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ORIGINAL ARTICLE

Influenza A Virus Surveillance Based on Pre-Weaning Piglet Oral Fluid Samples

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Summary

Influenza A virus (IAV) surveillance using pre-weaning oral fluid samples from litters of piglets was evaluated in four ~12 500 sow and IAV-vaccinated, breeding herds. Oral fluid samples were collected from 600 litters and serum samples from their dams at weaning. Litter oral fluid samples were tested for IAV by virus isolation, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), RT-PCR subtyping and sequencing. Commercial nucleoprotein (NP) enzyme-linked immunosorbent assay (ELISA) kits and NP isotype-specific assays (IgM, IgA and IgG) were used to characterize NP antibody in litter oral fluid and sow serum. All litter oral fluid specimens (n = 600) were negative by virus isolation. Twenty-five oral fluid samples (25/600 = 4.2%) were qRT-PCR positive based on screening (Laboratory 1) and confirmatory testing (Laboratory 2). No hemagglutinin (HA) and neuraminidase (NA) gene sequences were obtained, but matrix (M) gene sequences were obtained for all qRT-PCR-positive samples submitted for sequencing (n = 18). Genetic analysis revealed that all M genes sequences were identical (GenBank accession no. KF487544) and belonged to the triple reassortant influenza A virus M gene (TRIG M) previously identified in swine. The proportion of IgM- and IgA-positive samples was significantly higher in sow serum and litter oral fluid samples, respectively ($P < 0.01$). Consistent with the extensive use of IAV vaccine, no difference was detected in the proportion of IgG- and blocking ELISA-positive sow serum and litter oral fluids. This study supported the use of oral fluid sampling as a means of conducting IAV surveillance in pig populations and demonstrated the inapparent circulation of IAV in piglets. Future work on IAV oral fluid diagnostics should focus on improved procedures for virus isolation, subtyping and sequencing of HA and NA genes. The role of antibody in IAV surveillance remains to be elucidated, but longitudinal assessment of specific antibody has the potential to provide information regarding patterns of infection, vaccination status and herd immunity.

Introduction

Influenza A virus (IAV) is infectious for a wide variety of vertebrate hosts and is important as a cause of acute respiratory disease in humans and domestic animal species (Vincent et al., 2008). Historically, IAV has been associated with major epidemics in humans, horses, poultry and swine (Morens and Taubenberger, 2010). Influenza A virus is an enveloped RNA virus composed of eight negative-sense, single-stranded genomic segments that undergo rapid genetic evolution via point mutation and genetic reassortment (Brown, 2000). Genetically diverse, IAV subtype

classification is based on two external surface proteins, hemagglutinin (HA) and neuraminidase (NA). HA facilitates the attachment of the virus to epithelial cells in the respiratory tract via sialic acid (SA) molecules bound to galactose (Gal). Among its functions, NA cleaves SA from cells to release progeny virus. To date, 16 HAs and 9 NAs have been identified in avian species, with two additional subtypes (H17N10 and H18N11) recently identified in bats (Tong et al., 2012, 2013; Zhu et al., 2012; Mehle, 2014). HAs from avian IAVs preferentially bind to SAα2,3-Gal receptors, while HAs from mammalian IAVs have an affinity for SAα2,6-Gal receptors (Matrosovich et al., 1999; Gagneux et al., 2003). This is important because differences in the distribution of SA α2,3-Gal and SA α2,6-Gal receptors in the respiratory tract reflect host species susceptibility to IAVs. In quail (*Coturnix coturnix*), pheasants (ring-necked pheasants, *Phasianus colchicus*), chickens (*Gallus gallus*) and Peking ducks (*Anas platyrhynchos domestica*), both SAα2,3-Gal and SAα2,6-Gal receptors are present throughout the respiratory system (Yu et al., 2011). In contrast, SAα2,6-Gal receptors predominate in the upper respiratory tracts of humans and pigs and SAα2,3-Gal receptors in the lower (Shinya et al., 2006; Nelli et al., 2010).

Influenza A virus in swine is a public health issue because of concerns that reassortants originating in pigs could spill over to susceptible human populations. However, IAV in swine is also an economic issue because of its impact on productivity and a welfare issue because of its impact on pig health and productivity. Control of IAV in swine populations is complicated by the fact that the virus is endemic in contemporary herds and may circulate in any age group, including suckling pigs (Corzo et al., 2012). Commonly, more than one subtype circulates concurrently in a population (Corzo et al., 2013). Ultimately, the control of IAV in swine will rely on effective interventions based on a sound understanding of the ecology of IAV in contemporary swine production systems. This fundamental understanding must be based on data collected from commercial swine herds. Traditionally, IAV surveillance has been based on collecting and testing individual pig nasal swab and/or serum samples, but the labour and cost of this approach makes it unacceptable for routine use. A more efficient and effective surveillance system could be based on collecting and testing oral fluid specimens. Previous research showed that both IAV and anti-IAV antibody can be detected in oral fluid specimens using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively (Detmer et al., 2011; Ramirez et al., 2012; Romagosa et al., 2012; Goodell et al., 2013, 2014; Panyasing et al., 2014). Therefore, the purpose of the current study was to evaluate the feasibility of IAV surveillance in breeding herds using pre-weaning oral fluid samples from litters of piglets.

