RESEARCH REPORT
SWINE HEALTH

Title: Development of diagnostic assays for detecting PRRSV infection using oral fluid samples as an alternative to serum-based assays – **NPB #09-234**

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Industry Summary: Currently, diagnosis of PRRSV infection is by virus antigen, nucleic acid or antibody detection in serum samples. Thus, serum is the standard sample for diagnostic evaluation. However, blood sample collection is a labor-intensive procedure and may cause negative effect on animal health. In contrast, oral-fluid samples provide a cost effective and non-invasive alternative to serum samples. Particularly, it is more suitable for sampling in large epidemiologic studies. Since oral fluid contains lower amount of antibody, a more sensitive oral fluid-based assay is needed for detection of PRRSV infection. The fluorescent immunomicrosphere assay (FMIA) has advantage over traditional ELISA test format, including improved sensitivity and the ability of multiplex, i.e., detect PRRSV antigen and host antibody response to several viral proteins simultaneously. In this study, we developed a multiplexed fluorescence microsphere immunoassay (FMIA) for detection of PRRSV specific antibodies in oral fluid and serum samples. Recombinant nucleocapsid protein (N) and nonstructural protein 7 (nsp7) from both PRRSV genotypes (Type I and Type II) were used as antigen and covalently coupled to Luminex fluorescent microspheres. Based on an evaluation of 488 oral fluid samples with known serostatus, the oral fluid-based FMIAs were achieved greater than 92% sensitivity and 91% specificity. In serum samples (n = 1639), the FMIAs reached greater than 98% sensitivity and 95% specificity. The assay was further employed to investigate the kinetics of antibody response in infected pigs. In oral fluid, N protein was more sensitive for the detection of early infection (7 and 14 dpi), but nsp7 detected higher and longer antibody response after 28 days post infection. In serum, the antibodies specific to nsp7 and N proteins were detected as early as 7 days post infection, and the responses lasted more than 202 days. This study provides a framework from which a more robust assay could be developed to profile the immune response to multiple PRRSV antigens in a single test. The development of oral fluid-based diagnostic tests will revolutionize the way we survey for swine herds and improve our ability to cheaply, efficiently track PRRSV infections in both population and individual animals.
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Keywords: PRRSV, nsp7, N protein, fluorescent microsphere assay (FMIA), ELISA.

Scientific abstract: For effective disease surveillance, rapid and sensitive assays are needed to detect antibodies against PRRS virus (PRRSV) infection. In this study, we developed a multiplexed fluorescence microsphere immunoassay (FMIA) for detection of PRRSV specific antibodies in oral fluid and serum samples. Recombinant nucleocapsid protein (N) and nonstructural protein 7 (nsp7) from both PRRSV genotypes (Type I and Type II) were used as antigen and covalently coupled to Luminex fluorescent microspheres. Based on an evaluation of 488 oral fluid samples with known serostatus, the oral fluid-based FMIAs were achieved greater than 92% sensitivity and 91% specificity. In serum samples (n = 1639), the FMIAs reached greater than 98% sensitivity and 95% specificity. The assay was further employed to investigate the kinetics of antibody response in infected pigs. In oral fluid, N protein was more sensitive for the detection of early infection (7 and 14 dpi), but nsp7 detected higher and longer antibody response after 28 days post infection. In serum, the antibodies specific to nsp7 and N proteins were detected as early as 7 days post infection, and the responses lasted more than 202 days. This study provides a framework from which a more robust assay could be developed to profile the immune response to multiple PRRSV antigens in a single test. The development of oral fluid-based diagnostic tests will revolutionize the way we survey for swine herds and improve our ability to cheaply, efficiently track PRRSV infections in both population and individual animals.

Introduction

PRRS is still the most economically devastating disease in the swine industry. In the absence of effective vaccines and therapeutic drugs, one of the key approaches to achieve the "National PRRS Elimination" is to identify PRRSV infected pigs, so such pigs can be quarantined, isolated or removed from herds to block or reduce the transmission of infection to susceptible animals. Serum is a standard antimortem sample that is routinely collected for diagnostic evaluation to determine whether pigs have been exposed to the PRRSV. However, the blood collection is a labor-intensive procedure and may cause negative effect on animal health. Previous studies evaluated the use of oral fluid sampling as an efficient, cost-effective approach to PRRSV surveillance in swine population (Prickett et al., 2008a, b). Oral fluid is a complex mixture of saliva and

gingival crevicular fluid. Gingival crevicular fluid is an oral mucosal transudate derived from the passive transport of serum components through the oral mucosa into the gingival crevice of the mouth. It more closely resembles serum than salivary gland secretions. The use of oral-fluid samples as an inexpensive, safe, non-invasive alternative to blood in determining acute infection and prevalence of immunity has become well established for various human pathogens, such as human immunodeficiency virus, hepatitis A and B viruses, and rubella virus (Parry et al., 1987; Frerichs et al., 1992; Connell et al., 1993; Emmons et al., 1995). Oral fluid has been used in epidemiological studies of HIV infection in developing countries and potentially has a role in epidemiological studies of other human infectious agents (Frerichs et al., 1992; Nokes et al., 2001). The presence of PRRSV in oral fluids was first reported in 1997, in which virus was isolated from buccal swabs collected from experimentally inoculated young pigs on 7, 14, 21, 28, 35, and 42 days post inoculation (DPI) (Wills et al., 1997). A recent study by Prickett et al. (2008) reported that PRRSV was detected by real-time qRT-PCR in oral fluid for approximately 4 weeks post-inoculation. Low levels of anti-PRRSV antibody were detected in oral fluid samples by IDEXX ELISA and indirect fluorescent antibody tests. These reports suggested that porcine oral fluid samples could be used for diagnostic monitoring of PRRSV infection.

Using traditional immunoassays to detect host antibodies in oral fluid is less sensitive than in sera due to the lower concentration of host antibodies present in oral fluid (Prickett et al., 2008). In this study, we used a fluorescent microsphere immunoassay (FMIA) to improve the sensitivity of oral fluid-based assays. The FMIA is a newly emerging technology, which has tremendous potential in disease diagnosis. The FMIA uses multiple fluorescent microspheres (up to 100 color-coded bead sets), and each bead set conjugated to different antigens or antibodies as the solid phase for the detection of antibodies or antigens in biological samples.
An advantage of this technology is that FMIA allows uniform detection of multiple antigens or antibodies simultaneously within a small volume of a single sample. Therefore, the assay is less labor intensive and requires smaller amounts of samples.

Traditional antibody detection assays, such as IDEXX HerdChek® PRRS ELISA, are based on the PRRSV nucleocapsid (N) protein as the antigen. Our previous studies showed that certain nonstructural proteins, nsp1, nsp2 and nsp7 are highly immunogenic (Brown et al., 2009; Johnson et al., 2007). Serum

antibody specific to these proteins can be detected as early as 14 dpi, and lasted more than 202 dpi. Recently, we developed an nsp7-based ELISA for diagnose PRRSV infection in serum samples (Brown et al., 2009). In this study, we adapted the N protein and nsp7-based ELISAs into a multiplex FMIA format for diagnosis of PRRSV infection in oral fluid. As a comparison, a FMIA using serum samples is also developed.

Objectives: The objective of this proposed study is to develop and validate new diagnostic tests for PRRSV using oral fluid samples. The diagnostic performance (diagnostic sensitivity and specificity) of these new oral fluid-based tests will be evaluated using paired oral fluid and serum samples from animals with known PRRSV infection status.

Materials and Methods

Viruses and Cells: MARC-145 cells were cultured in Minimal Eagle's Medium (MEM; GIBCO BRL Life Technologies) with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 20 ug/ml streptomycin). Cells were maintained at 37 ^{0}C in a humidified 5% CO_2 incubator. PRRSV strains SD01-08 (Type I) and VR2332 (Type II) were propagated on MARC-145 cells using the method described previously (Kim et al., 1993).

Expression of recombinant nsp7 and N proteins: DNA fragments corresponding to NA-nsp7 and NA-N from Type II PRRSV strain VR2332, and EU-nsp7 and EU-N from Type I PRRSV strain SD01-08 were amplified by RT-PCR and cloned into a pET protein expression vector (Novagen). Primers used for amplification of NA-nsp7 or EU-nsp7 region were described previously (Ferrin et al., 2004; Brown et al., 2009). Recombinant proteins were expressed as His-tagged fusion proteins and purified as we described previously (Ferrin et al., 2004; Brown et al., 2009). Purified fusion proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity of the protein. Western blot was performed to confirm the specificity of the protein using an anti-His antibody (Novagen, Madison, WI).

