RESEARCH REPORT
SWINE HEALTH

Title: PEDV Diagnostic Approaches to Assess Sow Immunity and Piglet Protection. **NPB #14-038**

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Industry Summary:
Porcine epidemic diarrhea virus (PEDV) was first detected in the U.S. in May 2013 and spread rapidly across the country. PCR assays were quickly developed to detect the presence of PEDV RNA in intestinal contents or fecal material and these assays provide an important tool in control of the virus. The continuation of severe outbreaks of PEDV in North America highlights the need for additional well-validated diagnostic tests for the detection of recently infected animals and evaluation of their immune status to this virus. Specific serological tests can be used to detect the antibody response in animals following infection or vaccination. They can provide an important tool in the screening of replacement animals and certain tests, such as virus neutralization assays, may provide insight into the level of protective immunity in a given group of animals.

Various serological assays for PEDV have been previously described, but few were readily available in the U.S. Several U.S. laboratories quickly developed indirect fluorescent antibody (IFA) assays for the detection of antibodies to PEDV in swine serum, indicating prior exposure. However, the IFA has several disadvantages, including low throughput, moderate sensitivity and relatively subjective interpretation. Different serologic test formats have advantages and disadvantages, depending on the questions being asked, so a full repertoire of tests is useful. Therefore, the overall objective of this study was to complete the development, optimization and validation of selected serological assays for improved monitoring and control of PEDV. These assays were then used to assess the best methods to measure exposure and the development and duration of "protective" immunity in pigs. Specific objectives addressed included:

1. Finalize validation of our new PEDV fluorescent focus neutralization (FFN) assay and assessment of its ability to detect and quantify neutralizing or "potentially protective" antibodies in milk/colostrum as well as in serum samples.

2. Complete development of next generation assays such as specific ELISAs and fluorescent multiplex immunoassays (FMIA) to detect "potentially protective" antibodies in milk/colostrum, oral fluids and serum and determine correlation with functional virus neutralization, measured by the PEDV FFN.
3. Assess different tests and sample types (serum, milk and oral fluids) to determine the most accurate and cost effective strategies to predict duration of immunity and protection of piglets.

The PEDV FFN-based virus neutralization assay was optimized and validated for application with serum, milk and colostrum samples. Virus neutralization titers of serum samples from PEDV naïve pigs as determined by the FFN assay are generally <1:20, while most infected animals typically demonstrate titers of 1:40 to 1:1280 by 3 to 4 weeks post-exposure. Colostrum samples from recently infected sows tend to show substantially higher neutralizing antibody titers than serum samples from the same animals. Milk samples typically show neutralizing antibody levels similar to those observed in serum samples from the same sows. The PEDV FFN is now routinely offered by the South Dakota Animal Disease Research & Diagnostic Laboratory with over 10,000 samples tested to date.

Recombinant proteins including the PEDV nucleoprotein (NP) and the S1 region of the spike protein were expressed and purified for use in ELISA and FMIA tests. A variety of different assays were developed and validated by approved standards. Assessment involving over 1400 samples of known status demonstrated that the NP-based FMIA had the highest sensitivity and specificity at 98.2% and 99.2%, respectively, with a significant level of testing agreement among all assays. No cross-reactivity with the closely related coronaviruses, transmissible gastroenteritis virus (TGEV) or porcine respiratory coronavirus (PRCV) was noted. All assay formats detected seroconversion of naïve animals within 6-9 days post exposure.

In summary, the FFN assay allows relative quantitation of functional neutralizing antibodies in serum, milk or colostrum samples. Serum antibody levels detected by the FFN show good correlation with neutralizing antibody levels present in milk and colostrum. Measurement of neutralizing antibody responses using the FFN assay should provide a valuable tool for assessment of vaccine candidates and assessment of herd immunity. Well-validated, high throughput indirect ELISA, blocking ELISA and FMIA assays for the detection of PEDV antibodies were also developed and validated, showing good correlation with IFA and each other. Each assay format has advantages that dictate how they will be used in the field.

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Scientific Abstract:
Robust serological assays are needed for the improved control of PEDV. Therefore, the overall objective of this study was to develop and validate multiple serological assays for PEDV. These assays included a fluorescent focus neutralization assay (FFN) to measure functional virus neutralizing antibodies; an indirect ELISA (iELISA); a highly specific monoclonal antibody-based blocking ELISA (bELISA); and fluorescent microsphere immunoassays (FMIA) that can be multiplexed to monitor exposure to multiple antigens and pathogens simultaneously.

The FFN assay was optimized and evaluated using multiple panels of serum samples from PEDV naïve animals and herds sampled at various times post-exposure. Essentially all samples from naïve animals demonstrated serum FFN endpoint titers of <1:20 while most samples from PEDV positive herds had endpoint titers ranging from 1:40 to 1:1280. Additional sequential sample sets including serum, colostrum and milk were then evaluated. Mean colostrum titers were approximately 4-fold higher than serum titers at the time of farrowing while serum and milk titers were similar in magnitude, although substantial animal to animal variation was apparent.

A recombinant North American nucleoprotein (NP) based iELISA was developed and validated along with a bELISA using newly developed PEDV-NP specific biotinylated monoclonal antibodies and an FMIA using magnetic beads coupled with expressed PEDV-NP. Receiver operating characteristic (ROC) analysis was performed using swine serum samples (iELISA n=1486, bELISA n=1186, FMIA n=1420). The ROC analysis for the FMIA showed estimated sensitivity and specificity of 98.2% and 99.2%, respectively. The iELISA and bELISA showed a sensitivity and specificity of 97.9% and 97.6%; and 98.2% and 98.9%, respectively. Inter-rater (kappa) agreement was calculated to be 0.941 between iELISA and IFA, 0.945 between bELISA and IFA and 0.932 between FMIA and IFA. Similar comparative kappa values were observed between the iELISA, bELISA and FMIA, demonstrating significant agreement among assays. No cross-reactivity with the related coronaviruses, transmissible gastroenteritis virus (TGEV) or porcine respiratory coronavirus (PRCV) was noted. All three assays detected seroconversion of naïve animals within 6-10 days post exposure. FMIA tests using PEDV spike (S1) antigen preparations were also developed to compare IgA and IgG responses in serum, milk and colostrum with neutralizing antibody levels detected by the FFN. While moderate to strong correlations were noted among assays, the FFN appeared to provide the most consistent results in a cost-effective test format.

