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## Abstract

The ontogeny of PRRSV antibody in oral fluids has been described using isotype-specific ELISAs. Mirroring the serum response, IgM appears in oral fluid by 7 days post inoculation (DPI), IgA after 7 DPI, and IgG by 9 to 10 DPI. Commercial PRRSV ELISAs target the detection of IgG because the higher concentration of IgG relative to other isotypes provides the best diagnostic discrimination. Oral fluids are increasingly used for PRRSV surveillance in commercial herds, but in younger pigs, a positive ELISA result may be due either to maternal antibody or to antibody produced by the pigs in response to infection. To address this issue, a combined IgM-IgA PRRSV oral fluid ELISA was developed and evaluated for its capacity to detect pig-derived PRRSV antibody in the presence of maternal antibody. Two longitudinal studies were conducted. In Study 1 (modified-live PRRS vaccinated pigs), testing of individual pig oral fluid samples by isotype-specific ELISAs demonstrated that the combined IgM-IgA PRRSV ELISA provided better discrimination than individual IgM or IgA ELISAs. In Study 2 (field data), testing of pen-based oral fluid samples confirmed the findings in Study 1 and established that the IgM-IgA ELISA was able to detect antibody produced by pigs in response to wild-type PRRSV infection, despite the presence of maternal IgG. Overall, the combined PRRSV IgM-IgA oral fluid ELISA described in this study is a potential tool for PRRSV surveillance, particularly in populations of growing pigs originating from PRRSV-positive or vaccinated breeding herds.

## Keywords

Oral fluid, IgM-IgA, PRRSV, ELISA, Surveillance

## Disciplines

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# Detection of porcine reproductive and respiratory syndrome virus (PRRSV)-specific IgM-IgA in oral fluid samples reveals PRRSV infection in the presence of maternal antibody

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## ABSTRACT

The ontogeny of PRRSV antibody in oral fluids has been described using isotype-specific ELISAs. Mirroring the serum response, IgM appears in oral fluid by 7 days post inoculation (DPI), IgA after 7 DPI, and IgG by 9 to 10 DPI. Commercial PRRSV ELISAs target the detection of IgG because the higher concentration of IgG relative to other isotypes provides the best diagnostic discrimination. Oral fluids are increasingly used for PRRSV surveillance in commercial herds, but in younger pigs, a positive ELISA result may be due either to maternal antibody or to antibody produced by the pigs in response to infection. To address this issue, a combined IgM-IgA PRRSV oral fluid ELISA was developed and evaluated for its capacity to detect pig-derived PRRSV antibody in the presence of maternal antibody. Two longitudinal studies were conducted. In Study 1 (modified-live PRRSV vaccinated pigs), testing of individual pig oral fluid samples by isotype-specific ELISAs demonstrated that the combined IgM-IgA PRRSV ELISA provided better discrimination than individual IgM or IgA ELISAs. In Study 2 (field data), testing of pen-based oral fluid samples confirmed the findings in Study 1 and established that the IgM-IgA ELISA was able to detect antibody produced by pigs in response to wild-type PRRSV infection, despite the presence of maternal IgG. Overall, the combined PRRSV IgM-IgA oral fluid ELISA described in this study is a potential tool for PRRSV surveillance, particularly in populations of growing pigs originating from PRRSV-positive or vaccinated breeding herds.

## 1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in 1991 (Wensvoort et al., 1991). Assays for the detection of PRRSV serum antibody became widely available shortly thereafter, including immunoperoxidase monolayer (Wensvoort et al., 1992), immunofluorescence (Benfield et al., 1992), serum-virus neutralization (Benfield et al., 1992), and ELISA (Albina et al., 1992). The detection of PRRSV serum antibody using fluorescent microsphere-based assays has been reported under experimental conditions (Langenhorst et al., 2012). At the present time, the ELISA is the most common format for PRRSV antibody detection and commercial PRRSV antibody ELISA kits are widely available for serum and swine oral fluid specimens (Pejsak et al., 2017).

The ontogeny of PRRSV antibody in serum and oral fluids has been described using isotype-specific ELISAs. Kittawornrat et al. (2013), using paired samples collected over time post inoculation, showed that

the temporal appearance of antibody isotypes in serum and oral fluid was essentially identical in animals inoculated with viable, replicating PRRSV. That is, IgM was detectable by 7 days post inoculation (DPI), IgA after 7 DPI, and IgG by 9 to 10 DPI. Because of the higher concentration of IgG relative to other isotypes, commercial ELISA kits usually target the detection of IgG, although detection of IgM and IgA has been used in human diagnostic medicine.

In addition to antibody produced in response to PRRSV infection or vaccination, younger animals may also have ELISA-detectable PRRSV-specific passive antibody, primarily IgG, in serum and oral fluid (Biernacka et al., 2016; Goyal, 1993; Ramirez et al., 2011). In oral fluid-based testing, maternally-derived antibody creates a challenge in discerning whether a positive ELISA is the result of infection, vaccination, or maternal antibody. Therefore, the goal of this study was to develop a PRRSV combined IgM-IgA oral fluid ELISA and evaluate its performance under experimental and field conditions.

