RESEARCH REPORT
SWINE HEALTH

Title: Accurate ELISA test development: Evaluation of cysteine protease domain of non-structural protein 2 as a potential antigen **– NPB #05-155**

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Abstract

The objective of this research was to evaluate the cysteine protease domain (CP) of PRRSV Nsp2 as a potential new antigen for the development of a sensitive, specific and differential ELISA test. The CP regions of type 1 and type 2 PRRSV were expressed as recombinant proteins. Three hundred and fifty three serum samples from 32 individual pigs experimentally infected with Type 1 or Type 2 PRRSV were tested using Nsp2 CP-based ELISAs. Antibody specific to the CP domain can be detected as early as 14 dpi, and the antibody response lasted to 202 dpi. Receiver operating characteristic analysis based on the 81 known positive and 118 known negative samples showed good specificity (96.6%) and sensitivity (98.2%) of the Nsp2 CP-based ELISA. The capability of the CP-based ELISA for detecting serum antibody response from pigs infected with various genetically different field strains was determined. Nine hundred and seventy-nine serum samples submitted to the SDSU diagnostic laboratory were tested. The Nsp2 CP-based ELISA possessed 91.6% agreement with the IDEXX ELISA. In further testing of 202 IDEXX suspect false positive samples, our Nsp2 CP-based ELISA resolved 93.6% of the samples as negative. To differentiate Type 1 and Type 2 PRRSV, we developed an epitope-based ELISA using a conserved epitope, ES2 in the CP region of Type 1 PRRSV. The results showed that the ES2 epitope-based ELISAs are specific for identifying Type 1 PRRSV with 94.4% specificity and 94.5% sensitivity. This project addresses the "proof of concept" phase for new diagnostic assay development and more detailed "full validation" studies will be pursued based on the preliminary data generated from this project.

Introduction

PRRS is still the most economically devastating disease in the swine industry. Serological assays are popular diagnostic tests to determine if pigs have been exposed to the PRRS virus (PRRSV). In addition, serum is an antemortem sample that can be collected easily, in sufficient quantity for multiple tests, and swine practitioners are accustomed to the protocol for collecting serum. Currently, the IDEXX PRRS ELISA is the most widely used serological assay for determining the serostatus of swine herds. However, positive IDEXX ELISA results in otherwise seronegative herds causes concern for producers, necessitates the need for a variety

of follow-up assays to verify that the result is either positive or negative, which indicates that there is still a need for a reliable assay to identify the serological status of single reactors compared to herd reactors. While there is no standard protocol to verify false positive serological results for PRRSV, most diagnostic laboratories use either the indirect fluorescent antibody (IFA) assay and/or virus neutralization assays. However, the results from both of these assays are affected by antigenic variation and they may not detect a serological response against antigenically diverse PRRSV isolates, such as the European-like PRRSV strains, known as North American Type 1 isolates. The appearance of the Type 1 PRRSV isolates in the US also complicates the diagnosis of PRRSV as there is presently no serological assay that differentiates between both the Type 1 and Type 2 strains of PRRSV. The movement of the swine industry towards finding strategies to eliminate or eradicate PRRSV will require an adequate serological diagnostic assay that can detect acute and persistently infected pigs, detect various strains of PRRSV and have the capacity to differentiate between Type1 and Type2 PRRSV isolates.

Previous studies (Oleksiewicz et al., 2001a, 2001b; Murtaugh, 2004) and preliminary results generated in our laboratory demonstrated that the cysteine protease (CP) domain of PRRSV non-structural protein 2 (Nsp2) induces a strong antibody response. The purpose of this study was to evaluate the CP domain of PRRSV Nsp2 as a potential new antigen for the development of ELISA-based serological tests to increase the specificity and sensitivity of current ELISA tests. We investigated the detection time period of antibody responses to the CP domain. A panel of field serum samples, including the samples with suspected false positive IDEXX ELISA results, was tested on the Nsp2 CP-based ELISA. An ES2 epitope identified in the CP domain region, which is conserved among Type 1 PRRSV strains, was used to develop a peptide-based ELISA to specifically differentiate Type 1 and Type 2 isolates.

IV. Objectives 1). To *in vitro* express the CP domain and conserved ES2 epitope of Nsp2 of Type 1 and Type 2 PRRSV as ELISA antigens; 2). To determine the development of the serological response over time to the CP domain and ES2 epitope of Nsp2; 3). To assess anti-CP and anti-ES2 antibody responses against a broad spectrum of sera from pigs infected with different strains of PRRSV.