Oral fluid and serum samples: Four sets of oral fluid and serum samples were used in this study, which were collected from experimentally infected pigs. The first set of samples was paired oral fluid and serum samples from individual boars in a study conducted by Pig Improvement Company (PIC). A total of 72 boars ranging from 5 months to 4 years in age were experimentally inoculated with RespPRRS vaccine (group 1, n = 24),

Type I PRRSV (group 2, n = 24), or Type II PRRSV (group 3, n = 24). A total of paired oral fluid (n = 360) and serum (n = 360) samples were collected from all 72 boars on -7, 0, 7, 14, 21 days post infection (dpi). The second set of samples was a pen-based oral fluid and serum samples collected from 30 pigs. Pigs were 6-week-old at the time of inoculation and divided into three groups (n=10). Group one was inoculated with Type I PRRSV, group two was inoculated with Type II PRRSV, group three was mock infected. Serum and oral fluids were collected once a week at regular intervals for 49 days. These samples were used for establishing standards and diagnostic test validation. To investigate the time course appearance of antibody response, a panel of oral fluid samples was obtained from 1100 pigs that were experimentally inoculated with Type II viruses. Oral fluid was collected every week up to 90 dpi, and samples for each dpi were pooled together from individual pens. In addition, serum samples were obtained from 109 pigs experimentally infected with VR2332. Serum was collected at 7-day intervals for the first two weeks, then at 14-day intervals up to 202 dpi.

In addition to these samples obtained from experimentally infected pigs, a total of 772 field samples were obtained from the South Dakota Animal Disease Diagnostic Laboratory. Positive testing samples were used only from submitted cases having a herd history of PRRSV positive status and the IDEXX ELISA and IFA results of the entire case were shown to be positive. The negative testing samples were from the cases that have a herd history of PRRSV negative status and the IDEXX ELISA and IFA verified to be negative for the entire case.

Establishment of the test standards: For ELISA standards, a lot of internal quality control oral fluid or serum samples, generated from experimentally infected pigs, were established, which represents the standards of: high positive (optical density ~1.9-2.1), low positive (optical density ~0.6-0.7) and negative (optical density <0.2). The optimal dilution of recombinant protein was determined so that the control sample generated an optical density (OD) at the established standard. For FMIA standards, lots of both oral fluid and serum samples were collected form experimentally infected animals. The high-positive serological standard was established with a mean fluorescent intensity (MFI) between 25,000-29,000, and the low-positive serological standard was established with a MFI between 12,000-15,000. Negative control standards were collected from uninfected

animals and used to establish a baseline (with a MFI of between 800-1200), which had at least a 10 times lower MFI for optimum discrimination between positive and negative samples.

Antibody detection ELISAs: IDEXX ELISA was performed following the manufacture's instruction (IDEXX Laboratories). This assay is routinely conducted at South Dakota Animal Disease Research and Diagnostic laboratory under strict quality control guidelines. Results were quantified by reading at 405 nm with an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT) controlled by XChek Software (IDEXX Laboratories). The raw plate data was copied to an Excel spreadsheet to calculate the sample to positive (S/P) ratios using the following formula: S/P = (OD of sample – OD of buffer) / (OD of positive control – OD of buffer).

Covalent coupling of recombinant nsp7 and N proteins to fluorescent microspheres:

Fluorescent microsphere coupling was performed using the method described previously (Lawson et al., 2010). Briefly, 3.125 x 10^6 microspheres were washed twice with 250 ul activation buffer (0.1M NaH_2PO_4, pH 6.2) and sonicated for 60 s after each wash. Microspheres were activated for 20 min at room temperature in 500 ul activation buffer containing 2.5mg N-hydroxysulfocuccinimide (sulfo-NHS) and 2.5mg N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) (Pierce Chemical, Rockford, IL). Activated microspheres were washed twice with PBS and sonicated after each wash. Coupling was initiated by the addition of 250 ug of each of the four purified recombinant proteins EU-nsp7, NA-nsp7, EU-N or NA-N into a final volume of 500ul PBS and incubated in the dark for 3 h at room temperature. Coupled microspheres were washed once with 1ml of PBS + 0.05%NaN3 + 1.0% BSA (PBS-NB) and blocked with an additional 1ml of PBS-NB for 30 min to reduce nonspecific binding. Microspheres were then washed two times and re-suspended in PBS-NB to a final concentration of 2.0×10^6 antigen-coupled-microspheres/ml.

The amount of recombinant protein coupled to a microsphere was optimized by performing multiple coupling reactions with a various concentration of antigen. The amount of antigen was titrated against a fixed number of microspheres, and then tested to generate a maximal signal to noise ratio of MFI using standard oral fluid or serum.

To determine the coupling efficiency, 2.5 x 10^3 antigen-coupled microspheres were added to each column well of a 96-well microtiter filter plate that was pre-wetted with 20ul of PBS-NB. A solution of PBS-NB containing 1.0 mg/ml of each monoclonal antibody (anti-EU nsp7, anti-NA-nsp7, anti-EU nucleocapsid or anti-NA nucleocapsid) was serially titrated with 10 fold dilution. Fifty microliters of the serial diluted antigen specific monoclonal antibody (mAb) was added to corresponding wells containing coupled microspheres and allowed to incubate at room temperature for 1 h on a plate shaker. After washing the microspheres with PBST (PBS + 0.05% Tween-20) three times, 50ul of goat anti-mouse IgG-Strepavidin-R-Phycoerythrin (SAPE) (10ug/ml in PBS-NB, Invitrogen) was added to the antigen-antibody -microsphere mixture and incubated for 30 minutes at room temperature. Microspheres were washed with PBST three times and re-suspended in 125ul of PBST. The microspheres were then transferred to a 96- well polystyrene optical plate. Uncoupled microspheres were included as negative control. Microspheres were analyzed through a dual laser Bio-Rad, Bio-Plex 200® instrument. Data was analyzed with the Bio-Plex Manager software (version 6.0). The median fluorescent intensity for 100 microspheres was recorded at each titration point and a logarithmic regression curve was generated. Relative coupling efficiencies for each antigen coated microsphere were determined by analyzing the MFI at each dilution point and position under the linear portion of the curve.

Fluorescence microsphere immunoassays (FMIA): A 96-well hydrophilic membrane filter plate was blocked for 2 min with 150ul of PBS-NB, and then aspirated via a vacuum manifold and wetted with an additional 20ul of PBS-NB buffer. Fifty microliter of serum or oral fluid sample (diluted 1:50 or 1:3 respectively in PBS-NB) was added to duplicate filter plate wells along with 50 ul of PBS-NB containing 2.5×10^3 of each antigen-coupled microspheres (1.0×10^4 microspheres total per well for the 4-plex assay). Since the microspheres and reporter moiety are light sensitive, all incubations were performed in the dark by sealing the plate with foil. For the serum FMIA, the plate was incubated at room temperature for 1 h on a plate shaker rotating at 600 rpm. For the oral fluid FMIA, the plate was incubated at 4 ^{0}C overnight. The plate was washed three times with 150ul of PBST. For the serum FMIA, 50ul of biotinylated IgG (1:5000 dilution in PBS-NB, Jackson ImmunoResearch Laboratories) was added to the filter plate and incubated at room temperature for 1h. The oral fluid FMIA was treated the same as the serum FMIA except that biotinylated IgA and IgM (1:5000 dilution in PBS-NB, Bethyl

laboratories) was added along with the biotinylated IgG as secondary antibodies. After incubation with secondary antibodies, 50ul of SAPE (2.5 ug/ml in PBS-NB) was added to each well and incubated for 30 min at room temperature with shaking. The supernatant was aspirated and the plate was washed three times with PBST. Finally, the microspheres were re-suspended in 125ul of PBST per well and transferred to a clear 96-well polystyrene optical plate. Coupled microspheres were analyzed through the dual laser Bio-Rad, Bio-Plex 200® instrument. The MFI for 100 microspheres corresponding to each individual bead analyte was recorded for each well. All reported MFI measurements were normalized via F-Fo, where Fo was the background signal determined from the fluorescence measurement of the negative control sample, and F was the MFI for each serological test analyte.

