RESEARCH REPORT
SWINE HEALTH

Title: PEDV antibody-based diagnostic test improvement for evaluation of immunity in milk, feces, and serum **- NPB 14-175** revised

Investigator: Dr. Michael Murtaugh

Institution: University of Minnesota

Date Received: February 2, 2015

Industry Summary: Porcine epidemic diarrhea virus (PEDV), an enteric coronavirus related to transmissible gastroenteritis virus (TGEV), appeared suddenly in the United States in April 2013. Epidemic sow herd outbreaks, characterized by severe diarrhea, vomiting, and high mortality in nursing pigs for several weeks, continue to spread the disease. Virus can be detected in feces using a real-time PCR assay. Antibodies to the virus can be detected using an ELISA assay based on the nucleocapsid (N) protein. In house PEDV ELISAs are available through diagnostic laboratories to detect anti-PEDV antibodies and, recently, a commercial ELISA kit has become available (Biovet). In house ELISAs are able to detect antibodies to PEDV, but the sensitivity and specificity need to be improved. Our objective with this proposal was to create an improved ELISA with better sensitivity and specificity against PEDV. We also optimized the ELISA for use with milk, colostrum, and fecal samples. Previously, we were able to express and purify 4 PDCoV antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2) and ELISA assays were developed for each antigen. Colostrum, milk, and fecal samples were evaluated on all 4 ELISAs to determine antibody reactivity. To improve the ELISA, we expressed large amounts of protein, refolded the protein, and coupled the protein to an HRP substrate. We then examined the refolded protein for increased reactivity in the original ELISA format and we evaluated an alternative sandwich ELISA format. ELISA analysis of colostrum and milk samples using all 4 antigens and both IgG and IgA revealed that for IgA, antibodies to the spike antigens (S1, S2) were much more prevalent than anti-N antibodies. For IgG, mainly anti-N and S2 antibodies were present. Few fecal samples were antibody positive and when looking over a time course the data was un-interpretable. However, the key antibodies to look for in feces seem to be anti-N and anti-S2 of the IgA isotype. Further examination into sampling of feces at different times after infection needs to be performed for further optimization of this ELISA. Examination of antibody reactivity to refolded proteins compared to non-refolded protein preparations showed slight increases in ELISA OD values, but no statistical improvement in the ELISA sensitivity or specificity. HRP labeling was only achieved with N protein, but it did not specifically detect antibodies for unknown reasons. Other proteins could not be labeled since they were not soluble in the labeling reaction. However, a new antigen, which contains the putative antigenic regions of the spike protein is being expressed and purified for use in the ELISA and the alternative sandwich ELISA. Overall, an optimized ELISA was produced for use with milk and colostrum samples. A fecal ELISA was produced and with a time course of fecal sampling, this ELISA can be further evaluated for analysis of antibodies in fecal samples. Lastly, the alternative sandwich ELISA was not successful in improving sensitivity and specificity of the PEDV ELISA, but further examination into this problem is underway.

Dr. Cheryl Dvorak and Dr. Michael Murtaugh
dvora013@umn.edu, murta001@umn.edu
612-625-6735

Keywords: porcine epidemic diarrhea virus, PEDV, ELISA, nucleocapsid, spike, matrix, antibodies, protein purification, milk, colostrum, feces