Materials and Methods

Experimental design

In four IAV-vaccinated commercial swine herds, oral fluid samples were collected from 600 litters 24 h prior to weaning and serum samples from their dams ≤48 h later. Thereafter, samples were completely randomized within specimen type and tested. Oral fluid samples were assayed for IAV by virus isolation and qRT-PCR, followed by subtyping and sequencing on qRT-PCR-positive samples. Both sow serum and litter oral fluid specimens were tested for IAV nucleoprotein (NP) antibody using a commercial NP blocking ELISA (IDEXX influenza A Ab Test; IDEXX Laboratories, Inc., Westbrook, ME, USA) and NP isotype-specific indirect ELISAs (IgM, IgA and IgG). Descriptive and comparative statistical analyses were used to evaluate and compare quantitative and qualitative results in serum and oral fluid samples, as well as significant associations with animal and herd factors.

Animals and animal care

The study was conducted under the approval of the Iowa State University Institutional Animal Care and Use Committee (#8-11-7202-S). The study was performed in four ~12 500 sow-breeding herds located in Oklahoma, USA. Farm A was located ~0.8 km (0.5 miles) from Farm B and Farm C the same distance from Farm D. Farms A/B were ~9.7 km (6 miles) from farms C/D. Gilts sources, animals housing, feeding, handling and veterinary care were under the supervision of Seaboard L.L.C. Health Assurance and Welfare personnel. All four herds were considered to be endemically infected with IAV on the basis of their diagnostic history. Replacement gilts were routinely vaccinated with autogenous IAV vaccine (Newport Laboratories, Worthington, MN, USA) at approximately 9, 12 and 24 weeks of age and again 1-week post-farrowing.

Sample collections

Six-hundred pairs of sow serum and pre-weaning litter oral fluid samples were collected between May and August by farm personnel (Table 1). Piglets averaged 17 days of age (range: 15–19 days) at the time of collection. Oral fluid samples were collected from litters 1 day prior to weaning by suspending unbleached cotton rope (1.25 cm, 0.5 in) in the area of the heat mat and within access of the piglets. When the material was saturated (15 min to 2 h), oral fluid was extracted by manually squeezing the rope while inside a plastic bag. Thereafter, the sample was transferred into a tube, held at 4°C and shipped to the laboratory on the following day. At the laboratory, samples were aliquoted into 5-ml cryogenic vials and stored at −80°C. Within 48 h of

litter oral fluid collection, sows were bled using a single-use blood collection system (Corvac®, Tyco Healthcare Group LP, Mansfield, MA, USA) and samples were shipped overnight to the laboratory. At the laboratory, samples were centrifuged at 1000 ***g*** for 10 min, after which the serum was aliquoted into 5-ml cryogenic vials and stored at −80°C. When all samples had been collected, they were completely randomized and tested.

Virus isolation (oral fluid)

Confluent monolayers of MDCK cells were prepared in 48-well plates (Costar; Corning, Corning, NY, USA). Cell culture media were removed, and monolayers were washed three times with IAV wash solution composed of minimal essential medium with Earle's salts (MEM; Sigma-Aldrich, St. Louis, MO, USA), 3× antibiotic–antimycotic solution [penicillin (300 IU/ml; Sigma-Aldrich), streptomycin (300 μg/ml; Sigma-Aldrich), gentamicin (150 μg/ml; Sigma-Aldrich), amphotericin B (0.75 μg/ml; Gibco, Grand Island, NY, USA)] and TPCK-treated trypsin (2 μg/ml; Sigma-Aldrich). Prior to inoculation onto MDCK cells, 0.35 μl of antibiotic–antimycotic solution was added to each 1 ml of oral fluid, after which samples were held at room temperature for 1 h. Each oral fluid sample was divided among 3 wells, that is ~0.4 ml/well, and then incubated at 37°C with 5% CO_2 for 2 h, after which the inoculum was removed. Cell monolayers were rinsed three times with the IAV wash solution, and then, 0.4 ml IAV post-inoculation media composed of MEM with Earle's salts, 3× antibiotic–antimycotic solution and TPCK-treated trypsin (1.5 μg/ml) were added and cell cultures were incubated for up to 5 days. Cell cultures were evaluated for the appearance of cytopathic effect (CPE) daily. If CPE was present, cell culture fluid was tested for HA activity and HA-positive cell culture fluids were tested for IAV by qRT-PCR. Cells with no CPE were subjected to two freeze–thaw cycles (−80 and 37°C) and tested for HA activity. Samples negative for CPE and/or HA were subjected to a second cell culture passage by pooling the fluid from all three wells and then re-inoculating fresh confluent MDCK cells in three wells. Samples were considered negative if CPE and HA were negative after the second passage on cell culture. Contaminated cell culture fluids were considered 'not determined'.

