RESEARCH REPORT
SWINE HEALTH

Title: Development of reagents and serological assays for porcine deltacoronavirus (PDCoV) – NPB #14-184

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Industry Summary:
A novel porcine deltacoronavirus (PDCoV) was reported in China in 2012 and identified in the U.S. in early 2014. Since then, PDCoV has been identified in a number of U.S. states and linked with apparent clinical disease including acute diarrhea and vomiting in the absence of other identifiable pathogens. Since PDCoV was just recently linked with clinical disease, few specific antibody-based reagents were available to assist in diagnosis of PDCoV and limited serological capabilities were available to detect an antibody response to this virus. Therefore, the overall objective of this project was to develop and validate selected diagnostic reagents and assays for porcine deltacoronavirus (PDCoV) antigen and antibody detection. Specific objectives include:

1. Development of specific expressed protein and antibody reagents for diagnostic assay development and confirmation of virus isolation attempts, including critical reagents for immunohistochemistry (IHC), fluorescent antibody (FA) staining and serological and antigen capture assays.
2. Development and validation of an initial generation of serological assays for detection of antibody responses to PDCoV. These assays include indirect ELISA, fluorescent microsphere immunoassays (FMIA) and indirect immunofluorescence assays (IFA).

The nucleoprotein of PDCoV was expressed as a recombinant protein and purified for use as an antigen to immunize rabbits for specific antisera production. The resulting antisera was evaluated for use in fluorescent antibody staining methods to detect PDCoV infected cells following virus isolation attempts and for immunohistochemistry staining of intestinal tissues of infected pigs. These rabbit polyclonal antibodies appear to be specific for PDCoV and do not cross react with related viruses that cause similar disease syndromes, such as porcine epidemic diarrhea virus (PEDV) or transmissible gastroenteritis virus (TGEV). Therefore, they provide another valuable tool, in addition to PCR, for diagnostic laboratories and researchers to identify PDCoV

and differentiate it from other related viruses. These reagents have been widely distributed to assist with research studies and diagnosis of PDCoV.

The same expressed nucleoprotein was also used as an antigen to develop serological tests to detect the antibody response to PDCoV in pigs following infection. Several different types of tests were developed to determine the best assays for different applications. Serum samples from swine herds with recent documentation of PDCoV infection and samples from expected naïve herds were used for initial assay optimization. ELISA and FMIA tests were developed since these test formats allow for high-volume testing in diagnostic laboratories. The tests were optimized in a checkerboard fashion to reduce signal to noise ratios using samples of known status. Statistical analysis was performed to establish assay cutoff values and assess diagnostic sensitivities and specificities. At least 260 known negative serum samples and 180 known positive samples were evaluated on each assay. The ELISA showed test sensitivity of approximately 95% and specificity of approximately 94%. The FMIA showed a sensitivity of 95% and specificity of 98%. Both ELISA and FMIA detected seroconversion of challenged pigs between 8-14 DPI.

Once PDCoV was adapted to grow in laboratory cell culture systems, additional serology assays using cell culture adapted virus were developed. An IFA test to detect a general antibody response and a virus neutralization test to quantify neutralizing antibody responses were developed. All of these tests continue to be further optimized and validated. These new tests should allow producers to determine if animals have recently been infected with PDCoV. Since many aspects of PDCoV infection and transmission are still not fully understood, the reagents and assays developed in this project should provide valuable tools to help understand this disease and to aid in the control and surveillance of porcine deltacoronavirus outbreaks.

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Scientific Abstract:
A novel porcine deltacoronavirus (PDCoV) was reported in China in 2012 and in the U.S. in February 2014. Since then, PDCoV has been identified in a number of U.S. states and linked with apparent clinical disease including acute diarrhea and vomiting in the absence of other identifiable pathogens. PDCoV is currently diagnosed by real time PCR and clinical symptoms along with elimination of other viral pathogens known to cause similar disease. No specific antibody-based reagents were available to assist in diagnosis of PDCoV, and limited serological capabilities were available to detect an antibody response to this virus. Therefore, the objectives of this study were to develop readily available reagents for detection of PDCoV antigen in diagnostic tests, such as virus isolation, immunohistochemistry and fluorescent antibody techniques, and to develop and optimize several serological assays including indirect ELISA, fluorescent microsphere immunoassay (FMIA), indirect fluorescent antibody (IFA) and virus neutralization assays.