Scientific Abstract: Porcine epidemic diarrhea virus (PEDV), an enteric coronavirus related to transmissible gastroenteritis virus (TGEV), appeared suddenly in the United States in April 2013. Epidemic sow herd outbreaks, characterized by severe diarrhea, vomiting, and high mortality in nursing pigs for several weeks, continue to spread the disease. Virus can be detected in feces using a real-time PCR assay. Antibodies to the virus can be detected in serum samples using an ELISA assay based on the nucleocapsid (N) protein, but the sensitivity and specificity need to be improved. Our objective for this proposal was to create an improved ELISA for serum, optimize an ELISA for use with milk, colostrum, and feces, and examine these assays to determine predictors of protection for piglets and protection against re-exposure for sows. We were able to detect anti-PEDV antibodies in milk and colostrum for IgG and IgA isotypes. In milk, PEDV-specific IgA antibodies to the spike proteins are the most prevalent. In colostrum, both IgG and IgA antibodies are prevalent, again IgA antibodies are most reactive to the spike proteins and IgG antibodies are mainly to N and S2. Fecal IgA antibodies were observed in some samples, but were not consistently detected. Refolding of the N protein was able to slightly increase the antibody reactivity in serum, colostrum, and milk samples. Refolding of the other protein antigens was unsuccessful, but a spike protein fragment containing just the predicted antigenic region is being produced for further testing. An alternative sandwich ELISA coupling N antigen to HRP was produced but lacked specific reactivity. Other proteins may be promising if they can be produced in a soluble form under physiological conditions. Lactogenic immunity was examined as a predictor of piglet protection and consistently high levels of IgG antibodies were observed in colostrum to N and S2, while IgA antibodies were mainly observed in colostrum to S1 and S2. Milk contained IgG antibodies, but the levels decreased quickly suggesting that colostral spike antigens might be the best indicators of lactogenic immunity. Analysis of predictors of protection after re-exposure suggested that IFA is a better predictor of protection than ELISA, since ELISA values did not suggest the animals would be protected, when in fact, they were protected against disease. Overall, the PEDV N and S2 ELISAs seem to be the most useful predictors of protection in both IgG and IgA isotypes, refolding of the N protein is able to increase sensitivity of the assay, and serum and colostrum reactivity are mainly IgG antibodies against the N and S2 proteins, while milk reactivity is IgA antibodies against the spike proteins. This study has increased our knowledge of the antibody presence following infection or re-infection as well as the best ways to detect predictors of protection.

Introduction:

Porcine epidemic diarrhea virus (PEDV), an enteric coronavirus related to transmissible gastroenteritis virus (TGEV), appeared suddenly in the Unites States in April 2013. Epidemic sow herd outbreaks, characterized by severe diarrhea, vomiting, and high mortality in nursing pigs for several weeks, continue to spread the disease. Virus can be detected in feces using a real-time PCR assay. Antibodies to the virus can be detected using an ELISA assay based on the nucleocapsid (N) protein. In house PEDV ELISAs are available through diagnostic laboratories to detect anti-PEDV antibodies and recently a commercial ELISA kit has become available (Biovet). In house ELISAs are able to detect antibodies to PEDV in serum, but the sensitivity and specificity need to be improved. Our objective for this proposal was to create an improved ELISA for serum and optimize an ELISA for use with milk, colostrum, and feces.

An improvement in the sensitivity and specificity of the antibodies to PEDV using ELISA is necessary for the prediction of protection of piglets against PEDV in the farrowing room and protection of previously exposed sows to re-challenge. Prediction of protection will allow producers to determine if their herd needs go undergo feedback before farrowing in order to

protect newborn piglets in case of PEDV infection. Because feedback causes illness, it is not desirable to use unless it is necessary for protection of newborn piglets. We have created and optimized PEDV ELISAs for serum, colostrum, and milk and have created a fecal ELISA that can be optimized after further examination. These ELISAs can be used to detect antibodies to PEDV for the determination of protection against infection and the ability to pass the protective antibodies on to piglets.

Objectives:

Our objectives for this proposal were to:

1. Enhance PEDV ELISA for detection of milk and fecal antibodies.
2. Evaluate an alternative sandwich ELISA format for better anti-PEDV antibody detection.
3. Optimize the relative sensitivity and specificity of PEDV immunoassays in various substrates (serum, milk, colostrum, feces, oral fluids).
4. Assess immunoassay methods for ability to predict protection of piglets in the farrowing room and protection of previously exposed sows to re-challenge.

The goal here was to increase the value of immunodiagnostics for PEDV, especially to use antibody status as a predictor of piglet protection due to lactogenic immunity.

Materials & Methods:

Antigen production: The PEDV genomic sequence (Genbank ID KF272920) was used to clone the 4 PEDV candidate antigens; nucleocapsid (N), matrix (M), the spike protein subunits (S1 and S2) and the spike antigenic region (SA). The gene sequences for each antigen were xamined for signal peptides, transmembrane domains, hydrophobic regions and other factors, which were then removed due to difficulty in expression of proteins containing these motifs. The gene was then optimized for expression in E. coli and synthesized and cloned into a protein expression vector (Life Technologies). These constructs were then transformed into E. coli Rosetta2 (Millipore) and T7 express cells (NEB) for growth and expression. The proteins were purified over a cobalt affinity column (ThermoScientific) and purified proteins were visualized using SDS-PAGE (Bio-Rad) and quantified using both a Bradford assay (Bio-Rad) and spectrophotometry (Bio-Tek Epoch)(Figure 1).