Assay validation: (i) Cutoff determination, diagnostic sensitivity and specificity: To accurately assess the diagnostic sensitivity and diagnostic specificity, the assays were validated using the samples from two distinct animal populations. The negative-testing (non-infected) validation population was composed of 385 negative oral fluid and 368 negative serum samples. The positive-testing (infected) validation population was composed of 103 positive oral fluid and 892 positive serum samples. Receiver Operating Characteristic (ROC) was conducted for each assay to determine assay cutoffs and diagnostic performance using MedCalc® Version 10.4.0.0 (MedCalc® Software, Mariakerke, Belgium). **(ii) Measurement of repeatability**: The repeatability of each FMIA was assessed by running the same lot of internal quality control standard of oral fluid or serum multiple times on different plates. For both nsp7 & nucleocapsid assays, the intra-assay repeatability was calculated from 36 replicates on a single plate then repeated over a three day period for inter-assay repeatability assessment. Each assay was run as a 4-plex and mean fluorescent intensity values expressed as mean, standard deviation and percent coefficient of variation of repeated measurements. The percent coefficient of variation (%CV) was calculated using the method described earlier (Ferrin et al., 2004; Brown et al., 2009).

2.9. Statistical analysis: The comparison of means and determination of percent coefficient of variation between groups was performed using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA). In addition, a Pearson's correlation coefficient was calculated using the same software for the determination of relative coupling efficiencies and for the comparison of single-plex versus multi-plex.

Results

Expression of recombinant nsp7 and N proteins

To develop a FMIA multi-plex test, we initially expressed recombinant proteins of NA-nsp7 and NA-N from Type II PRRSV strain VR2332, and EU-nsp7 and EU-N from Type I PRRSV strain SD01-08 as His-tagged fusion proteins in *E. coli*. Both EU-nsp7 and NA-nsp7 were expressed at high levels and they were purified in soluble forms. In contrast, recombinant EU-N and NA-N formed inclusion bodies and a protein refolding step was performed. The purity of the recombinant proteins was evaluated using SDS-PAGE followed by Coomassie blue staining. As shown in Fig.1, all of the His-tagged recombinant proteins migrated according to their predicted sizes. Recombinant nsp7 proteins showed around 30 kD band with > 95% purity. The protein concentration was determined to be approximately 1.2 mg/ml. Recombinant N proteins showed as 17 kDa bands with > 90% purity and concentration of 1.8 mg/ml. The identity of each protein was further confirmed by Western blot analysis with anti-His antibody (data not shown).

Fluorescent microsphere immunoassay development

Establishment of control standard: Two set of internal control standards were established using the serum or oral fluid collected from experimental animals. Both serum and oral fluid standards were established as 'high positive', 'low positive' and 'negative' standard. The 'high positive' standard generates OD of 1.9-2.1 in ELISA, and MFI of 25,000-29,000 in FMIA. The 'low positive' standard generates OD of 0.6-0.7 in ELISA, and MFI of 12,000-15,000 in FMIA. The 'negative' standard generates OD < 0.2, and MFI of 800-1,200 in FMIA.

Test optimization: To determine the optimal concentrations of antigen coupling, a series of couplings were performed using various concentration of antigen and analyzed against control standards in order to determine the optimum amount of antigen per microsphere. Four sets of beads each containing 3.125×10^6 beads were incubated with various concentrations (500ug, 250ug and 100ug) of purified EU-nsp7, NA-nsp7, EU-N or NA-N recombinant protein. Based on the highest signal to noise ratio for detection of PRRSV-specific antibodies in standard oral fluid or serum, we determined that 250ug per coupling reaction (80ug protein/ $1x\ 10^6$

microspheres) was the optimal concentrations for the coupling of EU-nsp7, NA-nsp7, EU-N and NA-N protein. The coupling efficiency of the antigen coated beads was determined using antigen-specific monoclonal antibody in a $\log_{10}$ dilution. As shown in Fig. 2, relative coupling efficiency curves were generated and an average correlation coefficient (R^2) of 0.998 was calculated for all regression analytes within the linear portion of the curve.

The optimal serum and oral fluid dilution was determined by diluting serum and oral fluid samples in a $\log_2$ titration. Figure 3 shows a concentration dependent MFI signal. In oral fluid FMIA, it was determined that 1:3 dilution of oral fluid samples gave an optimal signal to noise ratio, while in serum FMIA, 1:50 dilution of serum samples gave an optimal signal to noise ratio.

Cutoff determination, diagnostic sensitivity, and diagnostic specificity: GRAPH ROC software was used for ROC analysis of each FMIAs to determine an optimized cutoff that maximizes both the diagnostic specificity and diagnostic sensitivity of each assay. Oral fluid samples from a known positive population (20 samples from Type I PRRSV infected animals and 83 samples from Type II PRRSV-infected animals) and oral fluid samples from a known negative population (385 samples from PRRSV free animals) were analyzed. As a comparison, serum samples from a known positive population (176 samples from Type I PRRSV infected animals and 716 samples from Type II PRRSV-infected animals) and serum samples from a known negative population (368 samples from PRRSV free animals) were analyzed. The optimal cutoff value, diagnostic sensitivity and specificity of each individual test are presented in Figure 4. Each test showed greater than 90% diagnostic sensitivity and specificity in oral fluid-based FMIAs, while greater than 95% diagnostic sensitivity and specificity were achieved in serum based FMIAs. Table 1 summarizes the results of ROC analysis. Diagnostic sensitivity and specificity was generally lower for the oral fluid based assays. This result is expected, since the antibody concentration is higher in serum than that of oral fluid (refer to Fig. 6, and detailed description below).

Assessment of test repeatability: The precision of each individual FMIA were determined using internal control standards. Table 2 shows the intra-assay or inter-assay repeatability for each test. Both intra-assay and inter-assay repeatability are less than 10% coefficient of variability for all the tests, which suggests that these FMIAs are highly repeatable in diagnostic applications.

Development of a multiplex assay: Once we validated each of individual nsp7 and N-based FMIA in single-plex format, we combined the single-plex format into a 4-plex assay. The 4-plex assay was compared with single-plex to determine whether there was any cross reactivity among bead sets. Each oral fluid and serum internal control standard along with their individual corresponding bead set were first tested in a single-plex format and then combined to test in a 4-plex format. Correlation coefficients were determined for comparison between each individual N and nsp7-based FMIA and the 4-plex assay. As shown in Figure 5, there is no statistical difference between multiplex and singleplex analytes at any time point for both oral fluid and serum-based assay. Both nsp7 and N protein-based FMIA demonstrated good correlation coefficients of greater than 0.98, indicating that there is very little cross reactivity with the presence of multiple protein-coupled microspheres.

Evaluation of the swine antibody response in oral fluid and sera samples

Once we validated the FMIA, we further tested the feasibility of applying this test in an experimental study. Initially, we compared the amount of antibody present in oral fluid and serum. Paired oral fluid and serum was collected at 21 dpi from the same individual pigs (n=21) and the relative anti-N and anti- nsp7 antibody concentrations were determined. As shown in Fig. 6, the Eu and NA nsp7-based FMIA showed a 4.0 times and 8.4 times greater concentration of anti-nsp7 antibody in serum than that of oral fluid, respectively. Both Eu and NA N protein-based FMIAs showed a 2.6 times difference on the level of anti-N antibody between serum and oral fluids.

The FMIA was further employed to investigate the kinetics of antibody response in oral fluid and serum samples. Initially, a panel of oral fluid samples from 1100 Type II PRRSV-infected pigs that collected at 7-day intervals was evaluated by NA nsp7 and NA N-based FMIAs. As shown in Fig7A, antibody response to nsp7 and N could be detected as early as 14 dpi, and lasted to 56 dpi (end of the study). The NA-N based FMIA was more sensitive for the detection of early infection (14 and 21 dpi), but NA-nsp7 based FMIA detected higher antibody response after 28 days post infection. We further evaluated the kinetics of the antibody response in serum samples (Fig. 7B). A serial bleed of serum samples (n = 1014) were obtained from 109 pigs

experimentally infected with Type II PRRSV. They were collected at 7-day intervals for the first two weeks and then at 14-day intervals for up to 202 days post inoculation. There is a similar peak antibody response detected by both NA nsp7 and NA N-based FMIAs. Interestingly, seroconversion could be detected as early as 7dpi in both tests, which would be normally detected at 14 dpi by ELISAs (Brown et al., 2009), suggesting the higher sensitivity of FMIA than that of ELISA.

