Development and Validation of an Assay To Detect Porcine Reproductive and Respiratory Syndrome Virus-Specific Neutralizing Antibody Titers in Pig Oral Fluid Samples

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Porcine reproductive and respiratory syndrome virus (PRRSV)-specific neutralizing antibodies (NA) are important for clearing the virus. Pen-based pig oral fluid samples for disease surveillance are gaining in importance due to the ease of collection and low cost. The aim of this study was to develop a PRRSV-specific NA assay to determine NA titers in pig oral fluid samples. At first, we standardized the PRRSV NA assay using pen-based pig oral fluid samples collected over a period of 3 months from a herd of swine that received a PRRSV modified live vaccine (PRRS-MLV), and we also used oral fluid and serum samples collected from individual boars that were vaccinated with PRRS-MLV or infected with a virulent PRRSV strain. Our results suggest that a PRRSV NA titer of >8 in oral fluid samples is virus specific and can be detected beginning at 28 days after vaccination or infection. To validate the assay, we used 104 pen-based pig oral fluid and five representative serum samples from each pen of unknown history, as well as 100 serum samples from repeatedly vaccinated sows and oral fluid samples of their respective litters belonging to four different swine-breeding farms. Our results demonstrated that PRRSV NA titers in oral fluid samples are correlated with serum sample titers, and maternally derived PRRSV-specific NA titers could be detected in litters at the time of weaning. In conclusion, we have standardized and validated the pig oral fluid-based PRRSV NA assay, which has 94.3% specificity and 90.5% repeatability. The assay can be used to monitor herd immunity against PRRSV in vaccinated and infected herds of swine.

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating disease of pigs worldwide. Clinical outcomes are characterized by reproductive failure in breeding animals and respiratory distress in pigs of all ages, which is associated with poor growth performance (1, 2). The etiological agent, PRRS virus (PRRSV), has a unique feature of causing severe clinical disease and maintaining persistent subclinical infections (3). Early after PRRSV exposure, the rapid production of virus-specific antibodies is detected from 1 week postinfection (p.i.), but the virus does not elicit a neutralizing antibody (NA) response until at least 3 or 4 weeks p.i. (4, 5). Although the protective ability of PRRSV NA is still not fully understood, the clearance of viremia has been documented by NA and is considered to be one of the important components of protective immunity (4, 6). An earlier report has established a relationship between PRRSV NA titers in pig serum and protection in a passive protection study, with an NA titer of 16 providing sterilizing immunity (7). These studies concluded that an NA titer of ≥16 should protect pigs from PRRS (even without including the host gamma interferon [IFN-γ]-induced protection). Therefore, an easy and cost-effective diagnostic tool to monitor PRRS NA titers in herds of swine is highly useful to evaluate herd immunity against PRRS in field situations. However, evaluating PRRSV herd immunity using individual serum samples in a statistically valid manner requires collecting blood samples from a large number of pigs, which is not feasible. Recently, oral fluid sample submissions for various disease surveillance and diagnosis efforts have increased due to the ease of the collection method and the cost-effectiveness of disease surveillance (virus or antibody) in large commercial herds of swine (8).

Oral fluid is a mixture of saliva and mucosal transudate that contains specific antibodies derived from serum (9) and salivary glands (10). Viruses, such as HIV (11), dengue virus (12), hepatitis A, B, and C (13), measles (14), and rubella (14), and virus-specific antibodies have been detected in human oral fluid samples. Studies have indicated that the antibody isotype IgG that is present in oral fluid has the potential to replace serum IgG in disease prevalence surveys (14). Several oral fluid-based viral antibody assays have been developed (14), and the US Food and Drug Administration has approved a rapid HIV oral fluid-based antibody detection assay for diagnostic purposes in humans. The virus-specific antibody is only detected in oral fluid samples when the antibody is present in the serum, and it is detected simultaneously in both serum and oral fluid but not in seronegative controls (10, 15). Studies have demonstrated viral NA activity in human oral fluid...
samples against cytomegalovirus and rhinovirus, which indicates immunological resistance in the mouth against certain viral infections (10, 15). The virus-specific NA in oral fluid persists for long periods (10). Two major antibody classes that operate in saliva are secretory IgA (sIgA) and IgG (16). sIgA is secreted by plasma cells in salivary glands, and most IgG in saliva is derived from serum, while some IgG is also locally produced (16).

