Probability of detecting influenza A virus subtypes H1N1 and H3N2 in individual pig nasal swabs and pen-based oral fluid specimens over time

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A B S T R A C T

The probability of detecting influenza A virus (IAV) by virus isolation (VI), point-of-care (POC) antigen detection, and real-time reverse-transcription polymerase chain reaction (rRT-PCR) was estimated for pen-based oral fluid (OF) and individual pig nasal swab (NS) specimens. Piglets ($n = 82$) were isolated for 30 days and confirmed negative for porcine reproductive and respiratory syndrome virus, *Mycoplasma hyopneumoniae*, and IAV infections. A subset ($n = 28$) was vaccinated on day post inoculation (DPI) −42 and −21 with a commercial multivalent vaccine. On DPI 0, pigs were intratracheally inoculated with contemporary isolates of H1N1 ($n = 35$) or H3N2 ($n = 35$) or served as negative controls ($n = 12$). OF ($n = 370$) was collected DPI 0–16 and NS ($n = 924$) DPI 0–6, 8, 10, 12, 14, 16. The association between IAV detection and variables of interest (specimen, virus subtype, assay, vaccination status, and DPI) was analyzed by mixed-effect repeated measures logistic regression and the results used to calculate the probability ($\hat{p}$) of detecting IAV in OF and NS over DPI by assay. Vaccination (p-value < 0.0001), DPI (p-value < 0.0001), and specimen-assay interaction (p-value < 0.0001) were significant to IAV detection, but virus subtype was not (p-value = 0.89). Vaccination and/or increasing DPI reduced $\hat{p}$ for all assays. VI was more successful using NS than OF, but both VI and POC were generally unsuccessful after DPI 6. Overall, rRT-PCR on OF specimens provided the highest $\hat{p}$ for the most DPIs, yet significantly different results were observed between the two laboratories independently performing rRT-PCR testing.

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1. Introduction

Once considered a seasonal infection with rapid onset and recovery (Van Reeth et al., 2012), influenza A virus (IAV) in contemporary commercial swine populations is a chronic, endemic disease with significant herd-level economic effects and broad public health implications. As a result of public health concerns, IAV monitoring in swine has been initiated in some areas, but primarily as a passive system based on testing convenience samples selected from routine case submissions to veterinary diagnostic laboratories (USDA, 2010). Ante mortem diagnostic assays for influenza virus detection include

real-time, reverse transcriptase polymerase chain reaction (rt-PCR), antigen-capture assays, and virus isolation. For both swine and humans, the standard sample type for these assays has been nasal (or oropharyngeal) swab samples from acutely infected individuals. Studies have shown that influenza A virus isolation is equally effective using either Madin Darby Canine Kidney (MDCK) cells or embryonated eggs (Bowman et al., 2013; Swenson et al., 2001). In humans, studies based on clinical cases reported that PCR was significantly more sensitive than either virus isolation (VI) or point of care (POC) assays for virus detection (Al Johani et al., 2011; Babin et al., 2011; Chartrand et al., 2012; Cheng et al., 2004; Fuenzalida et al., 2010; Ganzenmueller et al., 2010; Gao et al., 2012; Gimeno et al., 2010; Hurt et al., 2009; Liao et al., 2009, 2011; Pregliasco et al., 1998). In swine, research has focused on the pathogenesis of IAV and data on the performance of diagnostic assays or comparison of diagnostic specimens beyond day post inoculation (DPI) 6 is sparse.

If routine IAV surveillance of swine populations is to be achieved, simple, inexpensive, and reliable methods of sampling and testing are needed. Nasal swabs (NS), the traditional ante mortem specimen for IAV detection, do not meet these sampling criteria. Successful field detection of IAV using NS is a complex, stressful (for pigs and people), and labor-intensive process. Alternatively, oral fluids (OF), a specimen new to swine diagnostics, but well-characterized in human diagnostics (Prickett and Zimmerman, 2010), is easy to collect because pigs naturally investigate their environment by chewing (Kittawornrat and Zimmerman, 2011). To evaluate the potential of IAV surveillance based on OF sampling, the probability of detecting IAV in OF and NS specimens collected from vaccinated or unvaccinated pigs was compared for 16 days following inoculation with contemporary H1N1 or H3N2 isolates.

2. Materials and methods

2.1. Experimental design

IAV vaccinated and unvaccinated pigs were inoculated with subtypes H1N1 or H3N2. Pen-based oral fluid samples were collected day post inoculation (DPI) 0–16 and individual pig nasal swab samples were collected DPI 0–6, 8, 10, 12, 14, and 16. Specimens were tested for IAV by virus isolation (VI), a "point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc., Union City, CA), and two real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays. Statistical analyses were performed to define the effect of assay, specimen, virus subtype, vaccination status, DPI, and their interactions on virus detection.

2.2. Influenza A viruses

Isolate A/Swine/Ohio/511445/2007 γ H1N1 virus (provided by Dr. Amy Vincent, USDA National Animal Disease Center, Ames, IA) was recovered from an influenza outbreak at a county fair in Ohio. A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus (provided by Dr. Marie Gramer, University of Minnesota, St. Paul, MN) was isolated from

clinically-affected finishing pigs in Illinois. Both viruses were propagated on Madin-Darby Canine Kidney (MDCK) cells to achieve virus concentrations of approximately $1 \times 10^{6.5}$ median tissue culture infectious dose ($TCID_{50}$) per ml.

2.3. Animals and animal care

The study was conducted in compliance with the Iowa State University (ISU) Institutional Animal Care Use Committee (#11–09-6834-S) and the Institutional Biosafety Committee (09-I-0028-A) guidelines. ISU is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

IAV-negative piglets ($n = 82$) were obtained from one 600 sow farm at ~21 days of age and an average weight of 6.26 kg (13.8 lb). Ten days prior to receipt of study piglets, sows in breeding and gestation phases and non-study piglets on sows were bled based on 90% confidence of detecting 10% prevalence. Sow and non-study piglet serum were tested for antibodies against IAV, porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae* and confirmed negative. Likewise, serum samples were tested by PRRSV rRT-PCR using pooled serum samples (5 samples per pool) to confirm freedom from acute PRRSV infection.

The source farm routinely vaccinated piglets against porcine circovirus type 2 (PCV2) at 5 and 21 days of age using a commercial PCV2 vaccine (Circumvent®PCV Intervet/Shering-Plough Animal Health, Millsboro, DE). Pigs were fed age-appropriate, commercially-formulated diets throughout the study.

2.4. Study timeline and treatments

On DPI -43, the 82 ~21-day-old piglets were moved from the sow farm into one room of a disinfected isolation facility. Serum samples were collected on DPI -42, -21, -7, and 0 and tested for antibodies against IAV, PRRSV, and *M. hyopneumoniae* to document the continued negative status of the group. On DPI -42, each animal was visually inspected and ear tagged. Pigs were randomized to one of six treatments (Table 1) by first assigning ear tag numbers to treatments and then blindly taking tags out of a container as the tags were applied. Pigs were housed as one group in the isolation facility; therefore, ear tag color was used to differentiate pigs in IAV vaccinated ($n = 28$) and nonvaccinated ($n = 54$) treatment groups. Vaccinated pigs were intramuscularly administered a trivalent commercial IAV vaccine (Flu-Sure® XP, Pfizer Animal Health, Madison, NJ) [A/Swine/Iowa/110600/00 γ (H1N1), A/Swine/North Carolina/031/05 δ (H1N1), and A/Swine/Missouri/069/05(H3N2)] according to label instructions on DPI -42 and DPI -21. The γ H1N1 and Cluster 4 H3N2 components of the vaccine were 95.4% and 98.4% homologous to the hemagglutinin (HA) amino acid sequences of the H1N1 and H3N2 viruses used to inoculate pigs.

On DPI -10, the animals were moved to the ISU Livestock Infectious Disease Isolation Facility, placed 3 or 4 pigs per pen, and allowed to acclimate for ten days. To accommodate the number of pigs and pens, the H1N1 and

Table 1
Description of treatment groups and sampling.

