RESEARCH REPORT
SWINE HEALTH

Title: PDCoV ELISA development **- NPB 14-176**

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Industry Summary:

Porcine deltacoronavirus (PDCoV) was first identified in the United States in February 2014 in pigs with unexplained diarrheal disease in which PDCoV was later observed to be present. Diagnosis of PDCoV is performed using a real-time PCR assay to detect viral infection, but a serological test to identify viral antibodies, suggesting earlier infection and protection against PDCoV, has not been created. Our main objectives for this proposal were to develop and characterize a rapid, specific, and sensitive ELISA for PDCoV and to share the reagents and protocols with the swine diagnostic and research community. We were able to express and purify 4 PDCoV antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2). An ELISA assay was developed for each of these 4 antigens to examine the antibody reactivity to each antigen in serum from infected pigs. Serum from confirmed PDCoV-positive animals was difficult to acquire. Serum samples were obtained from 300 animals of unknown PDCoV status, that came from farms in which PDCoV had been observed. These serum samples were then run on our PDCoV ELISA with all 4 antigens. We observed a few animals from each farm that seemed to be antibody positive based on the ELISA results, mainly with reactivity to PDCoV N protein. In order to determine the specificity and cut-off values for the ELISAs, we examined serum samples from known PDCoV-negative (44 samples) and PRCV-positive animals (175 samples, a common coronavirus infection that may cross-react with PDCoV proteins). The negative samples gave low values, but surprisingly the PRCV positive samples showed higher overall values for the N protein than that of the samples with unknown PDCoV status. Comparison to ELISAs with M, S1 and S2 as antigens indicated that N results were false positives since all three of the other antigens gave consistently low ELISA results. Serum samples over a time course of infection would allow us to examine the dynamics of PDCoV antibody production and determine values for a true antibody positive sample.

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Scientific Abstract:

Porcine deltacoronavirus (PDCoV) was first identified in the United States in February 2014 in pigs with unexplained diarrheal disease. Further investigation revealed coronavirus-like particles in electron micrographs, which led to the identification of PDCoV by real-time PCR. Diagnosis of PDCoV is performed using a real-time PCR assay to detect viral infection, usually in fecal samples. Serological tests to identify viral antibodies were not available. However, serology is important and useful to identify previous viral infection, even after clearance of the virus, and to suggest that the animal has protection against PDCoV infection. Our main objectives for this proposal were to develop and characterize a rapid, specific, and sensitive ELISA for PDCoV and to share the reagents and protocols with the swine diagnostic and research community. We cloned, expressed and purified 4 PDCoV antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2). These antigens were then used to develop 4 separate ELISA assays to examine the antibody reactivity to each antigen in serum from infected pigs. Serum from confirmed PDCoV-positive animals was difficult to acquire. Serum samples were obtained from 300 animals of unknown PDCoV status, that came from farms in which PDCoV had been observed. These serum samples were then run on our PDCoV ELISA with all 4 antigens. We observed a few animals from each farm that seemed to be antibody positive based on the ELISA results, mainly with reactivity to PDCoV N protein. In order to determine the specificity and cut-off values for the ELISAs, we examined serum samples from known PDCoV-negative (44 samples) and PRCV-positive animals (175 samples). PRCV (porcine respiratory coronavirus), a close relative to transmissible gastroenteritis virus (TGEV), is a common coronavirus infection, and may have cross-reactive antibodies to PDCoV. The negative samples gave low values, but surprisingly the PRCV positive samples showed higher overall values for the N protein than that of the samples with unknown PDCoV status. Comparison to ELISAs with M, S1 and S2 as antigens indicated that N results were false positives, since all three of the other antigens gave consistently low ELISA results. Because of this cross-reactivity and the lack of known antibody positive samples, we are unable to truly test this ELISA. However, an ELISA is available in the event that PDCoV antibody positive samples are identified, which will allow for the optimization of the ELISA assay to decrease the cross-reactivity due to PRCV infection. Serum samples over a time course of infection would allow us to examine the dynamics of PDCoV antibody production and determine values for a true antibody positive sample.