In summary, well-validated iELISA, bELISA and FMIA assays for the detection of PEDV antibodies were developed and showed good correlation with IFA and each other. Each assay format has advantages that dictate how they will be used in the field. Measurement of neutralizing antibody responses using the FFN assay should provide a valuable tool for assessment of vaccine candidates or protective immunity. Ongoing field experience with the FFN assay should eventually allow better correlation of measured neutralizing antibody levels and expectations of protective immunity.

Introduction:
Porcine epidemic diarrhea virus (PEDV) was first described in Europe in the 1970s with more recent and severe outbreaks in Asia (Debouck & Pensaert, 1980; Paudel, et al. 2014). The virus was identified in the United States in May 2013, causing severe diarrhea and vomiting in pigs across age groups, with high mortality of up to 90%-95% in suckling pigs (Stevenson, et al. 2013). PEDV is an enveloped, single stranded RNA virus belonging to the *Coronaviridae* family within the order *Nidovirales*. Recently, the International Committee on Taxonomy of Viruses (ICTV) recognized four genera within the *Coronavirinae* subfamily: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Adams & Carstens, 2012). PEDV belongs to the genus *Alphacoronavirus* along with other swine viruses including transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV).

In response to the recent outbreaks of highly virulent PEDV in North America (NA), PCR assays were quickly developed to detect the presence of PEDV RNA in intestine or fecal material. These assays provide an important tool in control of the virus; however, well-validated, high-throughput assays to detect antibodies following infection would provide additional valuable diagnostic tools for the swine industry. The ability to detect and evaluate antibody responses using serologic tests is important in efforts to answer basic production related questions. These questions may include whether a production site is naïve or has historically experienced a PEDV exposure, even though a producer has not seen obvious clinical signs; the level of immune response sows may have in relation to vaccination, initial wild-type virus infection or intentional feedback exposure; and whether sow immunity is inadequate when clinical infection occurs in individual litters after initial PEDV exposure in a herd.

In the present study, a fluorescent focus neutralization (FFN) assay was developed and optimized for the rapid evaluation of PEDV neutralizing antibodies in serum, colostrum and milk samples. Highly purified, recombinant PEDV-nucleoprotein (NP) and the S1 region of the spike protein were prepared as antigens for the development of indirect ELISA (iELISA), blocking ELISA (bELISA) and fluorescent microsphere immunoassay (FMIA) platforms for the detection of PEDV antibodies in serum. These assays provide high throughput serological tests designed to address PEDV disease diagnostics. They were fully validated using a large number of serum samples of known status. Validation of tests was conducted using methods for the validation of serological assays for the diagnosis of infectious diseases previously described by Jacobson (1998) for the Office International des Epizooties.

Objectives:
The overall objective of this study was to complete the final optimization and validation of selected serological assays for improved monitoring and control of PEDV. These assays were then assessed to determine the best methods to measure exposure and the development and duration of "potentially protective" immunity in pigs. Specific objectives addressed in this study included:

1. Finalize validation of our new PEDV fluorescent focus neutralization (FFN) assay and assessment of its ability to detect and quantify neutralizing or "potentially protective" antibodies in milk/colostrum as well as in serum samples.

2. Complete development of next generation assays such as specific ELISAs and fluorescent multiplex immunoassays (FMIA) to detect "potentially protective" antibodies in milk/colostrum, oral fluids

and serum and determine correlation with functional virus neutralization, measured by the PEDV FFN.

3. Assess different tests and sample types (serum, milk and oral fluids) to determine the most accurate and cost effective strategies to predict duration of immunity and protection of piglets.

Materials & Methods:

To accurately assess the diagnostic sensitivity and specificity of the assays, samples of known serostatus for PEDV were used. This included sera from multiple animal populations including experimentally infected animals and serum samples from animals with known historical exposure to PEDV that were submitted to the South Dakota Animal Disease Research and Diagnostic Laboratory (ADRDL). PEDV negative sample sets included samples from PEDV negative control pigs used in experimental studies and selected high biosecurity herds with no history of PEDV. In addition, archived serum samples collected prior to the emergence of PEDV in the U.S., including samples testing positive for the related swine coronaviruses TGEV and PRCV (n=>50), were used. The exact number of positive and negative sera used for sensitivity and specificity calculations per assay with statistical testing agreement calculations based on serum numbers is listed in Table 1. The majority of these sera were identical among assays, but limited serum volume did not allow for use of all sera samples among all assays. For time course studies, serum samples from experimentally infected animals were obtained courtesy of Dr. Richard Hesse (Kansas State University Veterinary Diagnostic Laboratory, National Pork Board Grant #13-228). Additionally, colostrum, milk and oral fluid samples of know status were evaluated.

A PEDV virus neutralization assay using a FFN format was developed for rapid detection of neutralizing antibodies produced in response to PEDV infection. The FFN was evaluated using serum samples or rennet treated milk and colostrum samples. Heat-inactivated samples were diluted in a 2-fold dilution series starting at 1:10 in MEM plus 1.5 µg/ml TPCK-treated trypsin in 96-well plates. An equal amount of cell culture adapted PEDV stock at a concentration of 100 foci forming units/100µl was added to each well and plates incubated for 1 hour at 37 ºC. The virus/sample mixture was then added to washed confluent monolayers of Vero-76 cells and incubated for 2 hours at 37 ºC. Plates were washed again with MEM/TPCK-trypsin medium and incubated 20-24 hours to allow for replication of non-neutralized virus. Plates were then fixed with 80% acetone and stained with FITC conjugated mAb SD6-29 to allow visualization of infected cells. Endpoint neutralization titers were determined as the highest serum, milk or colostrum dilution resulting in a 90% or greater reduction in fluorescent foci relative to controls.

The development and validation of the iELISA, bELISA and FMIA testing platforms made use of recombinant expressed full length PEDV-NP or truncated spike proteins. The NP open reading frame (ORF) and selected regions of the spike protein of PEDV were amplified, cloned and expressed in *E. coli*. Expressed proteins were purified three times using nickel-NTA affinity column chromatography and refolded back to their native conformational state.

The ELISAs were performed by coating alternate wells of Immulon 1B, 96-well, microtiter plates with 250 ng/well of selected purified, expressed PEDV antigens. The optimal dilution of the recombinant protein and secondary detection antibody was determined by a checkerboard titration that gave the highest signal to noise

ratio. The raw data was normalized and transformed into Excel spreadsheets. For iELISA formats, sample to Positive (S/P) ratios were calculated using the following formula: S/P = optical density (OD) of sample - OD of buffer/OD of positive control - OD of buffer. For monoclonal antibody-based bELISA formats, a similar formula was used for calculation of percent inhibitions (PI).

