAV, while mixed infections containing an H1N1 and an H3N2 virus were found in 3 samples. The multiplex method used in this investigation of swine IAVs was robust and used to identify the majority of the MP-gene positive samples (56 out of 68), while it either partly identified or did not identify all the remaining 12.

NGS analysis of the HA genes revealed that of the eleven genetically and antigenically distinct HAs identified in North America, only five were detected in the North or South Carolina. Specifically, classical gamma H1, human-like delta 1 and delta 2 H1, pandemic H1 and cluster IV H3 HAs. This is in contrast, with the other swine-producing areas of the USA, such as the Midwest, where classical alpha and beta H1 HAs are frequently isolated (Corzo et al., 2013; Kitikoon et al., 2013). Consequently, the findings of this study are of significant importance for proper vaccine selection. Commercial swine IAV vaccines contain between two and six inactivated strains (Van Reeth and Ma, 2013), which offer protection against viruses within the same HA lineage. Additionally, possible mismatch between

vaccine and wild type HA may lead to vaccine-associated enhanced respiratory disease (Gauger et al., 2012; Rajao et al., 2016). Swine in North Carolina and South Carolina should be immunized with IAV vaccines containing the HA lineage strains that are circulating in the region. However, the recent epidemiological history of swine IAVs has shown that the prevalence of different virus strains can periodically change and viruses can rapidly spread on a regional level. For this reason, long-term IAV surveillance studies are needed for monitoring and selecting adequate prevention measures, but also for the detection of novel viruses that may potentially infect humans.

Finally, the genetic makeup of the pandemic H1 HAs analyzed in this study and their close relationship with seasonal human IAVs, strongly suggest that these viruses do not continuously circulate within the swine population, but are rather a result of a single or multiple reverse zoonotic events as it has been observed before (Nelson et al., 2015a). In order to avoid the constant reintroductions of pdmH1N1 viruses from humans to swine and possible reassortment between human and swine IAVs, it is important for the industry to discourage workers with influenza-like symptoms to come into contact with animals.

Conclusions

Active surveillance for IAV in swine in North Carolina and South Carolina revealed positivity rates of 3.6% on sample and 17.4% on herd levels, indicating continuous circulation of IAVs in swine year round. Overall, a higher prevalence of H1 over H3 IAVs was recorded, while only six out of eleven antigenically distinct haemagglutinin lineages identified in North America were detected, highlighting the importance of proper vaccine selection in order to match circulating and vaccine strains.

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References

- Anderson TK, Campbell BA, Nelson MI, Lewis NS, Janas-Martindale A, Killian ML, Vincent AL. Characterization of co-circulating swine influenza A viruses in North America and the identification of a novel H1 genetic clade with antigenic significance. *Virus Res.* 2015; 201:24–31. [PubMed: 25701742]
- Anderson TK, Nelson MI, Kitikoon P, Swenson SL, Korslund JA, Vincent AL. Population dynamics of cocirculating swine influenza A viruses in the United States from 2009 to 2012. *Influenza Other Respir Viruses.* 2013; 7(Suppl 4):42–51.
- Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014; 30:2114–2120. [PubMed: 24695404]
- Bowman AS, Sreevatsan S, Killian ML, Page SL, Nelson SW, Nolting JM, Cardona C, Slemons RD. Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A viruses at an Ohio agricultural fair, July 2012. *Emerg Microbes Infect.* 2012; 1:e33. [PubMed: 26038404]
- CDC. Influenza;1; (Flu). 2016. <http://www.cdc.gov/flu/>
- Chomczynski P, Wilfinger W, Kennedy A, Rymaszewski M, Mackey K. RNAzol [reg] BD: a reagent for the effective isolation of RNA from whole blood. *Nature Methods.* 2013

