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 (p̂) 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 p̂ 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 of OF specimens provided the highest p̂ for the most DPIs, yet significantly different results were observed between the two laboratories independently performing rRT-PCR testing.

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). Antemortem 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; Ganzennmueller et al., 2010; Gao et al., 2012; Gimeno et al., 2010; Hurt et al., 2009; Liao et al., 2009, 2011; Preglasco et al., 1998). In swine research, focus has remained 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 (#11–09-6834-5) and the Institutional Biosafety Committee (09-1-0028-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 $M. \text{hyopneumoniae}$ 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.

<table>
<thead>
<tr>
<th>Group designation</th>
<th>Negative control</th>
<th>H1N1 inoculated(a)</th>
<th>H3N2 inoculated(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV(\text{CTRL})</td>
<td>V(\text{CTRL})</td>
<td>UV(\text{H1})</td>
</tr>
<tr>
<td></td>
<td>V(\text{UV})</td>
<td>V(\text{H1})</td>
<td>V(\text{H3})</td>
</tr>
<tr>
<td>Vaccination(a)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Pigs</td>
<td>6</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Pens</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Of samples(e) (n = 370)</td>
<td>28</td>
<td>14</td>
<td>119</td>
</tr>
<tr>
<td>NS samples(e) (n = 924)</td>
<td>96</td>
<td>48</td>
<td>124</td>
</tr>
</tbody>
</table>

\(a\) A/Swine/Ohio/511445/2007 \(\gamma\) H1N1 virus.
\(b\) A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus.
\(c\) Vaccinated on DPI \(-24\) and \(-21\) with a trivalent, inactivated influenza vaccine (Flu-Sure\(\text{R}\) 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 \(2 \times 2\) 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\(\text{R}\) W5500 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 \(1 \times 10^5\) TCID\(\text{50}\) per ml of either A/Swine/Ohio/511445/2007 \(\gamma\) H1N1 or A/Swine/Illinois/02907/2009 cluster IV H3N2. A portion of each viral inoculum was stored \((-80^\circ\text{C})\) 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 \(4^\circ\text{C}\), after which sera were aliquoted into 5 ml tubes (BD Falcon\(\text{TM}\), Franklin Lakes, NJ) and stored at \(-20^\circ\text{C}\) 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 described 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 \((4^\circ\text{C})\), centrifuged at 13,000 \(\times\) g for 10 min at \(4^\circ\text{C}\), aliquoted into 5 ml snap cap tubes (BD Falcon, Fisher Scientific) and stored at \(-80^\circ\text{C}\).

### 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\(\text{TM}\) minitip flocked swab, Fisher Scientific No. 501CS01) was inserted ~8 cm into one nasal passage of the pig, rotated, and removed. The nasir 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\(\text{R}\)), and the samples were stored at \(-80^\circ\text{C}\).

### 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\(\text{R}\), 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 Multi-S-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, and 60 °C for 45 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 inoculated at 37 °C with 5% CO2 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

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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 α = 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 cefotiofur 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, Mariakerke, 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

<table>
<thead>
<tr>
<th>Assay and specimen</th>
<th>Negative control</th>
<th>H1N1 inoculated</th>
<th>H3N2 inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unvaccinated</td>
<td>Vaccinated</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>POC (15 min incubation)</td>
<td>0/23</td>
<td>0/10</td>
<td>23/75</td>
</tr>
<tr>
<td></td>
<td>0/23</td>
<td>0/10</td>
<td>28/75</td>
</tr>
<tr>
<td>POC (30 min incubation)</td>
<td>0/23</td>
<td>0/10</td>
<td>37/3</td>
</tr>
<tr>
<td>POC NS (15 min incubation)</td>
<td>0/84</td>
<td>0/46</td>
<td>67/192</td>
</tr>
<tr>
<td>Virus isolation oral fluids</td>
<td>0/26</td>
<td>0/14</td>
<td>26/114</td>
</tr>
<tr>
<td></td>
<td>0/26</td>
<td>0/14</td>
<td>22/23</td>
</tr>
<tr>
<td>Virus isolation nasal swabs</td>
<td>1/95</td>
<td>0/58</td>
<td>119/257</td>
</tr>
<tr>
<td>rRT-PCR OF Laboratory A</td>
<td>2/28</td>
<td>0/14</td>
<td>85/119</td>
</tr>
<tr>
<td>rRT-PCR OF Laboratory B</td>
<td>1/27</td>
<td>0/14</td>
<td>72/115</td>
</tr>
<tr>
<td>rRT-PCR NS Laboratory A</td>
<td>5/96</td>
<td>0/58</td>
<td>176/264</td>
</tr>
<tr>
<td>rRT-PCR NS Laboratory B</td>
<td>1/96</td>
<td>0/58</td>
<td>147/264</td>
</tr>
</tbody>
</table>

* “Point of care” (POC) rapid antigen test (VetScanTM, Abaxis Inc.), real-time reverse-transcription polymerase chain reaction (rRT-PCR).

a 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.