For FMIA testing, a two-step carbodiimide coupling procedure was used to couple selected protein antigens to Luminex™ microspheres. Test serum, or rennet treated milk or colostrum samples were incubated with antigen coated microspheres, washed and analyzed through a dual-laser Bio-Rad Bio-Plex 200 instrument as previously described (Lawson et al. 2010; Langenhorst et al. 2012). The median fluorescent intensity (MFI) for 100 microspheres corresponding to each individual bead analyte was recorded for each well. All reported MFI measurements were normalized via $F - F_0$, where F_0 was the background signal determined from the fluorescence measurement of a test sample in uncoated beads and F was the MFI for a serological test sample using antigen-coated beads.

To validate ELISA and FMIA tests, four serological reference serum sets were constructed as standards termed high, medium, low and negative to serve as internal quality control standards and to mathematically normalize individual samples for objective comparisons between testing platforms. To accurately assess the diagnostic sensitivity and specificity of the assays, samples of known serostatus for PEDV were used. This included sera from multiple animal populations including experimentally infected animals and serum samples submitted to the South Dakota ADRDL. PEDV negative sample sets included samples from selected high biosecurity herds with no history of PEDV and archived serum samples collected prior to the emergence of PEDV in the U.S., including samples testing positive for the related swine coronaviruses TGEV and PRCV. Known positive samples were collected from pigs that were naturally infected at least 3 weeks prior to collection and were previously positive by PCR. The negative-testing sample population (uninfected animals) consisted of maximally 980 PEDV negative serum samples, while the positive-testing (infected) population was composed of 516 serum samples. Receiver operating characteristic (ROC) analysis was calculated for each assay to assess diagnostic performance, which included determination of sensitivity, specificity and threshold cutoff using MedCalc version 11.1.1.0 (MedCalc software, Mariakerke, Belgium).

The repeatability of each assay was assessed by running the same internal quality control serum standards in multiple replicates within the same run or between runs. For the iELISA and the bELISA, the intra-assay repeatability was calculated for 48 replicates on 3 separate plates, then repeated over a 3-day period for inter-assay repeatability assessment. The values for each assay were expressed as a mean, standard deviation and percent coefficient of variation (CV%) for repeated measure.

To measure statistical testing agreement, multiple comparison, inter-rater agreement (kappa measure of association) was calculated among all four tests (bELISA, iELISA, FMIA and IFA) using IBM, SPSS version 20 software (SPSS Inc., Chicago, IL). The sample cohort used was a well-characterized set of serum samples collected from “positive testing” experimentally infected pigs over time courtesy of Dr. Richard Hesse (n=158) and from archived experimental control uninfected PEDV “negative testing” animals. The interpretation of kappa can be rated as follows: Kappa less than 0.0, “poor” agreement; between 0.0 and 0.20, “slight” agreement; between 0.21 and 0.40, “fair” agreement; between 0.41 and 0.60, “moderate” agreement; between 0.61 and 0.80, “substantial” agreement; and between 0.81 and 1.0, “almost perfect” agreement [28,29].

Results:

Objective 1. The FFN assay was initially evaluated using serum samples from experimentally inoculated piglets. Additional evaluation was conducted using 250 serum samples from known PEDV naïve herds and 250 samples from herds with documented PEDV exposure, collected at least 3 weeks after initial PCR diagnosis and whole herd feedback. Experimentally inoculated piglets demonstrated detectable seroconversion by 14 DPI (Figure 1). Essentially all samples from PEDV naïve animals had serum FFN endpoint titers of <1:20 while most samples from the PEDV positive set had endpoint titers ranging from 1:40 to 1:1280 (Figures 2 and 3). Further evaluation of the FFN included serum, milk and colostrum samples from 27 sows from a herd that had experienced an acute PEDV outbreak 6 to 7 weeks prior to farrowing. All animals were exposed to live virus twice within the first week of the outbreak, followed by one dose of Harrisvaccines Porcine Epidemic Diarrhea Vaccine, RNA (Harrisvaccines, Inc., Ames, IA) at 1 week pre-farrow. Serum and colostrum samples were tested at the time of farrowing, followed by serum and milk samples at 1 week and 2 weeks later. As shown in Figure 4, mean colostrum titers were approximately 4-fold higher than serum titers at the time of farrowing. At later time-points, serum and milk titers were similar in magnitude, although substantial animal to animal variation was apparent.

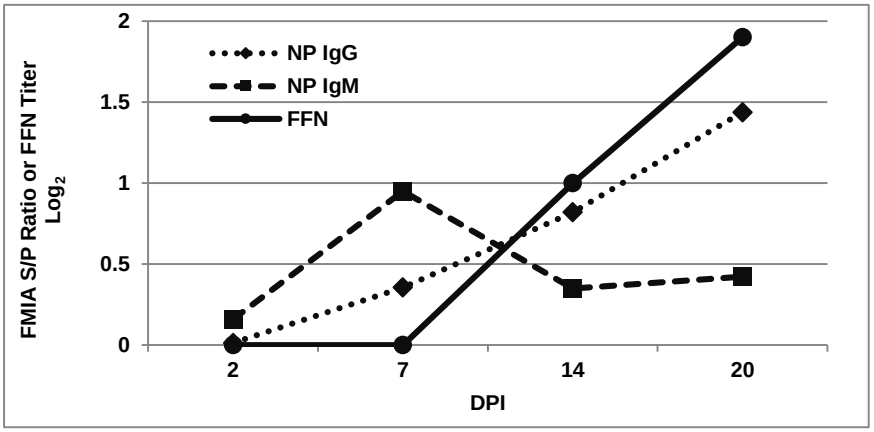

Figure 1. FFN antibody and FMIA isotype time course evaluation. Using serum collected over time from experimentally infected pigs, the FMIA demonstrates the kinetic nucleoprotein-directed, isotype-specific response of IgG and IgM in serum. In addition, the data show appearance of neutralizing antibodies by 14 DPI.