The full-length nucleoprotein (NP) of PDCoV was cloned and expressed in *E. coli* as a 41 kDa polyhistidine fusion protein. This protein was purified by nickel-NTA affinity column chromatography and is recognized in Western blotting and ELISA by convalescent serum from infected pigs. Both denatured and refolded versions of this protein were used to immunize rabbits for hyperimmune serum. Rabbit hyperimmune sera specifically recognize the NP and can be used in indirect fluorescent antibody staining and immunohistochemical staining procedures for the detection of PDCoV antigen. These reagents have been widely distributed to assist with research studies and diagnosis of PDCoV.

An IFA test for PDCoV serology was first developed using cell culture adapted virus. Serum samples from swine herds with recent documentation of PDCoV infection and samples from expected naïve herds were used for initial assay optimization. For development of ELISA and FMIA test formats, a refolded version of the same NP described above was used as an antigen. The tests were optimized in a checkerboard fashion to reduce signal to noise ratios using samples of known status. Receiver operator characteristic (ROC) analysis was performed to establish assay cutoff values and assess diagnostic sensitivities and specificities. Both FMIA and ELISA showed seroconversion of challenged pigs between 8-14 DPI. In addition, neutralizing antibody responses in serum are being quantified using a prototype fluorescent focus neutralization (FFN) assay. These new diagnostic tests provide additional tools to aid in the control and surveillance of porcine deltacoronavirus outbreaks.

Introduction:
Coronaviruses are enveloped, positive sense RNA viruses divided among several genera, including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and the recently described genus *Deltacoronavirus*. A novel porcine deltacoronavirus (PDCoV) was reported in China in 2012 (Woo, et al. 2012). Other important porcine coronaviruses include porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV); members of the genus *Alphacoronavirus* (Saif, et al. 2012). In February 2014, the Ohio Department of Agriculture announced the identification of PDCoV in the

U.S. Since then, PDCoV has been identified in a number of states and linked with apparent clinical disease including acute diarrhea and vomiting in the absence of other identifiable pathogens. PDCoV is currently diagnosed by real time PCR and clinical symptoms along with elimination of other viral pathogens known to cause similar disease.

Since no specific antibody-based reagents were available to assist in diagnosis of PDCoV, one purpose of this study was to develop readily available reagents for detection of PDCoV antigen in diagnostic tests, such as virus isolation, immunohistochemistry and fluorescent antibody techniques. Serological tests for the detection of antibody responses to PDCoV were also very limited. Therefore, another objective of this study was to develop and optimize several serological assays including an indirect ELISA, a fluorescent microsphere immunoassay (FMIA), and an indirect fluorescent antibody (IFA) test.

Both specific antibody-based reagents and serological tests are essential for the further study and control of PDCoV and the differentiation of PDCoV infection from other related diseases such as porcine epidemic diarrhea virus (PEDV) or transmissible gastroenteritis virus (TGEV). The tools developed during the course of this study can be applied to many ongoing and future studies to better understand and control PDCoV.

Objectives:

The overall objective of this proposal was to develop and validate selected diagnostic reagents and assays for porcine deltacoronavirus (PDCoV) antigen and antibody detection. Specific objectives include:

1. Develop specific expressed protein and antibody reagents for diagnostic assay development and confirmation of virus isolation attempts, including critical reagents for immunohistochemistry (IHC), fluorescent antibody (FA) staining, serological assays and antigen capture assays.
2. Develop and validate an initial generation of serological assays for detection of antibody responses to PDCoV. These assays include indirect ELISA, fluorescent microsphere immunoassays (FMIA) and indirect immunofluorescence assays (IFA).

Materials & Methods:

The full-length nucleoprotein (NP) of PDCoV was cloned and expressed in *E. coli* as a 41 kDa polyhistidine fusion protein. This protein was purified by nickel-NTA affinity column chromatography. Both denatured and refolded versions of this protein were used to immunize rabbits for hyperimmune serum production. Animal studies were approved by the South Dakota State University Institutional Animal Care and Use Committee (approval number 13-056A). Resulting antisera was evaluated for specific recognition of PDCoV NP or infected cells by Western blotting, fluorescent antibody staining methods, and immunohistochemistry on formalin fixed tissues of infected piglets. Specificity of reactivity was tested by Western blotting and fluorescent antibody staining using other swine coronaviruses including PEDV and TGEV.