- Corzo CA, Culhane M, Juleen K, Stigger-Rosser E, Ducatez MF, Webby RJ, Lowe JF. Active surveillance for influenza A virus among swine, midwestern United States, 2009–2011. *Emerg Infect Dis.* 2013; 19:954–960. [PubMed: 23735740]
- Decorte I, Steensels M, Lambrecht B, Cay AB, De Regge N. Detection and Isolation of Swine Influenza A Virus in Spiked Oral Fluid and Samples from Individually Housed, Experimentally Infected Pigs: Potential Role of Porcine Oral Fluid in Active Influenza A Virus Surveillance in Swine. *PloS one.* 2015; 10:e0139586. [PubMed: 26431039]
- Dykhuis Haden C P, Fangman T, Holtkamp D. Assessing production parameters and economic impact of swine influenza, PRRS and *Mycoplasma hyopneumoniae* on finishing pigs in a large production system. *Proceedings AASV American Association Swine Veterinarians Annual Meeting.* 2012:75–76.
- Gauger PC, Vincent AL, Loving CL, Henningson JN, Lager KM, Janke BH, Kehrli ME Jr, Roth JA. 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.* 2012; 49:900–912. [PubMed: 22461226]
- Goodell CK, Prickett J, Kittawornrat A, Johnson J, Zhang J, Wang C, Zimmerman JJ. Evaluation of Screening Assays for the Detection of Influenza A Virus Serum Antibodies in Swine. *Transbound Emerg Dis.* 2016; 63:24–35. [PubMed: 24571447]
- Gramer MR, Lee JH, Choi YK, Goyal SM, Joo HS. Serologic and genetic characterization of North American H3N2 swine influenza A viruses. *Can J Vet Res.* 2007; 71:201–206. [PubMed: 17695595]
- Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol.* 2001; 146:2275–2289. [PubMed: 11811679]
- Holtkamp DR, Garcia R. The economic cost of major health challenges in large U.S swine production systems. *Proceedings AASV American Association Swine Veterinarians Annual Meeting.* 2007:85–89.
- Hunt M, Gall A, Ong SH, Brenner J, Ferns B, Goulder P, Nastouli E, Keane JA, Kellam P, Otto TD. IVA: accurate de novo assembly of RNA virus genomes. *Bioinformatics.* 2015; 31:2374–2376. [PubMed: 25725497]
- Kitikoon P, Nelson MI, Killian ML, Anderson TK, Koster L, Culhane MR, Vincent AL. Genotype patterns of contemporary reassorted H3N2 virus in US swine. *J Gen Virol.* 2013; 94:1236–1241. [PubMed: 23695819]
- Kyriakis CS, Brown IH, Foni E, Kuntz-Simon G, Maldonado J, Madec F, Essen SC, Chiapponi C, Van Reeth K. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. *Zoonoses Public Health.* 2011; 58:93–101. [PubMed: 20042068]
- Lewis NS, Anderson TK, Kitikoon P, Skepner E, Burke DF, Vincent AL. Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. *J Virol.* 2014; 88:4752–4763. [PubMed: 24522915]
- Lewis NS, Russell CA, Langat P, Anderson TK, Berger K, Bielejec F, Burke DF, Dudas G, Fonville JM, Fouchier RA, Kellam P, Koel BF, Lemey P, Nguyen T, Nuansrichy B, Peiris JM, Saito T, Simon G, Skepner E, Takemae N, consortium, E. Webby RJ, Van Reeth K, Brookes SM, Larsen L, Watson SJ, Brown IH, Vincent AL. The global antigenic diversity of swine influenza A viruses. *Elife.* 2016; 5:e12217. [PubMed: 27113719]
- Mori I, Yokochi T, Kimura Y. Role of influenza A virus hemagglutinin in neurovirulence for mammals. *Med Microbiol Immunol.* 2002; 191:1–4. [PubMed: 12137193]
- Nelson MI, Stratton J, Killian ML, Janas-Martindale A, Vincent AL. Continual Reintroduction of Human Pandemic H1N1 Influenza A Viruses into Swine in the United States, 2009 to 2014. *J Virol.* 2015a; 89:6218–6226. [PubMed: 25833052]
- Nelson MI, Viboud C, Vincent AL, Culhane MR, Detmer SE, Wentworth DE, Rambaut A, Suchard MA, Holmes EC, Lemey P. Global migration of influenza A viruses in swine. *Nat Commun.* 2015b; 6:6696. [PubMed: 25813399]
- Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet.* 2003; 362:1733–1745. [PubMed: 14643124]