In pigs, shedding of PRRSV (17, 18) and the presence of virus-specific antibody in oral fluid samples have been reported (19). Recently, swine researchers have conducted pioneering studies to survey PRRSV and porcine circovirus 2 (PCV2) using oral fluid samples (19–24). Several research articles on the detection of high levels of PRRSV-specific IgG antibody in oral fluid samples have been published (21–23). So far, however, no attempts have been made to standardize a technique to detect NA titers in oral fluid samples against PRRSV or against any other swine virus, despite reports on the presence of NA activity against rhinovirus (15), influenza virus (25), mumps (26), cytomegalovirus (10), and herpesvirus 7 (27) in human oral fluid samples. A validated diagnostic tool for monitoring PRRSV NA activity in pen-based oral fluid samples from infected and/or vaccinated herds of swine may serve as a breakthrough to monitor herd immunity against PRRS. This approach might offer a significant advancement with respect to ease, timelines, cost of disease surveillance, and monitoring PRRS herd immunity in both large and small herds of swine and both wean-to-finish and breeding herds of swine following vaccination and/or after recovery from the natural infection. Therefore, the objective of this study was to standardize and validate an assay to detect PRRSV NA titers in pig oral fluid samples. To achieve our goal, PRRSV NA titers were analyzed in oral fluid and serum samples collected simultaneously from a large number of pens of commercial herds of swine with a history of PRRSV vaccination, infection, or no infection, and the results were compared. Our results are encouraging, and this diagnostic tool may help Swine farmers and veterinarians to maintain PRRS herd immune status in herds of swine, which may help the undertaking of appropriate control measures when on the verge of PRRS outbreaks.

MATERIALS AND METHODS

Cell lines and virus strains. MARC-145 cells, a clone of the African green monkey kidney cell line MA-104 (28), was used in this study. The cells were cultivated in Dulbecco’s modified Eagle medium (DMEM) (HyClone, MA) supplemented with 10% bovine growth serum (HyClone, MA) containing 0.1 mM HEPES and an antibiotic-antimycotic mixture at 37°C in a humidified atmosphere with 5% CO2.

In this study, three PRRSV strains, VR2332, MN184, and JA142 (ATP vaccine strain), which are phylogenetically highly divergent from each other (29, 30), were used in the NA assay, and MN184 strain was also used for the infected pig groups. Two modified live PRRS virus vaccines, Ingelvac PRRS-ATP (Boehringer Ingelheim) and Ingelvac PRRS-MLV (Boehringer Ingelheim), derived from a parental strain (JA142) and a North American prototype strain (VR2332), respectively, were used in pigs. The PRRSV strains used in the NA assay were propagated in MARC-145 cells, and aliquots whose titers were to be determined were stored at −80°C until used in the experiments. To determine virus titer, a 10-fold serial dilution was performed on PRRSV stocks, and dilutions were incubated with a confluent monolayer of MARC-145 cells in a 96-well microtiter plate for 48 h. Thereafter, dilutions were subjected to a standard indirect immunofluorescence assay (IFA), and the titer was calculated using the Reed and Muench method as described previously (31). The viral titer was expressed in 50% tissue culture infective dose (TCID50) per ml.

Experimental samples. Oral fluid samples were collected using cotton rope as previously described (19). Serum samples were collected using a single-use blood collection system (Vacutainer; Becton Dickinson, NJ) and serum separation tubes (Kendall, MA).

Samples for standardization. To standardize the PRRSV NA assay using oral fluid samples, the following clinical samples were used: (i) Five-week-old pigs (n = 1,100) in a PRRSV-negative commercial wean-to-finish herd of swine were vaccinated intramuscularly with PRRS-MLV, and oral fluid samples were collected from every pen (n = 37) in the barn at −2, 10, 15, 20, 28, 35, 41, 49, 56, 75, and 91 days postvaccination (d.p.v.). These samples were pooled on each day of collection (22). (ii) Oral fluid and serum samples were collected from 24 boars vaccinated with PRRS-MLV and 24 boars infected with a type 2 PRRSV (MN184 strain) (30). The samples that were collected at 0 and 21 d.p.v. or d.p.i. were used in the study.