	Negative control		H1N1 inoculated[a]		H3N2 inoculated[b]	
	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated
Group designation	UV_{CTRL}	V_{CTRL}	UV_{H1}	V_{H1}	UV_{H3}	V_{H3}
Vaccination[c]	No	Yes	No	Yes	No	Yes
Pigs	6	6	24	11	24	11
Pens	2	2	8	3	8	3
OF samples[d] ($n = 370$)	28	14	119	48	114	47
NS samples[e] ($n = 924$)	96	58	264	121	264	121

[a] A/Swine/Ohio/511445/2007 γ H1N1 virus.
[b] A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus.
[c] Vaccinated on DPI -42 and -21 with a trivalent, inactivated influenza vaccine (Flu-Sure® XP, Pfizer Animal Health, Madison, NJ).
[d] Oral fluid samples collected daily DPI 0–16.
[e] Nasal swabs collected on DPI 0–6, 8, 10, 12, 14, 16 from all inoculated pigs and DPI 0–2, 4, 8, 12 and 16 from negative control pigs.

H3N2 inoculant groups (35 pigs each) were each housed in two rooms: one room of 5 pens and one room of 6 pens. Vaccinates were housed with their inoculant group, but were penned separately from non-vaccinates. The control group was housed in four pens configured in a 2×2 arrangement in one room. Pigs were observed daily throughout the experiment. Manual contact was used to stimulate movement or assess lethargy, if animals were reluctant to rise. Individual animal weights were obtained at DPI -42 and DPI 0 using portable electronic scales (Siltec® WS500 Electronic Weighing Scale Bradford, MA) for the purpose of assessing general health and group uniformity.

On DPI 0, animals in inoculant groups H1N1 and H3N2 were intratracheally administered 2 ml of a solution containing $1 \times 10^{6.5}$ $TCID_{50}$ per ml of either A/Swine/Ohio/511445/2007 γ H1N1 or A/Swine/Illinois/02907/2009 cluster IV H3N2. A portion of each viral inoculum was stored ($-80\,^{\circ}C$) for back-titration.

2.5. Serum samples

Serum samples were collected on DPI -42, -21, -7, 0, 7. Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Samples were centrifuged at $1800 \times g$ for 10 min at $4\,^{\circ}C$, after which sera were aliquoted into 5 ml tubes (BD Falcon™, Franklin Lakes, NJ) and stored at $-20\,^{\circ}C$ until tested.

2.6. Oral fluid samples

Oral fluid samples were collected daily DPI -5 to -16. To familiarize pigs with the rope used for collecting oral fluid samples, 45 cm (18″) of 1.3 cm (1/2″) 3-strand, undyed, unbleached 100% cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL) was knotted and placed on the floor of each pen for 20 min in the morning and afternoon on DPI -6. Thereafter, collections were performed as describe elsewhere (Prickett et al., 2008a). To avoid contamination between treatment groups, three people collected and processed ropes, i.e., one person for each treatment group (negative control, H1N1, H3N2). Ropes were processed prior to leaving the treatment group housing area. Immediately following collection, samples were refrigerated ($4\,^{\circ}C$),

centrifuged at $13,000 \times g$ for 10 min at $4\,^{\circ}C$, aliquoted into 5 ml snap cap tubes (BD Falcon, Fisher Scientific) and stored at $-80\,^{\circ}C$.

2.7. Nasal swab samples

Nasal swabs were collected on DPI 0–6, 8, 10, 12, 14, and 16 from all inoculated pigs and DPI 0–4, 8, 12 and 16 from negative control pigs. To collect samples, a swab (Copan™ minitip flocked swab, Fisher Scientific No. 501CS01) was inserted ~8 cm into one nasal passage of the pig, rotated, and removed. The naris sampled was alternated each day, i.e., all left nares one day and all right nares the next. Following sampling, the swab was broken off into 3 ml of viral transport medium (BD Universal Transport Media with modified Hank's balanced salt solution, Fisher Scientific No. 220220). In the laboratory, the tubes containing swabs were vortexed, the media aliquoted into two ml cryovials (Corning®), and the samples were stored at $-80\,^{\circ}C$.

2.8. Testing procedures

Pre-inoculation samples: To verify the negative status of the group prior to inoculation with IAV, serum samples collected on DPI -42, -21, -7, 0, 7 were tested for evidence of infection with PRRSV, *Mycoplasma hyopneumoniae*, and IAV.

Indirect antibody ELISAs licensed for testing swine serum (IDEXX PRRS X3 Ab Test., IDEXX Laboratories, Inc., Westbrook, ME; IDEXX M hyo Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) were performed according to the manufacturer's instruction and analyzed using the manufacturer's software (xChek®, IDEXX Laboratories, Inc., Westbrook M.E). Negative and positive controls provided by the manufacturer were run on all plates and the performance of each plate was validated using the manufacturer's software. To detect acute PRRSV viremia, serum samples were pooled in groups of 4 or 5 and tested by PRRSV rRT-PCR (Eshelman, 2010a,b).

Serum samples were tested for IAV antibody using a blocking ELISA (IDEXX AI MultiS-Screen Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) using a cut-off of S/N = 0.67 (Ciacci-Zanella et al., 2010). The assay was conducted according to the manufacturer's instructions and the laboratory's standard operating procedure

(Boesenberg, 2012). Consistent with the other ELISAs, quality control included statistical process control (SPC) charting of in-house negative and positive controls (Northwest Analytic SPC, Portland, OR). Data management and calculations were performed using the software provided by the manufacturer. Results were reported as S/N (sample/kit negative control) ratios.

Post-inoculation IAV samples: Oral fluid and nasal swabs collected between DPI 0 and 16 were tested for IAV by rRT-PCR (Laboratories A and B), VI, and POC assays. Testing was blinded by complete randomization of samples prior to submission.

Laboratory A rRT-PCR protocols for matrix and hemagglutinin genes: OF or NS specimens (180 μl) were centrifuged (14,000 × g for 30 s) prior to extraction 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) on the QIAGEN® QIAcube® processor. The inhibition control (IC) was used as an extraction and PCR inhibition control for each sample. The rRT-PCR assay was performed using commercial reagents (Universal Influenza A Matrix MPX 2.0, Tetracore, Inc., Rockville, MD) 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) with the following cycling conditions: 50 °C for 30 min (reverse transcription), then 95 °C for 2 min (RT inactivation/initial denaturation), 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 was collected during the 60 °C step in the FAM™ and CY5 channels. A sample was considered positive for IAV matrix target if it yielded a Ct of <37. Matrix-positive samples were tested by rRT-PCR for H1 and H3 genes. The H1 assay was performed using a protocol described in detail elsewhere (WHO, 2009), with primers and probes obtained from Biosearch Technologies (Novato, CA), and the positive control from Integrated DNA Technologies, Inc. (Coralville, IA). The H3 assay was performed using a protocol described elsewhere (Richt et al., 2004), with primers and probes obtained from Integrated DNA Technologies, Inc. and a positive control consisting of a dilution of extracted H3 RNA from a previous study.