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## 2. Materials and methods

### 2.1. Experimental design

Two longitudinal studies were conducted to evaluate PRRSV oral fluid antibody ontogeny using isotype-specific ELISAs (IgM, IgA, IgG, IgM-IgA). In Study 1 (experimental data), oral fluid antibody isotype responses were evaluated in individual pigs following administration of a modified-live PRRSV vaccine. In Study 2 (field data), PRRSV antibody isotype responses were monitored in oral fluid samples collected from PRRS unvaccinated, group-housed pigs in commercial wean-to-finish farms in Iowa USA. In Study 2, wild-type PRRSV infection was determined by PRRSV real-time reverse-transcriptase PCR (RT-rtPCR) testing and sequencing. Studies were conducted with the authorization of the Iowa State University Office for Responsible Research and the permission of the producers.

### 2.2. Animals and animal care and PRRSV status

Study 1 was an experimental study conducted in a biosafety level 2 research facility located at Iowa State University and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The facility was designed with a single-pass, non-recirculating ventilation system, i.e., unidirectional flow from low contamination areas to high contamination areas. Each room was ventilated separately and humidity and temperature was strictly controlled. Zones of negative pressure prevented airborne contamination from area-to-area or room-to-room. Pigs were housed in individual pens (1.52 m × 1.83 m) in one room.

Partitions with evenly-spaced vertical bars allowed interactions between pigs in adjacent pens. Animal care, housing, and feeding were under the supervision of the research facility staff.

Pigs (n = 12; 50 kg) were sourced from a PRRSV-naïve commercial herd. To confirm PRRSV-naïve status, pigs were tested for PRRSV serum antibody at 19 and 11 days prior to arrival at the research facility and again prior to vaccination. Pigs were acclimated in the facilities for 5 days and then vaccinated with a modified-live PRRS vaccine on Day 0 of the study (Ingelvac PRRS<sup>®</sup> MLV, Boehringer Ingelheim Vetmedica Inc., St. Joseph Missouri). Individual oral fluid samples were collected twice daily from each of the 12 pigs from Day -7 through Day 42 using a protocol described elsewhere (Prickett et al., 2008a,b). In brief, one 100% cotton rope was hung in each pen for 30 min, during which time the pigs interacted (chewed on) the rope. Thereafter, the wet portion of the rope was inserted into a plastic bag and severed from the remainder of the rope. Oral fluid was extracted by passing the wet rope, still within the bag, through a portable towel wringer (Dynajet, Nürtingen, Germany). Samples were decanted into 50 mL centrifuge tubes and placed on crushed ice. The morning and afternoon oral fluid samples from each day were combined, aliquoted into 5 mL cryovial tubes and stored at -80 °C.

Study 2 was a field study conducted on three separate farms in one production system. Each farm (A, B, C) consisted of three curtain-sided, wean-to-finish barns (1, 2, 3) sited parallel to each other and spaced 10 m apart. Barns (13.4 m × 61.0 m) were designed with split-zone ventilation, independent control of curtains, and ridge ventilation by zone. Manure was collected in shallow pits beneath each barn and moved to an outdoor above-ground slurry storage tank via a scraper system. All farms were managed on an all-in-all-out basis, with buildings cleaned and disinfected between groups. Animal housing, handling, feeding, and health care were implemented by producers and with the assistance of production system veterinarians. For the purpose of implementing this study, producers and veterinarians did not vaccinate or move pigs between pens or barns during the 2-month sampling period.

Each barn contained 40 pens arranged as 20 pens on either side of a central walkway. On Farm A, pens (3 m × 6 m) were built with solid

concrete walls and partial slats. On Farm B and C, pens (3 m × 6 m) were built with gated walls and partial slats. During the collection period, all occupied pens held ~25 pigs. Barns were populated with weaned pigs (~21 days of age) sourced from one PRRSV-endemic breeding herd over the course of 7 to 14 days, but each farm's pigs came from a different sow herd. For all breeding herds, commercial modified-live PRRS vaccines were administered to replacement gilts during quarantine, but not to sows or pigs in other phases of production.

Oral fluid samples were collected weekly from every occupied pen (n = 36) in every barn (n = 3) on each farm (n = 3) using the procedure described above. Samples were decanted into 50 mL centrifuge tubes and placed on crushed ice for transport to the laboratory. Samples were aliquoted into 4 mL cryovials in the laboratory and stored at -20 °C.

After weekly oral fluid collection, blood samples were collected from 20 pigs in each barn by sampling 10 pigs from each of two pens. Pens selected were approximately 1/4 of the distance from each end of the barn on opposite sides of the walkway. The same pens were sampled each week, but not necessarily the same pigs (convenience sampling). Blood samples were collected using a single-use vacutainer system with 10 mL serum separation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Blood samples were placed on ice for transport to the laboratory. In the laboratory, samples were centrifuged (1000g for 10 min), aliquoted into 4 mL cryovials, and stored at -20 °C.

### 2.3. PRRSV ELISAs

Serum samples were tested for PRRSV antibodies using a commercial PRRSV ELISA (IDEXX PRRS ×3 Ab Test, IDEXX Laboratories, Inc., Westbrook ME USA) following the instructions provided by the manufacturer. Sample-to-positive (S/P) results ≥0.4 were considered positive for PRRSV antibody.