Influenza A virus oral fluid qRT-PCR (Laboratory 1)

RNA extraction was performed on oral fluid specimens at the Iowa State University Veterinary Diagnostic Laboratory using the MagMAXTM Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA, USA) using the high-volume-modified lysis (HVML) procedure. The lysis/binding solution for the HVML protocol was prepared using 45 ml lysis/binding solution with 200 μl carrier RNA without the addition of isopropanol. For the lysis step, 300 μl of sample was added to 450 μl of modified lysis/binding solution. XenoTM internal control RNA was added to the lysis/binding solution at 4000 copies per reaction prior to extraction to monitor PCR amplification and detect inhibition. The sample, lysis/binding solution, and internal control RNA were vortexed for 3 min and centrifuged at 2500 **g** for 6 min. A volume of 600 μl of lysate was added to 350 μl isopropanol with 20 μl magnetic bead mix prior to extraction and elution into 90 μl buffer. The HVML used 300 and 450 μl of wash solutions I and II, respectively. The HVML extraction was conducted using the Kingfisher program AM1836_DW_HV_v3.

Table 1. Count of matched sow serum and litter oral fluid (OF) samples and influenza A virus (IAV) qRT-PCR-positive OF samples

	Calendar week and month															
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	
Samples by farm[a]	May				June				July				August			*n*
Farm A – samples (serum and OF)	11	•	•	•	33	33	•	40	36	•	•	•	•	•	•	153
IAV qRT-PCR-positive OF samples	0	•	•	•	0	0	•	2	0	•	•	•	•	•	•	2
Farm B – samples (serum and OF)	18	5	17	•	•	15	•	•	42	24	24	•	•	•	•	145
IAV qRT-PCR-positive OF samples	1	0	0	•	•	0	•	•	14	0	0	•	•	•	•	15
Farm C – samples (serum and OF)	•	•	•	•	•	•	•	•	•	•	•	51	52	32	17	152
IAV qRT-PCR-positive OF samples	•	•	•	•	•	•	•	•	•	•	•	1	0	0	0	1
Farm D – samples (serum and OF)	•	•	•	•	•	•	•	•	•	•	30	30	35	42	13	150
IAV qRT-PCR-positive OF samples	•	•	•	•	•	•	•	•	•	•	5	0	0	1	1	7
Total samples (serum and OF)	29	5	17	0	33	48	0	40	78	24	54	81	87	74	30	600
IAV qRT-PCR-positive OF samples	1	0	0	0	0	0	0	2	14	0	5	1	0	1	1	25

• not applicable.

[a]All gilts were vaccinated with autogenous influenza A virus vaccine at approximately 9, 12 and 24 weeks of age and then 1-week post-farrowing.

Influenza A virus qRT-PCR was performed on nucleic acid extracts according to the manufacturer's instructions using PCR reagents with multiple primers and probes targeting different genomic regions (MagMAXTM Gold SIV Detection Kit; Life Technologies). One positive extraction control, one positive amplification control, one negative extraction control and a negative amplification control were included with each extraction and/or PCR run. Each oral fluid reaction included 12.5 μl of 2× multiplex RT-PCR buffer, 1.0 μl of 25× SIV primer probe mix, 2.5 μl of 10× multiplex RT-PCR enzyme mix and 1.0 μl of nuclease-free water. A final volume of 25 μl, consisting of 17 μl master mix and 8 μl of RNA extract, was placed in each well of a 96-well fast PCR plate (Life Technologies). The qRT-PCR was performed using an AB 7500 fast thermocycler: 1 cycle at 48°C for 10 min, 1 cycle at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 45 s. Amplification curves were analysed with commercial thermal cycler system software. The cycle threshold was set at 0.2, and the 'auto baseline' was used to determine fluorescence baselines. Samples with C_t values <38 were considered positive. Internal control XenoTM RNA C_t values were set at 10% of maximum.

Influenza A virus oral fluid qRT-PCR (Laboratory 2)

Oral fluid samples identified as IAV qRT-PCR positive (n = 22) at Laboratory 1 were submitted with qRT-PCR-negative oral fluid samples (n = 44) to Laboratory 2 for confirmatory testing (Tetracore®, Inc., Rockville, MD, USA).

Prior to RNA extraction, 180 μl of sample was centrifuged (14 000 ***g*** for 30 s), and then, 140 μl of the supernatant was manually lysed in a biosafety cabinet. Nucleic acids were extracted and purified from the lysate according to the manufacturer's recommendations using the QIAGEN® QIAamp® Viral Mini QIAcube® kit (Catalog #52926; Qiagen, Inc., Valencia, CA, USA) on the QIAGEN® QIAcube® processor (Qiagen, Inc.). The inhibition control (IC) was used as an extraction and PCR IC for each sample.

The qRT-PCR procedure was performed using commercial reagents (Universal Influenza A Matrix MPX 2.0; Tetracore®, Inc.), and the dry master mix was prepared according to the manufacturer's recommendations. The reactions were run (Applied Biosystems® 7500 Fast Real-Time PCR System; Applied Biosystems®, Foster City, CA, USA): 50°C for 30 min (reverse transcription), then 95°C for 2 min (RT inactivation/initial denaturation) and followed by 40 cycles of 95°C for 15 s, 52°C for 15 s and 60°C for 33 s (amplification). The thermocycler was run in 'standard' mode, and fluorescence data were collected during 60°C step in the FAMTM and CY5 channels. A sample was considered positive for the IAV matrix target if it yielded a C_t of <37.