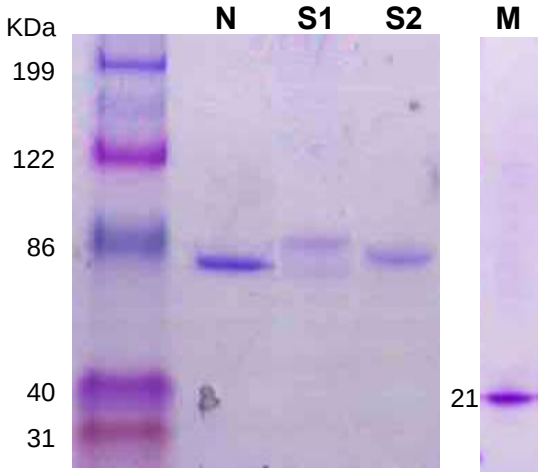

Figure 1. PEDV protein antigens. His-tagged PEDV N, S1, S2, and M proteins were expressed in bacteria and purified by cobalt-IMAC. Purity was assessed by SDS-polyacrylamide gel electrophoresis as shown.

Protein Refolding: Test refolding reactions were performed using the Pierce protein refolding kit (Pierce). Refolded protein from these small scale tests were then used for the refolded protein ELISA assays. Large scale refolding of the protein was performed based on the small scale test conditions. Proteins were then dialyzed into a sodium phosphate buffer to remove guanidine salts and were concentrated using Aquacide II (Millipore). Concentrated protein was then dialyzed into a sodium phosphate buffer and the final concentration was determined by Bradford assay (Bio-Rad).

ELISA: Four separate ELISA assays, using each of the purified antigens, were run following our PEDV ELISA protocol. ELISA plates were coated with 200 ng of antigen overnight at 4°C in carbonate buffer. Plates were then washed with PBST and blocked for 2h with PBST+5% NFDM, pH 9.6. Plates were washed and serum, diluted 1:50 in PBST+5% NFDM, pH 7.4, was incubated for 1h at room temperature. Plates were washed with PBST and secondary antibody (HRP-

conjugated goat anti-pig IgG, Bethyl), diluted 1:100,000 in PBST+5% NFDM, pH 7.4, was added and incubated for 1 h. Plates were washed with PBST and detected using TMB peroxidase substrate (KPL) for 15 minutes following the manufacturer's instructions. Plates were read at 450nm on an Epoch Microplate Reader (BioTek Instrument Inc. Winooski, VT) using Gen5 software. For the milk and colostrum ELISA, samples were diluted 1:50 and secondary antibody was either HRP-conjugated goat anti-pig IgG or IgA (Bethyl).

Pre-processing of fecal samples was performed by adding 200 mg feces to 800 ul of fecal antibody extraction buffer (PBS, pH 7.4, 1mM PMSF, 10mM EDTA, 1:1000 dilution of protease inhibitor cocktail (Sigma)), vortexing well, and spinning down the insoluble fraction. The soluble fraction was then used without dilution for the ELISA.

For the alternative sandwich ELISA, refolded N protein was coupled to HRP using the EZ-link Plus activated peroxidase kit (Thermo Scientific). The ELISA was performed similarly to the original PEDV ELISA above. Basically, 200 ng of N protein was coated on an ELISA plate, serum samples were diluted 1:50, HRP-N protein was added, and TMB peroxidase substrate was used for detection.

Anti-PEDV antibodies from serum were also examined by IFA at the University of Minnesota diagnostic laboratory.