Oral fluid samples were tested for PRRSV antibodies using a commercial PRRSV oral fluid antibody (IgG) ELISA (IDEXX PRRS OF Ab Test, IDEXX Laboratories, Inc.) following the instructions provided by the manufacturer. S/P results ≥0.4 were considered positive for PRRSV antibody. IgM, IgA, and IgM-IgA oral fluid ELISAs were performed as instructed by the manufacturer for the PRRSV OF Ab ELISA (IgG) with the following exceptions: the kit IgG conjugate was replaced with goat anti-pig IgM (A100-100P Bethyl Laboratories) diluted 1/5000 in IDEXX conjugate diluent; or goat anti-pig IgA (A100-102P Bethyl Laboratories) diluted 1/3000 in IDEXX conjugate diluent; or dual mixture of IgM (1/5000)-IgA (1/3000). Plate positive controls for the IgM, IgA, or IgM-IgA ELISAs were based on oral fluid samples of known positive PRRSV status diluted in kit sample diluent to produce optical density (OD) values between of 0.6 and 0.7. Tests were performed as recommended by the manufacturer and results reported as S/P ratios. Cutoffs for the IgM, IgA, and IgM-IgA oral fluid ELISAs were determined by receiver operator characteristic curve (ROC) analysis, as described in Section 2.6 (statistical analysis).

### 2.4. PRRSV RT-rtPCR

All samples were tested for PRRSV RNA at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) using standard protocols. Systematic bias was addressed by randomizing samples order prior to submission.

In Study 2 (field data), serum samples from the same pen were pooled by five and tested by PRRSV RT-rtPCR. Nucleic acid extraction was performed using the MagMAX<sup>™</sup> viral RNA isolation kit (Life Technologies, Carlsbad, CA, USA) and a Kingfisher 96 magnetic particle processor (Thermo-Fisher Scientific, Waltham, MA, USA) using a standard lysis procedure. A lysis/binding solution was prepared with 65 µL lysis/binding solution, 1 µL carrier RNA, 65 µL isopropanol and 2 µL Xeno<sup>™</sup> RNA template at 10,000 copies/µL. At the lysis step, 130 µL of

the prepared lysis/binding solution was added to 20  $\mu$ L of magnetic bead mix prior to extraction and elution into 90  $\mu$ L buffer. The standard lysis procedure used 150  $\mu$ L in wash I and 150  $\mu$ L in wash II. The extraction was performed using the Kingfisher AM1836\_DW\_50\_v3 program (Thermo-Fisher Scientific).

In Study 2, oral fluid samples were tested individually (not pooled) for PRRSV RT-rtPCR testing. Nucleic acid extraction was performed using the MagMAX™ viral RNA isolation kit and a Kingfisher 96 magnetic particle processor using a high-volume modified lysis (HVML) procedure. A modified lysis/binding solution was prepared with 120  $\mu$ L lysis/binding solution, 2  $\mu$ L carrier RNA, 120  $\mu$ L isopropanol and 2  $\mu$ L Xeno™ RNA template at 10,000 copies/ $\mu$ L. At the lysis step, 240  $\mu$ L of the prepared lysis/binding solution was added to 20  $\mu$ L of magnetic bead mix prior to extraction and elution into 90  $\mu$ L elution buffer. An additional modification for the HVML procedure was an increase in wash I and II solutions, i.e., the procedure used 300  $\mu$ L in wash I and 450  $\mu$ L in wash II. The extraction was performed using the Kingfisher AM1836\_DW\_HV\_v3 program.

Both serum and oral fluid samples were assayed using a commercial PRRSV real-time RT-rtPCR kit (EZ-PRRSV MPX 4.0 assay, Tetracore®, Rockville, MD, USA). For each run, positive controls for PRRSV Types 1 and 2 and a negative amplification control were included. For each control well, 17.25  $\mu$ L of EZ-PRRSV MPX 4.0 Reagent was added. The EZ-PRRSV MPX 4.0 Reagent includes buffer, primer and probes, 0.75  $\mu$ L Enzyme Blend, 0.25  $\mu$ L IC and 7  $\mu$ L of positive control (Type I or 2 IVT) or negative control (1  $\times$  TE). Each well contained 17.25  $\mu$ L of the EZ-PRRSV MPX 4.0 Reagent, which included buffer, primer, probes, 0.75  $\mu$ L Enzyme Blend and 7  $\mu$ L of the oral fluid extract. Plates were loaded onto the thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA, USA) and the following cycling conditions were used: one cycle at 48 °C for 15 min, one cycle at 95 °C for 2 min, 45 cycles of: 95 °C for 5 s, and 60 °C for 40 s. Samples with Ct values < 45 for Type 2 PRRSV were considered positive.

### 2.5. PRRSV sequencing

In Study 2 (field data), one RT-rtPCR-positive pooled serum sample (pool of 5) from each barn was selected for PRRSV sequencing each week. Approximately 1082 base pairs of open reading frame (ORF)5 region and the flanking regions within the PRRSV genome were amplified using forward primer 5'-AAG GTG GTA TTT GGC AAT GTG TC-3' and reverse primer 5'-GAG GTG AAT TTC CAG GTT TCT A-3' and the qScript™ Custom One-Step RT-rtPCR Kit (Quanta Biosciences, Gaithersburg, MD USA). The serum sequencing PCR setup reaction used 320 nM of each primer with 12.5  $\mu$ L 2X qScript™ One-Step master mix, 0.5  $\mu$ L qScript One-Step reverse transcriptase and 7.2  $\mu$ L nuclease-free water. The final PCR volume of 25  $\mu$ L consisted of 21  $\mu$ L of master mix and 4  $\mu$ L of RNA extract. One positive extraction control, one negative extraction control, and one negative amplification control were included with the reaction. The PCR was performed (Applied Biosystems® 2720 thermal cycler, Life Technologies Corporation) with the following cycling conditions: one cycle at 48 °C for 20 min, one cycle at 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 50 °C for 50 s, and 68 °C for 50 s. The final elongation step was 68 °C for 7 min. Detection of the RT-rtPCR product of the correct size (1082 bp) was performed on a QIAxcel® capillary electrophoresis system (Qiagen®) using a DNA screening cartridge and the AM420 method and purified with ExoSAP-IT® (Affymetrix, Santa Clara, CA USA) following the manufacturer's recommendations. Samples were submitted to the Iowa State University DNA Facility for sequencing and commercial software was used to compile sequences (Lasergene®, DNASTar, Madison, WI, USA).