An indirect fluorescent antibody test (IFA) was developed using cell culture adapted PDCoV provided by the National Veterinary Services Laboratories. Alternate rows of confluent swine testicular (ST) cells in 96-well plates were infected with PDCoV at approximately 100 fluorescent focus units (FFU)/well and remaining wells left as uninfected control wells. After 18-22 hr incubation at 37C, plates were fixed with 50% acetone/methanol at -20C, air dried and held at -20C until use. Test and control serum samples were added to paired wells covering a 2-fold dilution series from 1:20 through 1:160, incubated at 37C for 45 min, washed and incubated with fluorescein isothiocyanate-labeled goat anti-swine immunoglobulin G (Kirkegaard & Perry Laboratories, Inc.) for another 45 min. Plates were washed with PBS and cell monolayers examined by fluorescence microscopy for PDCoV-specific staining. Serum samples from swine herds with recent documentation of PDCoV infection and samples from expected naïve herds were used for initial assay optimization.

For development of indirect ELISA and FMIA test formats, both denatured and refolded versions of the PDCoV NP were initially evaluated, with the refolded version adapted for later optimization. ELISA microtiter plates were coated with 250 ng of antigen while Luminex microspheres for the FMIA were coupled at a concentration of 12.5 ug nucleoprotein per 6 $x10^6$ microspheres. The tests were optimized in a checkerboard fashion to reduce signal to noise ratios using expected seronegative and seropositive serum samples. The expected positive samples included samples from experimental infection and field serum samples from herds previously testing PDCoV positive by PCR. Expected negative samples included archived experimental serum collected prior to 2009 and field samples from high-health herds with no known history of PDCoV exposure. Initial receiver operator characteristic (ROC) validation and determination of diagnostic sensitivity and specificity of the PDCoV-NP indirect ELISA and FMIA assays was then conducted. ROC analysis was performed using MedCalc version 11.1.1.0 (MedCalc software, Mariakerke, Belgium).

Results:

The full-length NP of PDCoV was successfully expressed in *E. coli* as a 41 kDa polyhistidine fusion protein. This protein was purified by nickel-NTA affinity column chromatography and was recognized in Western blotting and ELISA by convalescent serum from infected pigs (Figures 1 and 2). Rabbit hyperimmune sera produced against denatured and refolded versions of the protein specifically recognized the NP and can be used in indirect fluorescent antibody staining at dilutions of 1:1000 to 1:5000 (Figure 3). The polyclonal antiserum was successfully used in immunohistochemical staining procedures for the detection of acutely infected villous enterocytes (Figure 4). Additionally, in a related project, monoclonal antibodies (mAbs) were produced to the PDCoV-NP to provide even more specific diagnostic reagents (Figure 5).

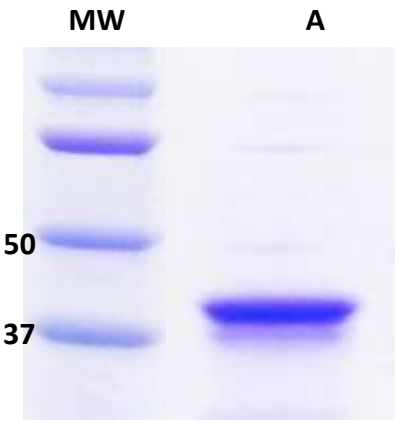


Figure 1. SDS-PAGE/Coomassie blue staining of expressed and purified PDCoV-NP antigen lane A.