Application of the 4-plex FMIA for detection of PRRSV infection in field samples.

The robustness of the FMIA was further evaluated in field samples. Since there is no oral fulid diagnostic sample available from the diagnostic laboratories, field serum samples were used to assess the capability of FMIA. Each sample was tested simultaneously for its reactivity with EU-nsp7, NA-nsp7, EU-N and NA-N antigen using 4-plex assay. A total of 772 field serum samples submitted to South Dakota Animal Disease Diagnostic Laboratory were evaluated. These samples were initially tested by IDEXX ELISA, and then tested by 4-plex FMIA. When comparing the FMIA with the IDEXX ELISA, 383/383 (100%) IDEXX ELISA positive samples tested as positive by both the nsp7 and N-based 4-Plex FMIA. Similarly, of the field samples originally testing as negative in IDEXX ELISA, 384/389 (98.7%) were tested to be negative by the nsp7-based FMIA, while 389/389 (100%) tested to be negative by the N protein-based FMIA (Table 3).

Discussion

Development of diagnostic tests that are able to detect PRRSV-specific antibodies in oral fluid offers an important tool for PRRS surveillance in commercial herds and boar studs. Oral fluid sampling has marked advantages over serum sampling, including lower labor / materials costs, noninvasive collection and lower biosecurity risks because samples can be collected by site personnel. This is more important in the PRRS surveillance program at a population level. The development of oral fluid-based diagnostic tests will be a significant breakthrough in our effort to control PRRSV, because it will be a major improvement in our ability to cheaply, efficiently track PRRSV infections in both populations and individual animals. Previous study in 10 wean-to-finish commercial barns showed that oral fluid sampling 6 of 42 pens in 1,100-head at two-week intervals effectively and efficiently detected circulation of PRRSV, PCV2 and SIV by PCR (Prickett et al.,

2008a, 2008b). In this study, we developed a multiplex FMIA using oral fluid. As a comparison, serum-based FMIA was also developed. These assays are based on the detection of antibodies against nsp7 and N protein of both genotypes of PRRSV. N protein was selected as the antigen because it is highly conserved among different strains of the PRRSV, and all current commercially available serological tests are based on this protein. Nsp7 was selected based on the highly immunogenic nature of the protein (Brown et al., 2009). Both antigens were prepared by expression as recombinant proteins in *E. coli*. It was noted that the generation of highly purified recombinant proteins and maintaining the native conformation of proteins are required for the test. High level expression of N protein resulted in the formation of inclusion bodies, which required the re-folding step to restore the native conformation of the protein. Nsp7 was expressed as a soluble recombinant protein in bacterial culture, which is convenient for antigen preparation, especially when applied to diagnostic settings requiring large volume of the antigen. However, the protein seems sensitive to degradation. It is important to ensure that the recombinant protein is being properly handled, such as preventing multiple freeze/thrown of the protein, keeping the protein under the cold temperature condition, and including the protease inhibitor in the recombinant protein extraction and purification reagents.

The FMIA using Type I PRRSV antigens seem to have slightly lower diagnostic sensitivity and specificity than that of FMIA using Type II PRRSV antigens. This could be caused by the small sample size of oral fluid from Type I PRRSV infected pigs, which may bias the statistical analysis. Since it is a pen-based sampling for oral fluid, it reduced the actual number of samples. The panel of Type I PRRSV oral fluid samples used in this study was generated from a recent pig study at SDSU. Unfortunately, this type of sample is not available from other diagnostic or research laboratories in the US. In addition, since oral fluid is not a routine sample for diagnostic evaluation, it prohibited us to test the feasibility of applying FMIA in the field oral fluid samples. Instead, serum samples were used for further validation of the FMIA in field settings. We expected that our initial studies with oral fluid, including current FMIA development study, will change the traditional serum based sampling methods, and oral fluid will be eventually become a standard sample routinely used for diagnostic evaluation.

In comparison of the oral fluid-based assay to serum-based assay, the oral fluid-based assay has slightly lower diagnostic sensitivity and specificity. This result is expected, since we have shown that lower concentration of antibodies was present in oral fluid samples. The markedly lower levels of antibody in oral fluid may be due to physiological and anatomical differences in how antibody is secreted through gingival crevicular tissues. On the other hand, it may be due to environmental factors such as high levels of proteases present in the bucal mucosa and oral fluid. The lower amount of antibody present in oral fluid samples also prevents us to successfully develop an oral fluid-based ELISA. In comparison to oral fluid-based ELISA, the oral fluid-based FMIA had a signal-to-noise ratio significantly higher than that observed in ELISA (9.9 and 2.4 for N protein based FMIA and ELISA, respectively). Although further optimization of oral fluid -based ELISA may be necessary to improve the test sensitivity, our results demonstrated the feasibility of using FMIA as an alternative to ELISA for serological detection of PRRSV infection in oral fluid.

The FMIA we developed with PRRSV nsp7 and N antigens provided important information regarding to the kinetics of antibody response of pigs to these proteins during the time course of infection. In comparison to the antibody response to different antigens, higher level of antibody response to N protein was observed in early infection (14 dpi), but antibody titer to nsp7 was higher after 28 days post infection, and was remained as high level to the end of experiment (56 dpi). Another interesting observation is that antibody response can be detected as early as 7 dpi by FMIA in serum samples. In our previous study, same panel of serum samples were tested by IDEXX ELISA and nsp7 ELISA. The earliest detection of antibody response was at 14 dpi (Brown et al., 2009). These results demonstrate a greater sensitivity of the FMIA over ELISA. In serum FMIA, antibody detection seemed to reach the maximal level after 28 dpi. There is a similar peak antibody titer to nsp7 and N protein, and the antibody response to both proteins sustained to 202-day post infection. These results will be important for future application of FMIA in PRRS surveillance program. Data on the proportion of a herd population that is immune or has been infected have many important epidemiologic applications for disease control, including 1) to determine the timing of infection/immune status; 2) to identify susceptible groups in the population, so that such animals can be quarantined or removed timely to prevent transmission to naïve herd; 3) to evaluate vaccine efficacy: since nsp7 is a nonstructural protein, it only expresses during the active viral

infection. It would have potential to differentiate the infected animals from the animals vaccinated with killed vaccines based on the presence or absence of nsp7 specific antibodies; 4) to use these data in mathematical modeling to predict disease outbreaks and design better management strategies.

In summary, we have developed a FMIA that is able to detect virus-specific antibodies in oral fluid. This study represents the "proof of concept" phase for new PRRS diagnostic tests development using oral fluid samples. The availability of the oral fluid-based assay will be translated into an improvement in our ability to conduct field studies and monitor herd health status in regional control programs. This technology can be applied to other swine pathogens, including PCV2, SIV, *M. hyopneumoniae*, which is currently under development in our laboratories. Our ultimate goal is to develop a rapid multiplex test to detect various swine pathogens simultaneously in a single test sample. We believe the success in this will revolutionize the way we survey for swine herds, a more efficient method as we move toward disease control and elimination.

Table 1. Summary of ROC analysis on oral fluid or serum-based FMIAs

Serum FMIA	**n = 1084 NA nsp7**	**n = 555 EU nsp7**	**n = 1084 NA N**	**n = 555 EU N**
Sensitivity	98.2	99.4	99.3	100
Specificity	95.1	96.3	98.9	99.7
Oral Fluid FMIA	**n = 374 NA nsp7**	**n = 114 EU nsp7**	**n = 374 NA N**	**n = 114 EU N**
Sensitivity	92.8	95.0	100	95.0
Specificity	93.1	91.5	97.5	95.7

Table 2. Assay repeatability of oral fluid and serum-based FMIAs

Repeatability assay	**nsp7**		**N**	
	NA	**EU**	**NA**	**EU**
Serum Intra-assay repeatability (%CV)	1.0	2.3	1.9	1.7
Serum Inter-assay repeatability (%CV)	3.2	4.2	4.7	3.4
Oral fluid Intra-assay repeatability (%CV)	5.8	6.9	3.2	1.5
Oral fluid Inter-assay repeatability (%CV)	7.8	9.1	3.0	1.4

Table 3. Comparison of FMIA with IDEXX ELISA on evaluation of field serum samples