Results:

ELISA detection in colostrum, milk, and feces

We obtained milk samples from PEDV positive and negative commercial farms to test the reactivity of the ELISA against N, S1 and S2 proteins for both the IgG and IgA isotypes. To determine positive and negative cut-off values for the milk ELISA, the range of OD values was examined (Figure 2). PEDV-specific IgG antibodies were basically not detectable in milk samples (Figure 2A). PEDV-specific IgA antibodies were observed in milk with S1 and S2 antibodies observed at higher levels than N (Figure 2B). The conservative cut-off for IgA N antibody positive samples in milk was determined to be an OD of 0.4, for S1 the cut-off was 0.2, and for S2 it was 0.3. Examination of a larger set of known negative and positive milk samples will confirm and optimize these cut-off values in order to validate the ELISA following quality control/quality assurance procedures.

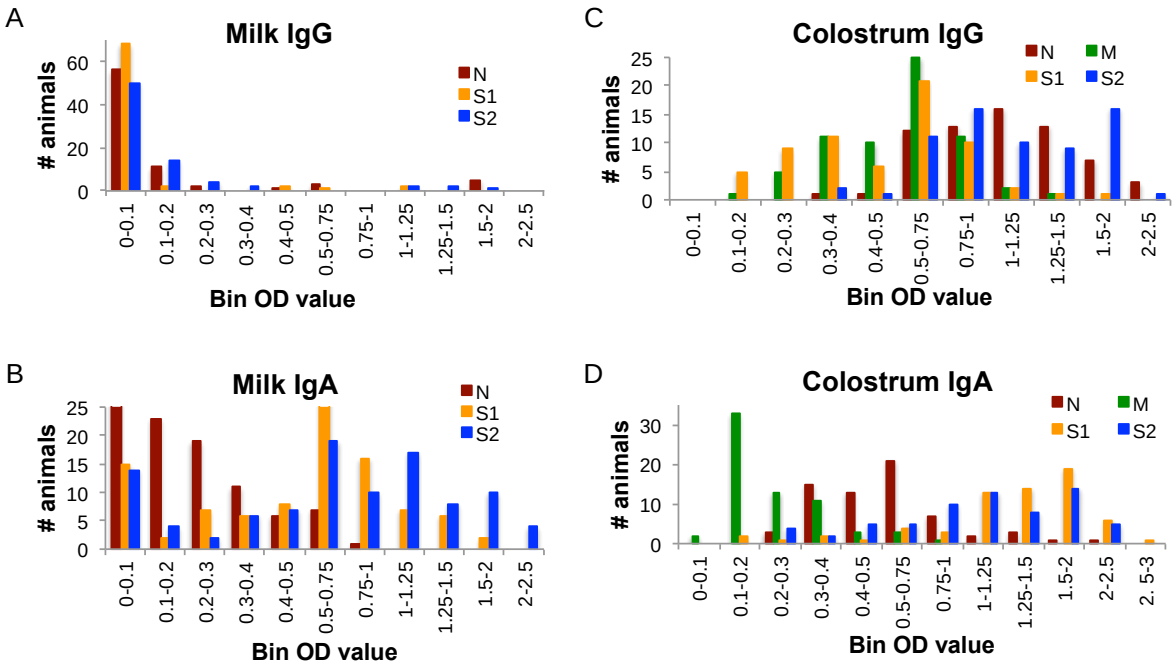

Figure 2. Colostrum and milk antibody distributions. Anti-PEDV N, M, S1, and S2 protein ELIS A OD values were determined for milk (A,B) and colostrum (C,D) for IgG (A,C) and IgA (B,D) isotype antibodies.

Colostrum samples from PEDV positive farms were examined using the PEDV N, M, S1, and S2 antigens for both IgG and IgA isotypes. The range of ELISA OD values was examined to

determine which antigens showed better anti-PEDV reactivity (Figure 2C, D). Anti-PEDV IgG antibodies in colostrum were most reactive to the S2 and N proteins, IgA antibodies were most reactive to the S1 and S2 antibodies and anti-M IgA antibodies were barely detectable in colostrum (Figure 2C, D). Conservative cut-off values were set at an OD of 0.2-0.4, but negative samples need to be obtained in order to truly determine PEDV-negative cut-off values and to validate the ELISAs.