Influenza A virus subtyping (Laboratory 1)

Hemagglutinin and NA subtyping was performed on qRT-PCR-positive IAV nucleic acid extracts using Swine Influenza Virus Subtyping RNA Reagents (Life Technologies). Separate RT-PCR reactions were used to detect the presence of H1 or H3 HA or N1 or N2 NA, respectively. Each oral fluid reaction included 12.5 μl of 2× multiplex RT-PCR buffer, 1.0 μl of 25× H1H3 or N1N2 Primer Probe Mix, 2.5 μl of 10× multiplex RT-PCR enzyme mix and 1.0 μl of nuclease-free water. Each subtyping plate included the same positive and negative controls used in the SIV general RT-PCR reaction. The IAV general RT-PCR cycling conditions were used for RT-PCR subtyping, and amplification curves were analysed with commercial thermal cycler system software using the same cycle threshold and 'auto baseline' determinants as the IAV general RT-PCR. Samples with C_t values <38 were considered positive.

Influenza A virus sequencing (HA, NA and M gene)

Whole genome HA, NA and matrix (M) genes were sequenced using conventional methods. Viral RNA was extracted using the Ambion MagMAXTM-96 AI/ND (Life Technologies) and a Kingfisher 96 instrument (Thermo Scientific). Specifically, 50 μl of sample, 100 μl of viral lysis/binding solution with carrier RNA and 20 μl of bead solution were used with 100 μl of the wash solution supplied with the kit. The final extracted sample elution volume was 50 μl. The extractions were performed using the Kingfisher program AM_1835_DW_NVSL. RT-PCR was conducted for each gene segment using the primers described in Table 2 and the FideliTaqTM RT-PCR Master Mix (2X) kit (Affymetrix, Cleveland, OH, USA). The sequencing RT-PCR setup reaction used 200 nM of each primer with 25 μl 2× RT-PCR master mix and 9 or 13 μl nuclease-free water for the HA or NA/M genes, respectively. The final volume of 50 μl consisted of 38 or 42 μl master mix and 12 or 8 μl of RNA extract for the HA or NA/M genes, respectively. One positive extraction control (H1 or H3), one negative extraction control and one negative amplification control were included with the reaction. RT-PCR was performed using an ABI 2720 thermal cycler: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 3 min, 50 cycles of 94°C for 30 s, 48°C for 30 s and 68°C for 120 s. The final elongation step was 68°C for 5 min. Detection of the RT-PCR product, HA at 1800 base pairs (bp), the NA at 1500 bp and M at 1100 bp, was performed on a QIAxcel® (Qiagen, Inc.) capillary electrophoresis system using a DNA-screening cartridge and the AM420 method and

purified with ExoSAP-IT PCR cleanup reagent (Affymetrix) following the manufacturer's recommendations. Samples were submitted to the Iowa State University DNA facility (Ames, IA, USA) for sequencing. Lasergene® software (DNAStar, Madison, WI, USA) was used to compile sequences.

Serum NP blocking ELISA

Sow serum samples were tested for NP antibodies using a commercial blocking ELISA performed as recommended by the manufacturer (IDEXX Influenza A Ab Test; IDEXX Laboratories, Inc.). Reactions were measured as optical density (OD) at a wavelength of 650 nm using an ELISA plate reader (BioTek® Instruments Inc., Winooski, VT, USA), operated with commercial software (GEN5, BioTek® Instruments Inc.). Sample-to-negative (S/N) ratios were calculated as described by the manufacturer, with S/N ratios ≤0.60 considered antibody positive (Goodell et al., 2014).sequences.

Serum NP isotype-specific indirect ELISAs

Sow serum samples were assayed for anti-NP IgM, IgA and IgG using indirect ELISAs. Plates and reagents (sample diluent, substrate, stop solution and wash solution) were from a commercial IAV blocking ELISA (IDEXX Influenza A Ab Test; IDEXX Laboratories, Inc.) and conjugate diluent from a commercial indirect ELISA (IDEXX PRRS X3 Ab Test; IDEXX Laboratories, Inc.). Detection of isotype-specific NP antibody utilized horseradish peroxidase (HRP)-conjugated goat anti-pig IgM (A100-100P), IgA (A100–102P), and IgG (A100–104P) antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) diluted in conjugate diluent. Duplicate in-house negative and positive serum controls (IgM, IgA and IgG) were used to validate plate performance and to calculate sample-to-positive (S/P) ratios.

To perform the assay, serum samples were diluted 1 : 50 (IgM), 1 : 10 (IgA) or 1 : 50 (IgG) and then plates were loaded (100 μl) with samples, negative and positive controls (IgM, IgA or IgG). After 2-h incubation at 37°C, plates were washed 3 times, and then, 100 μl of diluted HRP-conjugated goat anti-pig IgM (1 : 8000) or IgA (1 : 2000) or IgG (1 : 10 000) was added to each well, and the plates were incubated at 37°C for 1 h. Plates were then washed three times, after which 100 μl of 3, 3′, 5, 5′-tetramethyl benzidine (TMB) substrate was added. After 15-min incubation at room temperature, 100 μl of stop solution was added and plates were read at a wavelength of 650 nm. S/P ratios were calculated for each sample as:

$$\text{S/P ratio} = \frac{\text{sample OD} - \text{negative control mean OD}}{\text{positive control mean OD} - \text{negative control mean OD}}$$

Oral fluid NP blocking ELISA

Oral fluid samples were assayed for IAV antibody using a commercial blocking ELISA (IDEXX Influenza A Ab Test; IDEXX Laboratories, Inc.) performed as described elsewhere (Panyasing et al., 2012). Briefly, each plate was loaded with undiluted oral fluid samples (200 μl), kit negative and positive controls and in-house oral fluid controls (low, medium and high) and incubated for 16 h at 22°C. Thereafter, the assay was performed and S/N ratios calculated as described by the manufacturer.