V. Materials and Methods:

1. Antigen Production:

1.1. *In vitro* express CP domains of European-like and North American PRRSV:

The regions containing the protease domain sequence of European-like Type 1 isolate, 01-08 (nucleotide 1278 – nucleotide 1515 of ORF1a) and the North American Type 2 isolate, VR-2332 (nucleotide 1296 – nucleotide 1533 of ORF1a) were amplified from genomic RNA by RT-PCR using primers Ensp2-CPF / Ensp2-CPR for Type 1 virus and primers NAnsp2-CPF/NAnsp2-CPR for Type 2 virus (Table 1). The RT-PCR products were then cloned into the protein expression vector pBAD-Thio (Invitrogen). Recombinant proteins were expressed in *E. coli* Topo 10 cells to produce a fusion protein with the N-terminal fused to thioredoxin tag and C-terminal fused to six histidine residues. After treatment with 1mM IPTG, bacteria were sonicated, centrifuged and the fusion proteins were purified by nickel-affinity chromatography. The purified fusion proteins were analyzed by SDS-PAGE and Western-blotting as we described before (Ferrin et al., 2004).

1.2. *In vitro* expression of ES2 epitope as tandem repeat epitopes:

DNA sequence coding for the ES2 epitope, PEDDWASDYDLVQA, which covers nucleotides 1587 to 1628 of viral genome was amplified by RT-PCR. Two forward primers (Table 1) were used for constructing expression vectors with tandem repeat ES2 genes. Forward primer ES2F-1 contained a BglII restriction site, but without a link sequence, whereas the forward primer ES2F-2 contained not only a BglII restriction site, but also a link sequence, ggtggtggtggttcc, which coded for a flexible peptide consisting of four glycines and one serine. A flexible peptide linker consisting of glycines between epitopes can help to display the epitopes (Jimo and Jeremy, 1994). A reverse primer, ES2R (Table 1), was flanked by a BamHI and a HindIII restriction enzyme site. The ES2 region was first amplified with the forward primer ES2F-1 and reverse primer ES2R. The PCR product was digested with BglII and HindIII, then cloned into expression vector pET-28a (+) (Novagen) that was digested by BamHI and HindIII. This recombinant plasmid was named pET-28a-ES2 (+1).

The pET-28a-ES2 (+1) was digested with BamHI and HindIII, then ligated with the second copy of ES2 which was amplified by forward primer 2 and reverse primer and digested with BglII and HindIII. The following 4 copies of ES2 fragments were inserted the same way as the second copy of ES2. Fig. 1 shows the final construction of pET-28a-ES2 (+6). The recombinant protein with six ES2 epitopes was expressed in *E. coli* BL21 (DE3) to produce a fusion protein with six histidine residues at C-terminal. The proteins were purified by nickel- affinity chromatography and analyzed by SDS-PAGE.

Table 1. Primers for amplification of CP domains and ES2 epitope

Primer name	Sequences	Amplification regions
Ensp2-CPF	5'-gggtcttgtggttggcacgttcttg-3'	CP domain of Type 1 PRRSV
Ensp2-CPR	5'-agatctcacctctacttcccagtgaaccc-3'	CP domain of Type 1 PRRSV
NAnsp2-CPF	5'-gccgaagggaattgtggttgg-3'	CP domain of Type 2 PRRSV
NAnsp2-CPR	5'-gacagtccaatgctcaccttccag-3'	CP domain of Type 2 PRRSV
ES2F-1	5'-gcagatctccagaggatgattgggc-3'	ES2 epitope of Type 1 PRRSV
ES2F-2	5'-gcagatctggtggtggtggttccccagaggatgattgggc-3'	ES2 epitope of Type 1 PRRSV
ES2R	5'-atcccaagcttcggggatcccgcctgagcaag-3'	ES2 epitope of Type 1 PRRSV

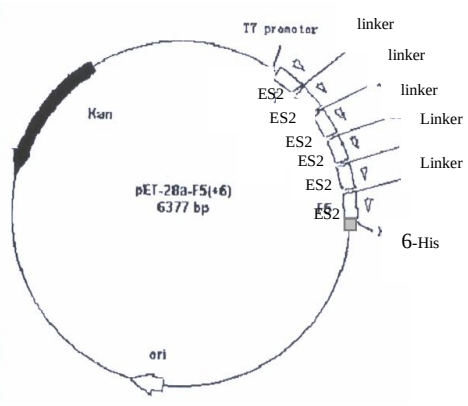

Figure 1. Construction of recombinant expression vector carrying tandem repeat ES2 epitopes. ES2: ES2 epitope; linker: linker sequence between epitopes; 6-His: six histidine tag for facilitating protein purification. Figure adapted from Sun et al., 2004, J. Virol. Methods. 119: 79-86.