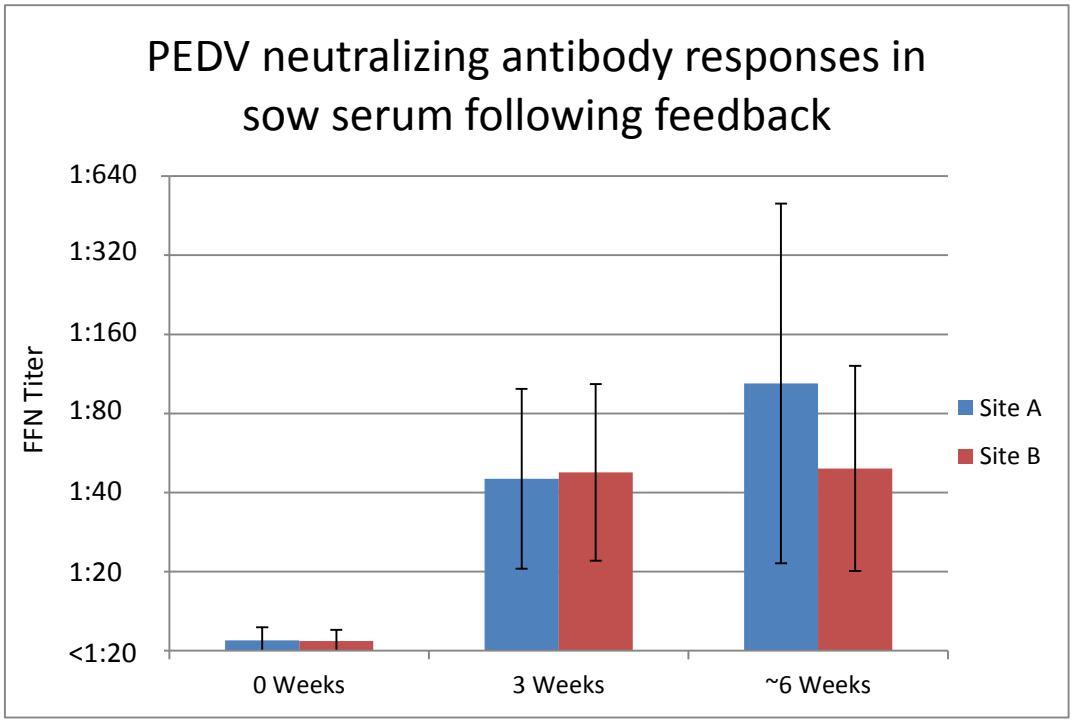

Figure 2. Neutralizing antibody responses in sow serum samples as determined by the FFN assay at the time of feedback, then 3 and 6 weeks later. Samples associated with NPB grant 13-263, Travis Clement, et al.

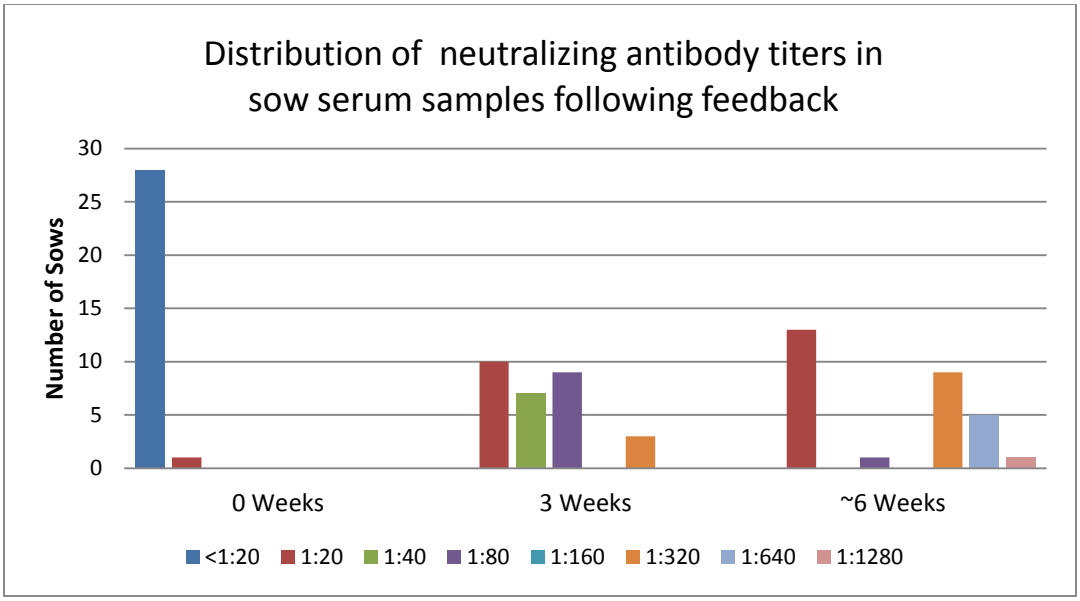

Figure 3. Distribution of neutralizing antibody titers within a single site as determined by the PEDV FFN assay. At the time of initial feedback essentially all animals showed no detectable neutralizing antibody in serum. All animals seroconverted by 3 weeks post-feedback and remained seropositive through 6 weeks. Substantial animal to animal variation in detectable neutralizing antibody levels was apparent in this case.

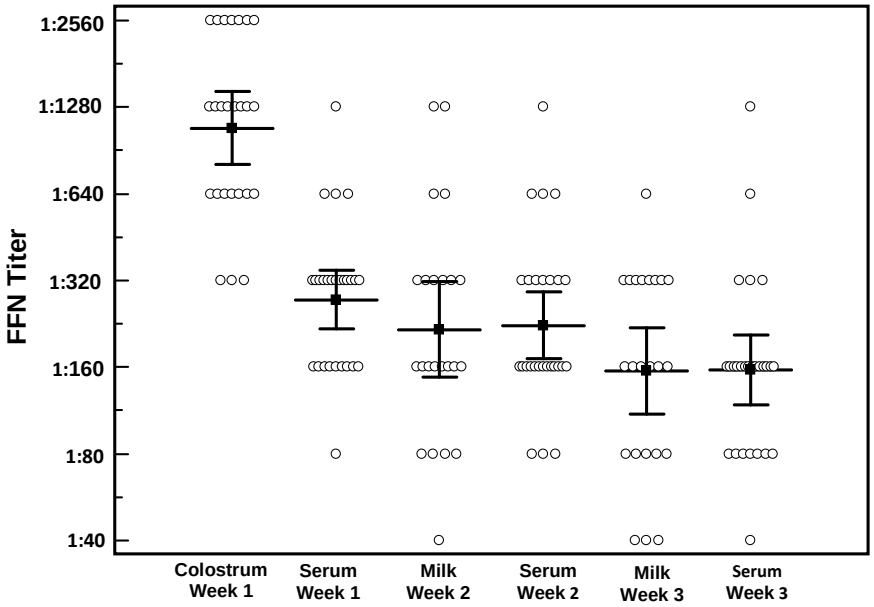

Figure 4: Assessment of neutralizing antibody titers in different sample matrices following PEDV exposure. FFN titers were detected in various sample matrices including colostrum (n=25), milk (n=23) and serum (n=27) collected at the time of farrowing and weekly for two weeks post-farrowing. Error bars indicate a 95% confident interval for mean titers indicated by horizontal lines.