Oral fluid NP isotype-specific indirect ELISAs

The oral fluid NP isotype-specific indirect ELISAs are fully described elsewhere (Panyasing et al., 2013). Briefly, oral fluid samples were assayed on ELISA plates manually coated with 1 μg/well of commercially produced NP antigen (Cat no. IMR-274; Imgenex © Corporation, San Diego, CA, USA). The reagents used in the ELISAs were identical to the serum NP-isotype-specific assays, with the exception of IgM, IgA and IgG conjugate concentrations. To perform the assays, plates were loaded with undiluted oral fluid specimens (200 μl) and pre-diluted negative (100 μl) and positive (100 μl) in-house plate controls (in duplicate) and incubated at 4°C for 16 h. After incubation, plates were washed three times. To detect IgM, IgA and IgG, 100 μl of HRP-conjugated goat anti-pig IgM

Table 2. Primer sets used for RT-PCR amplification of hemagglutinin (HA), neuraminidase (NA) and matrix (M) genes of influenza A viruses

Target gene	Primer	Primer sequence 5′-3′
H1	H1-F	AAGCAAAAGCAGGGGAAAATAA
	HR	AGTAGAAACAAGGGTGTTTTT
H3	H3-F	AGCAAAAGCAGGGGATAATTCT
	HR	AGTAGAAACAAGGGTGTTTTT
NA	NA-1F	TAT TGG TCT CAG GGA GCA AAA GCA GGA GT
NA	NA-1413R	ATA TGG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT
M	M-1F	TAT TCG TCT CAG GGA GCA AAA GCA GGT AG
M	M-1027R	ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT

(1 : 2000), IgA (1 : 2000) or IgG (1 : 1500) was added and the plates incubated at 37°C for 1 h. Plates were then washed three times, TMB substrate (100 μl) added, and the plates were incubated at room temperature for 15 min. Stop solution (100 μl) was then added, and the plates were read immediately thereafter. Reactions were measured at a wavelength of 650 nm, and S/P ratios calculated as described in 2.10.

Statistical analysis

Oral fluid qRT-PCR qualitative responses were analysed for significant differences among herds and sampling time points (Fisher's Exact test) and significant associations with sow parity, litter size, as well as the qualitative responses of IgM, IgA, IgG and blocking ELISA (Wilcoxon–Mann–Whitney test) using commercial software (SAS® 9.2; SAS® Institute Inc., Cary, NC, USA). The M gene nucleotide sequences were compared with a variety of classical and recent North American swine-derived IAV full-length M gene sequences available in the GenBank database. A phylogenetic tree was generated by the distance-based, neighbour-joining method using MEGA5.2 software (Tamura et al., 2011). Serum and oral fluid S/P and S/N antibody responses were analysed for significant associations with sow parity, litter size, herd, sampling time point and qRT-PCR response, and their interactions were analysed by analysis of variance (ANOVA). The relationship between IgM, IgA and IgG S/P ratios in serum versus oral fluid samples was evaluated by correlation analysis (Pearson's correlation coefficient).

Results

Detection of Influenza A virus in oral fluid

All oral fluid specimens ($n = 600$) were negative by virus isolation. IAV qRT-PCR testing in Laboratory 1 identified 22 (3.7%) positive oral fluid samples (Table 3). Ten of the 22 positive samples were successfully subtyped, with most shown to be mixed infections (H1, H3, N1 and N2). To confirm the qRT-PCR results, the 22 positive samples plus 44 randomly selected qRT-PCR-negative samples were submitted to Laboratory 2. Testing at Laboratory 2 identified 18 qRT-PCR-positive samples, including 3 that had previously tested negative at Laboratory 1. Among the cumulative total of 25 qRT-PCR-positive samples reported by Laboratory 1 and/or Laboratory 2, 18 were available for sequencing, that is 7 samples had been depleted. None of the attempts to sequence HA and NA genes was successful, but M gene sequences were obtained for all 18 samples. Genetic analysis revealed that the 18 M gene nucleotide sequences were 100% identical to each other (GenBank Accession number KF487544) and to the M gene of a previous GenBank submission (JX444793/A/swine/Ohio/A01203624/2012(H3N2) (Fig. 1).

As shown in Table 1, the cumulative proportion of IAV qRT-PCR-positive oral fluid samples by farm (high to low) was as follows: Farm B (15/145, 10.3%), Farm D (7/150, 4.7%), Farm A (2/153, 1.3%) and Farm C (1/152, 0.7%). Pairwise comparisons showed that the proportion of qRT-PCR positives in Farm B differed significantly from Farms A and C (Fisher's Exact Test, $P < 0.001$), but not from Farm D ($P = 0.07$). In Farm B, 14 of the 15 total qRT-PCR-positive oral fluid samples were recovered at one sampling point, that is calendar week 28. Similarly in Farm D, five of the seven total qRT-PCR-positive oral fluid samples were detected in the samples collected at week 30. No association was detected between IAV qRT-PCR oral fluid status and sow parity (Wilcoxon–Mann–Whitney test, $P = 0.10$) or the number of pigs weaned (Wilcoxon–Mann–Whitney test, $P = 0.07$).