Serum group	**IDEXX ELISA results**	**Total no. of samples**	**No. of IDEXX positive**	**No. of EU & NA nsp7 positive**	**No. of EU & NA nsp7 negative**
1	positive	383	383	383	383
2	negative	389	389	384	389

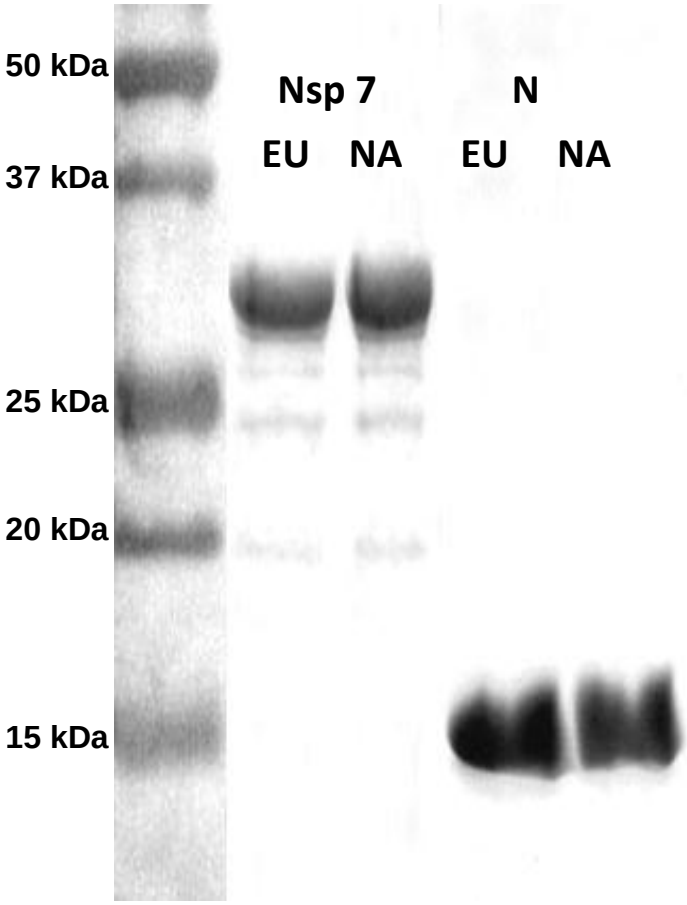


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant PRRSV protein preparations, followed by Coomassie blue staining. The left lane shows the protein molecular mass standard; the remaining lanes represent EU nsp7, NA nsp7, EU N and NA N protein preparations as indicated. NA, North American genotype (Type II); EU, European genotype (Type I).

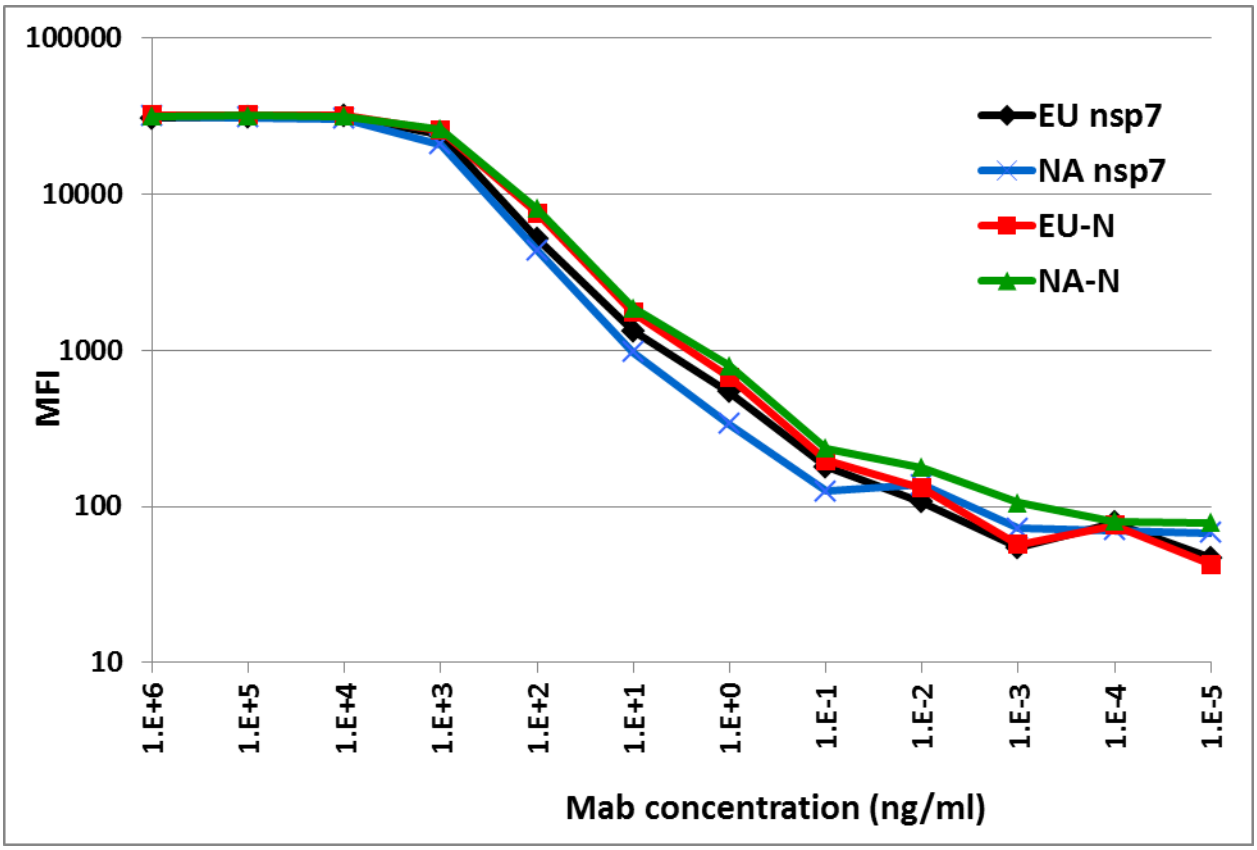


Figure 2. Coupling efficiency of the antigen coated beads determined using antigen-specific monoclonal antibody (mAb). Note, an antigen-specific mAb was detected at 1 ng/ml in each FMIA. MFI: Mean Fluorescence Intensity.

