RESEARCH REPORT
SWINE HEALTH

Title: Development and validation of diagnostic testing detection for antigen and antibody detection for PEDv - **NPB #13-239**

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Date Submitted: 26 June 2014

Industry Summary: Since the introduction of porcine epidemic diarrhea virus (PEDV) in the United States, millions of piglets have died. Key items are needed to be address to further initial PEDV research and development of effective control and prevention strategies include development and validation of ELISA assays for serological and environmental testing to determine history of exposure, efficacy of feedback, and duration of immunity. This project was implemented to produce PEDV viral protein antigens, produce an ELISA assay, and validate it for routine diagnostic analysis. The goal was achieved.

Keywords: PEDV, porcine epidemic diarrhea virus, ELISA, nucleocapsid, spike protein, matrix protein

Scientific Abstract: Porcine epidemic diarrhea virus (PEDV) is major cause of severe diarrhea and dehydration in pigs. Belonging to the *Coronaviridae* family, PEDV is an enveloped, positive-sense, single-stranded RNA virus with a genome size of approximately 28kb. The first detection of PEDV was reported in 1971 from England while Japan, China, South Korea, and Thailand also have reported PEDV infections. The United States first detected PEDV in May 2013 (1-5). The veterinary diagnostic laboratories quickly

development sensitive and specific real time RT-PCR (RRT-PCR) assays to detect PEDV in a variety of porcine and environmental samples. However, lack of a reliable method for evaluation of immunity has hindered progress in control and prevention of PEDV. We developed an ELISA using nucleocapsid protein expressed in bacteria and purified. When coated on microtiter plates, antibodies bind to nucleocapsid from immune pigs specifically. Validation was carried out to determine the ranges of test values for negative and positive samples so that evidence of previous exposure to PEDV could be reliably predicted.

Introduction: Classified as a member of the *Coronaviridae* family, porcine epidemic diarrhea virus (PEDV) was first reported in the United Kingdom in 1971. PEDV caused clinical signs resembling transmissible gastroenteritis virus. PEDV was subsequently reported in Hungary, Italy, Germany, France, Switzerland, and the Czech Republic *(1-7).* In addition, countries in Asia have reported PEDV outbreaks including China, South Korea, Thailand, and Vietnam. The initial reports of PEDV were mild compared to the 2010 and present Chinese reports. Recently, PEDV was detected in the United States in April 2013.

Objectives:

1. Development and standardization of viral propagation techniques to produce virus for use in diagnostic testing

2. Development of standardized reference samples that can be utilized by VDLs for diagnostic test validation.

3. Validation of the current PCR diagnostic tests

Materials & Methods:

A PEDV isolate was obtained from the Minnesota Veterinary Diagnostic Laboratory and genomic RNA was extracted and frozen at -80. The genome sequence of the virus was obtained from Genbank. Primers were designed for amplification of nucleocapsid (N), matrix protein (M), and S1 and S2 domains of the major spike protein. These genes were targeted because they are immunogenic in other coronaviruses and have shown

evidence of antigenicity in reports from labs in Asia. PCR reactions were carried out successfully and amplicon products were cloned into a bacterial expression vector and used to transform competent *E. coli* cells.

Recombinant clones were obtained, purified, and re-cloned into a *E. coli* BL21(DE3) Rosetta cells. Pure colonies were obtained and test expression was carried out. Large-scale expression was performed in Fernbach flasks. Cell pellets were lysed and antigen was recovered in inclusion body preparations. Proteins were solubilized in guanidine-containing buffer and passed over a cobalt-containing affinity column. Recombinant protein was isolated by the binding of a 6x-histidine sequence to the metal, washing and elution at reduced pH. Protein quality was assessed by gel electrophoresis and quantity determined by light absorption at OD_{280} and Coomassie blue dye-binding assay.

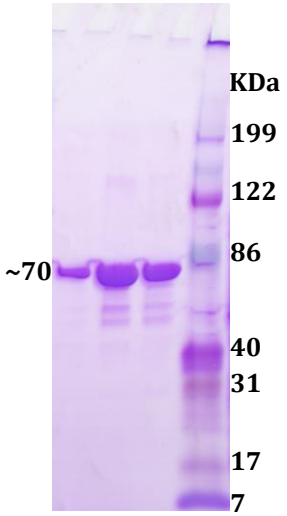
ELISA was performed by coating microtiter plates with antigen, blocking with nonfat dry milk, addition of test serum, and detection of bound antibodies by reaction with goat anti-swine IgG heavy and light chains conjugated to horseradish peroxidase (HRP). HRP catalyzed oxidation of tetramethylbenzidine to a blue color that is converted to yellow by pH reduction.