Laboratory B rRT-PCR protocols for matrix and hemagglutinin genes: OF (300 μl) or NS (50 μl) samples were assayed using a commercial kit performed as instructed by the manufacturer (Swine Influenza Virus RNA Test Kit Document part number 4444272 Rev B, Applied Biosystems®). Briefly, nucleic acid from OF and NS samples was extracted and purified using MagMAX™ Pathogen RNA/DNA Kit (part no.4462359). IAV rRT-PCR was performed using 2× Multiplex RT-PCR Enzyme Mix, SIV Primer probe mix, Xeno® RNA control (Applied Biosystems®, Foster City, CA, part no. 4415200) and SIV-Xeno® RNA Control Mix. The reactions were run (Applied Biosystems® 7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA) with the following cycling conditions: 48 °C for 10 min

(reverse transcription), then 95 °C for 10 min (RT inactivation/initial denaturation), followed by 40 cycles of 95 °C for 15 s, and 60 °C for 45 s (amplification). The thermocycler was run in "standard" mode and fluorescence data was collected during the 60 °C annealing/extension stage. Analysis was performed using the control-based threshold setting, with thresholds for SIV-RNA set at 5% of the positive control dRN at cycle 40. A sample was considered positive for IAV if it yielded a Ct of <38. Matrix-positive samples were tested by rRT-PCR for H1 and H3 genes using non-commercial in-house reagents with one primer/probe mix amplifying both H1 and H3 RNA in a single multiplex reaction. The H1 probe was labeled with a VIC® fluorophore (Applied Biosystems®) and the H3 was labeled with FAM™ (Applied Biosystems®). Using the previously extracted RNA, rRT-PCR was performed using 2× Multiplex RT-PCR buffer (Applied Biosystems®), enzyme mix (Applied Biosystems®), and H1H3 primer probe mix (Applied Biosystems®).

Virus Isolation: Confluent monolayers of MDCK cells were prepared in 48-well plates (Costar, Corning, Corning, NY). Cell culture media was removed and monolayers were washed 3 times with IAV wash solution composed of minimal essential medium with Earle's salts (MEM; Sigma–Aldrich, St. Louis, MO), 3× antibiotic–antimycotic solution [(penicillin (300 IU/ml; Sigma–Aldrich, St. Louis, MO), streptomycin (300 μg/ml; Sigma–Aldrich, St. Louis, MO), gentamicin (150 μg/ml; Sigma–Aldrich. St. Louis, MO) and amphotericin B (0.75 μg/ml; Gibco, Grand Island, NY)], and TPCK-treated trypsin (2 μg/ml; Sigma–Aldrich. St. Louis, MO). Prior to inoculation on to MDCK cells, 0.35 μl of antibiotic–antimycotic solution was added to each 1 ml oral fluid and nasal swab sample, after which samples were held at room temperature for 1 h. Each OF and NS sample was divided among 3 wells, i.e., ~0.4 ml per well, and then incubated at 37 °C with 5% CO_2 for 2 h, after which the inoculum was removed. Cell monolayers were rinsed 3 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) was 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 by for IAV by rRT-PCR. Cells with no CPE were subjected to two freeze–thaw cycles (−80 °C 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 3 wells and then re-inoculating on to fresh confluent MDCK cells in 3 wells. Samples were considered IAV 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 point-of-care (POC) antigen test: The POC assay (VetScan®, Abaxis Inc.) evaluated was a rapid, immunochromatographic avian IAV antigen assay USDA licensed for chicken, turkey, and duck tracheal, oropharyngeal, and cloacal swab specimens. Oral fluid and nasal swab samples were tested according to the manufacturer's instructions for avian samples. In brief, 100 μl of sample

was added to the device and it was sealed closed. Nasal swab results were read at 15 min and oral fluid results were read at both 15 and 30 min. Results were interpreted as described by the manufacturer.

2.9. Analysis

A mixed-effect repeated measures logistic regression model (Proc GLIMMIX, SAS® Version 9.3, SAS® Institute, Inc., Cary, NC) was used to analyze the association between the detection of IAV and the variables of interest: sample specimen (OF or NS), virus subtype (H1N1 or H3N2), assay (VI, POC, rRT-PCR), vaccination status (yes/no), and DPI. Within the limitations of the dataset, interactions of specimen with other variables of interest were tested for significance. Random effects included sample and pen. Fixed effects were considered significant at $\alpha = 0.05$. The probability of detecting IAV in OF and NS over time post inoculation was estimated for rRT-PCR, VI, and POC tests from the mixed-effect repeated measures logistic regression model. Likewise, the model was used to calculate the probability of detection for both rRT-PCR laboratories over time. A nonlinear mixed model (Proc NLMIXED, SAS® Version 9.3) was used to estimate mean rRT-PCR Ct values for each laboratory as a function of vaccination status and specimen over DPI. Ct cutoff values for each laboratory served as boundary constraints.

3. Results

General observations: All serum samples collected on DPI -42, -21, -7, and 0 were ELISA negative for *M. hyopneumoniae*, PRRSV and IAV antibodies, as well as negative for PRRSV by rRT-PCR. At the time of inoculation (DPI 0), all animals appeared clinically normal and no statistically significant difference in pig weight by treatment group was detected. Back titration of the H1N1 and H3N2 inocula yielded estimates of $1 \times 10^{6.5}$ and $1 \times 10^{5.7}$ TCID$_{50}$/ml for A/Swine/Ohio/511445/2007 γ H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2, respectively. Pigs were reluctant to move on DPI 1, but not on DPI 2 or later. One pig was treated for polyarthritis with ceftiofur sodium (Excede® for swine, Pfizer Animal Health, 5 mg/kg) on DPI 4, but no animals required treatment for respiratory disease and no animals were removed from the study. A total of 370 OF samples and 924 NS samples were collected for testing (Table 1). Results are summarized by assay and treatment group in Table 2.

Receiver operator characteristic (ROC) analyses (MedCalc® Version 12.3.0.0, MedCalc Software, Maria-kerke, Belgium) of the two OF POC protocols (15 min versus 30 min incubation) for DPIs 1–6 showed that longer incubation resulted in improved overall diagnostic sensitivity, i.e., 41.6% (47/113) with 15 min incubation versus 51.3% (58/113) with 30 min incubation (*p*-value = 0.0015). Prolonged incubation did not affect the specificity (100%, 113/113) of the POC for OF. Because the 30 min incubation POC assay was shown to be more diagnostically sensitive, the 30 min results were used in the statistical model.

An assessment of the association between the number of NS positive pigs within the pen and the detection of IAV in pen-based OF is given in Table 3 by assay. For all assays, the likelihood of IAV detection in OF increased as the number of NS positive pigs in the pen increased. Most notably, the likelihood of an rRT-PCR result was >70% with one NS rRT-PCR positive pig in the pen, but the probability was >30% even when zero NS positive pigs were detected.

Analysis of the data in a mixed-effect logistic regression model identified the variables significant to IAV detection as:

Table 2
Summary of influenza A virus test results (positive/number tested) by assay, specimen, and treatment group.

Assay[a] and specimen[b]	Negative control		H1N1 inoculated[d]		H3N2 inoculated[e]	
	Unvaccinated	Vaccinated[c]	Unvaccinated	Vaccinated[c]	Unvaccinated	Vaccinated[c]
POC OF (15 min incubation)	0/23	0/10	23/75 30.7%	2/30 6.7%	22/66 33.3%	0/27
POC OF (30 min incubation)	0/23	0/10	28/75 37.3%	7/30 23.3%	24/66 36.4%	0/27
POC NS (15 min incubation)	0/84	0/46	67/192 34.9%	10/88 11.4%	83/191 43.5%	0/88
Virus isolation oral fluids	0/26	0/14	26/114 22.3%	1/47 2.1%	7/107 6.5%	1/47 2.1%
Virus isolation nasal swabs	1/95	0/58	119/257 46.3%	28/114 24.6%	119/258 46.1%	1/119 0.8%
rRT-PCR OF Laboratory A	2/28 7.1%	0/14	85/119 71.4%	28/48 58.3%	103/114 90.4%	18/47 38.3%
rRT-PCR OF Laboratory B	1/27 3.7%	0/14	72/115 62.6%	21/48 43.8%	93/114 81.6%	13/47 27.7%
rRT-PCR NS Laboratory A	5/96 5.2%	0/58	176/264 66.7%	60/121 49.6%	181/264 68.6%	42/121 34.7%
rRT-PCR NS Laboratory B	1/96 1.0%	0/58	147/264 55.7%	45/121 37.2%	156/262 59.5%	13/121 10.7%

[a] "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.), real-time reverse-transcription polymerase chain reaction (rRT-PCR).