To date, over 10,000 samples have been tested by the PEDV FFN assay. This assay has been fully optimized and is now offered to the swine industry through the South Dakota Animal Disease Research & Diagnostic

Laboratory and is being used in numerous vaccine and research trials. The test has been evaluated with serum, milk and colostrum samples and shows a high level of consistency. Traditional CPE-based virus neutralization assays were also compared to the FFN but they did not demonstrate the dynamic range or consistency of the FFN (Figure 5).

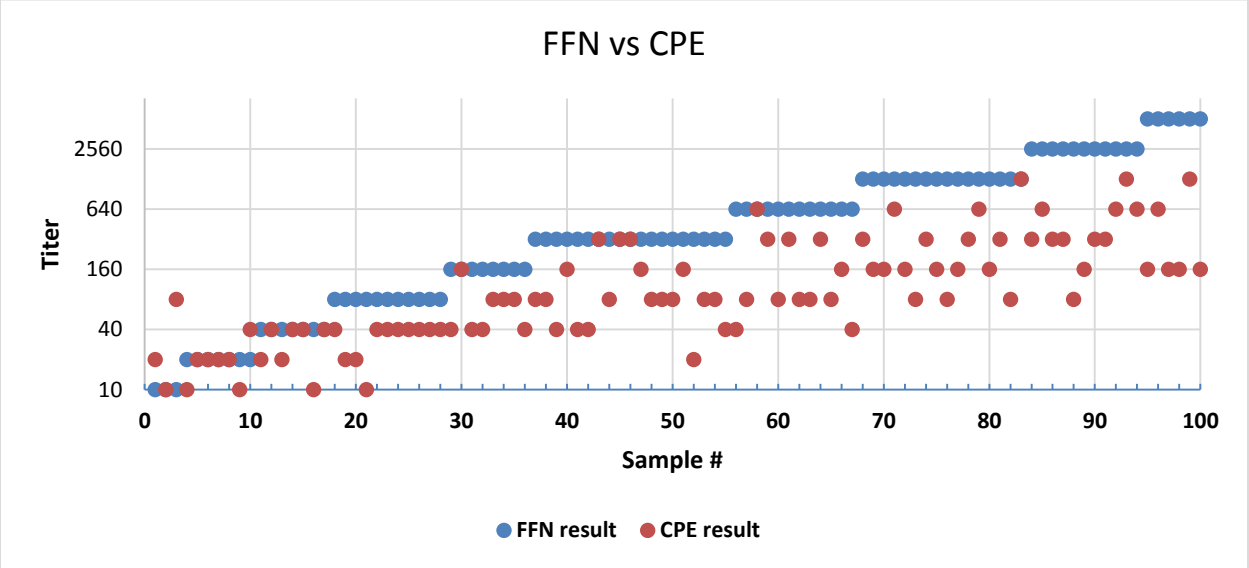

Figure 5. Comparison of FFN and traditional CPE-based virus neutralization results for a subset of 100 serum samples.

Objective 2. To optimize the ELISA and FMIA assays, various antigen and serum dilutions were used to determine optimum concentrations. All tests were optimized in a checkerboard fashion to maximize signal-to-noise ratios. It was determined by antigen titration that the optimal coating of Luminex™/FMIA microspheres was achieved at a concentration of 12.5 µg protein per 3.125 x 10^6 microspheres. Similarly, the optimum coating of both the iELISA and bELISA plates was achieved at a concentration of 250 ng/well. In addition, to determine the optimum serum dilution for each of the testing platforms, a well-characterized PEDV "high" positive serum standard was serially diluted in a $\log_2$ titration against antigen coated microspheres (FMIA) or antigen coated ELISA wells at a fixed concentration. Figure 6 shows concentration-dependent OD or MFI signals of various serum standards. Overall, sample absorbance increased inversely proportional to the serum dilution. However, based upon the highest signal-to-noise ratio, it was determined that the optimal serum dilution for the bELISA was 1/3, while the iELISA and FMIA each demonstrated an optimum dilution of 1/50 as indicated by arrows (Figure 6).

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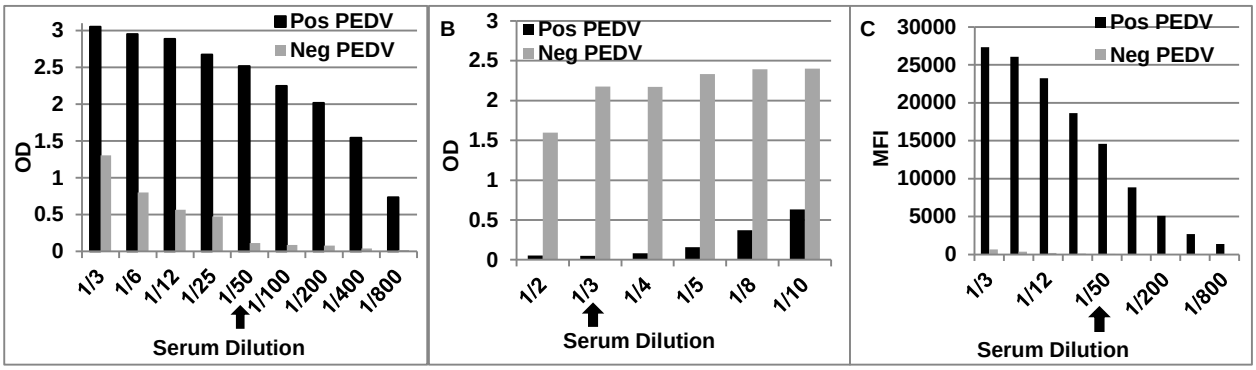

Figure 6. Serum dilution optimization for both ELISA assays and FMIA. Reference serum standard was titrated 2-fold in antigen coated wells at a fixed concentration in order to gauge a maximum signal-to-noise ratio for each assay (A) iELISA, (B) bELISA, (C) FMIA. Arrows show the optimum dilution of swine serum from which the highest signal to noise ratio was achieved.