Introduction:

Porcine deltacoronavirus (PDCoV) was first identified in the United States in February 2014. PDCoV is a deltacoronavirus in the Coronaviridae family, a member of the Nidovirales. It is related to other swine viruses, PEDV, TGEV, and PRCV, which are alphacoronaviruses. PDCoV is an enveloped virus containing a positive single strand RNA genome of approximately 26 kb. Symptoms of PDCoV are similar to that of PEDV and TGEV, causing diarrhea and vomiting in all age groups and mortality in nursing piglets. PDCoV seems to be a milder disease than PEDV and control and prevention of PEDV works for PDCoV, as well. Diarrheal disease due to unknown origin (not due to PEDV, TGEV, rotavirus, or *Salmonella)* may be due to PDCoV.

Diagnosis of PDCoV is performed using a real-time PCR assay to detect viral infection, usually in fecal samples. Serological diagnostics to identify the presence of viral antibodies to PDCoV are not available. Diagnosis of previous viral infection, even if the virus has been cleared from the animal, can be performed by serology. Serology identifies the presence of virus-specific antibodies. Serology can also identify protective antibody levels that are effective against re-infection and can often be passed on to piglets through colostrum and milk to protect them from infection.

Our objectives for this proposal were to develop and characterize a rapid, specific, and sensitive ELISA for PDCoV and to share the reagents and protocols with the swine diagnostic and research community. We cloned, expressed and purified 4 PDCoV antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2). These antigens were then used to develop 4 separate ELISA assays to examine the antibody reactivity to each antigen in serum from infected pigs. Serum from confirmed PDCoV-positive animals was difficult to acquire. Serum samples were obtained from 300 animals of unknown PDCoV status, which came from farms in which PDCoV had been observed. These serum samples were then run on our PDCoV ELISA with all 4 antigens. We observed a few animals from each farm that seemed to be antibody positive based on the ELISA results, mainly with reactivity to PDCoV N protein. In order to determine the specificity and cut-off values for the ELISAs, we examined serum samples from known PDCoV-negative (44 samples) and PRCV-positive animals (175 samples). PRCV (porcine respiratory coronavirus), a close relative to transmissible gastroenteritis virus (TGEV), is a common coronavirus infection, and may have cross-reactive antibodies to PDCoV. The negative samples gave low values, but surprisingly the PRCV positive samples showed higher overall values for the N protein than that of the samples with unknown PDCoV status. Comparison to ELISAs with M, S1 and S2 as antigens indicated that N results were false positives, since all three of the other antigens gave consistently low ELISA results. Because of this cross-reactivity and the lack of known antibody positive samples, we are unable to truly test this ELISA. However, an ELISA is available in the event that PDCoV antibody positive samples are identified, which will allow for the optimization of the ELISA assay to decrease the cross-reactivity due to PRCV infection. Serum samples over a time course of infection would allow us to examine the dynamics of PDCoV antibody production and determine values for a true antibody positive sample. Unfortunately, a time course study with archived serum samples obtained at Kansas State University were not provided in spite of repeated requests.

Objectives:

Our objectives for this proposal were to:

1. Develop a rapid, specific, and sensitive ELISA for PDCoV antibodies in serum, feces, milk, colostrum and oral fluids.
2. Characterize ELISA performance on archived serum samples from herds with and without a PCR history of PDCoV.
3. Compare the ELISA to other assays, including IFA or SN. (planned when PDCoV cell culture becomes available)
4. Share the reagents and protocols with the swine diagnostics and research community.

The goal here was to apply our experience of rapid ELISA development for PEDV to produce ELISA tests for PDCoV that can be applied to diagnostics, epidemiology, and immunology investigations.

Materials & Methods:

Antigen production: The PDCoV genomic sequence 8734/USA-IA/2014 (Genbank ID KJ567050) was used to clone the 4 PDCoV candidate antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2). The gene sequences for each antigen were examined 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). This construct was 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), visualized using SDS-PAGE electrophoresis (Bio-Rad), and quantified using both a Bradford assay (Bio-Rad) and spectrophotometry (Bio-Tek Epoch).