### 2.6. Statistical analysis

Receiver operating characteristic curve (ROC) analyses were performed in R 3.3.0 (R Core Team, 2013) with the objective of calculating

the cutoffs and associated diagnostic sensitivity and specificity for each oral fluid antibody isotype-specific ELISA assay. PRRSV IgM, IgA, and IgM-IgA ELISA ROC analyses were done separately for Study 1 and Study 2.

In Study 1 (experimental data), sample status (positive/negative) was defined relative to the day of vaccination (Day 0, Ingelvac PRRS® MLV). For the PRRSV IgM and IgM-IgA ELISA ROC analyses, samples from days -7 to 5 were considered negative and samples from days 10 to 28 as positive. For the PRRSV IgA and IgG ELISA ROC analyses, days -7 to 7 were classified as negative and days 11 to 28 as positive.

In Study 2 (field data), the infection status for pen-based samples was determined by RT-rtPCR testing. For the ROC analyses, oral fluid samples from a pen were considered IgM and IgA antibody negative up to, and including, the first positive PRRSV RT-rtPCR result from the pen. Thereafter, samples were considered positive for IgM beginning with the next weekly sampling and for four subsequent weekly samplings. For IgA and IgM-IgA ELISA ROC analyses, samples were considered positive for all weekly samplings after the first RT-rtPCR positive result. The IgG ELISA results were not analyzed due to the presence of maternal PRRSV antibody.

For both Study 1 and 2, transformation of S/P values ( $x^{3/7}$ ) was performed to fulfill the assumption of normality for the IgM, IgA, and IgM-IgA data. Thereafter, a linear mixed model was fitted to the data, with PRRSV infection status as the explanatory variable and pen as the random effect. After obtaining the fixed parameter estimates and standard deviation, point estimates, variance, and confidence intervals for diagnostic sensitivity and specificity were calculated based on the Normal model. Because of the correlated structure of the data, i.e., repeated measures at the sampling level, binomial exact confidence intervals were calculated using model-based estimation of diagnostic sensitivity and specificity. Asymptotic logit transformation was used to avoid confidence intervals beyond [0, 1]. That is, diagnostic sensitivity and specificity point estimates were first logit transformed, then confidence intervals were calculated, after which the final confidence intervals were obtained by back transformation.

## 3. Results

All samples were randomized prior to PRRSV ELISA or RT-rtPCR testing. All samples were tested once and no retests were performed.

### 3.1. Study 1 (experimental data)

Study 1 followed the PRRSV oral fluid antibody isotype response of 12 pigs following vaccination with a modified-live PRRSV vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim Vetmedica, Inc.) under experimental conditions. All pigs were confirmed free of PRRSV infection by PRRSV RT-rtPCR and PRRSV ELISA testing. From DPV -7 to 42, oral fluid samples were collected twice daily from individual pigs for a total of 600 oral fluid samples. At the end of the trial, all samples were tested for PRRSV antibody using isotype-specific ELISAs (IgM, IgA, IgG, and IgM-IgA). The oral fluid isotype-specific ELISA mean S/P values and the percent of IgM-IgA ELISA samples with S/P ratios  $\geq 0.40$  by DPV are shown in Fig. 1.

### 3.2. Study 2 (field data)

Study 2 was conducted on 3 commercial farms (A, B, C) in one production system, each with 3 wean-to-finish barns (1, 2, 3). Oral fluid samples were collected weekly from the 36 occupied pens (~25 pigs per pen) in each of the 3 barns, i.e., 108 samples per week, for a total of ~972 oral fluid samples per farm. In addition, 20 serum samples from two pens in each barn were collected at each weekly sampling for a total of 1620 serum samples per farm. The PRRSV status of barns and farms was determined on the basis of RT-rtPCR testing and reflected the endemic circulation of virus in the production system. No PRRSV