IAV NP antibody in sow serum and litter oral fluid samples

Qualitative results of IAV NP antibody testing of sow serum and litter oral fluid are shown in Table 4. The proportion of IgM, IgA, IgG or blocking ELISA-positive sow serum or litter oral fluid samples did not differ among herds or within farms by calendar week. A comparison of sow serum and litter oral fluids qualitative results found no difference in the proportion of IgG or blocking ELISA positives, but the proportion of IgM and IgA positives was higher in sow serum and litter oral fluid, respectively (Fisher's Exact Test, $P < 0.01$). No association was detected between IAV qRT-PCR oral fluid results and IgM, IgA, IgG or blocking ELISA qualitative results in sow serum or litter oral fluid samples.

Sow serum and litter oral fluid IgM, IgA, IgG and blocking ELISA quantitative responses (S/P and S/N ratios) are shown in Fig. 2. No significant association was detected between sow serum or litre oral fluid antibody responses and farm, sow parity, number of pig weaned, calendar week, or IAV qRT-PCR oral fluid results. Evaluation of the association between litter oral fluid and sow serum antibody responses showed a significant association between IgG S/P ratios ($P = 0.01$, $r = 0.10$), but not between IgM, IgA or blocking ELISA responses.

Discussion

Influenza A virus surveillance is necessary to monitor viral evolution and support the development of improved diagnostic tests and more efficacious vaccines (Vincent et al., 2014). More narrowly, research on the ecology of IAV in swine populations is motivated by concerns that

Table 3. Summary of test results on influenza A virus qRT-PCR-positive litter oral fluid samples and matched sow serum

			Sow serum			Pre-weaning litter oral fluid samples						
Farm	Sow parity	Pigs weaned	IgM (S/P)	IgA (S/P)	IgG (S/P)	Lab1[a] C_t qRT-PCR	Lab2[b] C_t qRT-PCR	Virus subtyping	*M* gene sequencing	IgM (S/P)	IgA (S/P)	IgG (S/P)
A	3	11	0.63	0.18	2.92	>38.0	36.62	ND	Y	0.40	0.01	2.75
A	2	12	0.17	0.58	2.51	37.70	>37.0	Untypable	Y	0.02	0.12	2.87
B	4	10	0.52	0.75	2.62	30.70	32.32	H1, H3, N1 and N2	Y	0.10	0.01	2.77
B	3	11	0.58	0.77	2.67	37.80	35.51	Untypable	QNS[c]	0.04	0.05	2.52
B	6	11	0.56	0.37	2.42	28.70	29.67	H1, H3, N1 and N2	Y	0.08	0.02	2.70
B	6	11	0.51	0.32	2.44	33.0	30.99	H1	Y	0.22	0.03	2.63
B	6	12	0.02	0.54	2.19	33.30	34.12	H1	Y	0.09	0.07	2.41
B	3	11	0.11	0.56	2.41	32.10	31.82	H1, H3	QNS	0.17	0.15	1.37
B	3	11	0.73	0.65	2.39	31.60	32.31	H1, N2	QNS	0.12	0.03	2.58
B	1	11	0.02	0.44	2.26	36.10	>37.0	Untypable	Y	0.06	0.42	3.00
B	4	11	0.82	0.44	2.48	34.70	33.08	Untypable	Y	0.14	0.12	2.52
B	4	12	0.12	0.01	2.52	24.20	26.66	H1, H3, N1 and N2	Y	0.02	0.08	2.63
B	6	11	0.44	0.21	2.42	35.90	36.21	Untypable	Y	0.16	0.11	2.57
B	2	11	0.59	0.17	1.65	28.10	31.25	H1, H3, N1 and N2	Y	0.12	0.00	2.15
B	6	10	0.02	0.57	2.56	32.00	32.14	Untypable	Y	0.16	0.07	2.59
B	6	12	0.91	0.36	2.43	34.60	33.08	H1	Y	0.03	0.02	2.68
B	2	11	0.27	0.73	1.88	28.70	>37.0	H1, H3, N1 and N2	Y	0.08	0.12	2.37
C	2	9	0.05	0.21	2.14	>38.0	29.64	ND	Y	0.30	1.35	1.93
D	3	10	0.33	0.58	2.78	>38.0	35.48	ND	Y	0.05	0.04	2.26
D	3	10	0.95	0.49	2.65	36.00	36.88	Untypable	QNS	0.16	0.06	2.22
D	2	11	0.00	0.26	2.47	36.87	>37.0	Untypable	QNS	0.16	0.03	1.66
D	5	11	0.55	0.68	2.63	33.60	>37.0	Untypable	QNS	0.19	0.09	1.09
D	1	10	1.00	0.53	2.21	37.40	>37.0	Untypable	Y	0.00	0.44	1.60
D	4	10	0.89	0.49	2.56	36.90	36.58	Untypable	Y	0.08	0.03	2.61
D	1	12	0.22	0.54	2.13	37.70	>37.0	Untypable	QNS	0.09	0.06	2.76

[a]Influenza A virus qRT-PCR C_t values <38 were positive in Laboratory 1.