2. Serum samples:

A panel of serum samples from animals challenged with four different European-like Type 1 PRRSV isolates was obtained from the previous NPB project #04-186. Serum samples from pigs infected with North American Type 2 PRRSV strain, VR-2332 were obtained from the USDA PRRSV Integrated project "Creation of a sample resource for the research community and identification of diagnostic targets for the analysis of immune protection and viral persistence." All of these serum samples were generated from challenged piglets, serially bled at regular intervals for a duration of at least 2 months. An extensive database including all serological data is available for correlation with the Nsp2 CP and ES2 ELISA results. To assess anti-CP and anti-ES2 antibody responses against a broad spectrum of sera from pigs infected with different field strains of PRRSV, 979 serum samples were obtained from the SD Animal Disease Research and Diagnostic Laboratory (ADRDL). These samples were collected from 216 cases submitted from 13 different states during the year 2005-2006. All were initially evaluated using the IDEXX PRRSV ELISA. In addition, 202 IDEXX ELISA suspected false positive samples were also obtained from the SD ADRDL.

3. Detection of anti-CP or anti-ES2 antibodies in serum by Nsp2 CP-ELISA:

The 96-well Immulon 2 HB plate was coated with expressed Nsp2 CP protein or tandem ES2 epitope for 1 hour at 37 ^{0}C, excess protein binding sites were blocked with 10% milk in 1X PBS with 0.05% Tween 20 (PBST buffer) overnight at 4 ^{0}C. Test sera were applied at selected dilutions. After 1-hour incubation at 37 ^{0}C, plates were washed and horseradish peroxidase-conjugated goat anti-swine Ig was added to bind to any PRRSV serum antibodies that bound to the antigen on the plates. After one hour incubation at 37 ^{0}C, the conjugate was removed, plates were washed and the substrate ABTS was added for color development. Results were quantified by reading at 405 nm with an EL800 microplate reader (BioTek Instruments Inc.) controlled by

XCheck software (IDEXX Laboratories). The raw plate data were copied to an Excel spreadsheet to calculate the sample to positive (S/P) ratios using the following formula:

S/P = (OD of sample – OD of buffer) / (OD of positive control – OD of buffer)

4. Establishment of the cut-off value:

Populations of known negative and positive sera were examined to establish appropriate assay cut-off values. One hundred and eighty-one known positive and 225 known negative sera samples from the animal challenge studies (NPB project #04-186 and PRRSV CAP project) were used in this study. Receiver Operating Characteristic (ROC) analysis methodology assessment was performed using GRAPH ROC software (Version 2.0; [http://members.tripod.com/refstat/ GraphROC.htm]) to obtain the cut-off value.

Results:

1. To *in vitro* express the CP domain and conserved ES2 epitope of Nsp2 of Type 1 and Type 2 PRRSV as ELISA antigens

1.1. *In vitro* express CP domains of Type 1 and Type 2 PRRSV:

The nickel-affinity chromatography purified recombinant protein was analyzed for purity using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 2, Western blot analysis revealed that anti-CP monoclonal antibody specifically recognized the recombinant proteins. Western blot showed a sharp band around 25 kd of molecular weight, which is the predicted size of the fusion proteins (predicted CP domain of VR2332 as 8.6 kd; predicted CP domain of 01-08 as 8.9 kd; N and C-terminal tag as 16 kd).