[b] 370 oral fluid (OF) and 924 nasal swab (NS) samples (DPI 0 to 16) were assayed by rRT-PCR and VI. 231 OF and 689 NS samples (DPI 0 to 10) were tested by POC. Differences between the number collected and the number tested represents missing data.

[c] Vaccinated on DPI -42 and -21 with a trivalent, inactivated influenza vaccine (Flu-Sure® XP, Pfizer Animal Health).

[d] A/Swine/Ohio/511445/2007 γ H1N1 virus.

[e] A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus.

Table 3
Detection of influenza A virus (IAV) in pen-based oral fluid (OF) samples as a function of the number of nasal swab (NS)-positive pigs within a pen.[a]

Assay used to test OF and NS samples	Number of NS-positive pigs within a pen			
	0	1	2	≥3
Virus isolation	0/123	3/16	7/26	23/47
	0%	19%	27%	49%
POC[b] (OF with 30 min incubation)	7/101	12/17	9/10	29/29
	7%	71%	90%	100%
rRT-PCR[c] Laboratory A	23/59	18/25	18/19	112/114
	39%	72%	95%	98%
rRT-PCR[c] Laboratory B	28/86	31/35	9/10	82/82
	33%	89%	90%	100%

[a] Table based on days in which both nasal swab and oral fluid samples were collected from pens of IAV-inoculated pigs, i.e., DPI 0–6, 8, 10, 12, 14, 16.
[b] "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.) comparison based on samples collected from DPI 1–6, 8, 10.
[c] Real-time reverse-transcription polymerase chain reaction (rRT-PCR).

vaccination status (p-value < 0.0001), DPI (p-value < 0.0001), and specimen $\times$ assay (p-value < 0.0001). Virus subtype was not significant to IAV detection (p-value $= 0.89$); therefore, these data were subsumed into a single variable for subsequent analyses. The probability of IAV detection (p) over time by specimen (p_{OF}, p_{NS}) was calculated using Eqs. (1) and (2), with results for unvaccinated and vaccinated pigs shown in Figs. 1 and 2, respectively.

$$\lambda_{\text{specimen type}} = \text{Logit}(P(X)) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \quad (1)$$

α = intercept; β_1 = regression coefficient for vaccination status (referent: vaccinated); β_2 = regression coefficient for DPI 1–16 (referent: DPI = 1); β_3 = regression coefficient for assay (referent: POC).

$$\text{Probability specimen type} = (p_{OF,NS}) = \frac{e^{(\lambda)}}{1 + e^{(\lambda)}} \quad (2)$$

The calculated probability ($\hat{p}$) differed significantly between unvaccinated and vaccinated pigs (Table 4). In
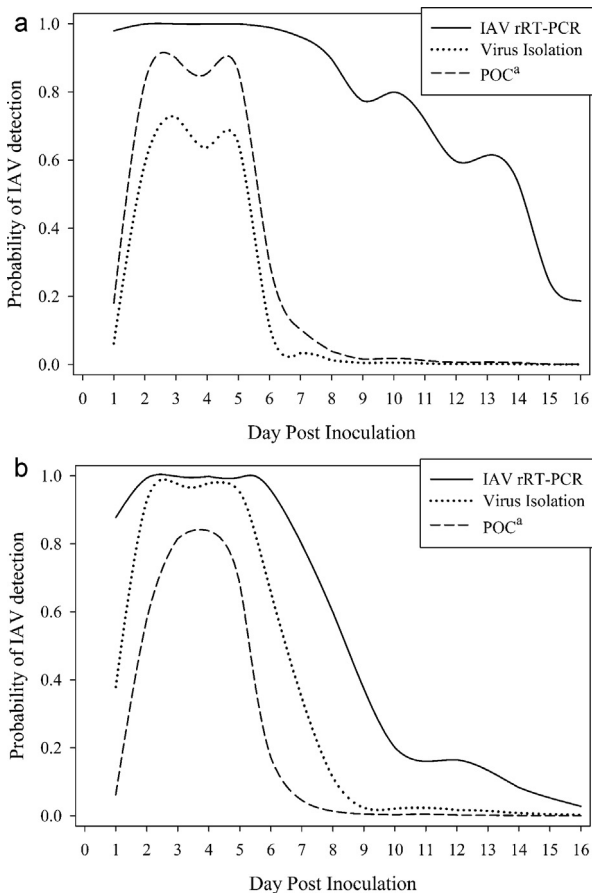
Fig. 1. Probability of detecting IAV in pen-based OF (a) or individual NS (b) from unvaccinated pigs by assay over time ([a]"Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.).
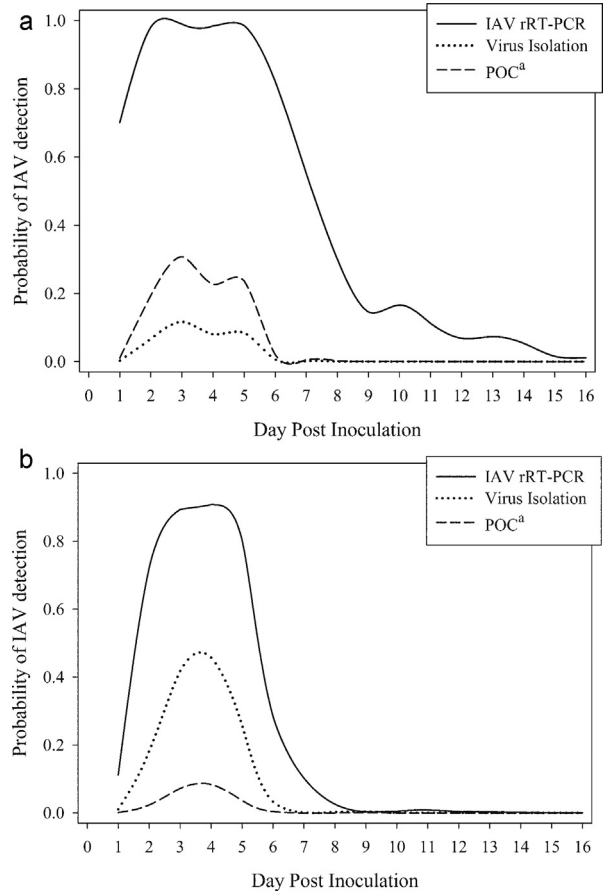
Fig. 2. Probability of IAV detection in pen-based OF (a) or individual NS (b) from vaccinated pigs by assay over time ([a]"Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.).

Table 4
Probability of detecting IAV over time in inoculated animals by vaccination status, assay,[a] and specimen.[b]