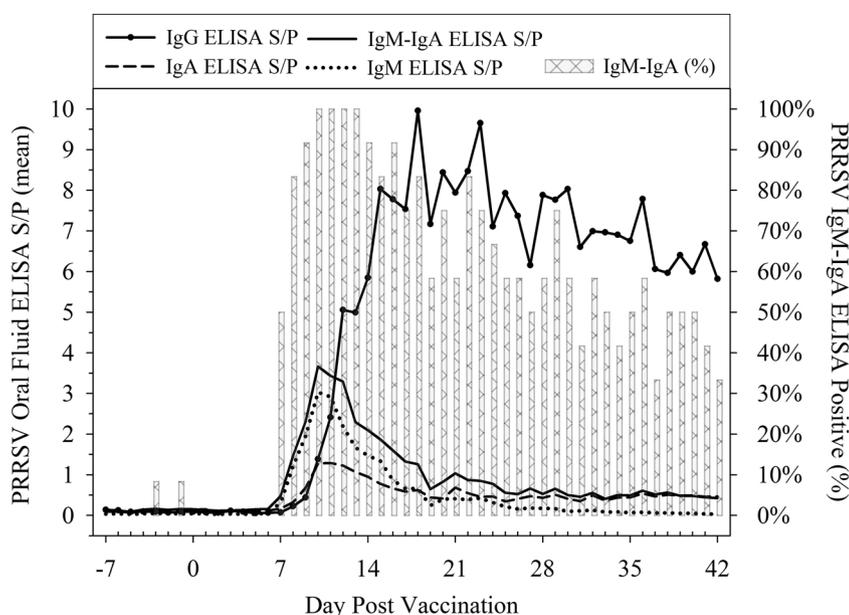


Fig. 1. PRRSV antibody ontogeny in oral fluid samples collected from 12 pigs vaccinated with a modified-live virus vaccine over the course of 49 days (Study 1).

vaccine was used in the pigs or their dams during the production cycle.

The PRRSV RT-rtPCR results for oral fluid samples are shown in Fig. 2. In Farm A, PRRSV RT-rtPCR-positive oral fluid samples were observed in one or more of the three barns at every sampling period (week 0 through 8). Of the 972 oral fluid samples collected on Farm A, 425 samples were positive. At the last sampling, all oral fluid samples collected from all Farm A ( $n = 108$ ) were positive. No RT-rtPCR-positive oral fluid samples were observed in Farm B and in Farm C one oral fluid sample tested positive at week 8.

PRRSV RT-rtPCR-positive serum samples (pooled by fives) were found in Farm A on sampling weeks 4 through 8. No RT-rtPCR-positive serum samples were detected at any time in Farms B and C. In Farm A, PRRSV sequencing was attempted on RT-rtPCR-positive pooled serum samples collected weeks 4 through 8. To optimize sequencing success and collect sequencing data over time, the pool with lowest Ct was selected each week from each barn. A total of 14 pooled serum samples were submitted for sequencing and 10 ORF 5 sequences were obtained. Sequence analysis provided evidence of the circulation of wild-type PRRSV (Fig. 3).

All oral fluid samples collected in Study 2 ( $n = 2916$ ) were tested for PRRSV antibody using isotype-specific ELISAs (IgM, IgA, IgG and IgM-IgA). The mean S/P values for the IgG ELISA and IgM-IgA ELISA are shown in Fig. 4. The percent of IgM-IgA ELISA samples with S/P ratios  $\geq 0.40$  by sampling week are given in Fig. 2.

### 3.3. ROC analysis

For both Study 1 (experimental data from individual pigs) and Study 2 (field data from pens of pigs), the diagnostic sensitivities, specificities, and 95% correlated confidence intervals were calculated for the IgA, IgM, and IgM-IgA ELISAs over a range of S/P cutoffs of 0.20, 0.30, 0.40, 0.50, and 0.60 (Table 1). For Study 1, the oral fluid isotype-specific ELISA mean S/P values and the percent of IgM-IgA ELISA samples with S/P ratios  $\geq 0.40$  by DPV are shown in Fig. 1. Two of 156 samples collected between DPV -7 to 5 had S/P values  $\geq 0.40$ . Specifically, one sample collected on DPV -3 had an S/P value of 0.46 and one sample collected on DPV -1 had an S/P value of 0.53. These values were accounted for in the ROC analysis (Table 1), resulting in a diagnostic specificity of 99%.

## 4. Discussion

Various economic studies have uniformly shown that PRRSV inflicts major losses on swine health and productivity (Holtkamp et al., 2013; Nathues et al., 2017; Neumann et al., 2005; Nieuwenhuis et al., 2012; Zhang and Kono, 2012). In Europe and North America, the cost of PRRSV to the industry in terms of hog marketed has been estimated at \$6.25 to \$15.25 per pig (Holtkamp et al., 2013; Nathues et al., 2017). On-going losses at this level are unacceptable in terms of animal welfare and the public's perception of swine production, but the solution to this dilemma is not apparent. Calvin Schwabe in 1982 recommended that veterinary practitioners use surveillance to understand the patterns of disease and establish baselines against which the effect of control interventions could be measured. Schwabe's vision was never realized, but the complex, dynamic, global nature of contemporary swine production mandates that on-going, near-real-time surveillance be part of the PRRSV solution.

PRRSV surveillance can be based on the detection of nucleic acid and/or antibody: each has its strengths and weaknesses. The time to RT-rtPCR-detectable viremia differs among PRRSV isolates, but the majority of animals are viremic within 48 h (Pepin et al., 2015). Under experimental conditions,  $\sim 50\%$  of pigs were still nucleic acid-positive at  $\sim 50$  DPI and  $\leq 10\%$  of animals remained positive at  $\sim 100$  DPI (Hortner et al., 2002; Molina et al., 2008). This unusually long duration of viremia makes nucleic acid detection a viable option for PRRSV surveillance. However, serum-RT-rtPCR-negative animals can still harbor infectious PRRSV. That is, after the immune response clears virus from the circulatory system, infectious PRRSV can still be recovered from lymphoid tissues, e.g., tonsils of the soft palate (Hortner et al., 2002; Wills et al., 2003).