Samples for validation. To validate the standardized oral fluid-based NA assay, the following samples were used. (i) From two commercial wean-to-finish barns (barns A and B) in a herd of swine in the Midwest United States, 104 pen-based oral fluid samples (25 pigs per pen, each pig weighing 66 to 99 lbs) and five representative serum samples from each pen were collected on the same day. The PRRSV vaccination and infection statuses of the pigs were not known at the time of sample collection (Table 1). (ii) From a herd of swine in the Midwest United States, 100 serum samples from sows and oral fluid samples from their respective litters (immediately after preweaning, at −3 weeks of age) were collected on the same day. Those samples were from four different sow farms (25 serum and 25 litter oral fluid samples from each farm). In all four swine farms, gilts were vaccinated with PRRS-MLV twice before being transferred to sow herds. In each sow herd, a different PRRSV vaccination regimen was followed, receiving up to 2 to 3 doses of additional vaccination (PRRS-MLV and ATP in combination) (Table 2).

PRRS virus neutralization test by indirect immunofluorescence assay. All filtered oral fluid and serum samples (0.2-μm filter) used in the NA assay were treated with UV light (254 nm) at a distance of two inches from the samples in plates for 45 min and were then heat inactivated for 30 min at 56°C. Each test sample was 2-fold serially diluted (1:2 to 1:128) in serum-free DMEM (100 μl per well) and incubated with an equal volume of 100 TCID50 of one of the PRRSV strains (VR2332, MN184, or ATP virus) (30) for 1 h at 37°C. After incubation, 100 μl of the supernatant was transferred into a 96-well microtiter plate containing a confluent monolayer of MARC-145 cells; each sample was run in duplicate. After 1 h of incubation, 100 μl of DMEM containing 2% horse serum and an antibiotic-antimycotic mixture was added, and the plate was incubated for 48 h at 37°C in a CO2 incubator. Cells were fixed using an acetone–Milli-Q water (8:2) mixture for 10 min at room temperature (−20°C), and plates were dried completely before being immunostained as described previously (31). Cells were treated with anti-PRRSV nucleocapsid protein-specific monoclonal antibody (SDOW17) (Rural Technologies, Inc., SD) (1:5,000) for 2 h at 37°C, followed by treatment with Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen, CA) secondary antibody (1:3,000). The plate was examined under a fluorescence microscope after mounting with glycerol–phosphate-buffered saline (PBS) (6:4). The virus-neutralizing antibody (NA) titer was determined to be the reciprocal dilution ratio of the sample at which >90% inhibition in the PRRSV-induced immunofluorescence was observed.

Diagnostic specificity and sensitivity. To determine the optimal PRRSV NA titer for a positive/negative cutoff value with the highest diagnostic specificity and sensitivity using pig oral fluid samples, three sets of samples were used: (i) four pooled (n = 37) reference pen-based oral fluid samples collected from pigs vaccinated with PRRS-MLV at −2, 10, 15, and 20 d.p.v., (ii) 24 individual boar oral fluid and 24 respective boar serum samples vaccinated with PRRS-MLV at 0 and 21 d.p.v., and (iii) 47 and 57 pen-based oral fluid samples from wean-to-finish pigs belonging to barns A and B, respectively.
Repeatability. To test the repeatability of the assay, a total of 411 oral fluid samples used for assay validation were tested in duplicate in 2-fold serial dilutions (1:2 to 1:32). The number of mismatches between duplicates was tabulated to get an indication of repeatability, as described previously (32).

Detection of PRRSV isotype-specific antibody by ELISA. Both serum and oral fluid samples were tested for anti-PRRSV antibodies using a commercial ELISA kit (HerdChek X3 PRRS enzyme-linked immunosorbent assay [ELISA]; IDEXX Laboratories, Inc., Portland, ME). Levels of isotype-specific total IgM, IgA, and IgG antibodies (not PRRSV-specific) were quantified using commercial ELISA kits (Bethyl Laboratories, Montgomery, TX). Serum samples were tested according to the manufacturer’s protocol, and oral fluid samples were tested as described previously (22).

Detection of PRRSV RNA by real-time PCR. Total nucleic acid in test samples was extracted using a Thermo Electron KingFisher automated magnetic particle processor and using a MagMAX-96 pathogen RNA/DNA kit (Applied Biosystems). Real-time PCR (RT-PCR) was performed with a commercially available reagent set (TaqMan North American reagents and TaqMan North American controls; Applied Biosystems). Reactions were performed according to the manufacturer’s instructions using the ABI 7500 real-time PCR system.