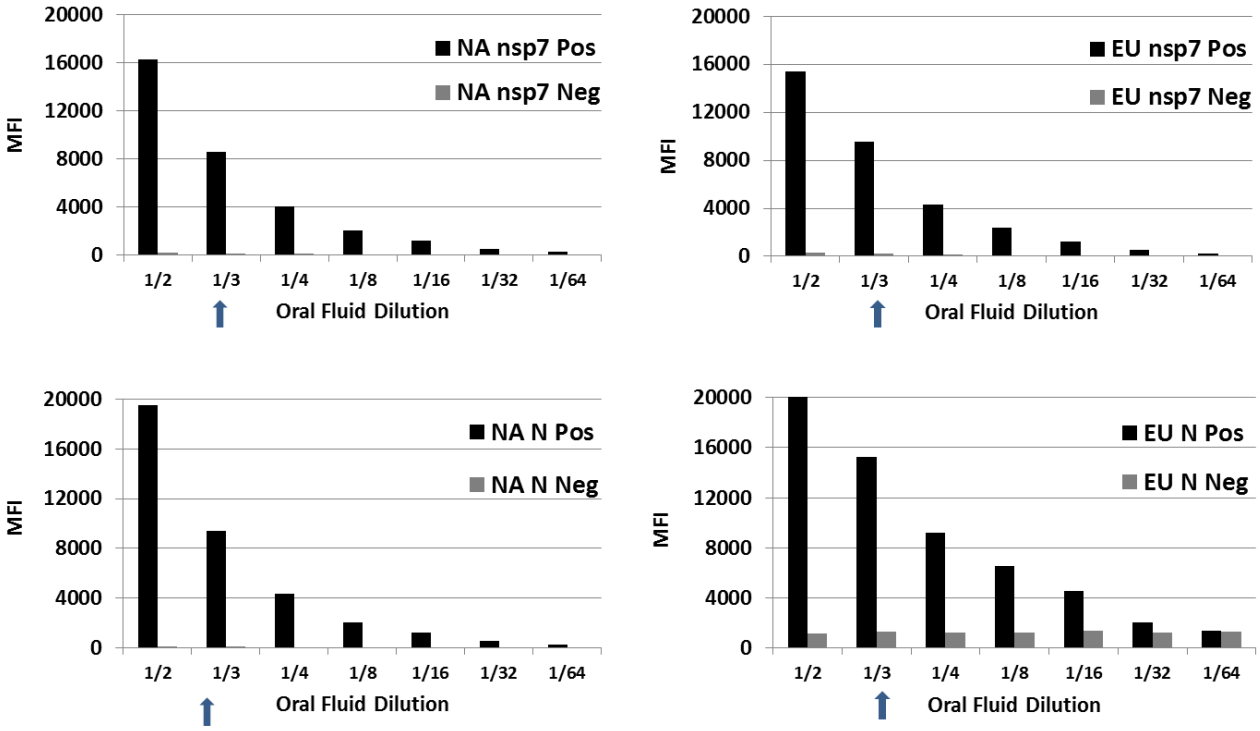


Figure 3A

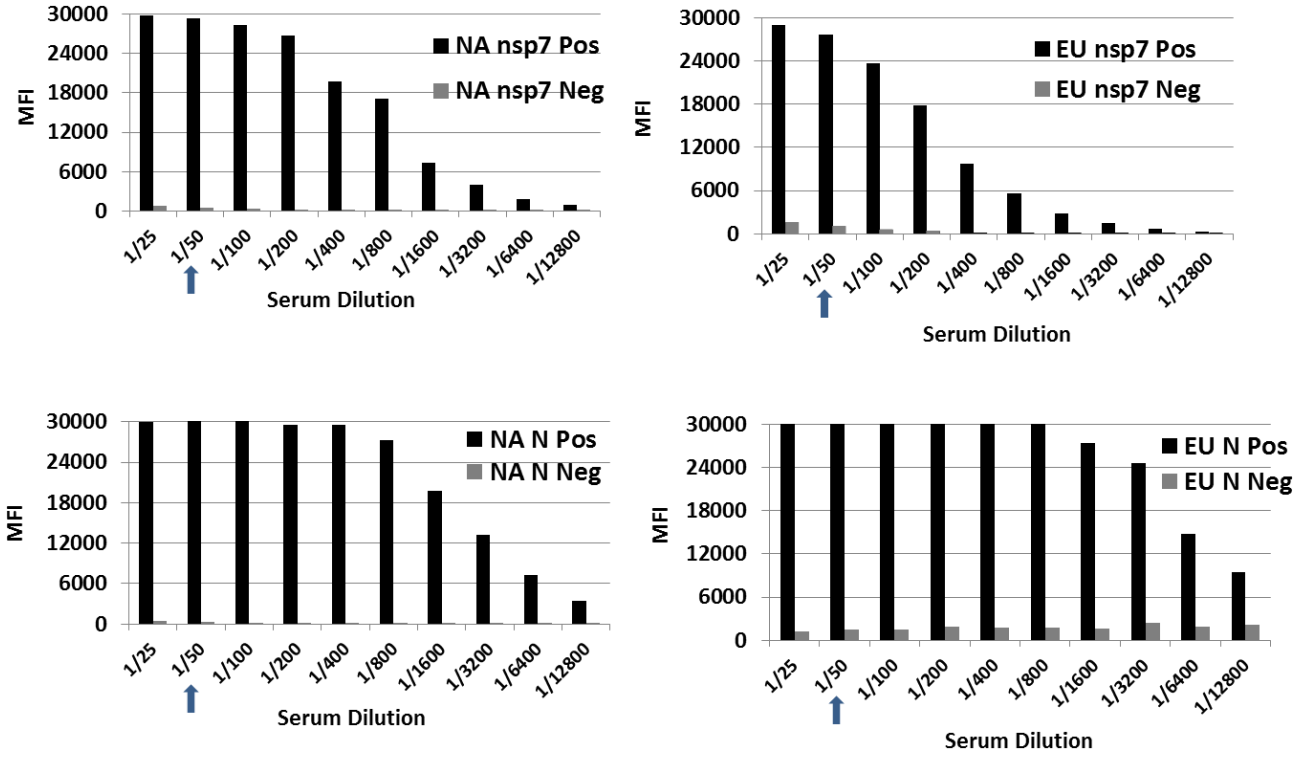


Figure 3B.

Figure 3. Optimization of the amount of oral fluid (A) or serum (B) for fluorescence microsphere immunoassay. The amount of internal standard sample was two-fold titrated against a fixed number of antigen/microsphere complex, and then tested in FMIA to generate a maximal signal to noise ratio of mean fluorescence intensity (MFI).

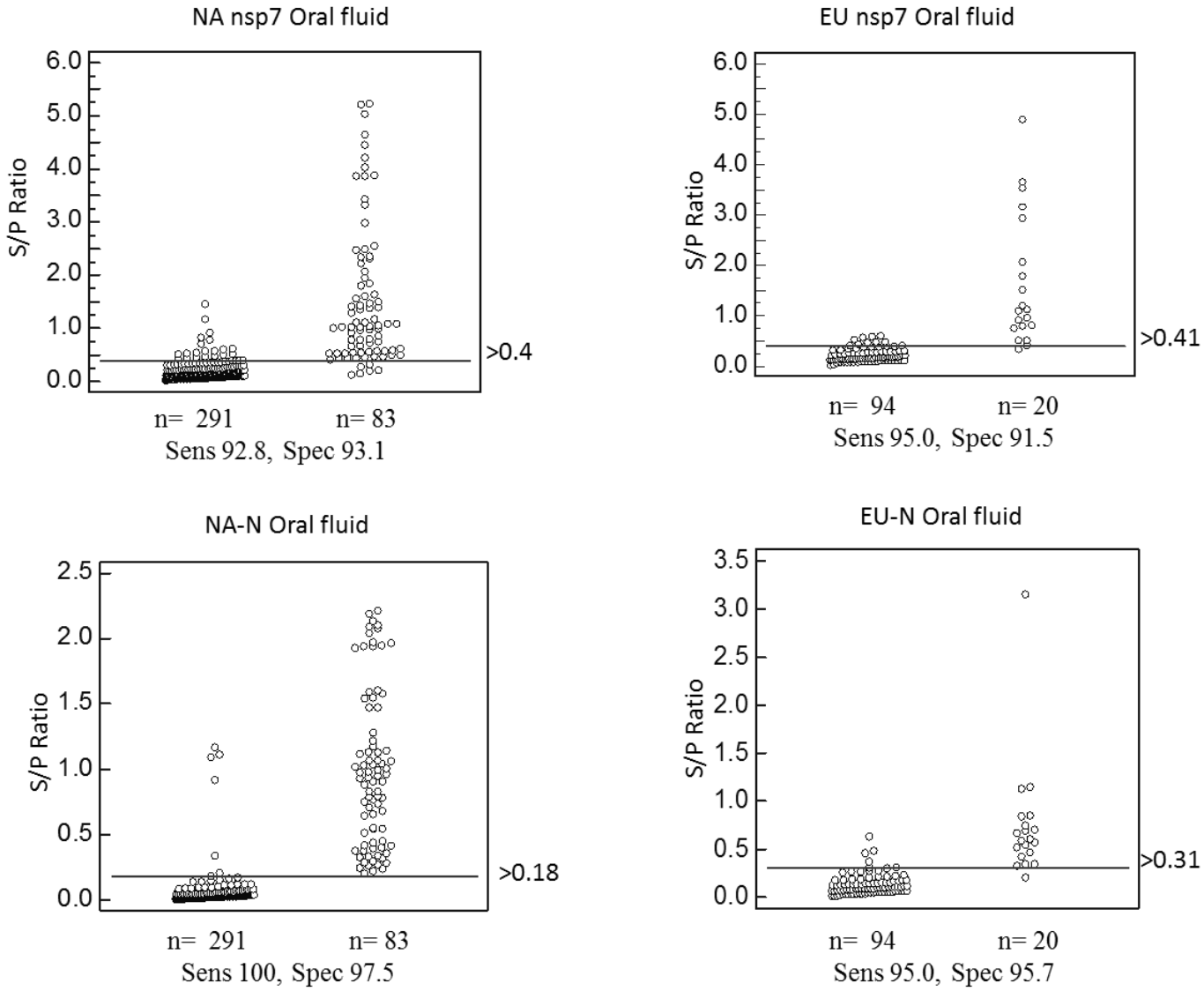

NA nsp7 Oral fluid
S/P Ratio
6.0
5.0
4.0
3.0
2.0
1.0
0.0
>0.4
n= 291
n= 83
Sens 92.8, Spec 93.1
EU nsp7 Oral fluid
S/P Ratio
>0.41
n= 94
n= 20
Sens 95.0, Spec 91.5
NA-N Oral fluid
S/P Ratio
2.5
2.0
1.5
1.0
0.5
0.0
>0.18
n= 291
n= 83
Sens 100, Spec 97.5
EU-N Oral fluid
S/P Ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
>0.31
n= 94
n= 20
Sens 95.0, Spec 95.7