The humoral immune response against a variety of PRRSV proteins has been described (Molina et al., 2008) and a variety of serum antibody detection platforms are available in diagnostic laboratories, e.g., ELISA, IFA, IPMA and neutralizing antibody assays (Decorte et al., 2014; Ouyang et al., 2013; Pejsak et al., 2017; Yoon et al., 1995). ELISA is compatible with high-throughput laboratories, is technically simple, and is a widely used assay for PRRSV antibody detection (Pejsak et al., 2017). Kittawornrat et al. (2012a, 2012b) in a study involving 12 laboratories found that the results produced by a PRRSV oral fluid ELISA were highly repeatable within laboratories and highly reproducible between laboratories. When used in surveillance, PRRSV serum and oral fluid ELISAs can provide useful data concerning herd immunity

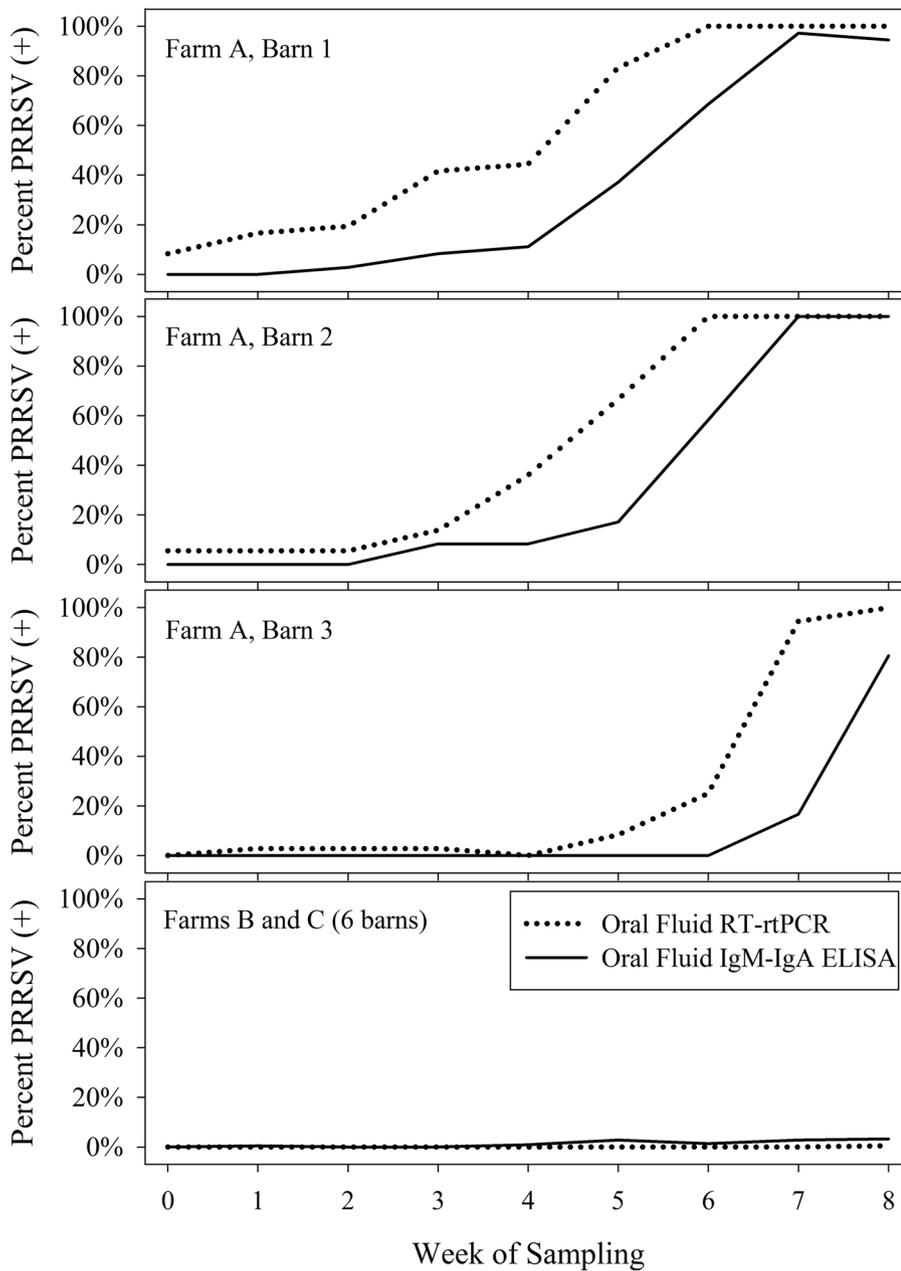


Fig. 2. Oral fluid testing results (% positive) in PRRSV-positive (A) and PRRSV-negative (B, C) wean-to-finish farms (Study 2).

and exposure history. However, PRRSV maternal antibody may be present in pigs up to 10 weeks old (Yoon et al., 1996; Houben et al., 1995). The presence of maternal IgG antibody complicates the interpretation of ELISA results because, in younger pigs, a positive result

may represent maternal antibody or antibody produced by the pig in response to PRRSV infection.