Statistical analysis and data interpretation. All data were expressed as the mean value ± the standard error of the mean (SEM). Results of the PRRSV NA assay were expressed in actual NA titers. Statistical analyses were performed using GraphPad InStat 5.0 Prism software by applying the Welch corrected unpaired t test and paired t test to determine the statistical significance (P < 0.05) between the two indicated test groups.

RESULTS

Standardization of NA assay to determine PRRSV NA titers in pig oral fluid samples. To standardize the pen-based oral fluid PRRSV-specific NA assay, oral fluid samples were heat and UV treated before use, so as to inactivate microorganisms and innate antiviral factors that are present in the oral fluid (33–35). To determine PRRSV NA titers in oral fluid samples, we prepared standards by 2-fold dilutions of the virus from a starting virus amount of 100 TCID50 per well and infected confluent monolayers of

| TABLE 1 Summary of anti-PRRSV immune statuses in serum and oral fluid samples of a commercial herd of swine |
|---------------------------------------------------------------|
| Sample characteristics                                           | Results from barn: | |
|                                                               | A                  | B                  |
| Serum No./total no. (%) of samplesa                            | 10/235 (4.3)       | 225/235 (96)       | 285/285 (100) |
| NA titer range (mean ± SEM)                                    | 0                  | 9.4–32b (20.0 ± 0.8) | 0 |
| Antibody S/P ratio (mean ± SEM)                                | 2.3 ± 0.2          | 2.3 ± 0.1          | 0 ± 0.2       |
| PRRSV RNA-positive result                                      | ND                 | ND                 | ND            |
| Oral fluid No./total no. (%) of samplesc                        | 29/47 (61.7)       | 18/47 (38.3)       | 48/57 (84.2)  |
| NA titer range (mean ± SEM)                                    | 3–8d (7 ± 0.3)     | 12–32d (17.8 ± 1.6) | 0–8e (6 ± 0.3) |
| Antibody S/P ratio (mean ± SEM)                                | 3.1 ± 1.4          | 3.6 ± 0.1          | 0             |
| PRRSV RNA-positive result (no./total no.)                      | 2/2                | 32/45              | 0             |

a There were five serum samples from each pen, i.e., 235 from barn A and 285 from barn B.  
b Average NA titer of five serum samples.  
c Number of positive or negative samples irrespective of serum NA results.  
d NA titers in pen-based oral fluid samples from each pen (n = 25 pigs per pen).  
e ND, not determined.  
f S/P, sample-to-positive ratio.

| TABLE 2 PRRSV vaccination program of sows and sample collection schedule from four sow farms |
|---------------------------------------------------------------|
| Farm* Jan Feb Mar Apr May Jun Jul Aug                           |
| a                A A*                                             |
| b                A A M                                           |
| c                A A                                             |
| d                d M                                             |

a All gilts were vaccinated with PRRS-MLV twice before they were moved into sow herds.  
b A, vaccination of sows with PRRS-ATP.  
c M, vaccination of sows with PRRS-MLV.  
d In the first week of January, farm d sows were vaccinated with both PRRS-ATP and PRRS-MLV on the same day.  
e Double-sided arrows indicate the periods when serum and oral fluid samples were collected from the 25 sows and their respective litters in each farm, and both samples were collected on the same day immediately after weaning from sows and litters.
MARC-145 cells, and these were used for comparison with the NA activity of test samples. The NA titers of test samples were expressed as the reciprocal of the highest dilution ratio of the oral fluid that inhibited >90% of the virus-induced immunofluorescence activity (Fig. 1). PRRSV NA titers against VR2332 strain in PRRS-MLV-vaccinated reference standard oral fluid samples at all indicated days postvaccination were determined. At 0 to 20 d.p.v., the NA titers were all <8, and from 28 to 91 d.p.v., the NA titers increased steadily and reached a peak titer of 32 (Fig. 2).

Further, PRRSV NA titers in the serum and oral fluid samples collected from individual boars vaccinated with PRRS-MLV or infected with the virulent type 2 PRRSV strain MN184 were analyzed. In serum samples, the NA titers of all 24 boars at 0 d.p.v. were negative, and at 21 d.p.v., only four vaccinated animals had NA titers of four (n = 3) and eight (n = 1) (Fig. 3A). The NA titers in the oral fluid samples of all 24 boars at both 0 and 21 d.p.v. were <8 (Fig. 3B). In the serum of the 24 boars infected with the MN184 strain, no NA titers were detected either at 0 or 21 days postinfection (d.p.i.) (Fig. 3A), and the respective oral fluid samples of individual boars also had NA titers of <8 (Fig. 3B). Together, these results and the reference standard oral fluid sample data (Fig. 2) suggested that a positive/negative cutoff value of eight could be used to determine PRRSV-specific NA titers in oral fluid samples of pigs that were vaccinated or infected with PRRSV.