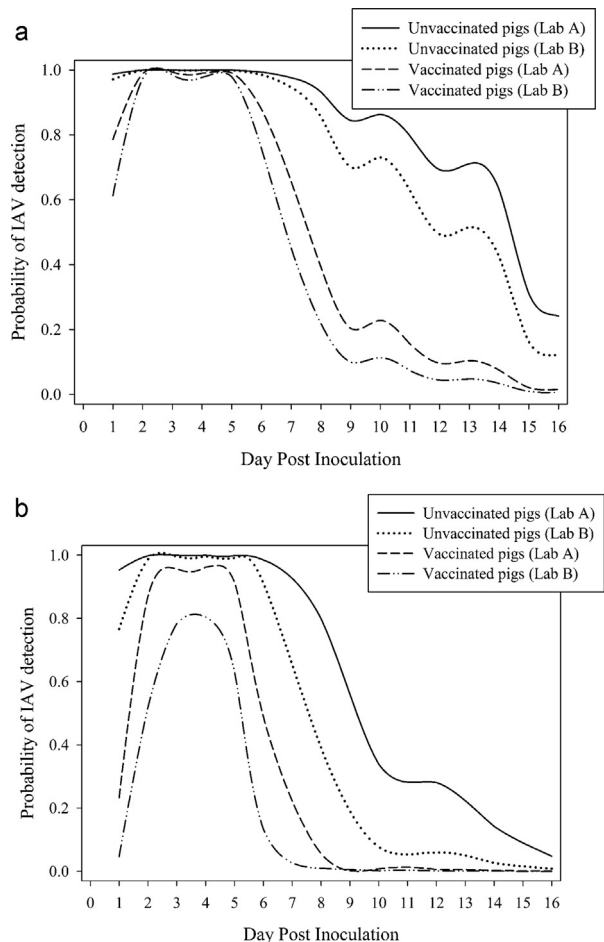| DPI	Unvaccinated, IAV-inoculated						Vaccinated, IAV-inoculated					
	rRT-PCR		Virus isolation		POC		rRT-PCR		Virus isolation		POC[a]	
	OF	NS	OF	NS	OF	NS	OF	NS	OF	NS	OF	NS
1	0.98	0.88	0.06	0.38	0.18	0.06	0.70	0.11	0.00	0.01	0.01	0.00
2	1.00	0.99	0.59	0.93	0.83	0.58	0.98	0.72	0.07	0.18	0.19	0.02
3	1.00	1.00	0.73	0.98	0.90	0.81	0.99	0.89	0.12	0.42	0.31	0.07
4	1.00	1.00	0.64	0.98	0.85	0.84	0.98	0.91	0.08	0.46	0.23	0.08
5	1.00	1.00	0.65	0.95	0.86	0.68	0.99	0.80	0.08	0.26	0.24	0.04
6	0.99	0.96	0.11	0.66	0.30	0.17	0.82	0.28	0.01	0.03	0.02	0.00
7	0.96	na	0.03	na	0.10	na	0.55	na	0.00	na	0.01	na
8	0.90	0.60	0.01	0.11	0.04	0.01	0.30	0.03	0.00	0.00	0.00	0.00
9	0.77	na	0.00	na	0.02	na	0.15	na	0.00	na	0.00	na
10	0.80	0.20	0.01	0.02	0.02	0.00	0.17	0.00	0.00	0.00	0.00	0.00
11	0.72	na	0.00	na	0.01	na	0.11	na	0.00	na	0.00	na
12	0.60	0.16	0.00	0.02	0.01	0.00	0.07	0.00	0.00	0.00	0.00	0.00
13	0.61	na	0.00	na	0.01	na	0.07	na	0.00	na	0.00	na
14	0.53	0.08	0.00	0.01	0.01	0.00	0.05	0.00	0.00	0.00	0.00	0.00
15	0.24	na	0.00	na	0.00	na	0.02	na	0.00	na	0.00	na
16	0.19	0.03	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00

[a] Real-time reverse-transcription polymerase chain reaction (rRT-PCR), virus isolation and "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.).
[b] Oral fluid (OF) and nasal swab (NS) samples.
na: No sample collected.

unvaccinated pigs, rRT-PCR $\hat{p}_{OF}$ and $\hat{p}_{NS}$ were equivalent through DPI 6, then higher in OF through DPI 16. VI was more successful using NS than OF, but VI $\hat{p}$ was significantly lower than rRT-PCR $\hat{p}$. The POC assay $\hat{p}$ was greater in pen-based OF (30 min incubation) from DPI 1–6 than individual NS in unvaccinated groups. After DPI 6, both VI and POC were generally unsuccessful in unvaccinated animals. Vaccination significantly reduced the $\hat{p}$ for all assays. For example, the rRT-PCR $\hat{p}$ on DPI 1 in vaccinated pigs was 70% for pen-based OF and 11% for NS versus 98% and 88%, respectively for samples from unvaccinated pigs. The highest $\hat{p}$ of isolating virus from OF was $\leq$12% at any time and $\leq$46% for individual NS. After DPI 6, $\hat{p}$ approached zero in all assay-specimen combinations except for OF specimens tested by rRT-PCR.

IAV rRT-PCR performance: In unvaccinated animals, significant differences between laboratories were observed after DPI 7 (Fig. 3a and b), whereas differences were observed at DPI 1 and after DPI 6 in vaccinated animals (Fig 3a and b). As given in Table 5, within laboratory estimated mean Ct values were essentially equivalent between OF and NS from DPI 0–5 in unvaccinated pigs, after which estimated mean Ct values were lower, i.e., virus RNA concentrations were higher, in OF than NS through DPI 16. Similar observations were made in vaccinated pigs, with sporadic detection in OF after DPI 9.

Results of hemagglutinin subtyping (H1 or H3) on rRT-PCR IAV matrix gene positive samples are reported in Table 6. In both OF and NS samples, correctly identifying subtype ("matching") was significantly related to DPI (*p*-value < 0.0001) and laboratory (*p*-value < 0.0001). That is, matching was more successful in early infection and Laboratory B was significantly more likely than Laboratory A to match matrix positive samples to subtype from either OF or NS. Specifically, Laboratory A reported 129 of 605 (21%) of matrix-positive samples as "undetermined"

Fig. 3. Probability of detecting IAV in OF (a) and NS (b) by rRT-PCR by laboratory in unvaccinated and vaccinated pigs over time.

Table 5
Estimated mean Ct values of rRT-PCR positive samples by vaccination status, laboratory, and specimen.[a]

DPI	Unvaccinated, IAV-inoculated				Vaccinated, IAV-inoculated			
	Laboratory A		Laboratory B		Laboratory A		Laboratory B	
	OF	NS	OF	NS	OF	NS	OF	NS
	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)	$\bar{x}$ (95% CI)
1	27 (26, 28)	30 (29, 31)	32 (31, 33)	35 (34, 36)	37 (36, 38)	>37	>40	>40
2	18 (17, 19)	21 (20, 22)	23 (22, 23)	26 (25, 27)	27 (26, 29)	31 (30, 32)	32 (31, 33)	35 (35, 36)
3	16 (15, 17)	19 (18, 20)	21 (20, 22)	24 (23, 25)	26 (25, 27)	29 (28, 30)	30 (29, 31)	33 (33, 34)
4	16 (15, 17)	19 (18, 20)	20 (20, 21)	24 (23, 24)	25 (24, 26)	29 (28, 30)	30 (29, 31)	33 (32, 34)
5	17 (16, 18)	20 (20, 21)	22 (21, 23)	25 (24, 26)	27 (26, 28)	30 (29, 31)	32 (31, 33)	35 (34, 36)
6	22 (21, 23)	25 (24, 26)	27 (26, 28)	30 (29, 31)	32 (31, 33)	35 (34, 36)	37 (36, 38)	40 (39, 41)
7	27 (25, 28)	na	32 (30, 33)	na	37 (35, 38)	na	>40	na
8	31 (30, 32)	34 (34, 35)	36 (35, 37)	39 (38, 40)	>37	>37	>40	>40
9	33 (31, 34)	na	37 (36, 39)	na	>37	>37	>40	>40
10	35 (34, 36)	>37	>40 (39, 41)	>40	>37	>37	>40	>40
11	33 (32, 35)	na	38 (36, 40)	na	>37	>37	>40	>40
12	36 (35, 38)	>37	>40	>40	>37	>37	>40	>40
13	34 (32, 36)	na	39 (37, 40)	na	>37	>37	>40	>40
14	>37 (36, 38)	>37	>40	>40	>37	>37	>40	>40
15	>37 (36, 40)	na	>40	na	>37	>37	>40	>40
16	>37	>37	>40	>40	>37	>37	>40	>40

[a] Oral fluid (OF) and nasal swab (NS) samples.
na: No sample collected.

Table 6
Influenza A virus rRT-PCR hemagglutinin subtyping (H1 or H3) results for rRT-PCR IAV matrix gene-positive samples.