Prior research demonstrated that inoculation with type 1, type 2, or MLV PRRS viruses produced detectable levels of PRRSV IgM and IgA in

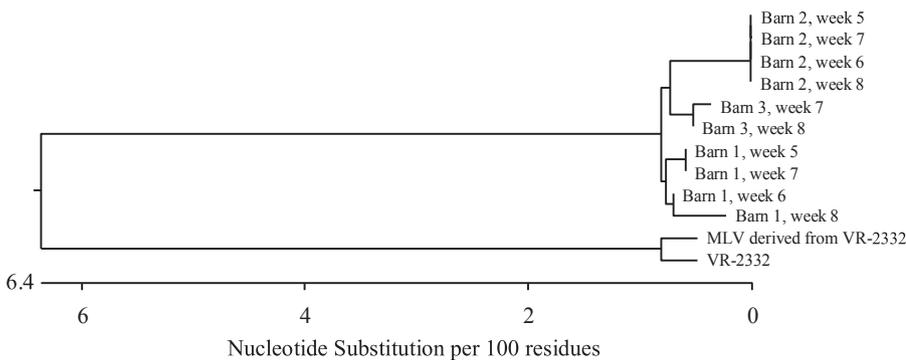


Fig. 3. Phylogenetic analysis (ORF 5 nucleotide level) of wild-type PRRS viruses circulating in Farm A (Study 2).

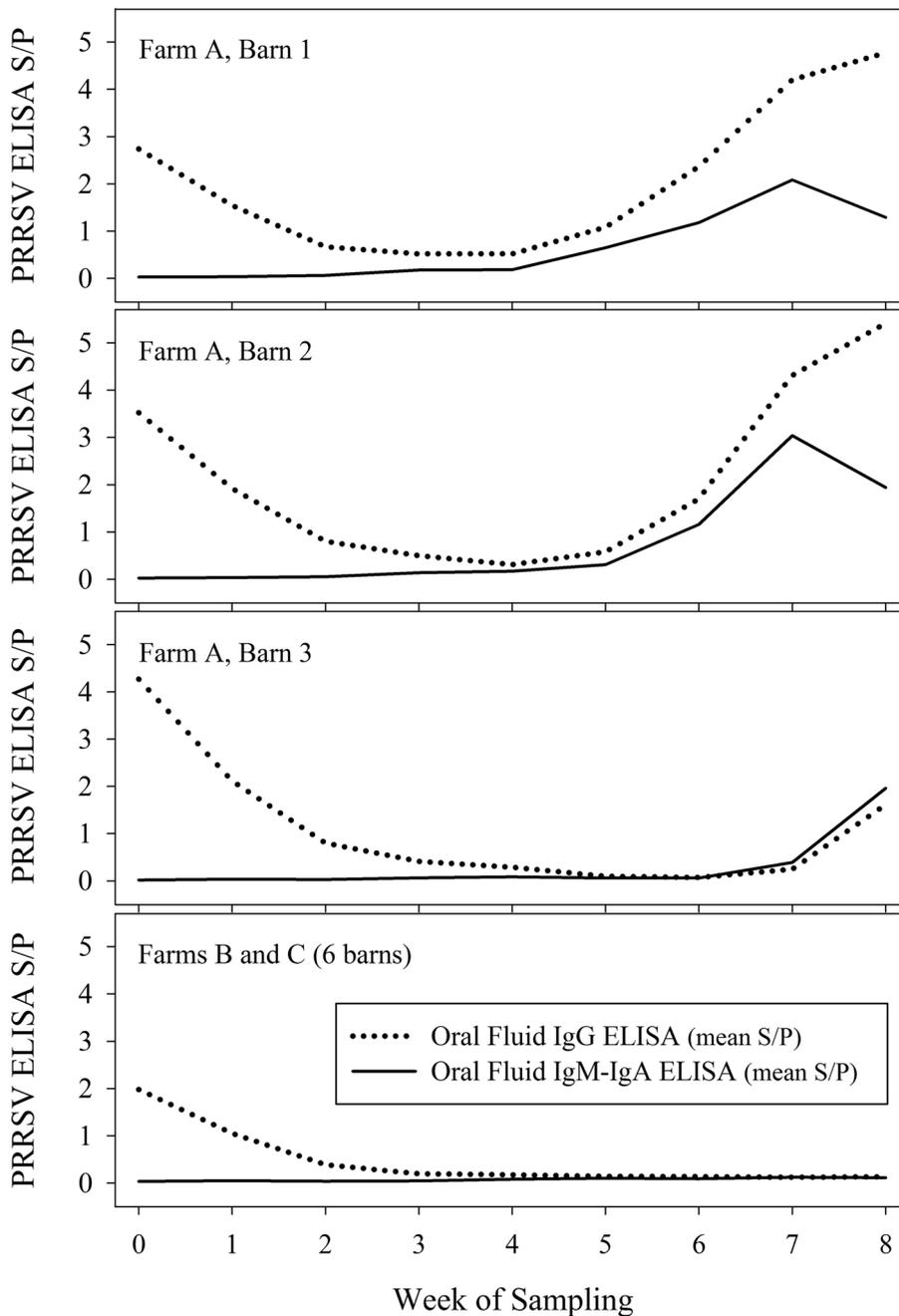


Fig. 4. Oral fluid testing results (mean S/P) in PRRSV-positive (A) and PRRSV-negative (B, C) wean-to-finish farms (Study 2).

both serum and oral fluid (Kittawornrat et al., 2013). The goals of the present research were to develop a combined IgM-IgA oral fluid ELISA, evaluate its performance using experimental and field samples, and determine whether the assay could detect PRRSV-specific IgM and IgA produced by pigs in response to infection, even in the presence of maternal IgG antibody.