Fecal samples were obtained from commercial and experimental sows to optimize the PEDV fecal ELISA protocol. The majority of samples examined were antibody negative, but a few animals were sampled over a time course and some of the time points showed antibody reactivity. Three animals were examined at approximately day 0, 4, and 7 post-farrowing on IgA isotype antibodies for N, M, S1, and S2 antigens (Figure 3). Interestingly, some days showed high antibody reactivity and other days had low reactivity. Since antibody levels usually do not vary that much in a few days, this suggests that sampling and preparation of fecal samples needs to be examined further.

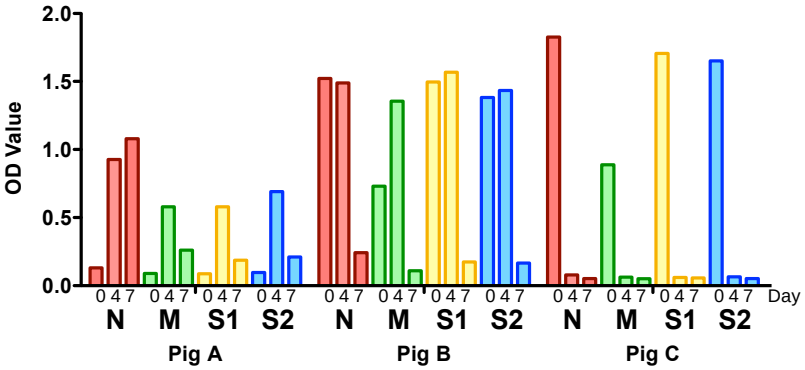

Figure 3. Anti-PEDV fecal antibody levels over time. Antibodies were extracted from sow feces and examined by ELISA for antibodies to PEDV-N, M, S1, and S2 proteins on the day of farrowing and approximately 4 and 7 days post-farrow.

Refolded antigen ELISA performance

The PEDV N protein was expressed and purified and a high concentration of protein was obtained. Large scale refolding and removal of denaturants for the PEDV N protein was successful, yielding an 85% recovery. Neither the M nor the S2 proteins were able to be successfully refolded and the S1 protein was unable to be expressed to high enough concentration for refolding. A clone containing just the antigenic regions of the spike proteins (based on the published literature) was ordered to determine if a smaller protein fragment will be more suitable for expression and refolding.

To determine if refolding of the N protein increased its antibody reactivity, equal amounts (200ng) of N and refolded N protein was used to coat ELISA plates. The antibody reactivity was compared between refolded protein and that of the original denatured protein preparations using serum, milk, and fecal samples for both the IgG and IgA isotype (Figure 4). All fecal samples were negative for PEDV antibodies. Refolding the N protein increased antibody reactivity by 25-35%. However, only in one instance did this increase the OD value enough to move the sample from negative to suspect positive.

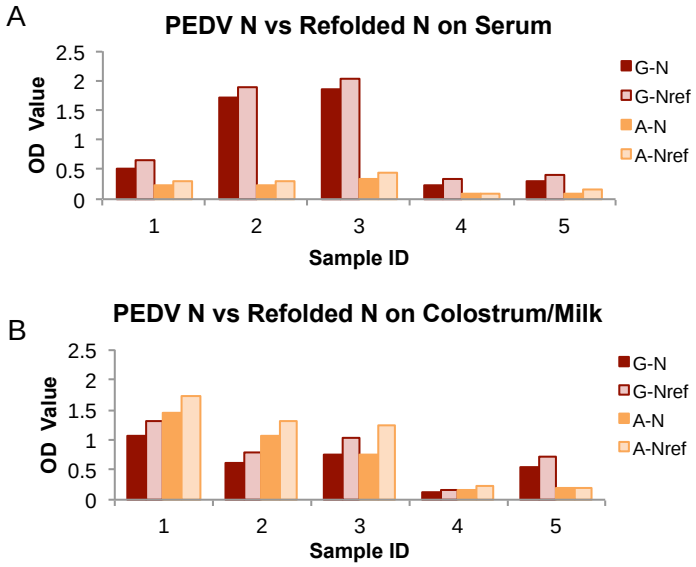

Figure 4. Antibody reactivity to N antigen before and after refolding. PEDV N protein was coated on ELISA plates before and after refolding to compare reactivity to (A) serum and (B) milk or colostrum antibodies. Reactivity to IgG antibodies are shown in maroon and IgA antibodies are shown in gold. The original PEDV N protein reactivity is shown in solid dark bars and reactivity to refolded protein is shown in lightly shaded bars.