DPI	Laboratory A								Laboratory B							
	Oral fluids[a]				Nasal swabs[a]				Oral fluids[a]				Nasal swabs[a]			
	N	M	MM	UD	N	M	MM	UD	N	M	MM	UD	N	M	MM	UD
1	18	13	0	5	50	36	3	11	17	17	0	0	33	33	0	0
2	20	19	0	1	65	61	1	3	19	17	0	2	58	58	0	0
3	18	16	0	2	69	64	0	5	17	16	0	1	61	61	0	0
4	18	17	1	0	67	65	1	1	18	17	1	0	59	59	0	0
5	19	18	0	1	65	58	3	4	17	17	0	0	62	62	0	0
6	21	20	0	1	63	51	2	10	21	20	1	0	53	53	0	0
7	21	18	1	2	na[b]	na	na	na	18	18	0	0	na	na	na	na
8	18	13	0	5	46	24	1	21	14	14	0	0	19	19	0	0
9	14	8	0	6	na	na	na	na	11	11	0	0	na	na	na	na
10	15	6	1	8	15	6	1	8	12	11	1	0	6	6	0	0
11	12	8	0	4	na	na	na	na	9	9	0	0	na	na	na	na
12	11	7	0	4	10	3	0	7	8	8	0	0	6	4	2	0
13	12	6	2	4	na	na	na	na	8	8	0	0	na	na	na	na
14	11	5	0	6	8	2	4	2	6	5	0	1	1	1	0	0
15	4	2	0	2	na	na	na	na	3	2	0	1	na	na	na	na
16	3	0	0	3	3	0	0	3	1	1	0	0	0	0	0	0

[a] "N" gives the number of rRT-PCR matrix gene-positive samples by DPI. Match (M) indicates the number of samples correctly identified by subtype, e.g. H1 or H3. Mis-match (MM) indicates the number of samples in which the subtype was incorrectly identified and undetermined (UD) indicates the number of samples in which it was not possible achieve subtype identity.
[b] No nasal swab samples collected on DPI 7, 9, 11, 13, or 15.

versus 5 of 554 (1%) from Laboratory B. Of the 129 samples with an undetermined subtype from Laboratory A, 33 (26%) were from vaccinated pens. Two of the 5 OF samples (40%) classified as undetermined from Laboratory B were from vaccinated pens.

4. Discussion

The detection of IAV in pen-based oral fluid samples and individual nasal swabs was compared for the first 16 days after intratracheal inoculation using three diagnostic assays (VI, POC, rRT-PCR). The timeline allowed for comparison of the onset, magnitude, and duration of virus shedding for two IAV subtypes in vaccinated and unvaccinated pigs. In recognition of the dynamic nature of IAV, the viruses selected for this study represented contemporary H1N1 (A/Swine/Ohio/511445/2007 γ H1N1) and H3N2 (A/Swine/Illinois/02907/2009 Cluster IV H3N2) isolates (Vincent et al., 2009; WHO, 2011). The viruses shared 95.4% and 98.4% HA amino acid homology to two of the IAV viruses (A/Swine/Iowa/110600/00 γ H1N1 and A/Swine/Missouri/069/05 H3N2) in the inactivated trivalent commercial vaccine. The three assays evaluated were chosen purposefully: (1) virus isolation is the historical standard for IAV diagnosis, although time consuming and not necessarily diagnostically sensitive

(Cheng et al., 2010; Ganzenmueller et al., 2010); (2) the POC assay is an animal-side test, but its performance has not been established for swine; and (3) rRT-PCR is the contemporary method of IAV diagnosis, but assay performance estimates are lacking.

A mixed-effect logistic regression model examining the association between IAV detection in specimen (OF or NS), virus subtype (H1N1 or H3N2), assay (VI, POC, rRT-PCR), vaccination status (yes/no), and DPI identified vaccination status (p-value < 0.0001), DPI (p-value < 0.0001), and the interaction of specimen and assay (p-value < 0.0001) as significant. The effect of vaccination and DPI is consistent with current knowledge regarding immunity against IAV whereas the specimen-assay interaction addresses the relationship between diagnostic specimens and assay performance.

IAV infection in healthy, naïve animals produces an anti-IAV immune response that results in a rapid reduction in viral replication and shedding (Van Reeth et al., 2012). Acquired immunity produced by IAV infection includes both cell mediated immunity that functions in viral clearance and heterosubtypic IAV immunity (Flynn et al., 1998; Nguyen et al., 1999; Takada et al., 2003; Webster and Askonas, 1980), and anti-IAV antibody on respiratory mucosal surfaces that prevents viral attachment and entry into pulmonary epithelial cells (Larsen et al., 2000). In addition to immunity resulting from primary infection, maternal antibody and immune priming produced by vaccination can also affect the course of infection. Maternally derived antibody (MDA) has been shown to reduce clinical signs in IAV-infected piglets (Kitikoon et al., 2006; Loeffen et al., 2003) and affect detection and transmission of IAV in both homologously and heterologously IAV-challenged piglets (Allerson et al., 2013; Kitikoon et al., 2006).

The ability of vaccine to reduce the magnitude and duration IAV shedding has been reported previously in both mammals and birds (Bikour et al., 1996; Bos et al., 2008; Jones et al., 2011; Paillot et al., 2013; Vincent et al., 2010). This is significant because a reduction in IAV shedding reduces the rate of transmission in pig populations (Allerson et al., 2013; Romagosa et al., 2011, 2012; Torremorell et al., 2012). For reasons that are unclear, the degree of immunity conferred by vaccination is variable. That is, protection is not entirely explained by the vaccine and challenge virus hemagglutinin amino acid homology (Vincent et al., 2010; Kyriakis et al., 2010; Van Reeth et al., 2004). The γ H1N1 and Cluster 4 H3N2 components of the commercial vaccine used in this study were 95.4% and 98.4% homologous to the HA amino acid sequences of the H1N1 and H3N2 viruses. Although amino acid homology cannot be directly correlated with protection, the effect of vaccination differed between virus subtypes. As shown in Table 2, IAV was isolated from 28 of 114 (24.6%) NS samples collected from H1N1 inoculated, vaccinated pigs (group V_{H1}) versus 1 of 119 (0.8%) NS samples collected from H3N2 inoculated, vaccinated pigs (group V_{H3}).

The interaction of specimen and assay underscores the fact that both the assay and the specimen affect the likelihood of detecting IAV. Virus isolation is time consuming and costly, but VI is currently the only option

if it is necessary to recover IAV isolates for further use or analysis. In both unvaccinated and vaccinated pigs, the probability of isolating IAV from NS or OF was highest at DPI 4, with the success of VI declining rapidly thereafter. Isolation was significantly less likely in OF than NS, particularly in vaccinated animals. We hypothesize that this reflects the presence of anti-IAV antibody (both IgG and IgA) in OF (Panyasing et al., 2012).

A marked advantage of the POC is rapid turnaround (30 min) for either OF or NS specimens. The POC assay detected IAV in swine in the first five days of infection with a positive predictive value of 100% (no false positives). However, the assay's diagnostic sensitivity for OF specimens for DPIs 1–5 was 51.3%, with better performance in unvaccinated pigs (64.6%) than vaccinated pigs (20.6%). Thus, the test was relatively insensitive and markedly affected by the presence of anti-IAV antibodies.

Relative to VI and POC, rRT-PCR was the most likely to detect IAV for the longest time post inoculation (Figs. 1 and 2), particularly in OF (Table 4). A comparison of rRT-PCR results from the two independent laboratories showed that the pattern of Ct values was affected by DPI, specimen, and vaccination status. Estimated mean OF and NS Ct values were similar within laboratory until DPI 6 (Table 5), after which estimated OF Ct values trended lower (indicating a higher virus concentration). Since IAV replicates in bronchial epithelial cells, the higher and more prolonged detection of viral RNA in OF could be explained by physical expulsion of virus from the lungs via normal pulmonary clearance mechanisms, such as coughing (Levandowski et al., 1985). Vaccination status affected detection, but the relationship remained the same, i.e., OF estimated mean Ct values were usually equal to, or less than, NS estimated mean Ct values.