Positive and negative test sera were obtained from various sources for validation purposes. Negative samples were gilts from a PED-negative source (n=25), and 20 boar serum samples from a PED-negative boar stud. Positive samples were diagnostic submissions from six sow herds subjected to feedback 3 to 4 weeks prior to serum collection (10 to 39 samples per herd, 156 total serum samples), and two sow herds subjected to feedback 6 weeks prior to sample collection (9 and 36 samples, 45 total serum samples).

Results:

Successful protein expression and purification was achieved for N, S1, and S2, but not for M. Yields of S1 and S2 were low and not sufficient for large-scale needs required for diagnostic ELISA use. By contrast, N yields were in the mg/L range so that hundreds of thousands of assays could be performed, and cobalt column purification produced high quality antigen (Fig. 1).

Fig 1. SDS-polyacrylamide gel showing purified N samples used as coating antigen for ELISA. Three samples are shown.

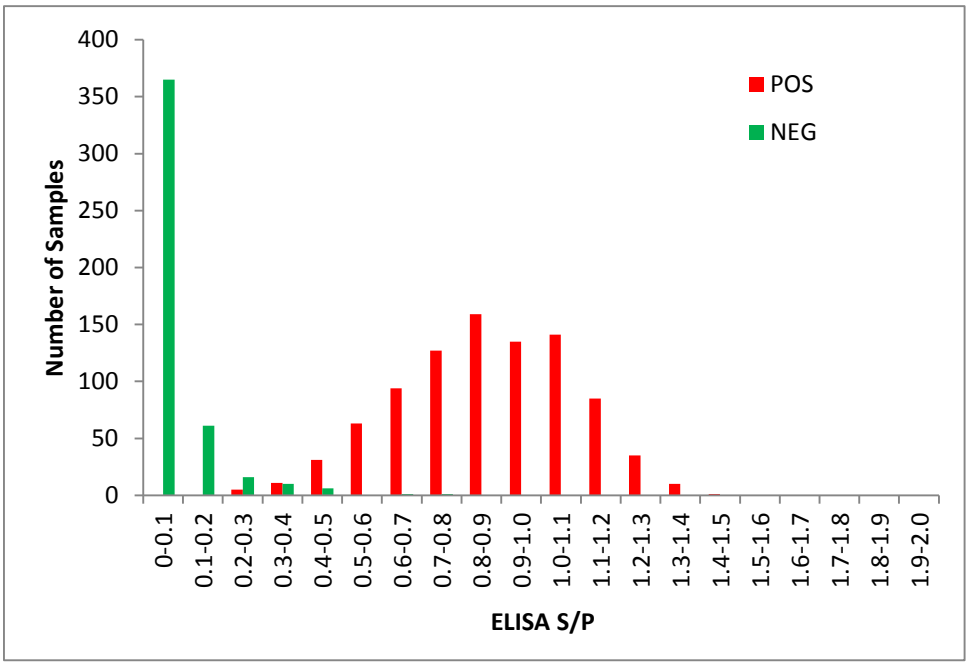


ELISA conditions were evaluated by coating microtiter plates with various amounts of purified N ranging from 50 to 500 ng per well, and incubating with various dilutions of positive and negative serum. Testing of putative positive samples resulted in 143/156 positives (sensitivity = 0.92; 95% confidence interval of 0.86-0.95) and 22/45 positives at 6 weeks post-feedback (sensitivity = 0.49; 95% confidence interval of 0.35-0.63). Specificity was 100% (0 positives in 45 samples). Specificity was further evaluated by incubating with serum positive for transmissible gastroenteritis virus and porcine respiratory coronavirus.

Final validation of this test was performed with 897 samples from a PEDV-positive population and 460 samples from several PEDV-negative populations. Samples from the positive population were from sows that had been feedback-exposed three weeks prior to sampling. Samples from negative populations were from gilts, sows and boars from farms that had not experienced clinical PED, some of which are also monitored by fecal PED PCR.