ROC analysis to determine sensitivity, specificity and threshold cut-off levels was performed using large numbers of swine serum samples and demonstrated excellent agreement (>0.91 kappa scores) between assays with good intra and inter assay repeatability (Table 1). None of the known positive TGEV or PRCV samples tested was shown to cross-react.

Table 1. Evaluation of statistical agreement among serological testing platforms. Multiple comparison, inter-rater agreement (kappa association) was calculated among all four tests. Kappa values shown represent a statistical measure of test agreement and were calculated using MedCalc version 11.1.1.0.

	FMIA	bELISA	Indirect ELISA	IFA
IFA	0.932	0.945	0.941	1
iELISA	0.919	0.923	1	0.941
bELISA	0.941	1	0.923	0.945
FMIA	1	0.941	0.919	0.932
Number Positive Serum Samples	158	158	158	158
Number Negative Serum Samples	361	361	361	361
Total Serum Samples Tested	519	519	519	519

The optimal cutoff values and corresponding sensitivity and specificity of each individual test are presented in Figure 7. Specifically, ROC analysis for the iELISA and bELISA showed similar sensitivity and specificity of 97.9% and 97.6%; and 98.2% and 98.9%, respectively. The ROC analysis for the FMIA showed estimated sensitivity and specificity of 98.2% and 99.2%, respectively. Although the FMIA showed an identical sensitivity as the bELISA, it demonstrated the highest degree of specificity of all three assays at 99.2%. This observation was not surprising given that FMIA technology inherently imparts greater sensitivity and a larger dynamic range than the ELISA platform (Christopher-Hennings, et al. 2013).

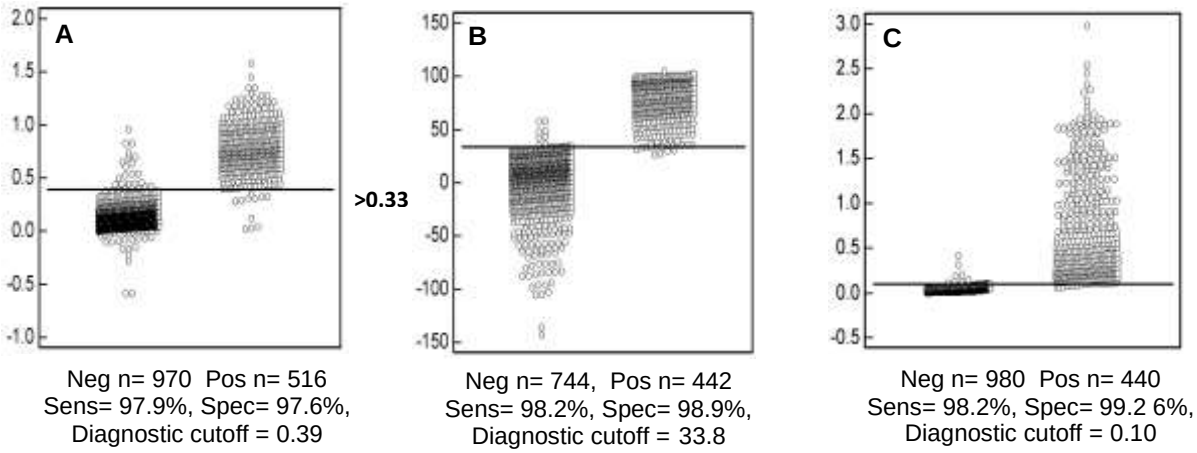

Figure 7. Receiver operator characteristic (ROC) validation and determination of diagnostic sensitivity and specificity of the PEDV-NP iELISA, bELISA and FMIA assays. Diagnostic sensitivity and specificity were calculated using serum samples from a known PED-uninfected and PED-infected population. ROC analysis was performed using MedCalc version 11.1.1.0 (MedCalc software, Mariakerke, Belgium). In each panel, the dot plot on the left represents the negative testing population, and the dot plot on the right represents the positive population. The horizontal line bisecting the dot plots represents the cutoff value that gives the optimal diagnostic sensitivity and specificity. (A) Serum iELISA, (B) Serum bELISA, (C) Serum FMIA.

In addition to determining cutoff values, sensitivities and specificities, multiple comparison tests were performed to calculate the degree of agreement among the ELISA, FMIA and IFA tests. Specifically, the Kappa test demonstrated all diagnostic platforms had kappa values greater than 0.91, which demonstrates that all 4 tests are in strong agreement with each other.

The iELISA and bELISA demonstrated slightly lower %CVs than the FMIA with 3.7%, 6.8%, 10.7% intra-assay variability for bELISA, iELISA and FMIA respectively. Inter-assay %CVs were 5.0%, 5.6% and 7.7% for the bELISA, iELISA and FMIA respectively. Nonetheless, all the CVs were 10.7% or less, which demonstrated that the tests were highly repeatable in a diagnostic application.

As shown in Figure 8, a mean antibody response to PEDV-NP could be detected as early as 9 DPI for both the iELISA and bELISA. The FMIA detected PEDV-NP antibodies slightly earlier at 6 DPI. All 3 tests detected the duration of antibody out to the 43 DPI time-point in this study but demonstrated a decline in detectable antibody after 21 DPI.

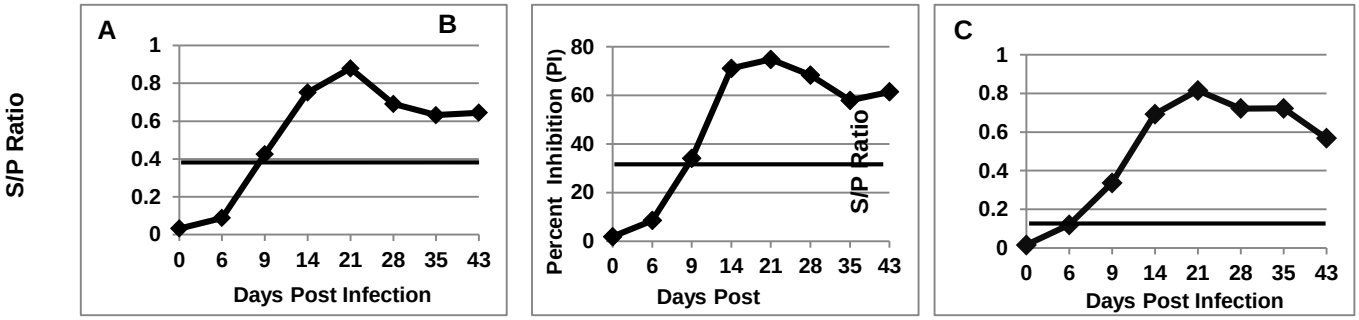

Figure 8. Kinetic time course antibody evaluation. Antibody time course kinetics were calculated for each of the ELISAs and FMIA using serum samples from experimentally infected pigs collected at weekly intervals. The horizontal line indicates the diagnostic cutoff for each test. All three tests demonstrate similar kinetic curve responses via their calculated S/P values. **(A)** Antibody kinetic time course via iELISA, **(B)** Antibody kinetic time course via bELISA, **(C)** Antibody kinetic time course via FMIA.