A direct statistical comparison of Ct values between laboratories was not possible because of differences in threshold and cutoff values, but it was possible to determine that the probability of detection differed between laboratories. Specifically, probability estimates ($\hat{p}$) based on logistic regression analysis revealed that Laboratory A was significantly more likely to report rRT-PCR positive OF and NS specimens than Laboratory B (Fig. 3a and b), while Laboratory B was more likely to correctly identify virus subtype in rRT-PCR-positive samples (Table 6). These results are of concern because surveillance requires highly reproducible and repeatable assays; otherwise, results come to be viewed with doubt and skepticism. Resolution of this issue should be a high priority.

Influenza A virus-associated morbidity and mortality is an economically significant problem in commercial swine populations. For producers and veterinarians, the prevention and/or control of IAV is dependent upon the degree of match between herd immunity (passive and acquired) and the virus strain infecting the herd. Maintaining herd protection is complicated by the constant emergence of new antigenic variants as IAV circulates within, and moves between, susceptible host populations. For example, pH1N1, first identified in April 2009 in humans (Garten et al., 2009), is now globally endemic in swine, co-circulating with established subtypes and

creating novel reassortants (Ali et al., 2012; Chen et al., 2013; Starick et al., 2012).

To respond in a timely fashion to this rapidly evolving scenario, swine producers and veterinarians need population-based sampling methods that provide for the continual assessment of IAV. Historically, ante mortem IAV detection has relied on NS specimens. While NS are the best sample for VI (Table 4), the process of collecting NS specimens is both labor intensive and dependent upon fortuitously selecting a pig in the first 7 days of IAV infection, when virus is still present in nasal secretions (Van Reeth et al., 2012). As an alternative, pen-based OF are easily collected and the probability of detecting IAV infection by rRT-PCR was actually shown to be higher than individual pig NS specimens in this study and elsewhere (Romagosa et al., 2012). Previously, shown to be an effective diagnostic specimen for a variety of swine pathogens (Detmer et al., 2011; Kittawornrat et al., 2010; Prickett et al., 2008a,b; Ramirez et al., 2012), OF would appear to be the specimen of choice for the surveillance of IAV.

Conflict of interest

Acknowledgments

References

Ali, A., Khatri, M., Wang, L., Saif, Y.M., Lee, C.W., 2012. Identification of swine H1N2/pandemic H1N1 reassortant influenza virus in pigs, United States. Vet. Microbiol. 158, 60–68.

Al Johani, S.M., Al Balawi, M., Al Alwan, B., Al Hefdhi, R., Hajeer, A., 2011. Validity of two rapid point of care influenza tests and direct fluorescence assay in comparison of real time PCR for swine of origin Influenza virus. J. Infect. Public Health 4, 7–11.

Allerson, M., Deen, J., Detmer, S.E., Gramer, M.R., Soo Joo, H., Romagosa, A., Torremorell, M., 2013. The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations. Vaccine 31, 500–505.

Babin, S.M., Hsieh, Y.H., Rothman, R.E., Gaydos, C.A., 2011. A meta-analysis of point-of-care laboratory tests in the diagnosis of novel 2009 swine-lineage pandemic influenza A (H1N1). Diagn. Microbiol. Infect. Dis. 69, 410–418.

Bikour, M.H., Cornaglia, E., Elazhary, Y., 1996. Evaluation of a protective immunity induced by an inactivated influenza H3N2 vaccine after an intratracheal challenge of pigs. Can. J. Vet. Res. 60, 312–314.

Boesenberg, K., 2012. Procedure for AIV MultiS-Screen ELISA. Doc 9.1330.In: Quality Systems, Veterinary Diagnostic Laboratory. Iowa State University, Ames, Iowa.

Bos, M.E., Nielen, M., Koch, G., Stegeman, A., De Jong, M.C., 2008. Effect of H7N1 vaccination on highly pathogenic avian influenza H7N7 virus transmission in turkeys. Vaccine 26, 6322–6328.

Bowman, A.S., Nelson, S.W., Edwards, J.L., Hofer, C.C., Nolting, J.M., Davis, I.C., Slemons, R.D., 2013. Comparative effectiveness of isolation techniques for contemporary Influenza A virus strains circulating in exhibition swine. J. Vet. Diagn. Invest. 25, 82–90.

Chartrand, C., Leeflang, M.M., Minion, J., Brewer, T., Pai, M., 2012. Accuracy of rapid influenza diagnostic tests: a meta-analysis. Ann. Intern. Med. 156, 500–511.

Chen, Y., Zhang, J., Qiao, C., Yang, H., Zhang, Y., Xin, X., Chen, H., 2013. Co-circulation of pandemic 2009 H1N1, classical swine H1N1 and avian-like swine H1N1 influenza viruses in pigs in China. Infect. Genet. Evol. Infect. Genet. Evol. 13, 331–338.

Cheng, P.K., Wong, K.K., Mak, G.C., Wong, A.H., Ng, A.Y., Chow, S.Y., Lam, R.K., Lau, C.S., Ng, K.C., Lim, W., 2010. Performance of laboratory diagnostics for the detection of influenza A(H1N1)v virus as correlated with the time after symptom onset and viral load. J. Clin. Virol. 47, 182–185.

Cheng, S.M., Vainionpaa, R., Zhao, P., Li, F., Hu, A., Forrest, B., Rappaport, R., 2004. Detection of influenza B in clinical specimens: comparison of high throughput RT-PCR and culture confirmation. Virus Res. 103, 85–90.

Ciacci-Zanella, J.R., Vincent, A.L., Prickett, J.R., Zimmerman, S.M., Zimmerman, J.J., 2010. Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. J. Vet. Diagn. Invest. 22, 3–9.

Detmer, S.E., Patnayak, D.P., Jiang, Y., Gramer, M.R., Goyal, S.M., 2011. Detection of Influenza A virus in porcine oral fluid samples. J. Vet. Diagn. Invest. 23, 241–247.

Eshelman, R., 2010a. Procedure for Ambion AgPath-ID Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Real-time PCR. Doc ID: [9.496] v3.In: Quality Systems, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

Eshelman, R., 2010b. Procedure for Kingfisher96 or 24 MagMAX Viral RNA Extraction. Doc ID: [9. 459]In: Quality Systems, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

Fuenzalida, L., Blanco, S., Prat, C., Vivancos, M., Dominguez, M.J., Mòdol, J.M., Rodrigo, C., Ausina, V., 2010. Utility of the rapid antigen detection BinaxNOW Influenza A&B test for detection of novel influenza A (H1N1) virus. Clin. Microbiol. Infect. 16, 1574–1576.

Flynn, K.J., Belz, G.T., Altman, J.D., Ahmed, R., Woodland, D.L., Doherty, P.C., 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity 8, 683–691.

Ganzenmueller, T., Kluba, J., Hilfrich, B., Puppe, W., Verhagen, W., Heim, A., Schulz, T., Henke-Gendo, C., 2010. Comparison of the performance of direct fluorescent antibody staining, a point-of-care rapid antigen test and virus isolation with that of RT-PCR for the detection of novel 2009 influenza A (H1N1) virus in respiratory specimens. J. Med. Microbiol. 59, 713–717.

Gao, F., Loring, C., Laviolette, M., Bolton, D., Daly, E.R., Bean, C., 2012. Detection of 2009 pandemic influenza A(H1N1) virus Infection in different age groups by using rapid influenza diagnostic tests. Influenza Other Respi. Viruses 6, e30–e34.

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., López-Gatell, H., Olivera, H., López, 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., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I., Cox, N.J., 2009. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 325, 197–201.

Gimeno, C., Bravo, D., Ocete, D., Tormo, N., Navalpotro, D., Costa, E., de Lomas, J.G., Navarro, D., 2010. Comparison of BinaxNOW Influenza A&B assay and real-time reverse transcription polymerase chain reaction for diagnosis of influenza A pandemic (H1N1) 2009 virus infection in adult patients. Diagn. Microbiol. Infect. Dis. 68, 456–458.