Alternative sandwich ELISA

The refolded N protein was coupled to HRP for antibody detection in ELISA. Similar to the original PEDV ELISA, N protein was coated on ELISA plates followed by serum antibody binding. In the alternative ELISA instead of detecting bound antibodies using an anti-pig IgG-HRP

conjugated antibody, bound antibodies are detected using HRP conjugated N protein. This should increase specificity of the ELISA since the antibody has to bind the N protein twice in order to be detected by ELISA.

ELISA analysis of pig serum samples using the alternative sandwich ELISA was performed. Unfortunately, when this alternative sandwich ELISA was performed with the N-HRP protein, the background values were extremely high. Every well gave similar reactivity. After further analysis, we concluded that the N-HRP must be able to either bind directly to the N protein coated on the ELISA plate or to the plate or other material in the well. Thus, this is not a viable option for increasing the specificity for the N protein ELISA. If other PEDV proteins were able to be successfully refolded and coupled to HRP, they might be better candidates for the alternative sandwich ELISA.

Prediction of protection of piglets

Lactogenic immunity is the major defense against infection in newborn piglets. In order to predict if piglets would be protected against PEDV infection, we examined colostrum and milk samples for anti-PEDV antibodies using all 4 antigen ELISAs and both IgG and IgA isotypes. We obtained colostrum (farrowing, day 0) and milk samples (day 3, 12, and 19) and examined these for PEDV N, S1, and S2 antibodies for both IgG and IgA isotypes for colostrum and IgA for milk (Figure 5). Colostrum IgA antibodies, not surprisingly, have higher antibody levels than in milk. Interestingly, for N and S2, IgG antibody levels were higher or similar to IgA antibody levels. Antibodies were present for all 3 antigens, but S1 and S2 had higher antibody levels (for IgA) and colostrum contained the majority of antibodies. Thus, the colostral spike antigens might be the best indicators of lactogenic immunity.

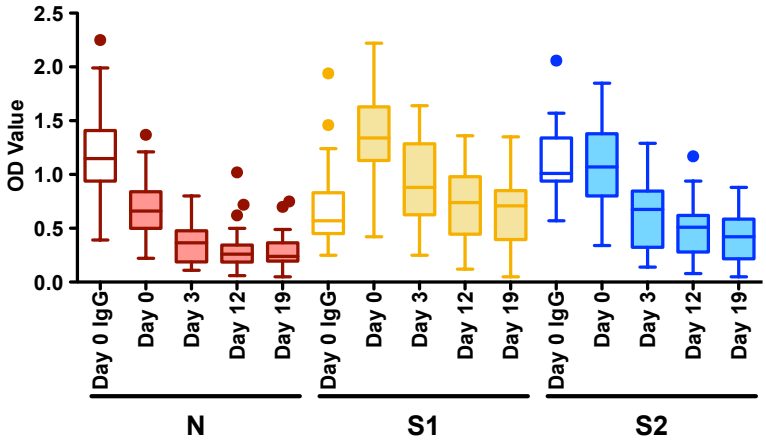

Figure 5. Colostrum and milk antibodies over time. Colostral antibodies were examined for IgG (open boxes) and IgA (shaded) isotypes against PEDV N, S1, and S2 antibodies. Milk was examined for IgA isotype antibodies against N, S1, and S2 at day 3, 12, and 19 post-farrow. Distribution of antibody levels from 38 sows is shown as box-whisker plots using the Tukey determination of whiskers.