Figure 4A

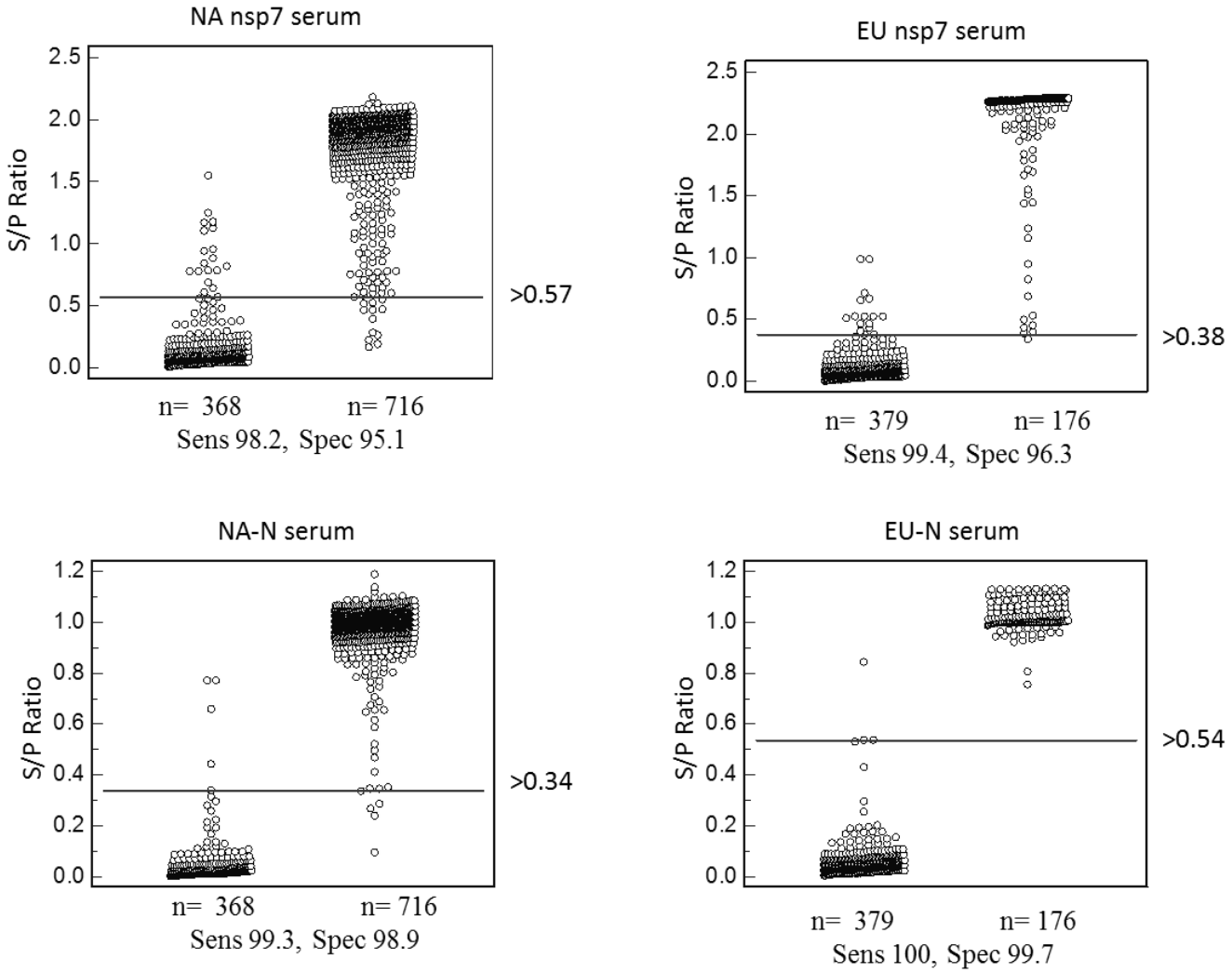


Figure 4B

Figure 4. Determination of diagnostic sensitivity and specificity by receiver operating characteristic (ROC) analysis for oral fluid-based FMIA (A) or serum-based FMIA (B). Diagnostic sensitivity and specificity were calculated using samples from known PRRSV-infected swine population (103 oral fluid and 892 serum) and known PRRSV negative swine population (385 oral fluid and 368 serum). ROC analysis was performed using MedCalc® Version 10.4.0.0 (MedCalc® Software, Mariakerke, Belgium). In each panel, the dot plot at left side represents negative population, and the dot plot at the right side represents positive population. A cross line between the positive and negative population represents the cut off value that gives the optimal diagnostic sensitivity and specificity.

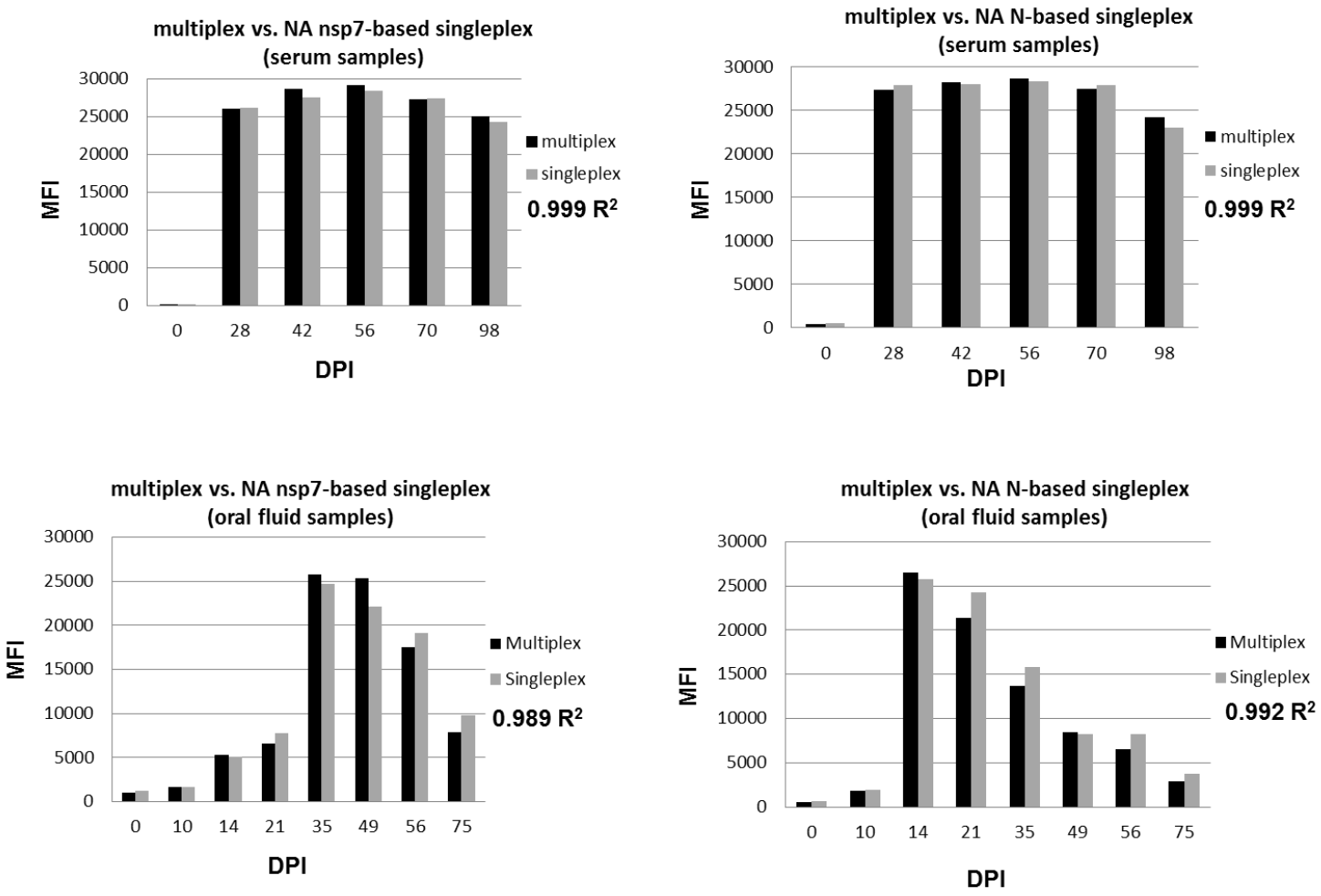


Figure 5. Development of 4-plex fluorescence microsphere immunoassay. Each individual corresponding bead set (indicated in each panel) was first tested in a single-plex format and then combined to test in a 4-plex format. Correlation coefficient (R2) as indicated in each panel was determined for comparison between each single-plex with the 4-plex FMIA.