Validation of NA assay to determine PRRSV NA titers in pen-based oral fluid samples. To validate the oral fluid-based PRRSV NA assay, pen-based (~25 pigs per pen) oral fluid and five representative serum samples from each pen were used. These samples were collected from two swine barns in the Midwest United States (barn A, n = 47 pens; barn B, n = 57 pens) with unknown PRRSV infection and vaccination histories. PRRSV in both barn A and B swine was also not isolated; therefore, we analyzed NA titers of individual boars also had NA titers of <8 (Fig. 3B).}

![FIG 1](image1.png) Representative pictures showing PRRSV neutralizing antibody (NA) titers in pen-based pig oral fluid samples. The NA titer of a sample is expressed as the reciprocal of the highest dilution ratio of the oral fluid that inhibited >90% of the virus-induced immunofluorescence activity. One hundred TCID<sub>50</sub> of the PRRSV was back-titrated to show the virus-induced effect.

![FIG 2](image2.png) Kinetics of PRRSV NA titers against the virus strain VR2332 in the reference PRRS-MLV-vaccinated pig oral fluid samples. Pooled pen-based oral fluid samples from 37 pens collected at -2, 10, 15, 20, 28, 35, 41, 49, 56, 75, and 91 d.p.v. in a PRRSV-negative commercial wean-to-finish herd of swine of 1,100 pigs (aged 5 weeks). PRRSV-specific NA titers were determined by the immunofluorescence assay. Each bar represents the average NA titer of triplicate samples ± SEM. The horizontal line indicates the positive/negative cutoff value of eight.

![FIG 3](image3.png) PRRSV NA titer against a homologous virus in the serum samples and the respective oral fluid samples collected from boars. From 24 serum and respective boar oral fluid samples vaccinated with PRRS-MLV or infected with MN184 strain collected at 0 and 21 d.p.v or d.p.i, respectively. Test serum (A) and oral fluid (B) samples were analyzed for NA titers. The horizontal bars represent the average NA titer from 24 each of serum (A) and oral fluid (B) samples ± SEM.
against the VR2332 strain of PRRSV. In barn A, 95.7% (225/235) of serum samples had NA-positive results, with titers ranging from 9.4 to 32 (Table 1). The mean NA titers of pen-based oral fluid samples in barn A corresponding to negative and positive serum NA activity were 3.5 and 11.5, respectively (data not shown). Out of 47 pen-based oral fluid samples in barn A, 34 (72.3%) were positive for PRRSV RNA. In barn B, all of the serum samples (n = 285) tested negative for PRRSV NA activity and for viral RNA, and the mean NA titer of the respective pen-based oral fluid samples (7.5) was also negative (Table 1). In barn A, only 38.3% (18/47) of pen-based oral fluid samples tested positive (NA titer >8) in the NA assay, with a mean titer of 17.8 (range, 12 to 32), and the remaining 61.7% (29/47) of samples were negative, with an average titer of 7 (range, 0 to 8). In barn B, 84.2% (48/57) of the oral fluid samples had negative results for NA titers (<8; average, 6), and the remaining 15.8% of oral fluid samples were NA positive (titer of >8; average, 15.1) (Table 1). In both barns, NA titers from five representative serum samples and pen-based oral fluid samples were compared separately, and a statistically significant difference (P < 0.001) was found in comparing NA titers from serum samples between barns A and B; a significant difference (P < 0.01) was also found in oral fluid samples between the two barns (Fig. 4A, B).

Detection of maternally derived PRRSV NA activity in oral fluid samples from litters. The transfer activity of maternal PRRSV NA antibodies to litters from sows that were vaccinated repeatedly with two types of live PRRSV vaccines (PRRS-MLV and PRRS-ATP) was determined (Table 2). For this analysis, individual sow serum samples (n = 25 per farm, n = 4 farms) and pen-based oral fluid samples from respective litters (n = 100 from 4 farms) collected on the same day immediately after weaning (~3 weeks) were used. Our results indicated that all 100 sow serum samples were negative for PRRSV RNA by RT-PCR and thus were negative for PRRSV viremia. PRRSV NA activity in sows indicated that 51% and 100% of the serum samples were positive for NA titers against the VR2332 and ATP strains, respectively. Of the oral fluid samples, 84% and 76% of litters were positive (>8) for NA titers against the PRRSV strains VR2332 and JA142, respectively (Fig. 5A, B; Table 3).