Hurt, A.C., Baas, C., Deng, Y.M., Roberts, S., Kelso, A., Barr, I.G., 2009. Performance of influenza rapid point-of-care tests in the detection of swine lineage A(H1N1) influenza viruses. Influenza Other Respi. Viruses 3, 171–176.

Jones, F.R., Gabitzsch, E.S., Xu, Y., Balint, J.P., Borisevich, V., Smith, J., Smith, J., Peng, B.H., Walker, A., Salazar, M., Paessler, S., 2011. Prevention of influenza virus shedding and protection from lethal H1N1 challenge using a consensus 2009 H1N1 HA and NA adenovirus vector vaccine. Vaccine 29, 7020–7026.

Kitikoon, P., Nilubol, D., Erickson, B.J., Janke, B.H., Hoover, T.C., Sornsen, S.A., Thacker, E.L., 2006. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet. Immunol. Immunopathol. 112, 117–128.

Kittawornrat, A., Prickett, J., Chittick, W., Wang, C., Engle, M., Johnson, J., Patnayak, D., Schwartz, T., Whitney, D., Olsen, C., Schwartz, K., Zimmerman, J., 2010. Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? Virus Res. 154, 170–176.

Kittawornrat, A., Zimmerman, J., 2011. Toward a better understanding of pig behavior and pig welfare. Anim. Health Res. Rev. 12, 25–32.

Kyriakis, C.S., Gramer, M.R., Barbé, F., Van Doorsselaere, J., Van Reeth, K., 2010. Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. Vet. Microbiol. 144, 67–74.

Larsen, D.L., Karasin, A., Zuckermann, F., Olsen, C.W., 2000. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. Vet. Microbiol. 74, 117–131.

Levandowski, R.A., Gerrity, T.R., Garrard, C.S., 1985. Modifications of lung clearance mechanisms by acute influenza A infection. J. Lab. Clin. Med. 106, 428–432.

Liao, R.S., Landt, O., Hill, J.T., 2011. Comparison of a laboratory-developed RT-PCR and the CDC RT-PCR protocol with rapid immunodiagnostic testing during the 2009 H1N1 influenza A pandemic. Diagn. Microbiol. Infect. Dis. 70, 236–239.

Liao, R.S., Tomalty, L.L., Majury, A., Zoutman, D.E., 2009. Comparison of viral isolation and multiplex real-time reverse transcription-PCR for confirmation of respiratory syncytial virus and influenza virus detection by antigen immunoassays. J. Clin. Microbiol. 47, 527–532.

Loeffen, W.L., Heinen, P.P., Bianchi, A.T., Hunneman, W.A., Verheijden, J.H., 2003. Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. Vet. Immunol. Immunopathol. 92, 23–35.

Nguyen, H.H., Moldoveanu, Z., Novak, M.J., van Ginkel, F.W., Ban, E., Kiyono, H., McGhee, J.R., Mestecky, J., 1999. Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. Virology 254, 50–60.

Paillot, R., Prowse, L., Montesso, F., Huang, C.M., Barnes, H., Escala, J., 2013. Whole inactivated equine influenza vaccine: efficacy against a representative clade 2 equine influenza virus. IFNgamma synthesis and duration of humoral immunity. Vet. Microbiol. 162, 396–407.

Panyasing, Y., Goodell, C.K., Wang, C., Kittawornrat, A., Prickett, J.R., Schwartz, K.J., Ballagi, A., Lizano, S., Zimmerman, J.J., 2012. Detection of influenza A virus nucleoprotein antibodies in oral fluid specimens from pigs infected under experimental conditions using a blocking ELISA. Transbound. Emerg. Dis., http://dx.doi.org/10.1111/tbed.12019.

Pregliasco, F., Mensi, C., Camorali, L., Anselmi, G., 1998. Comparison of RT-PCR with other diagnostic assays for rapid detection of influenza viruses. J. Med. Virol. 56, 168–173.

Prickett, J., Simer, R., Christopher-Hennings, J., Yoon, K.J., Evans, R.B., Zimmerman, J.J., 2008a. Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. J. Vet. Diagn. Invest. 20, 156–163.

Prickett, J., Simer, R., Yoon, K.J., Kim, W.I., Zimmerman, J.J., 2008b. Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J. Swin. Health Prod. 16, 86–91.

Prickett, J.R., Zimmerman, J.J., 2010. The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim. Health Res. Rev. 11, 207–216.

Ramirez, A., Wang, C., Prickett, J.R., Pogranichniy, R., Yoon, K.J., Main, R., Johnson, J.K., Rademacher, C., Hoogland, M., Hoffmann, P., Kurtz, A., Kurtz, E., Zimmerman, J., 2012. Efficient surveillance of pig populations using oral fluids. Prev. Vet. Med. 104, 292–300.

Richt, J.A., Lager, K.M., Clouser, D.F., Spackman, E., Suarez, D.L., Yoon, K.J., 2004. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. J. Vet. Diagn. Invest. 16, 367–373.

Romagosa, A., Allerson, M., Gramer, M., Joo, H.S., Deen, J., Detmer, S., Torremorell, M., 2011. Vaccination of influenza a virus decreases transmission rates in pigs. Vet. Res. 42, 120.

Romagosa, A., Gramer, M., Joo, H.S., Torremorell, M., 2012. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza Other Respi. Viruses 6, 110–118.

Starick, E., Lange, E., Grund, C., Grosse Beilage, E., Döhring, S., Maas, A., Noé, T., Beer, M., Harder, T.C., 2012. Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J. Gen. Virol. 93, 1658–1663.

Swenson, S.L., Vincent, L.L., Lute, B.M., Janke, B.H., Lechtenberg, K.E., Landgraf, J.G., Schmitt, B.J., Kinker, D.R., McMillen, J.K., 2001. A comparison of diagnostic assays for the detection of type A swine influenza virus from nasal swabs and lungs. J. Vet. Diagn. Invest. 13, 36–42.

Takada, A., Matsushita, S., Ninomiya, A., Kawaoka, Y., Kida, H., 2003. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine 21, 3212–3218.

Torremorell, M., Allerson, M., Corzo, C., Diaz, A., Gramer, M., 2012. Transmission of Influenza A Virus in Pigs. Transbound. Emerg. Dis., http://dx.doi.org/10.1111/j.1865-1682.2011.01300.x.

USDA July, 2010. Influenza surveillance in swine – procedures manual. http://www.aphis.usda.gov/animal_health/animal_dis_spec/swine/downloads/influenza_surv_swine_proc_manual.pdf (accesssed 12.12.12).

Van Reeth, K., Brown, I., Essen, S., Pensaert, M., 2004. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus. Res. 103, 115–124.

Van Reeth, K., Brown, I.A., Olsen, C.W., 2012. Ch 40 influenza virus. In: Zimmerman, J.J., Karriker, L., Ramirez, A., Schwartz, K., Stevenson, G. (Eds.), Diseases of Swine. 10th ed. John Wiley & Sons Inc, Ames, IA, pp. 557–572.

Vincent, A.L., Swenson, S.L., Lager, K.M., Gauger, P.C., Loiacono, C., Zhang, Y., 2009. Characterization of an influenza A virus isolated from pigs during an outbreak of respiratory disease in swine and people during a county fair in the United States. Vet. Microbiol. 137, 51–59.

Vincent, A.L., Ciacci-Zanella, J.R., Lorusso, A., Gauger, P.C., Zanella, E.L., Kehrli Jr., M.E., Janke, B.H., Lager, K.M., 2010. Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs. Vaccine 28, 2782–2787.

Webster, R.G., Askonas, B.A., 1980. Cross-protection and cross-reactive cytotoxic T cells induced by influenza virus vaccines in mice. Eur. J. Immunol. 10, 396–401.

World Health Organization, 2009. CDC protocol of realtimeRT-PCR for swine influenza A(H1N1). http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html (accessed 12.12.12).

World Health Organization, 2011. Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. Wkly. Epidemiol. Rec. 86, 469–480.