[b]C_t values <37 were positive in Laboratory 2.

[c]Quantity not sufficient (QNS) for testing.

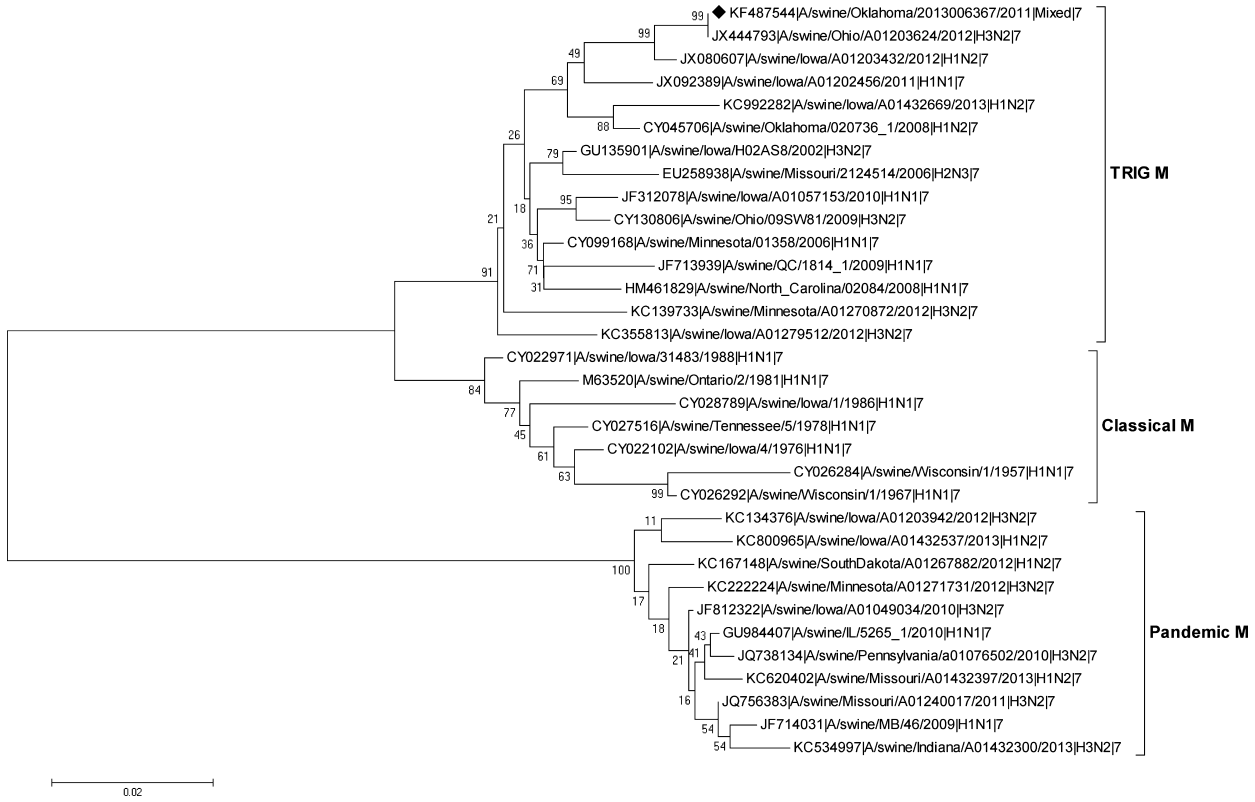

Fig. 1. Phylogenetic tree based on an analysis of influenza A virus matrix (M) genes.

reassortants originating in pigs could prove to be virulent in humans and by the need to ameliorate the negative effects of IAV on pig welfare and health. Historically, the collection of longitudinal infectious disease data in commercial herds has been constrained by the expense and inconvenience of collecting and testing specimens from individual animals. As an alternative, we evaluated the feasibility of conducting IAV surveillance using oral fluid samples collected from litters of piglets prior to weaning. This age group was selected under the premise that surveillance data from this group would allow producers to identify and respond to health issues in the breeding herd and anticipate post-weaning disease issues in growing pig populations. Serum samples collected from each litter's dam provided for comparisons with litter oral fluid testing results. Oral fluid and serum samples were collected by farm personnel and then sent to the laboratory for testing. This approach avoided the biosecurity risks invariably associated with sending outside personnel into a herd to collect samples.