The average NA titers of the pen-based oral fluid samples from 25 litters in all four sow farms against the VR2332 virus strain were comparable (Fig. 5C), and these were also comparable against the ATP strain, but with overall lower titers (Fig. 5D). In serum samples, the average NA titer in farm b was significantly higher (>12) than those for farms a and d (≦8) (Fig. 5C). However, against the ATP strain, NA titers in sows serum were high (>28) and comparable among all four farms (Fig. 5D). In the sows of farm b, >70% (17/25) of sows tested NA positive and correlated with increased average serum NA titers against VR2332 strain, compared to 40% in farms c and d and 55% in farm a (Fig. 5A, C). Interestingly, all 100 serum samples from all four sow farms were NA positive against ATP virus (Fig. 5B). The percentages of litters that were positive for NA activity against the VR2332 and ATP strains were approximately 80% and 70%, respectively, except in farm d against the ATP strain, where the NA positive litters were <60% (Fig. 5A, B). This may be due to the administration of a single dose of ATP vaccine compared to 2 to 3 doses in the other three farms (Table 2).

All serum and oral fluid samples from each of the four farms had comparable levels of individual (not PRRSV-specific) antibody isotypes (IgM, IgA, and IgG) (Table 4). However, in the oral fluid samples, only the IgG antibody isotype was detected, while high levels of PRRSV-specific antibodies were detected in both oral fluid and serum samples from all sows and their litters (Table 4).

Diagnostic specificity and sensitivity of the NA assay in oral fluid samples. In the NA assay, oral fluid samples had higher background activity than serum samples. Thus, the NA titer of eight was considered an appropriate positive/negative cutoff to identify PRRSV-specific NA titers. This was based on the results of four reference standard samples collected at 2 to 20 d.p.v. (Fig. 2), 96 boar samples (Fig. 3), and 57 field samples (barn B) with unknown PRRS histories (Fig. 4). Our results indicated a specificity of 94.3% (148/157) for our NA assay. Although all the reference standard oral fluid samples from vaccinated pigs indicated 100% sensitivity, due to the lack of individual pig serum and oral fluid samples collected >4 weeks postvaccination or postinfection, the diagnostic sensitivity of the assay could not be estimated at that time.

Repeatability. We performed an NA assay of all the validation samples in duplicate wells in four 2-fold dilutions (1:4, 1:8, 1:16, and 1:32), and 411 oral fluid samples were able to be used for the repeatability analysis. In 131 samples (31.9%), there was one-dilution-step difference between the duplicates in NA activity (>90% reduction in the PRRSV-induced immunofluorescence). There were 39 samples in which the qualitative result (positive/negative) would have been different if the result were to be based on either of the duplicate well results; thus, based on qualitative results, the repeatability of our assay was 90.5%.

DISCUSSION

A PRRSV NA assay to test pen-based pig oral fluid samples was developed, and it was found to have 94.3% specificity and 90.5%...
repeatability. The detection of a PRRSV-specific antibody response in pen-based oral fluid samples by ELISA provides an indication that pigs are exposed to virus through either disease or vaccination, but it does not suggest the herd immune status. However, the application of a PRRSV NA-based assay on pen-based oral fluid samples might provide cost-effective monitoring of PRRS herd immune status in both wean-to-finish and breeding herds of swine. In addition, it also helps to determine the levels of maternal anti-PRRSV NA activity in litters, which is important because virus-specific NA activity in oral fluid persists for long