Objective 3. The final objective involved the further assessment of the various assays developed here with different sample types including serum, colostrum, milk and oral fluids. Oral fluid samples presented substantial challenges as a sample type for consistent antibody detection using any of our current prototype assays. They were prone to demonstrating high background levels while having lower levels of detectable specific antibodies.

The detection and quantitation of neutralizing antibodies in serum, milk and colostrum using the PEDV FFN test proved to be a very robust method. Therefore, we placed more emphasis on application of this assay and trying to identify correlations with higher throughput assays such as the FMIA using spike protein-based antigens.

During evaluation of sequential serum samples, high levels of PEDV-NP specific IgM antibodies were observed at 7 DPI compared to IgG (Figure 1). However, the IgM antibodies decreased to barely detectable levels by 20 DPI. IgG continued to increase linearly to 20 DPI. There is a concomitant appearance of neutralizing antibodies by 14 DPI. Further evaluation of milk samples demonstrated moderate to high levels of correlation between FFN results and FMIA results using selected PEDV antigens to target IgA and IgG responses as shown in Table 2 and Figures 9 through 11. In milk samples, the PEDV FMIA targeting spike (S1) IgA demonstrated the highest level of correlation with virus neutralizations as determined by the FFN.

Table 2. Pearson correlations between the PEDV FFN and FMIA in milk samples when targeting IgA or IgG antibodies directed agains NP or spike (S1) antigens.

Milk		FMIA NP IgA	FMIA NP IgG	FMIA Spike IgA	FMIA Spike IgG
FFN	Correlation Coefficient	0.774	0.609	0.815	0.498
	Significance Level P	<0.001	<0.001	<0.001	<0.001
	n	190	191	191	191

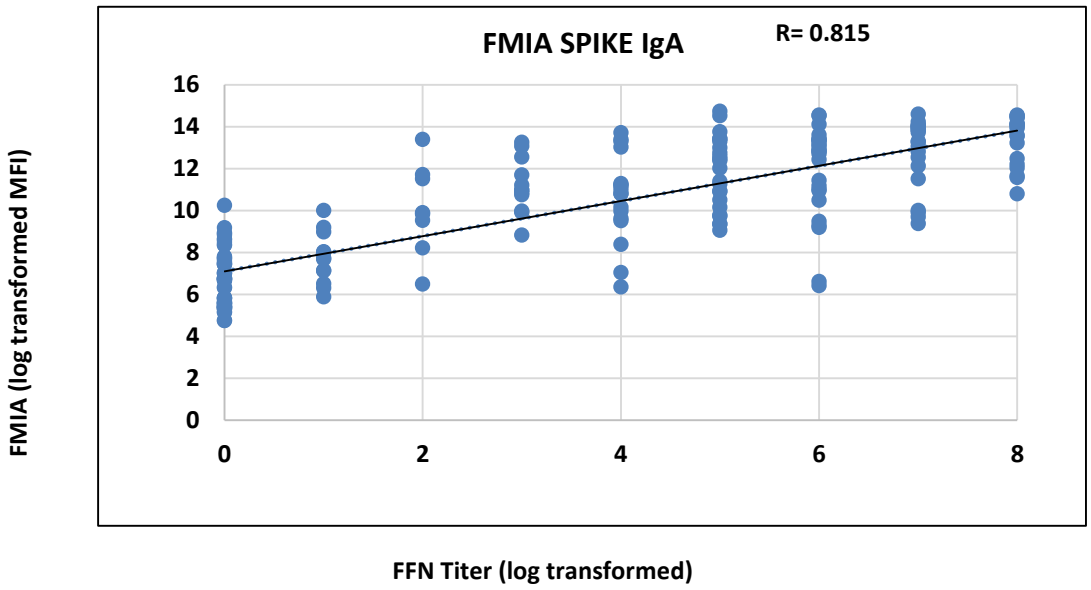

Figure 9. Corelation between PEDV anti-S1 IgA in milk measured by FMIA and neutralizing antibodies in milk measured by FFN.

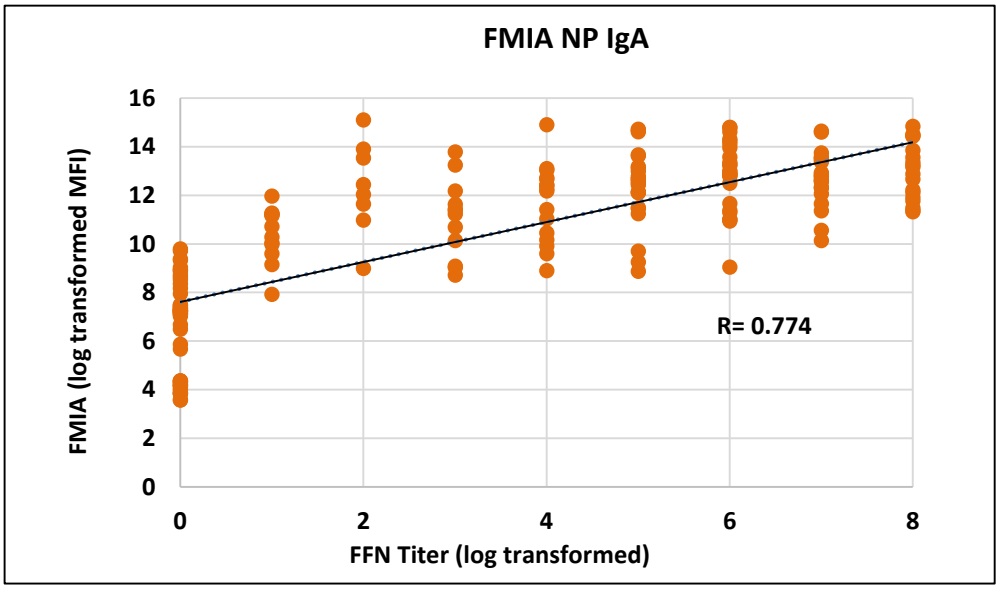

Figure 10. Corelation between PEDV NP IgA in milk measured by FMIA and neutralizing antibodies in milk measured by FFN.