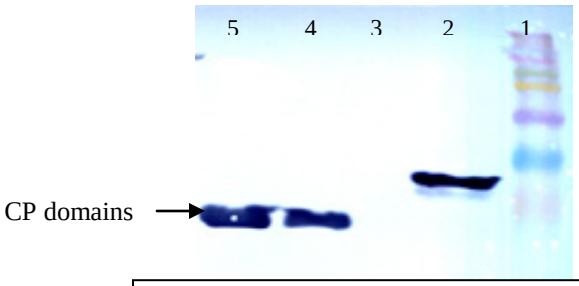

Fig 2. Western blot showing expression of cysteine protease of PRRSV Nsp2. Proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was probed with HRP anti-His mAb. Lane 1, molecular mass marker; Lane 2, positive control; Lane 3, negative control; Lane 4, nt 1297 – 1533 of VR2332; Lane 5, nt 1279 – 1515 of SD01-08.

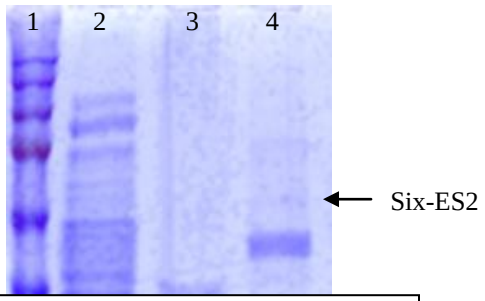

Fig. 3. SDS-PAGE stained with coomassie blue, which shown purified six ES2 recombinant protein. Lane 1, molecular mass marker; Lane 2, flow throw; Lane 3, wash; Lane 4, elution of purified six-ES2 recombinant protein.

1.2. *In vitro* expression of ES2 epitope as tandem repeat epitopes:

According to the study of Oleksiewicz et al. (2001), the ES2 epitope in the Nsp2 protein produced the highest serum antibody response and was located in the highly conserved region of Type 1 PRRSV isolates, which makes ES2 as a good antigen candidate for ELISA development. However, a single peptide with low molecular weight often can not be expressed efficiently without fusion to a foreign protein such as GST. Due to the possibility of non-specific reactivity against the fused foreign protein, an immunoassay based on such a fusion protein may not be site specific. Therefore, in our study, we used an approach based on the diagnostic antigen development for foot-and-mouth disease (Sun et al., 2004) to construct proteins with tandem repeat ES2 epitopes. It has been shown that antigens with polyvalent epitopes could result in greater sensitivity than those with monovalent epitopes when used in immunoassay (Tam and Zavala, 1989). As shown in Fig. 1, six copies of ES2 epitope, PEDDWASDYDLVQA, and a 6-Histidine tag were constructed in the protein expression vector, pET-28a (Novagen). A flexible peptide linker, GGTGGTGGTGGTTCC, was added between the epitopes for helping display the epitopes. Fig. 3 shows the results of SDS-PAGE analysis of purified six-ES2 recombinant protein.

2. To determine the development of the serological response over time to the CP domain and ES2 epitope of nsp2

We used *in vitro* expressed CP protease domains from Objective 1 as ELISA antigen to determine the time frame of detecting anti-CP serum antibody response. One hundred and fifty serum samples, as sequential bleeds from 0 dpi to 85 dpi, were obtained from a group of pigs experimentally infected with four different Type 1 PRRSV isolates, SD01-08, SD02-11, SD01-07, and SD03-15 (NPB project #04-186). In addition, 203 serum samples were obtained from a group of pigs experimentally infected with type 2 PRRSV, VR2332 from 0 dpi to 202 dpi (USDA CAP project). As shown in Fig. 4, the CP-based ELISA can detect the antibody response as early as 14 dpi, and the antibody response lasts more than 84 dpi for Type 1 PRRSV or 202 for Type 2 PRRSV.

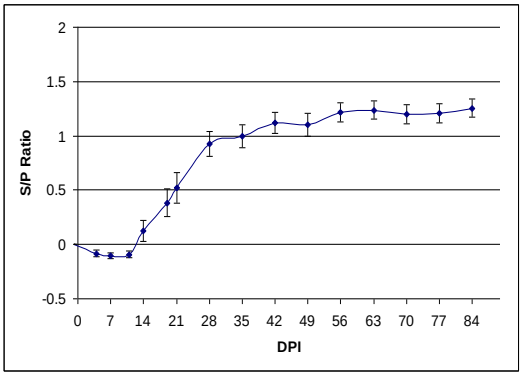

Fig. 4A. Mean S/P values of CP-based ELISA results for serum samples collected from 20 pigs over 85 DPI period. At 0 DPI, four groups of five pigs each were infected with Type 1 PRRSV strains 03-15, 01-08, 01-07, and 02-11.