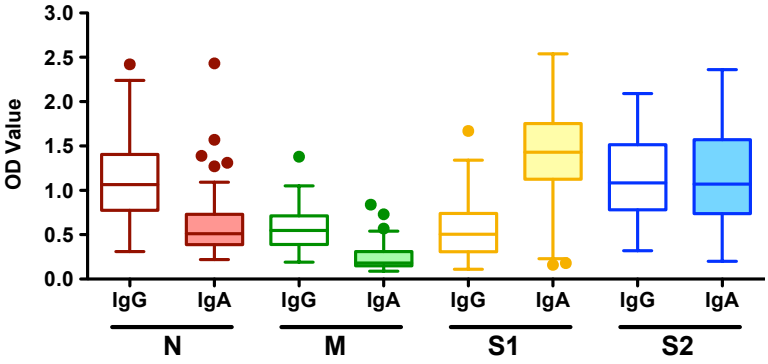

Figure 6. Colostrum antibody levels. Colostrum samples from 66 sows were examined by ELISA for N (maroon), M (green), S1 (yellow), and S2 (blue) protein antibodies and IgG (open boxes) and IgA (shaded) isotypes. Distribution of antibody is shown as box-whisker plots using the Tukey determination of whiskers.

To examine colostral antibody levels further, we examined 66 colostrum samples from 6 sample sets (a total of 5 separate farms) for N, M, S1, and S2 antigens and both IgG and IgA isotypes (Figure 6). The highest reactivity was observed for IgA against S1 and S2 and for IgG against N and S2. The S1 antigen was antibody negative in a few samples that showed antibody positive in both S2 and N. The anti-M antibodies were at low levels, if detectable at all. The S2 antigen reactivity was similar for both IgG and IgA and seems to be the best choice for detecting PEDV antibodies in colostrum.

Prediction of protection of sows to re-challenge

In order to examine the response of sows after re-exposure to PEDV, we examined a sow farm that was re-exposed to PEDV using feedback, approximately 4 months after their first feedback. The gilts at the time of re-exposure were all naïve. A total of 30 sows and 10 gilts were examined for antibody levels in serum and viral presence in feces over a time course following feedback (feedback = day 0, then day 7 and 35 post-feedback). After re-exposure no disease symptoms were observed in any of the animals, suggesting that they were all protected against re-infection. Antibody levels were examined using the PEDV N ELISA, IFA, and viral presence by PCR. ELISA analysis showed that at the time of re-exposure, about half of the sows had detectable anti-PEDV antibodies (gold), while 2 of the gilts (maroon) had detectable antibody levels (Figure 7A). At 7 and 35 days post-feedback antibody levels increased in both sows and gilts. At day 35 post-feedback, all gilts had detectable levels of antibodies. According to PCR (Figure 7B), all gilts and some sows were viremic 2-7 days after feedback, but by day 35 all virus was cleared from the animal. Since half of the sows were not viremic for PEDV, it suggests that they were protected against re-infection, even though antibody levels were not detectable by ELISA. Thus, we examined the presence of protective antibodies using IFA. We observed that 80% of previously exposed sows were antibody positive on the day of feedback, which increased to 100% of sows (and gilts) at 35 days post-feedback (Figure 7C). This suggests that IFA is a more accurate measure of protection than that of PEDV-N ELISA. Perhaps the addition of the S2 protein ELISA would further increase the predictive power of the PEDV ELISA on antibody protection.