ELISA development and validation: Four separate ELISA assays, using each of the purified antigens, were run following our PEDV ELISA protocol. Basically, ELISA plates were coated with 200ng of antigen, serum was diluted 1:50, and bound serum antibodies were detected using HRP-conjugated anti-Swine IgG (1:100,000 dilution, Bethyl labs) and TMB peroxidase substrate (KPL). The University of Minnesota Diagnostic laboratory provided 300 serum samples of unknown PDCoV status from 10 farms in which at least one oral fluid sample had tested positive for PDCoV and 175 serum samples from farms that were PDCoV negative and PRCV positive. A total of 44 PDCoV negative serum samples were obtained over the last 6 years from different laboratories at the University of Minnesota.

Growth in cell culture: An intestinal homogenate sample from an infected piglet that was positive for PDCoV by PCR was used to grow PDCoV in cell culture. The cell culture protocol for growing PEDV in cell culture was used for PDCoV growth. Basically, a small amount of filter sterilized intestinal homogenate was grown on VERO 81 cells for 24-48h, harvested, and grown for 2 more passages. PDCoV growth was confirmed by PCR at the University of Minnesota Diagnostic Laboratory.

Results:

Antigen production: PDCoV nucleocapsid (N), matrix (M), and spike protein subunits (S1 and S2) were synthesized and cloned into the pRSETa vector, which contains a His-tag (Life Technologies). Protein antigens were expressed in E. coli and purified using metal affinity chromatography on a cobalt column. Visualization of the purity of each antigen was determined by PAGE (Figure 1) and concentration was determined by Bradford assay and spectrophotometry.

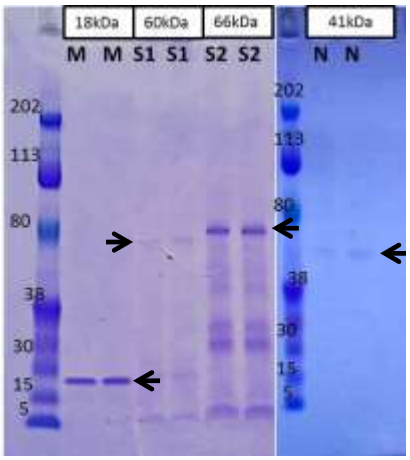

Figure 1. Purified PDCoV antigens. PDCoV proteins were expressed in E. coli and purified over a cobalt column. Purified proteins were visualized by SDS-PAGE. Purified protein bands are indicated by the arrows with the predicted size of each protein labeled at the top of the gel. The protein marker is labeled (KDa) in the first lane of the gel.

ELISA development and validation: Each antigen was used separately to develop an ELISA assay to determine the presence of PDCoV antibodies against each antigen. Serum samples of unknown PDCoV status (300 samples) from 10 different farms were analyzed using all 4 antigens (Figure 2). Each farm had at least one PDCoV PCR positive oral fluid sample. Unfortunately, the dynamics of when the farm was infected in regards to when the samples were obtained was not known and the same animals that were sampled for oral fluids were unlikely to be the same animals in which the serum was obtained. Even if the animals were PCR positive for PDCoV, it may be too early for the detection of anti-PDCoV antibodies. Overall, the N protein seems to have the most antibody reactivity. There was no correlation between the samples with the highest OD values in the 4 different ELISA assays.