![FIG 5 PRRSV NA titers in sows and their respective litters. Number of samples with positive NA titers against PRRSV VR2332 strain (A and C) and ATP strain (B and D) from sow serum and oral fluid samples of respective litters collected from four swine-breeding farms. A total of 100 each of serum and oral fluid samples from four farms that followed different PRRSV vaccination regimes were used (Table 2). Each bar represents the average NA titer from 25 samples ± SEM. Asterisks denote a statistically significant difference ($P < 0.05$) between sera from farm b and farm a or d.](cvi.asm.org)

| TABLE 3 Summary of PRRSV NA titers in the serum of sows and oral fluid of litters |
|-----------------------------------------------|---------------|---------------|---------------|---------------|
| Titer characteristic by sample strain and type | a | b | c | d |
| NA titers against VR2332 strain | | | | |
| Serum samples | | | | |
| No. of samples | 14 | 11 | 17 | 8 |
| NA titer range (mean ± SEM) | 3–32 (7 ± 2) | 0 | 3–32 (18 ± 3) | 0 |
| Oral fluid samples | | | | |
| No. of samples | 19 | 6 | 22 | 3 |
| NA titer range (mean ± SEM) | 12–32 (28 ± 2) | 4–8 (7 ± 1) | 12–32 (26 ± 2) | 3–8 (6 ± 2) |
| NA titers against ATP strain | | | | |
| Serum samples | | | | |
| No. of samples | 25 | 0 | 25 | 0 |
| NA titer range (mean ± SEM) | 16–32 (30 ± 1) | 0 | 16–32 (31.0 ± 1) | 0 |
| Oral fluid samples | | | | |
| No. of samples | 20 | 5 | 20 | 5 |
| NA titer range (mean ± SEM) | 12–32 (26 ± 2) | 0–8 (5 ± 2) | 12–32 (21 ± 2) | 4–8 (6 ± 1) |

* Oral fluid samples were measured based on an NA titer of 1:8 as the positive/negative cutoff value.
TABLE 4 Summary of immune status in the serum samples of sows and oral fluid samples of litters

| Immune response type | Immune status in farms: |
|----------------------|-------------------------|
|                      | a | b | c | d |
| **Sow serum samples** |   |   |   |   |
| IgA                  | 0.3 ± 0.1 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 |
| IgM                  | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.1 |
| IgG                  | 1.1 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 |
| Antibody ELISA<sup>a</sup> | 1.1 ± 0.2 | 0.8 ± 0.1 | 1.1 ± 0.2 | 0.9 ± 0.2 |
| **Litter oral fluid samples** |   |   |   |   |
| IgA<sup>a</sup>     | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.0 |
| IgM<sup>a</sup>     | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| IgG<sup>a</sup>     | 2.9 ± 0.3 | 2.2 ± 0.2 | 1.9 ± 0.3 | 2.2 ± 0.2 |
| Antibody ELISA<sup>a</sup> | 5.3 ± 0.5 | 4.3 ± 0.4 | 3.8 ± 0.5 | 4.4 ± 0.5 |

<sup>a</sup> Mean ± SEM of total (not PRRSV-specific) individual swine isotype antibody optical density values by ELISA.

<sup>b</sup> Mean ± SEM of PRRSV-specific antibody S/P values by ELISA (IDEXX Laboratories, Inc., ME).

periods (10). Such information may contribute to PRRSV vaccination compliance and herd immunity parameters in large commercial herds of swine, as well as surveillance of PRRSV infections in disease elimination programs (22).

Our study identified a PRRSV NA titer of >8 to be virus specific in pen-based oral fluid samples of vaccinated or infected herds of swine, and this appears only after 4 weeks postvaccination or postinfection. This is consistent with the detection of serum NA titers in PRRSV-vaccinated or -infected pigs, which appear only after 3 to 4 weeks (4, 36, 37). This detection level was found to be true in pen-based oral fluid samples of pigs of all ages and also in litters; however, there is a sensitivity difference between serum- and oral fluid-based NA assays. Our study suggests that an NA titer of <8 in oral fluid samples was influenced by background effects, such as the hypotonic nature of saliva, innate antiviral substances that are present in oral fluid (33–35), etc. In PRRSV-infected and -vaccinated pigs, both virus and NA are detectable at a certain time point and there may be an interference of UV light with NA activity. Therefore, the effect of UV light needs to be investigated by subjecting oral fluid samples to the presence or absence of UV irradiation; however, it is necessary to passage samples through a 0.2-μM filter to remove microorganisms that are present before applying them on live cells.