Table 4. Influenza A virus nucleoprotein (NP) antibody enzyme-linked immunosorbent assay (ELISA; IgM, IgA, IgG and blocking) qualitative testing results

		Count of ELISA-positive sow serum samples				Count of ELISA-positive litter oral fluid samples			
Farm[a]	*n*	IgM[b]	IgA[b]	IgG[b]	Blocking[c]	IgM[d]	IgA[d]	IgG[d]	Blocking[e]
A	153	16	5	153	148	0	13	152	145
B	145	16	3	144	135	4	10	145	134
C	152	22	4	152	142	1	12	152	142
D	150	12	4	150	143	1	4	150	143
Total	600	66	16	599	568	6	39	599	564

[a]All gilts were vaccinated with autogenous influenza A virus vaccine at approximately 9, 12 and 24 weeks of age and then 1 week post-farrowing.
[b]Indirect ELISA S/P ratios ≥0.84 (IgM), ≥0.75 (IgA) or ≥0.60 (IgG) were considered antibody positive (Panyasing et al., 2013).
[c]Blocking ELISA S/N ratios ≤0.60 were considered antibody positive (Goodell et al., 2014).
[d]Indirect ELISA S/P ratios ≥0.50 (IgM), ≥0.60 (IgA) or ≥0.60 (IgG) were considered antibody positive (Panyasing et al., 2013).
[e]Blocking ELISA S/N ratios ≤0.60 were considered antibody positive (Panyasing et al., 2012).

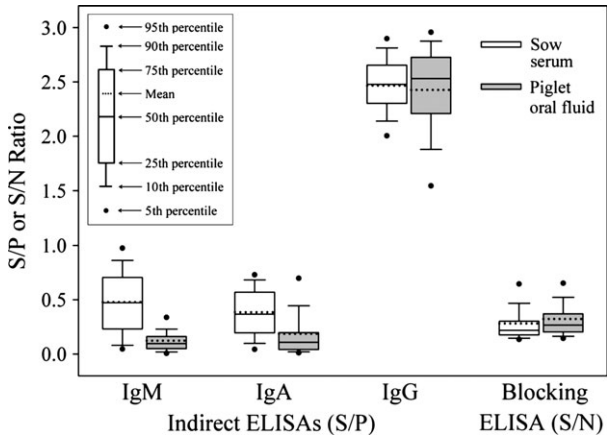

Fig. 2. Summary of influenza A virus nucleoprotein (NP) antibody testing results.

The results of the study showed that IAV could be detected by qRT-PCR, subtyped and sequenced using oral fluid specimens collected from neonatal pigs. Virus isolation from oral fluids was unsuccessful, but virus subtyping revealed that infections were commonly a mix of subtypes H1, H3, N1 and N2. These results were compatible with previous reports. Evaluating the detection of IAV in oral fluids by qRT-PCR as a function of within-pen prevalence, Romagosa et al. (2012) estimated the probability of detecting one acutely infected pig in a pen of 11 by qRT-PCR at 69% and 2 infected pigs at 99%. Detmer et al. (2011) reported successful subtyping, HA gene sequencing and IAV isolation from oral fluid field samples, albeit virus isolation is generally more successful using nasal swabs (Goodell et al., 2013). Cumulatively, the results showed a pattern of intermittent subclinical IAV infections in litters from IAV-vaccinated dams, with infrequent episodes involving larger numbers of litters. Thus, these data support the view that IAV circulates throughout the year in contemporary swine populations (Van Reeth et al., 2012). The absence of clinical losses was consistent with reports that maternal immunity can moderate fever, reduce clinical signs and prolong virus shedding, but not prevent infection (Loeffen et al., 2003; Kitikoon et al., 2006; Allerson et al., 2013).

Although the study was not designed to compare assay reproducibility, serial testing of a subset of samples showed that false negative results occurred in both laboratories performing qRT-PCR testing. That is, IAV M gene sequencing was successful on all qRT-PCR-positive samples submitted for sequencing (n = 18, Table 3), even if the sample tested negative in one of the two laboratories. HA and NA gene sequences were not obtained, but analysis of M gene sequences (n = 18) showed 100% nucleotide identity to each other and a previous GenBank submission in the TRIG M cluster (JX444793/A/swine/Ohio/A01203624/2012 (H3N2)). The length and genetic diversity of the HA (~1700 nucleotides) and NA (~1400 nucleotides) genes may account for the difficulty in sequencing these genes, as opposed to the highly conserved and shorter M gene (~1000 nucleotides; Lamb and Krung, 2001). This problem may be resolved in the future as assays are improved.

Previous research described the ontogeny of IAV NP antibody responses over time in oral fluid (Panyasing et al., 2012), serum, bronchoalveolar and nasal lavage fluid (Heinen et al., 2000) from pigs under experimental conditions. In the present study, nearly all serum and oral fluid samples were positive for NP IgG antibody due to the extensive use of IAV vaccine and the concomitant circulation of wild-type IAV in these populations. Although IgM and/or IgA-positive maternal serum and piglet oral fluid samples were identified, no association was detected between IAV qRT-PCR results and NP antibody profiles (IgM, IgA, IgG and blocking ELISAs). In part, the ability to detect patterns of antibody response to infection was compromised by the experimental design, that is samples were collected at a single point in time from each litter or sow.

Overall, the present study supports the use of oral fluid sampling as a means to conduct IAV surveillance in pig populations. In particular, oral fluid offers the potential to conduct surveillance with fewer samples than required for individual pig testing. Future work on IAV oral fluid diagnostics should focus on improved procedures for virus isolation, subtyping and sequencing of HA and NA genes. The role of antibody in IAV surveillance remains to be determined, but the assessment of IAV antibody responses over time in commercial swine populations could provide a cost-effective source of data on infection, vaccination status and herd immunity.

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