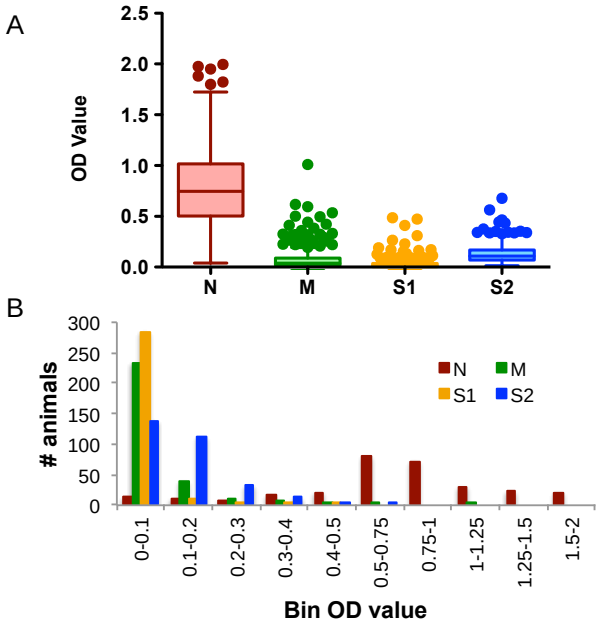

Figure 2. PDCoV ELISA analysis of pig serum. Pig serum from a farm that tested positive for PDCoV in at least one oral fluid sample was examined for the presence of antibodies to PDCoV N (maroon), M (green), S1 (gold), and S2 (blue) proteins. (A) Distribution of antibody levels is shown as box-whisker plots using the Tukey determination of whiskers. (B) Distribution of ELISA OD values for each PDCoV protein.

In order to determine which samples are truly positive and which might be due to background or cross-reactivity, a second set of samples was analyzed on all 4 antigen ELISAs. The first set of samples (44 samples) was from older serum samples banked in laboratory freezers (Fig. 3A,C) and the second set of samples (175 samples) was from PRCV positive and PDCoV negative farms (Fig. 3B,D). The negative samples gave low or no reactivity against any of the PDCoV proteins (Fig. 3A). Interestingly, the highest outlier in all 4 ELISAs is from the same animal. An examination of the distribution of OD values for each ELISA (Fig. 3C) suggests that a cut-off value for negative samples would be around an OD of 0.3-0.4. Cross-reactivity of these ELISAs was examined using the 175 PRCV positive (PDCoV negative) samples (Fig. 3B). PRCV is an alphacoronavirus that is an endemic infection on many farms. Unfortunately, these PRCV samples, which should be negative, gave higher overall values on the N-protein ELISA than that of the samples of unknown PDCoV status and based on the distribution of OD values, this seems to be true reactivity against the N protein (Fig.3D). This suggests that there is cross-reactivity between PRCV and PDCoV N. Comparison to ELISAs with M, S1 and S2 as antigens indicated that N results were false positives since all three of the other antigens gave consistently low ELISA results. Cross-reactivity with PRCV was not expected as the PDCoV and PRCV nucleocapsid proteins are not very similar (less than 25%).

Another way to test the sensitivity and specificity of the ELISA would be to examine samples over a time course following infection. We expected to accomplish this using a set of serum sample from a pathogenesis study conducted at Kansas State University. Unfortunately, we did not receive these samples despite our numerous requests. Because of the cross-reactivity with the PRCV positive samples, the lack of known antibody positive samples or samples over a time course of infection, we are unable to truly test this ELISA. However, an ELISA is available in the event that PDCoV antibody positive samples are identified. This will allow for the optimization of the ELISA assay and validation according to quality control/quality assurance critera, and

allow us to determine the best way to decrease the ELISA cross-reactivity due to PRCV infection.