For validation purposes, we made use of 104 pen-based oral fluid samples from two barns along with serum samples from only 20% of pigs from each pen with unknown PRRS histories. In barn A, only 38% of pen-based oral fluid samples had positive NA titers compared to in serum (Table 1). Unlike in the boar sample data, due to the lack of individual pig oral fluid and serum samples from barn A, we could not establish any relationship between serum and oral fluid NA titers. However, possible reasons for observed differences in NA activity between serum and oral fluid samples of barn A might be due to the differences in NA activity of the antibody isotype, that is present in serum and oral fluid samples; this is because in serum, the IgG isotype has the principal NA activity, and in oral fluid, the IgA isotype has this role (10, 15). Further, IgA in oral fluid is secreted locally and not through the leakage of the serum antibodies (10, 15). In addition, virus-specific NA activity in oral fluid is of a lower titer than in the serum samples, NA activity in oral fluid is detectable for a long period (10), and antibody is detected in serum before it appears in oral fluid (15).

Moreover, the relationship between oral fluid and serum samples with regard to viral NA titers has not yet been established and requires further investigations. While in barn B, 84% of oral fluid samples tested negative for NA activity, and the presence of 16% of NA-positive samples might be due to antiviral substances that are present in the saliva, as PRRSV RNA or virus-specific antibodies were absent in all of the barn B samples.

In the four sow farms, animals were vaccinated 4 to 5 times with modified live virus vaccines (MLV and ATP) before being bred (Table 1). The aim of such procedures was to elicit strong maternal immunity in the litters. There was no difference in the levels of either anti-PRRSV antibody response or individual antibody isotypes (not PRRSV-specific) in serum and oral fluid samples of sows from all four farms, despite significant variations found in the levels of virus-specific NA titers. So far, we lack any cost-effective and easy tool to measure the efficacy of vaccines in sows and their litters. Therefore, we analyzed individual sow serum and their respective litter oral fluid samples for PRRSV NA titers. The average NA titer in oral fluid samples of litters remained high (>16), irrespective of sows being vaccinated with 1 to 3 doses of PRRS-ATP along with 2 to 4 doses of PRRS-MLV, suggesting the presence of persistently high levels of maternal NA titers; this is consistent with an earlier report that NA activity in oral fluid persists for long periods (10). However, the number of litters that had NA activity was found to be dependent on the timing and type of the vaccine combination used in the sows.

However, in sow serum samples, the average NA titers against the VR2332 strain in three farms (a, c, and d) were low (close to 4), with >50% of the sows testing negative for NA activity (Fig. 5A and B). In farm b however, >70% of the sows were NA positive against VR2332 and 100% were positive against ATP, and the vaccination history indicated that farm b had received at least two doses of ATP and three doses of MLV, with MLV used as a last booster. Against the ATP virus, three farms (a, b, and c) had high average NA titers (>16) in both the sows and litters, but in farm d, which received just one dose of ATP vaccine, 9/25 litters were negative for NA activity. This suggests that there are a few cross-neutralizing NA epitopes present in PRRS-MLV and ATP viruses, and at least two doses of ATP vaccine in combination with PRRS-MLV appears to be an ideal strategy to elicit adequate maternal immunity in litters.

All 100 sow serum samples had NA activity with increased NA titers against the ATP virus, but only 50 were NA positive and also had lower titers against the VR2332 virus. This appears to be due to the boosting of conserved epitopes that are present in both PRRS-MLV and ATP vaccine viruses. However, close to 75% of the oral fluid samples from the litters from all four farms had NA activity, with comparable high titers against both VR2332 and ATP viruses, irrespective of their different vaccine regimens. While the average NA titers in serum samples of sows were low and almost comparable among the four farms against VR2332 virus, all sow serum samples had NA activity with high titers against the ATP virus. At this stage, we do not know the precise reason for such a significant difference in NA activity against two vaccine viruses in sow serum samples. However, in the litters, the oral fluid IgA fraction was not detected (Table 4), suggesting that the observed NA activity in the litters oral fluid samples is mostly
mediated by the maternally derived PRRSV-specific IgG isotype; this matter requires further investigation.

In conclusion, standard ELISA helps mainly in disease surveillance but does not determine the protective immune status in vaccinated and/or infected pigs. In contrast, the pen-based PRRSV NA assay provides information on PRRS immune status, provided that appropriate viral samples are applied in the assay. This diagnostic tool has the potential to monitor PRRS herd immunity in large commercial finisher and breeding herds, including in litters. Further, this assay could be applied to evaluate cross-reactive NA responses to predict protection against more contemporary strains. However, more investigations on pen-based oral fluid samples collected from a large number of vaccinated and/or infected herds of swine with European and North American genotype viruses are required to implement this diagnostic tool in field use.

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