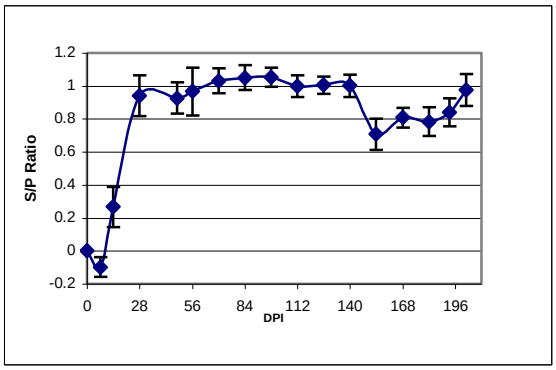

Fig. 4B. Mean S/P values of CP-based ELISA results for serum samples collected from 12 pigs infected with North American Type 2 VR 2332 during 0 to 202 DPIs.

At the same time, one hundred and fifty serum samples from NPB project #04-186 were also tested using the six-ES2 based ELISA. The antibody specific to six-ES2 recombinant protein can be detected as early as 14 dpi, and the antibody response lasted more that 85 dpi (Fig. 5). At the same time, ninety-six serum samples from a group of pigs experimentally infected with Type 2 PRRSV, VR2332 from 0 dpi to 85 dpi were also tested using six-ES2 based ELISA. The results showed that no anti-ES2 antibodies were detected in sera samples from Type 2 PRRSV infected animals (Fig. 5). ROC plot analysis based on the 100 known positive and 107 known negative samples showed good specificity (94.4%) and sensitivity (94.5%) for the Type 1 PRRSV. These results suggested that ES2 epitope-based ELISAs are specific for identifying Type 1 PRRSV, which could be used as a differential test to differentiate Type1 and Type2 PRRSV infection. Future studies are required for further validation of this test.

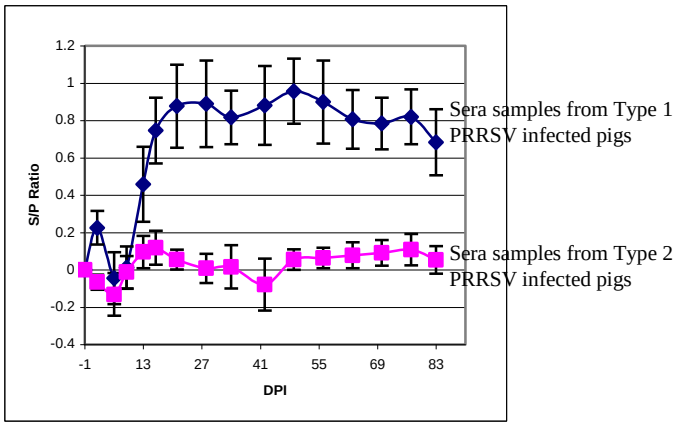

Fig. 5. ES2 epitope-based ELISA test of sera samples from experimental pigs infected with Type 1 and Type 2 PRRSV.

3. Assessment of anti-CP and anti-ES2 antibody responses against a broad spectrum of sera from pigs infected with different field strains of PRRSV.

We used a broad spectrum of serum samples submitted to our diagnostic laboratory to determine if the CP antigen and six-ES2 antigen based ELISAs could be applicable for detecting serum antibody response from pigs infected with various genetically different field strains. Since the source of field sera samples was unknown (whether pigs infected by Type 1 or Type 2 PRRSV), we used mixed antigen, including CP domains of Type 1 and Type 2 viruses and six-ES2 tandem epitopes. We designated this test as Nsp2-CP ELISA. Using 81 known positive and 118 know negative samples, ROC analysis determined a cutoff value of 0.473 with 98.2% sensitivity (conf. interval: 0.96-1.00) and 96.6% specificity (conf. interval: 0.92-0.98) (Fig. 6). Based on this cutoff value, 979 field sera samples were tested. Comparing with the IDEXX ELISA results, 525 out of 571 (91.9%) IDEXX positive samples were tested as positive by Nsp2-CP ELISA, and 372 out of 408 (91.2%) IDEXX negative samples were tested as negative by Nsp2-CP ELISA (Table 2).