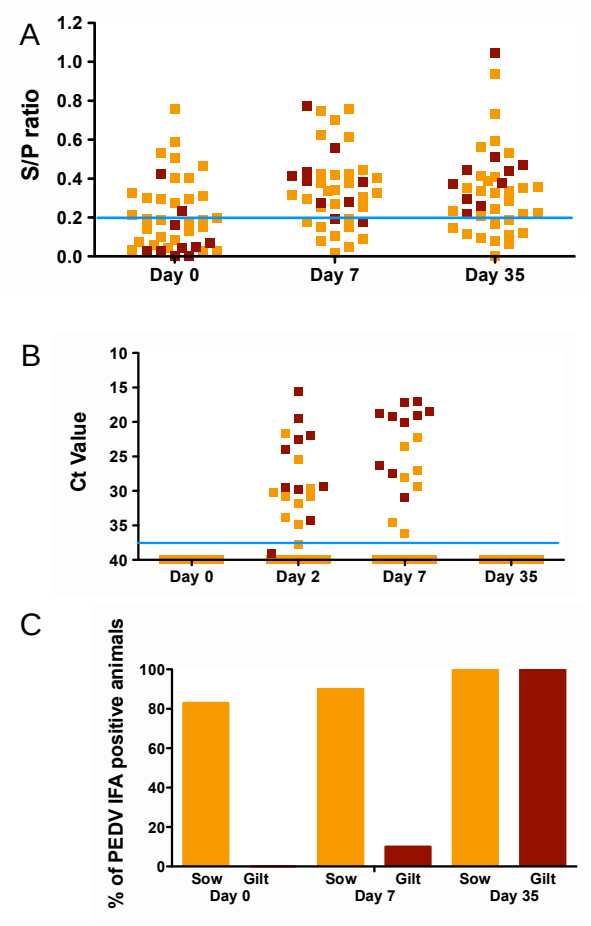

Figure 7. Sow serum antibody and viral levels following re-exposure. Serum from immune sows (gold) and naïve gilts (maroon) were examined by both **(A)** ELISA and **(C)** IFA. For ELISA, S/P ratios above the blue line are considered positive. A significant difference between antibody levels at day 0 and both day 7 and day 35 was observed. **(B)** qRT-PCR detection of PEDV from fecal samples at day 0, 2, 7, and 35 post-feedback. PEDV positive samples are shown as data above the blue line. Samples below the line and above 40 are considered suspect. A significant difference is observed between both day 0 and 35 and that of days 2 and 7.

Discussion:

PEDV appeared suddenly in the US causing epidemic sow herd outbreaks and devastating producers with the loss of entire groups of piglets, not to mention the loss of income. PEDV outbreaks were controlled through feedback, but the duration of protection was not known. The presence of protective antibodies can be examined using ELISA on serum samples, but the current assay does not have an optimal sensitivity and specificity and has not been tested on other sample types. In this project we examined ways to increase the sensitivity and specificity of the current assay, as well as examined other antigens for detection of antibodies to PEDV in serum, milk, colostrum, and feces. We were able to slightly increase the sensitivity of the assay by refolding the PEDV N protein before using it in the ELISA, but this was not significant. We were unable to create an alternative sandwich ELISA using the N-protein coupled to HRP to specifically bind serum antibodies a second time, as opposed to detecting with anti-pig IgG-HRP, which only requires serum antibodies to specifically bind to PEDV proteins one time. However, a spike antigenic

region is being produced to determine if this would be a better antigen for this alternative sandwich ELISA.

The presence of anti-PEDV antibodies in milk, colostrum, and fecal samples was examined by ELISA using the N, M, S1, and S2 proteins, as well as detecting IgG and IgA antibody isotypes. Both IgA and IgG antibodies were present in colostrum, while milk was mainly IgA isotype antibodies. IgG antibodies were observed to be predominantly towards the N and S2 proteins, while IgA antibodies reacted more to the S1 and S2 proteins. Fecal antibodies were rarely observed and were not consistent in day to day reactivity.

Predictors of protection of piglets by lactogenic immunity or sows by serum antibodies was examined. If protection can be predicted by detection of antibody levels, producers will be able to make informed decisions as to whether they need to treat animals by feedback in order to protect their farms from the devastating effects of a PEDV outbreak. We determined that colostrum IgG antibodies to N or S2 or colostral IgA antibodies to S1 or S2 were observed at high levels in sows in which the piglets on the farm were protected against PEDV. Milk contained antibodies mainly to S1 and S2, but at lower levels than that found in colostrum. Serum antibody levels were examined as predictors of protection after re-exposure by ELISA and IFA. IFA was observed to be a better predictor of protection than the PEDV N ELISA. All of the animals were protected against re-infection, but only 50% showed positive antibody levels by ELISA while 80% were positive by IFA.

In this project we were able to slightly increase the reactivity of the PEDV N ELISA, determine the best antigens and isotypes to use for antibody detection in colostrum and milk, and predict protection against PEDV re-infection and piglet infection. If and when the next wave of PEDV comes through the US, we will have more knowledge on how to quickly determine if a farm will be protected against an outbreak or if feedback should be performed on the farm if PEDV infection is expected to occur.

Acknowledgements:

We would like to thank the following veterinarians and producers for their generous contributions towards this research: Dr. Matthew Turner, Dr. Sara Hough, Prestage farms, Dr. Ian Levis, Iowa Select Farms, Dr. Tim Snider, Schafer Farms, Dr. Laura Greiner, and Carthage Veterinary Service.