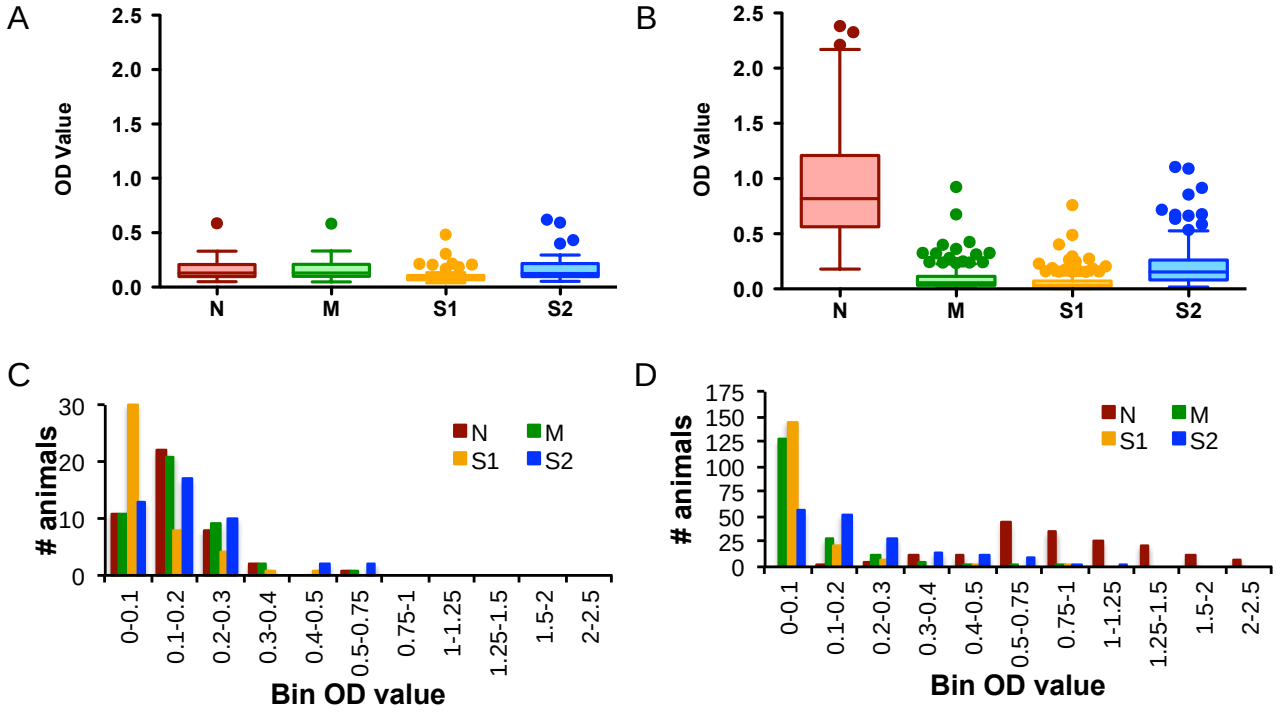

Figure 3. PDCoV ELISA cross-reactivity. Pig serum from (A) PDCoV negative farms and (B) PDCoV negative/PRCV positive farms were examined by PDCoV N (maroon), M (green), S1 (gold), and S2 (blue) protein ELISAs. (A,B) Distribution of antibody levels is shown as box-whisker plots using the Tukey determination of whiskers. (C,D) Distribution of ELISA OD values for each PDCoV protein.

Viral growth in cell culture: In order to develop an IFA or serum neutralization assay for PDCoV, a cell culture system for viral growth was needed. Since PEDV and PDCoV have similar growth in the pig, the PEDV cell culture system was used for proliferation of PDCoV. Growth of PDCoV was successful using intestinal homogenate that contained a very high level of virus. Cells were observed to die (CPE) due to viral infection within 24-48h post-infection and high levels of virus were obtained from infected cell cultures. The virus was then given to the University of Minnesota diagnostic laboratory for further testing and establishment of an IFA or serum neutralization PDCoV assays.

Discussion:

We have developed a PDCoV ELISA assay using 4 different PDCoV antigens; nucleocapsid (N), matrix (M), and the spike protein subunits (S1 and S2). All 4 antigens have been expressed and purified proteins are available for ELISA analysis. We examined serum samples from farms suspected to have PDCoV and observed reactivity mainly in the N protein ELISA. Analysis of negative samples suggested cut-off values at an OD of 0.4 for the N protein suggesting that the majority of the samples of unknown status are indeed PDCoV positive. However, when PRCV positive samples were examined to determine possible cross-reactivity in this assay, the N protein ELISA values were even higher in the PRCV positive samples than they were in the samples that were thought to be PDCoV positive. Comparison to ELISAs with M, S1 and S2 as antigens indicated that N results were false positives since all three of the other antigens gave consistently low ELISA results. However, due to this cross-reactivity the samples of unknown PDCoV status cannot be confirmed as antibody positive at this time.

Further optimization and validation of these 4 PDCoV ELISAs are needed, especially since PRCV cross-reactivity may be a problem. Ideally, examination of samples over a time course following infection would be performed on all 4 ELISAs. However, since pigs do not seem to be

getting sick with PDCoV currently, an optimization and validation of the assay is not an immediate concern. If PDCoV becomes a problem in the future, this research ELISA is available, which would hopefully be able to be quickly optimized once positive samples become available.