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


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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 ($p_{NS}, p_{OF}$) was calculated using Eqs. (1) and (2), with results for unvaccinated and vaccinated pigs shown in Figs. 1 and 2, respectively.

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

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

The calculated probability ($\hat{p}$) differed significantly between unvaccinated and vaccinated pigs (Table 4). In

**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.

<table>
<thead>
<tr>
<th>Assay used to test OF and NS samples</th>
<th>Number of NS-positive pigs within a pen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Virus isolation</td>
<td>0/123</td>
</tr>
<tr>
<td>POC$^b$ (OF with 30 min incubation)</td>
<td>0%</td>
</tr>
<tr>
<td>rRT-PCR$^b$ Laboratory A</td>
<td>7/101</td>
</tr>
<tr>
<td>rRT-PCR$^b$ Laboratory B</td>
<td>7%</td>
</tr>
</tbody>
</table>

$^a$ 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).

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unvaccinated pigs, rRT-PCR $\hat{p}_{\text{OF}}$ and $\hat{p}_{\text{NS}}$ 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”
Table 5
Estimated mean Ct values of rRT-PCR positive samples by vaccination status, laboratory, and specimen.  

<table>
<thead>
<tr>
<th>DPI</th>
<th>Unvaccinated, IAV-inoculated</th>
<th>Laboratory B</th>
<th>Vaccinated, IAV-inoculated</th>
<th>Laboratory B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laboratory A</td>
<td>OF NS</td>
<td>Laboratory B</td>
<td>OF NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27 (26, 28)</td>
<td>30 (29, 31)</td>
<td>32 (31, 33)</td>
<td>35 (34, 36)</td>
</tr>
<tr>
<td>2</td>
<td>18 (17, 19)</td>
<td>21 (20, 22)</td>
<td>23 (22, 23)</td>
<td>26 (25, 27)</td>
</tr>
<tr>
<td>3</td>
<td>16 (15, 17)</td>
<td>19 (18, 20)</td>
<td>21 (20, 22)</td>
<td>24 (23, 25)</td>
</tr>
<tr>
<td>4</td>
<td>16 (15, 17)</td>
<td>19 (18, 20)</td>
<td>20 (21, 22)</td>
<td>24 (23, 24)</td>
</tr>
<tr>
<td>5</td>
<td>17 (16, 18)</td>
<td>20 (21, 20)</td>
<td>22 (21, 23)</td>
<td>25 (24, 26)</td>
</tr>
<tr>
<td>6</td>
<td>22 (21, 23)</td>
<td>25 (24, 26)</td>
<td>27 (26, 28)</td>
<td>30 (29, 31)</td>
</tr>
<tr>
<td>7</td>
<td>27 (25, 28)</td>
<td>na</td>
<td>32 (30, 33)</td>
<td>na</td>
</tr>
<tr>
<td>8</td>
<td>31 (30, 32)</td>
<td>34 (34, 35)</td>
<td>36 (35, 37)</td>
<td>39 (38, 40)</td>
</tr>
<tr>
<td>9</td>
<td>33 (31, 34)</td>
<td>na</td>
<td>37 (36, 39)</td>
<td>na</td>
</tr>
<tr>
<td>10</td>
<td>35 (34, 36)</td>
<td>&gt;37</td>
<td>&gt;40 (39, 41)</td>
<td>&gt;40</td>
</tr>
<tr>
<td>11</td>
<td>33 (32, 35)</td>
<td>na</td>
<td>38 (36, 40)</td>
<td>na</td>
</tr>
<tr>
<td>12</td>
<td>36 (35, 38)</td>
<td>&gt;37</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>13</td>
<td>34 (32, 36)</td>
<td>na</td>
<td>39 (37, 40)</td>
<td>na</td>
</tr>
<tr>
<td>14</td>
<td>&gt;37 (36, 38)</td>
<td>&gt;37</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>15</td>
<td>&gt;37 (36, 40)</td>
<td>na</td>
<td>&gt;40</td>
<td>na</td>
</tr>
<tr>
<td>16</td>
<td>&gt;37</td>
<td>&gt;37</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

* Oral fluid (OF) and nasal swab (NS) samples.

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

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

* "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 to 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

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(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, naive 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

The authors declared the potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Authors R. Rauh, W. Nelson, C. O’Connell, and A. Burrell are employed by private industry. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

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