- Ozawa M, Kawaoka Y. Cross talk between animal and human influenza viruses. *Annu Rev Anim Biosci.* 2013; 1:21–42. [PubMed: 25387011]
- Rajao DS, Chen H, Perez DR, Sandbulte MR, Gauger PC, Loving CL, Shanks GD, Vincent A. Vaccine-associated enhanced respiratory disease is influenced by haemagglutinin and neuraminidase in whole inactivated influenza virus vaccines. *J Gen Virol.* 2016; 97:1489–1499. [PubMed: 27031847]
- Rajao DS, Gauger PC, Anderson TK, Lewis NS, Abente EJ, Killian ML, Perez DR, Sutton TC, Zhang J, Vincent AL. 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.* 2015; 89:11213–11222. [PubMed: 26311895]
- Richt JA, Lager KM, Clouser DF, Spackman E, Suarez DL, Yoon KJ. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. *J Vet Diagn Invest.* 2004; 16:367–373. [PubMed: 15460317]
- Rutvisuttinunt W, Chinnawirotpisan P, Simasathien S, Shrestha SK, Yoon IK, Klungthong C, Fernandez S. Simultaneous and complete genome sequencing of influenza A and B with high coverage by Illumina MiSeq Platform. *J Virol Methods.* 2013; 193:394–404. [PubMed: 23856301]
- Simon G, Larsen LE, Durrwald R, Foni E, Harder T, Van Reeth K, Markowska-Daniel I, Reid SM, Dan A, Maldonado J, Huovilainen A, Billinis C, Davidson I, Agüero M, Vila T, Herve S, Breum SO, Chiapponi C, Urbaniak K, Kyriakis CS, consortium, E. Brown IH, Loeffen W. European surveillance network for influenza in pigs: surveillance programs, diagnostic tools and Swine influenza virus subtypes identified in 14 European countries from 2010 to 2013. *PloS one.* 2014; 9:e115815. [PubMed: 25542013]
- Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol.* 2002; 40:3256–3260. [PubMed: 12202562]
- USDA. Hog and Pig Farming Highlights - Census of agriculture 2012. 2012
- Van Reeth, K., Brown, IH., Olsen, CW. Influenza Virus. In: Zimmerman, JJ. Karriker, LA. Ramirez, A. Schwartz, KJ., Stevenson, GW., editors. *Diseases of Swine.* Wiley-Blackwell; 2008. p. 557–571.
- Van Reeth K, Ma W. Swine influenza virus vaccines: to change or not to change—that's the question. *Curr Top Microbiol Immunol.* 2013; 370:173–200. [PubMed: 22976350]
- Vincent A, Awada L, Brown I, Chen H, Claes F, Dauphin G, Donis R, Culhane M, Hamilton K, Lewis N, Mumford E, Nguyen T, Parchariyanon S, Pasick J, Pavade G, Pereda A, Peiris M, Saito T, Swenson S, Van Reeth K, Webby R, Wong F, Ciacci-Zanella J. Review of influenza A virus in swine worldwide: a call for increased surveillance and research. *Zoonoses Public Health.* 2014; 61:4–17. [PubMed: 23556412]
- Vincent AL, Ma W, Lager KM, Gramer MR, Richt JA, Janke BH. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. *Virus Genes.* 2009; 39:176–185. [PubMed: 19597980]
- Wahlgren J. Influenza A viruses: an ecology review. *Infect Ecol Epidemiol.* 2011; 1
- Watson SJ, Langat P, Reid SM, Lam TT, Cotten M, Kelly M, Van Reeth K, Qiu Y, Simon G, Bonin E, Foni E, Chiapponi C, Larsen L, Hjulsgaard C, Markowska-Daniel I, Urbaniak K, Durrwald R, Schlegel M, Huovilainen A, Davidson I, Dan A, Loeffen W, Edwards S, Bublot M, Vila T, Maldonado J, Valls L, consortium, E. Brown IH, Pybus OG, Kellam P. Molecular Epidemiology and Evolution of Influenza Viruses Circulating within European Swine between 2009 and 2013. *J Virol.* 2015; 89:9920–9931. [PubMed: 26202246]
- Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. Evolution of swine H3N2 influenza viruses in the United States. *J Virol.* 2000; 74:8243–8251. [PubMed: 10954521]
- Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual review of biochemistry.* 1987; 56:365–394.
- Zhang J, Harmon KM. RNA extraction from swine samples and detection of influenza A virus in swine by real-time RT-PCR. *Methods in molecular biology.* 2014; 1161:277–293. [PubMed: 24899437]

Highlights

- ▶ Active surveillance for IAVs in swine showed a positivity rate 17.4% on herd level.
- ▶ Higher prevalence of H1 over H3 viruses was recorded.
- ▶ Phylogenetic analysis of pdmH1 HAs revealed the reverse zoonotic events.
- ▶ Genetic analyses of circulating strains suggest the use of region-specific vaccines.

Figure 1.
Classification of hemagglutinin genes by North American lineages.

B **Figure 2.**

Figure 3.

Table 1

Reference sequences used for hemagglutinin classification.

HA Type	Lineage		Reference Virus	HA accession no.
H1	Classical	Alpha	A/swine/Minnesota/02053/2008	CY099119
		Beta	A/swine/Texas/01522/2007	CY157999
		Gamma	A/swine/North Carolina/02403/2008	CY158217
	Human-like	Delta 1	A/swine/Ohio/A01279503/2012	KC355809
		Delta 2	A/swine/Indiana/A01049964/2011	JN652518
	Pandemic		A/California/07/2009	NC026433
H3	Cluster	I	A/swine/Minnesota/593/1999	AF251427
		II	A/swine/Colorado/23619/1999	AF268128
		III	A/swine/Illinois/21587/1999	AF268124
		IV	A/swine/Iowa/01700/2007	CY099027
	Novel human-origin		A/swine/Missouri/A01476459/2012	KP137795

Table 2

Swine influenza A viruses identified by multiplex RRT-PCR.

Number of samples (percent)	
Single infection:	
H1N1	24 (35.3)
H3N2	11 (16.2)
H1N2	16 (23.5)
Mixed infection:	
H1N1 and H3N2	3 (4.4)
H1N1 and H1N2	2 (2.9)
Partial subtype:	
H1Nx	2 (2.9)
H3Nx	2 (2.9)
HxN1	1 (1.5)
HxN2	1 (1.5)
No subtype identified:	6 (8.8)

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