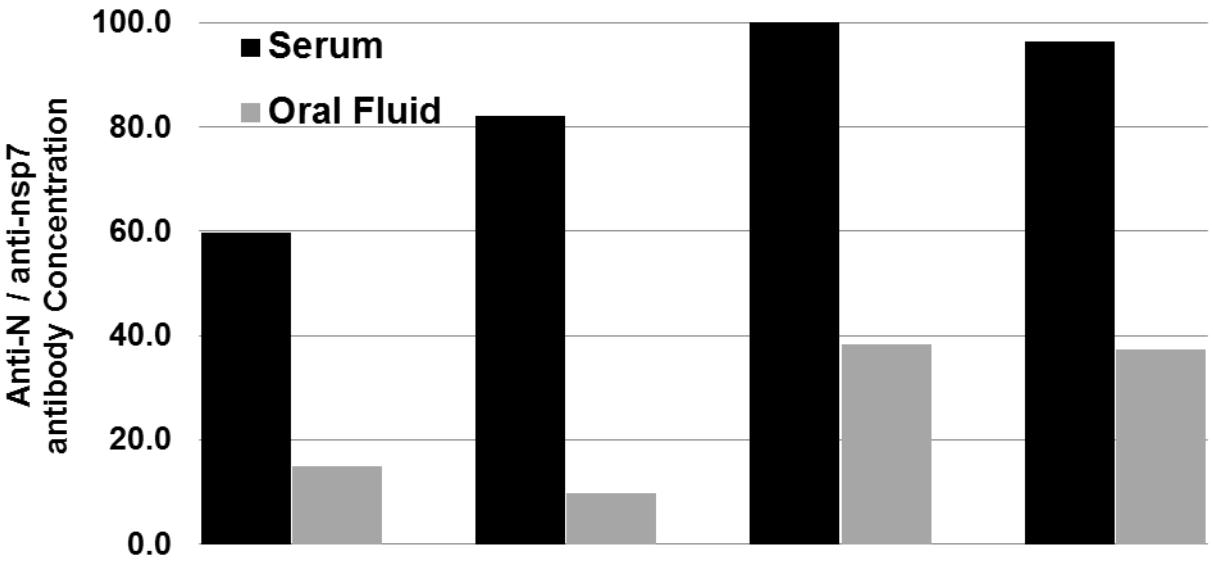
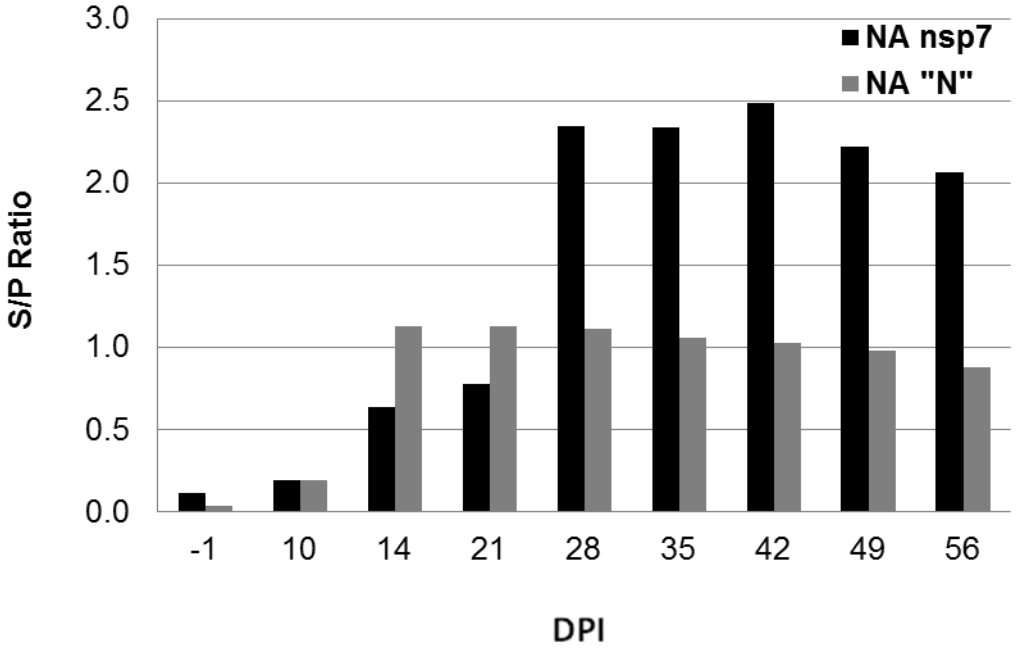


Figure 6. Comparison of the amount of antibody present in oral fluid and serum. Paired oral fluid and serum was collected at 21 dpi from the same individual pigs (n=21) and the anti- N and anti- nsp7 antibody concentrations were determined by 4-plex FMIA.

7A



7B

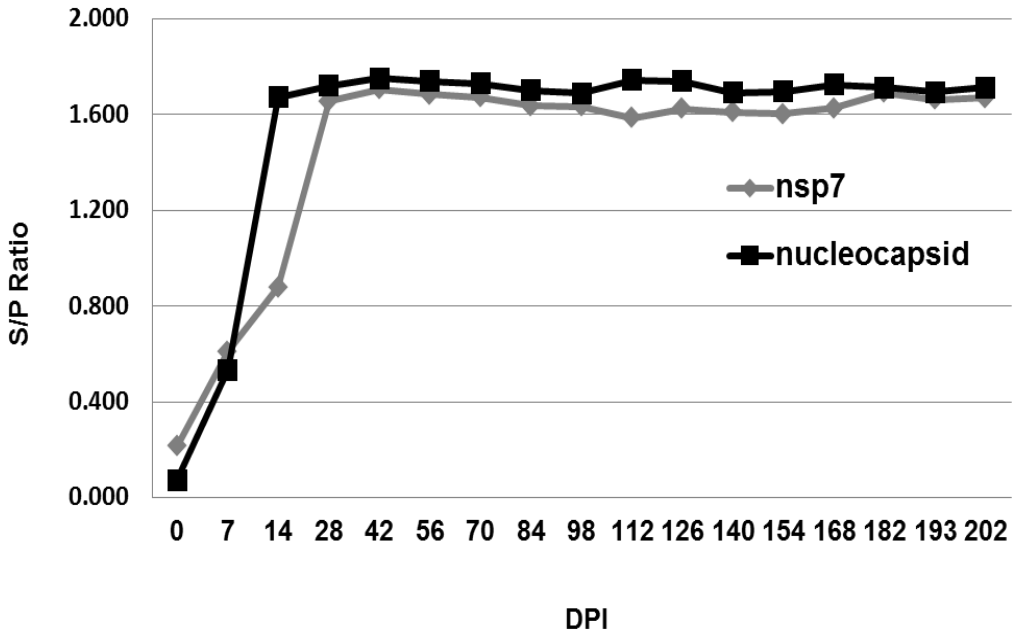


Figure 7. Kinetics of antibody response in serum and oral fluid samples. **A)**. A panel of pooled oral fluid samples (n = 9) from 1100 Type II PRRSV-infected pigs collected at 7-day intervals was evaluated by FMIA; **B)**. A total of 1014 serum samples obtained from 109 pigs experimentally infected with Type II PRRSV were tested by FMIA. They were collected at 7-day intervals for the first two weeks and then at 14-day intervals for up to 202 dpi. S/P ratio was calculated by (MFI of sample – MFI of buffer) / (MFI of positive control – MFI of buffer).