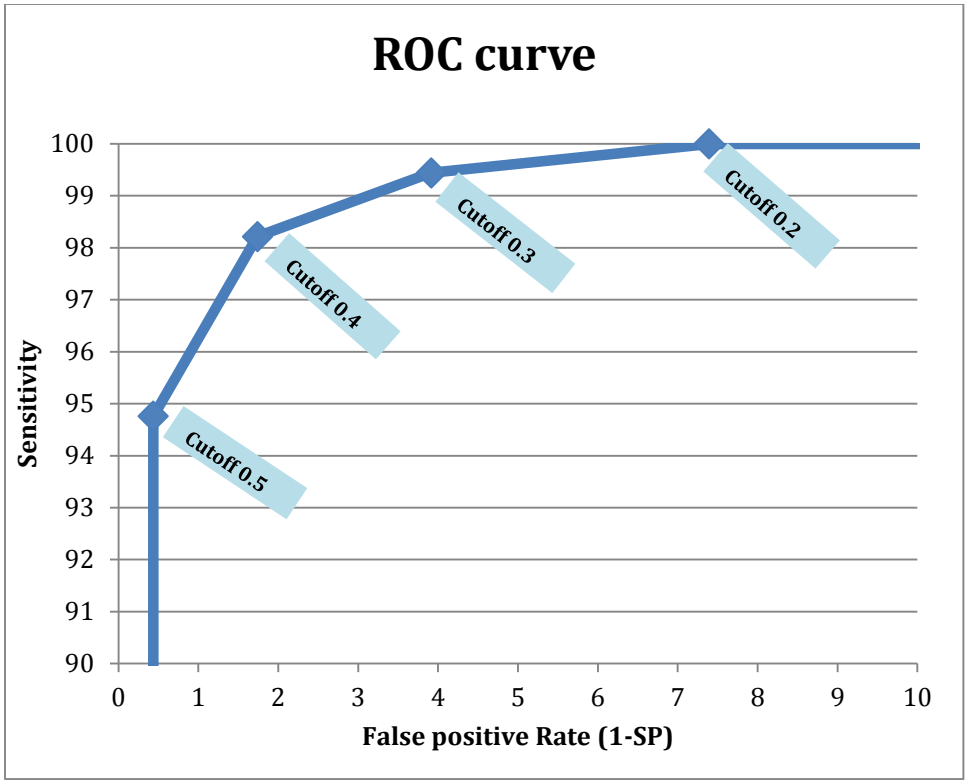
The ELISA test proved sensitive and specific, with a marked differentiation of S/P values from negative and positive populations (Fig 2). The test has been validated to detect antibodies in sow serum at 2-4 weeks post-feedback exposure. Other uses such as antibody detection after natural exposure, maternal antibody detection, antibody detection for longer periods of time, etc. have not been included in this validation and need to be evaluated further.

Fig 2. ELISA S/P ratios of serum samples from negative and positive sows.



Sensitivity and specificity determinations were made using receiver-operator characteristics (ROC) analysis. As shown in Figure 3, at an S/P ratio of ≥0.5 the ELISA had a sensitivity of about 95% and a specificity of 99.6%, meaning a predicted false-positive rate of 0.4%. Thus, at this level, about 5% of true positives would be read as negative, but only rarely would a negative be called positive. At a cut-off ratio of 0.2 the sensitivity was 100% and the specificity was 92.6%, meaning that all positives would be identified, and about 7.4% of putative positives on average would actually be negative.

Fig 3. Receiver-operator characteristics (ROC) analysis, The cutoff value for this test has been set at 0.5. With this cutoff value, sensitivity and specificity estimates and 95% confidence intervals are: SE = 94.8 (93.1 – 96.0) SP = 99.6 (98.4 – 99.9).



Discussion: The ELISA assay was implemented in the MN VDL using a recommended sample/positive (S/P) cutoff of 0.5 to distinguish negative and positive results. At this level, approximately 5% of true values would be recorded as negative. At a cutoff of 0.3 the false negative rate would be 4%. The test has proven to be reliable but conservative, since a small proportion of sows that are expected to behave as positive in feedback situations test seronegative at a 0.5 S/P ratio cutoff.

The role of the clinical diagnostic laboratories is to provide high sensitivity and specificity assay to help prevent and control pathogens and many assays must be evaluated before choosing the best assay to support the swine industry. Here we have provided one test that accomplished this goal.

We note that the results and discussion do not closely adhere to the stated goals of the project. At the time of the funding award, this project was part of a separate NPB proposal, funded as project 13-238, that was focused on molecular and virological diagnostics. We were asked to provide a separate budget for immunodiagnostics. The result was a second contract, 13-239, which was carried out as described in this report.

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