Using samples from MLV-vaccinated pigs and type 2 field virus-infected pigs, the results confirmed prior reports of IgM, IgA, and IgG antibody ontogeny in oral fluids following exposure to the virus. Testing of oral fluids from pigs originating from sow herds endemically infected with PRRSV found abundant PRRSV IgG, but no evidence of maternally-derived IgM or IgA in oral fluid specimens.

A comparison of IgM, IgA, and IgM-IgA ELISAs showed that the combined IgM-IgA assay provided better performance than detection of either isotype alone (Table 1). The authors were unable to locate other examples of combined antibody isotype ELISAs with which to compare these data, but there are examples of combining the results of two

different isotype assays to establish infection status. For both Dengue virus and Crimean-Congo hemorrhagic fever virus, performing IgM and IgG ELISAs in parallel and interpreting a positive result on either as indicative of infection significantly improved diagnostic sensitivity (Dowall et al., 2011; Vaughn et al., 1999). While the combined IgM-IgA oral fluid ELISA demonstrated adequate diagnostic sensitivity and high diagnostic specificity, higher performance may be achievable through further assay optimization and/or the removal of IgG. Removal of IgG from specimens using anti-IgG or protein G has been described as a method to improve detection of IgM and IgA (Ankerst et al., 1974; Martins et al., 1995). For example, Dowall et al. (2011) showed that removal of IgG from diagnostic samples resulted in increased both the diagnostic sensitivity and specificity of a Crimean-Congo hemorrhagic fever virus IgM ELISA.

Overall, the combined PRRSV IgM-IgA oral fluid ELISA described in this proof-of-concept study is a promising tool for PRRSV surveillance, particularly in populations of growing pigs originating from PRRSV-

**Table 1**  
PRRSV isotype-specific ELISA diagnostic sensitivity and specificity based on experimental and field data.

PRRSV ELISA antibody target	Cutoff (S/P)	Study 1 (Experimental data from individual pigs)		Study 2 (Field data from pens of pigs)	
		Diagnostic sensitivity (95% CI)	Diagnostic specificity (95% CI)	Diagnostic sensitivity (95% CI)	Diagnostic specificity (95% CI)
IgA	0.2	0.78 (0.76, 0.80)	0.87 (0.86, 0.88)	0.76 (0.75, 0.76)	0.94 (0.94, 0.94)
	0.3	0.63 (0.59, 0.66)	0.97 (0.97, 0.97)	0.69 (0.68, 0.70)	0.97 (0.97, 0.97)
	0.4	0.48 (0.44, 0.52)	0.99 (0.99, 0.99)	0.66 (0.65, 0.67)	0.98 (0.98, 0.98)
	0.5	0.40 (0.37, 0.44)	0.99 (0.99, 0.99)	0.63 (0.62, 0.64)	0.99 (0.99, 0.99)
	0.6	0.34 (0.31, 0.38)	0.99 (0.99, 0.99)	0.60 (0.59, 0.61)	0.99 (0.99, 0.99)
IgM	0.2	0.69 (0.67, 0.71)	0.99 (0.99, 0.99)	0.63 (0.62, 0.64)	0.99 (0.99, 0.99)
	0.3	0.61 (0.58, 0.63)	0.99 (0.99, 0.99)	0.56 (0.55, 0.57)	1.0 (0.99, 1.0)
	0.4	0.54 (0.51, 0.57)	1.0 (NA <sup>a</sup> )	0.50 (0.49, 0.52)	1.0 (0.99, 1.0)
	0.5	0.47 (0.44, 0.50)	1.0 (NA <sup>a</sup> )	0.45 (0.43, 0.46)	1.0 (0.99, 1.0)
	0.6	0.40 (0.38, 0.43)	1.0 (NA <sup>a</sup> )	0.42 (0.41, 0.44)	1.0 (0.99, 1.0)
Combined IgM-IgA	0.2	0.93 (0.93, 0.94)	0.85 (0.83, 0.86)	0.80 (0.80, 0.81)	0.95 (0.95, 0.95)
	0.3	0.84 (0.83, 0.85)	0.96 (0.96, 0.96)	0.77 (0.77, 0.77)	0.98 (0.98, 0.98)
	0.4	0.77 (0.75, 0.79)	0.99 (0.99, 0.99)	0.74 (0.73, 0.74)	0.99 (0.99, 0.99)
	0.5	0.68 (0.66, 0.71)	0.99 (0.99, 0.99)	0.72 (0.72, 0.73)	0.99 (0.99, 0.99)
	0.6	0.64 (0.61, 0.67)	1.0 (NA <sup>a</sup> )	0.69 (0.68, 0.70)	1.0 (0.99, 1.0)

<sup>a</sup> Confidence intervals not calculable.

positive or vaccinated breeding herds because of its ability to detect pig-derived IgM and IgA antibody in the presence of maternal IgG.

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## Conflict of interest statement

The authors declare no conflicts of interest with respect to the conduct, authorship, and/or publication of this study. Co-authors Baum, Giménez-Lirola, and Zimmerman have consulted with IDEXX Laboratories, Inc. on areas of diagnostic medicine independent of this research.

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