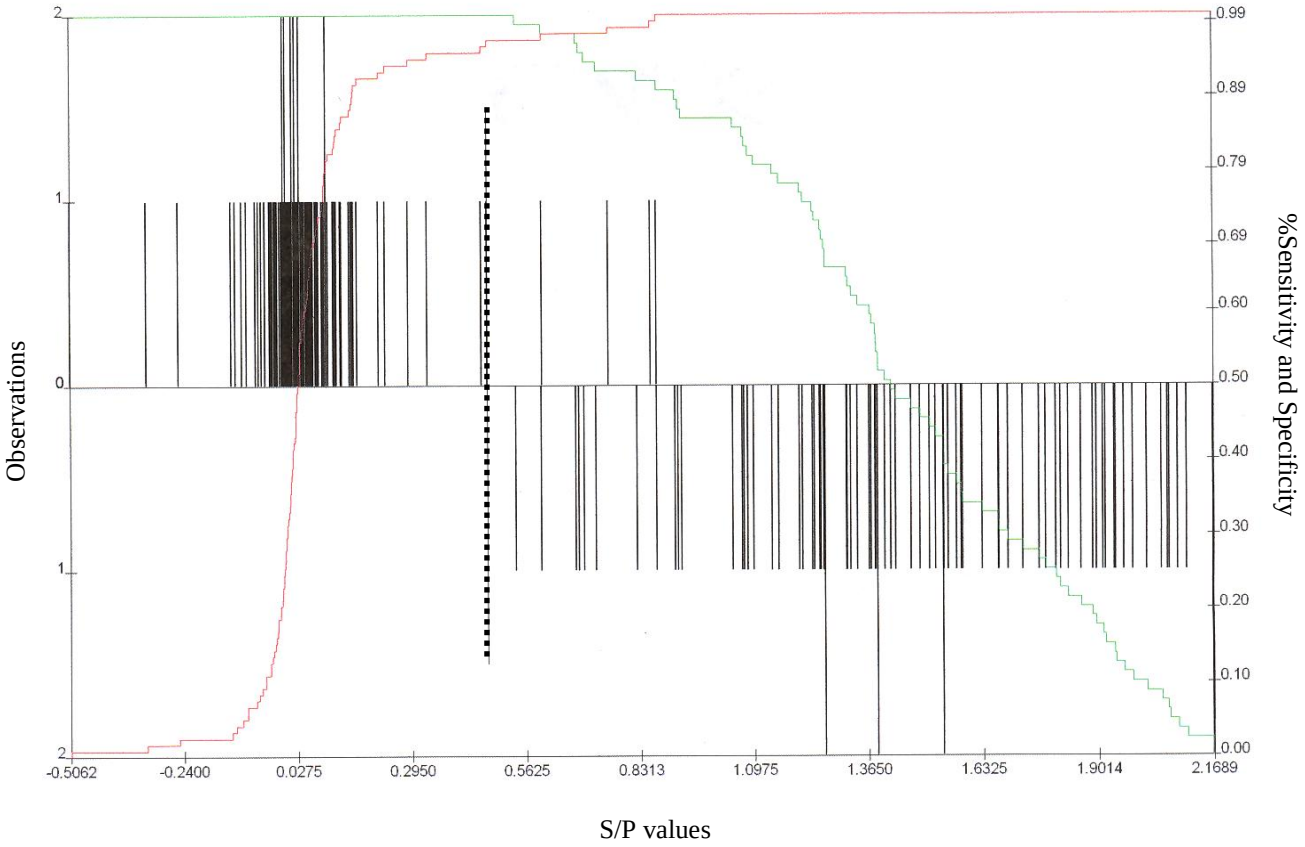

Fig. 6. ROC plot of the Nsp2-CP ELISA. The graph was calculated using the 199 individual animal serum samples and GraphROC software. The upward-pointing histogram on the left side of the figure represents the uninfected animals. The downward-pointing histogram on the right represents the PRRSV-infected animals. The red line represents the changes of sensitivity of the assay as the cutoff is moved from low to high S/P ratios. The green line represents the changes in the specificity of the assay as the cutoff is moved from low to high S/P ratios. The black dashed vertical line represents the optimized cutoff value.

Table 2. Relative comparison between nsp2-CP ELISA and IDEXX ELISA for detection of antibodies to PRRSV in field serum samples

IDEXX ELISA results	nsp2-CP ELISA		
	Positive	Negative	Total no. of serum samples
Positive	525	46	571
Negative	36	372	408
Total	561	418	979

We further investigated the feasibility of using the Nsp2-CP ELISA to determine the PRRSV serostatus of individual animals with unexpected positive IDEXX ELISA results. Two hundred and two samples with suspected false positive IDEXX ELISA results were tested by Nsp2-CP ELISA. One hundred and eighty-nine samples (189 of 202, 93.56%) demonstrated negative test results.