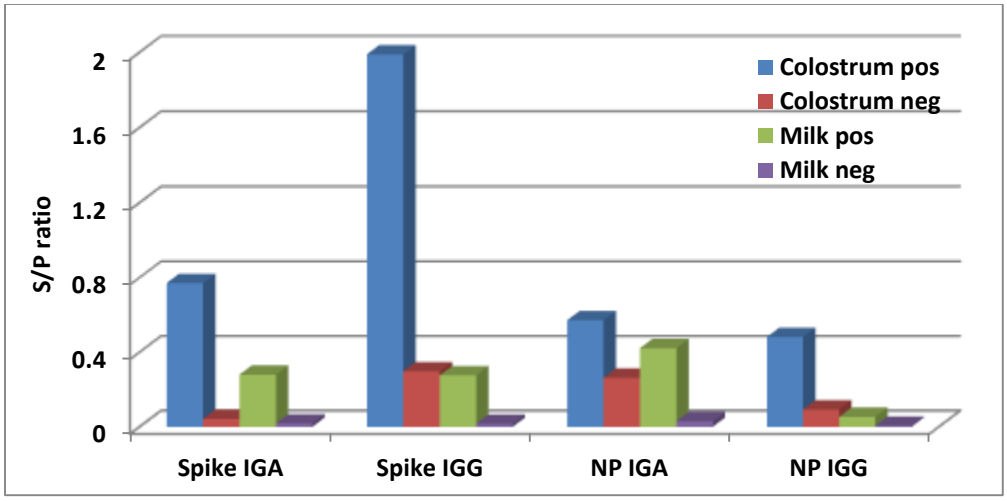

Figure 11. Relative levels of antigen specific antibodies in milk and colostrum measured via FMIA. Comparison of PEDV spike (S1) and NP IgA and IgG levels in known seropositive and sero-negative milk and colostrum samples.

Discussion:

The overall goal of this project was to develop and validate a variety of serological assays for the detection of PEDV antibodies. Each assay format has advantages that dictate how they will be used in the field. Measurement of neutralizing antibody responses using the FFN assay should provide a valuable tool for assessment of vaccine candidates or protective immunity. The FFN assay allows relative quantitation of functional neutralizing antibodies in serum, milk or colostrum samples. Serum antibody levels detected by the FFN show good correlation with neutralizing antibody levels present in milk and colostrum. Measurement of neutralizing antibody responses using the FFN assay should provide a valuable tool for assessment of vaccine candidates and assessment of herd immunity. Well-validated, high throughput indirect ELISA, blocking ELISA and FMIA assays for the detection of PEDV antibodies were also developed and validated, showing good correlation with IFA and each other.

Serological testing with IFA, iELISA, bELISA or FMIA is useful in determining whether pigs were previously infected with PEDV, or if piglets have acquired antibodies through colostrum (eg. passive antibody transfer). However, tests that evaluate the functionality of the antibodies such as the FFN are needed to determine if the detected immune response could be helpful in providing protection to nursing piglets. Neutralizing antibodies may be protective through actions including blocking uptake of the virus into cells, preventing virus binding to receptors on cells, preventing uncoating of the virus genomes in endosomes and/or causing aggregation of virus particles. For enveloped viruses, such as PEDV, lysis of the virus may also occur when antiviral antibodies and serum complement disrupt the viral membrane. For these reasons, an FFN-based virus neutralization assay was developed to assess levels of PEDV neutralizing antibodies in serum, milk or colostrum samples. The FFN provides a more rapid determination of neutralizing antibody levels than is possible with traditional virus neutralization assays that rely on visualization of virus-induced CPE after three or more days incubation to allow for full development of PEDV CPE. The direct observation of fluorescent stained infected cells, or lack of

stained infected cells in the case of virus neutralization, allows for simple endpoint determination. This feature is particularly valuable when dealing with a fastidious, trypsin-dependent virus such as PEDV where CPE-based endpoints may not be obvious or may be confused with trypsin-induced CPE in the cell monolayer. Although neutralizing antibodies present in the serum would not be expected to provide direct protection from a strictly enteric infection such as PEDV, our data suggest a correlation between detectable neutralizing antibody levels in the serum and those present in milk and colostrum of previously exposed or vaccinated sows. Specifically, FMIA-speciated IgA and IgG antibodies against recombinant PEDV-spike (S1) were detected at similar concentrations in milk. PEDV-spike (S1)-specific IgA antibodies demonstrated a higher correlation with FFN neutralizing titers than nucleocapsid-specific IgA antibodies in milk. Also, although high levels of anti-PEDV IgG antibodies were detected in milk via FMIA, a corelation could not be established with significant neutralizing antibody titers in milk measured by FFN. Additional evaluation of the FFN and other assays in the field, especially in cases of known PEDV challenge, will provide further insight into the assessment of protective immunity levels.

The ROC analysis for the NP- based IgG FMIA showed estimated sensitivity and specificity of 98.2% and 99.2%, respectively. The iELISA and bELISA showed a sensitivity and specificity of 97.9% and 97.6%; and 98.2% and 98.9%, respectively. Inter-rater (kappa) agreement was calculated to be 0.941 between iELISA and IFA, 0.945 between bELISA and IFA and 0.932 between FMIA and IFA. Similar comparative kappa values were observed between the iELISA, bELISA and FMIA, demonstrating significant agreement among assays. No cross-reactivity with the related coronaviruses, transmissible gastroenteritis virus (TGEV) or porcine respiratory coronavirus (PRCV) was noted. These three assays detected seroconversion of naïve animals within 6-10 days post exposure. FMIA tests using PEDV spike (S1) antigen preparations were also developed to compare IgA and IgG responses in serum, milk and colostrum with neutralizing antibody levels detected by the FFN. While moderate to strong correlations were noted among assays, the FFN appeared to provide the most consistent results in a cost-effective test format.

In summary, these well-validated PEDV iELISA, bELISA, FMIA and FFN assays are useful for a range of serological investigations. They can serve as a complement to nucleic acid detection and determine the PEDV status of asymptomatic individuals for cost-effective tools in management strategies and monitoring virus exposure within the herd. The FMIA will be useful for isotyping the antibody responses and in multiplexing for determining exposure to multiple pathogens simultaneously. In addition, the FFN is useful for determining whether the antibodies measured are providing a biological function of blocking virus infectivity.

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