VII. Discussion and Future Prospects:

Monitoring the serostatus of PRRSV-negative or low-prevalence herds is important to the swine industry. When the IDEXX ELISA is used as a screening tool, unexpected positive results from samples in negative-testing herds may require additional tests to resolve the problem. In comparison to the IDEXX ELISA, out of 979 diagnostic samples tested by the Nsp2 CP-based ELISA, 879 samples tested were in agreement with the IDEXX ELISA results with 98.2% sensitivity and 95.5% specificity. Most of the samples (82 samples) with inconsistent results from these two ELISA tests have S/P ratios around the cutoff value. Therefore, more detailed "full validation" studies should be pursued based on these preliminary data generated in this project, which includes optimization of the assay conditions and the cutoff value, determination of diagnostic sensitivity and specificity and monitoring the repeatability of the assay. In addition, our preliminary results demonstrated that the Nsp2 CP domain based antigen can detect serum antibody responses from 14 dpi to over 202 dpi. Nsp2 may have potential to use for detecting the persistence stage of the PRRSV infection. The ES2 epitope-based ELISAs showed good sensitivity and specificity for identifying Type 1 PRRSV, which could be used as a differential test to differentiate Type1 and Type2 PRRSV.

VIII. Lay statement:

Currently, in the absence of effective vaccines and therapeutic drugs, one of the key approaches to achieve the "National PRRS Elimination" is to identify PRRSV infected pigs, so such pigs can be quarantined, isolated or removed from herds. Sensitive and specific diagnostic assays are essential for the assessment of pigs suspected of being infected and for prevention of spread of the virus within and between herds. Currently, the IDEXX HerdChek® PRRS ELISA is widely used for the detection of antibodies to either North American Type 2 or European-like Type 1 PRRSV. Concerns with suspect false positive IDEXX ELISA results in otherwise seronegative herds, have necessitated the use of a variety of follow-up serological assays to confirm the true status of individual animals. However, the sensitivity of common follow-up serological assays, such as the indirect fluorescent antibody (IFA) and virus neutralization assays are affected by viral antigenic variation and may not detect a serological response against antigenically diverse PRRSV isolates, such as Type 1 isolates. In this study, we evaluated the feasibility of developing an ELISA based diagnostic assay using the cysteine protease (CP) domain region and the conserved ES2 epitope of non-structural protein (Nsp2) as antigens. The CP regions of Type 1 and Type 2 PRRSV were expressed as recombinant proteins. Three hundred and fifty three serum samples from 32 individual pigs experimentally infected with Type 1 or Type 2 PRRSV were tested using CP-based ELISAs. Antibody specific to the CP domain can be detected as early as 14 dpi, and the antibody response lasted to 202 dpi. Receiver operating characteristic analysis based on the 81 known positive and 118 known negative samples showed good specificity (96.6%) and sensitivity (98.2%) of Nsp2 CP-based ELISA. The capability of the Nsp2 CP-based ELISA for detecting serum antibody response from pigs infected with various genetically different field strains was determined. Nine hundred and seventy-nine serum samples submitted to the SDSU diagnostic laboratory were tested. The Nsp2 CP-based ELISA possesses 91.6% agreement with the IDEXX ELISA. In further testing of 202 IDEXX suspect false positive samples, our Nsp2 CP-based ELISA resolved 93.56% of the samples as negative. To differentiate Type 1 and Type 2 PRRSV, we developed an epitope-based ELISA using a conserved epitope, ES2 in the CP region of Type 1 PRRSV. The results showed that the ES2 epitope-based ELISAs are specific for identifying Type 1 PRRSV with 94.4% specificity and 94.5% sensitivity. This project addresses the "proof of concept" phase for new diagnostic assay development and more detailed "full validation" studies will be pursued based on the preliminary data generated from this project. For detailed information about this project, please contact Dr. Ying Fang, Center for Infectious Disease Research and Vaccinology, Veterinary Science Department, South Dakota State University, Brookings, SD 57007; e-mail: ying.fang@sdstate.edu; Phone: 605-688-6647.