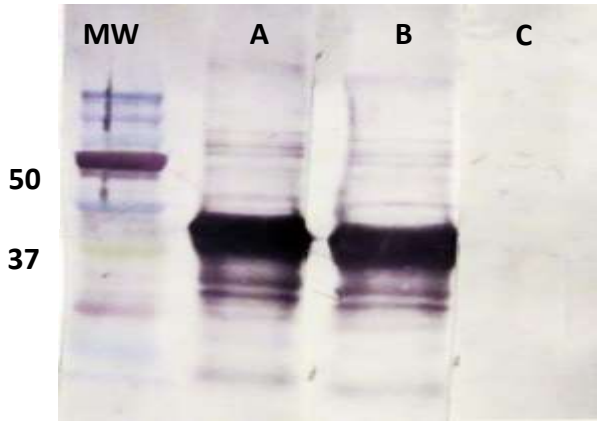


Figure 2. Western Blot showing serological detection of expressed PDCoV nucleoprotein and no cross reactivity with PEDV+ convalescent sera.
MW=Molecular weight ladder
A = anti-polyhistidine mAb
B = PDCoV+ convalescent swine serum.
C = PEDV+ convalescent swine serum.

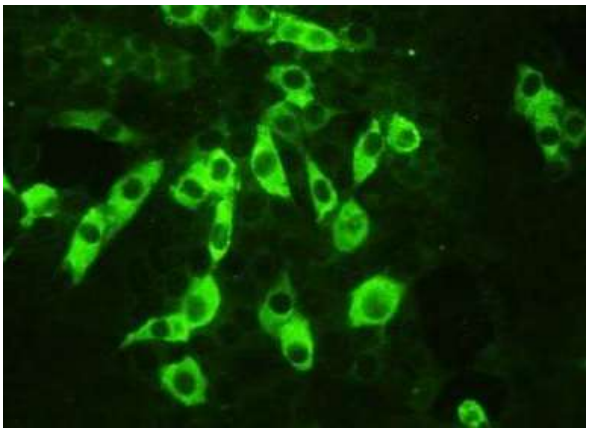


Figure 3. Indirect fluorescent antibody staining of PDCoV infected ST cells with rabbit anti-NP polyclonal antibody produced against refolded antigen.

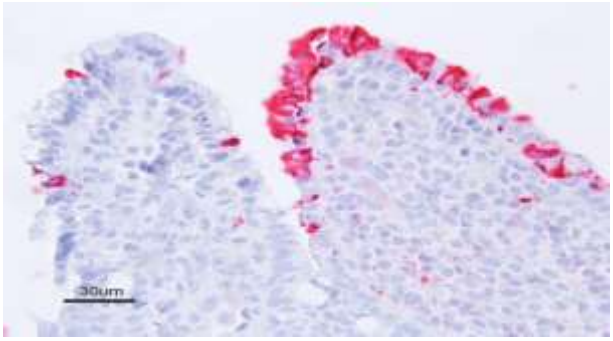


Figure 4. Immunohistochemistry staining of intestinal enterocytes with rabbit anti-NP in an acutely infected piglet (courtesy Dr. S. Vitosh, UN-L. NPB Project 14-182).

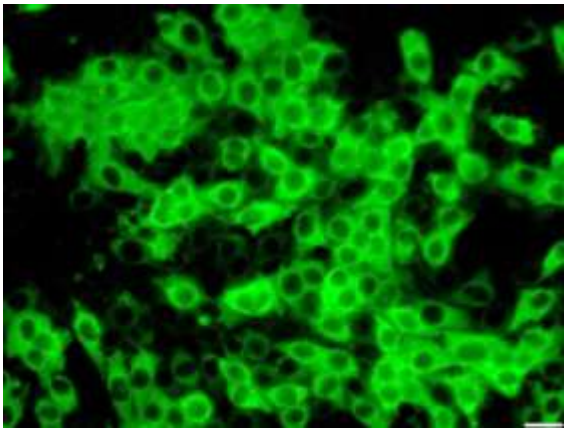


Figure 5. Indirect fluorescent antibody staining of PDCoV infected ST cells with PDCoV anti-nucleoprotein monoclonal antibody SD-55-197.

The indirect ELISA and FMIA serological tests were optimized and preliminary validation was initiated according to the five-stage ongoing process described by Jacobson (1998). Both denatured and refolded versions of the PDCoV NP antigen were evaluated. Slightly greater antibody capture efficacy was demonstrated using expressed antigen that was refolded back to a more native conformational state (Figure 6). Serological reference standards and internal quality control standards were also prepared and evaluated for each assay (Figure 7).

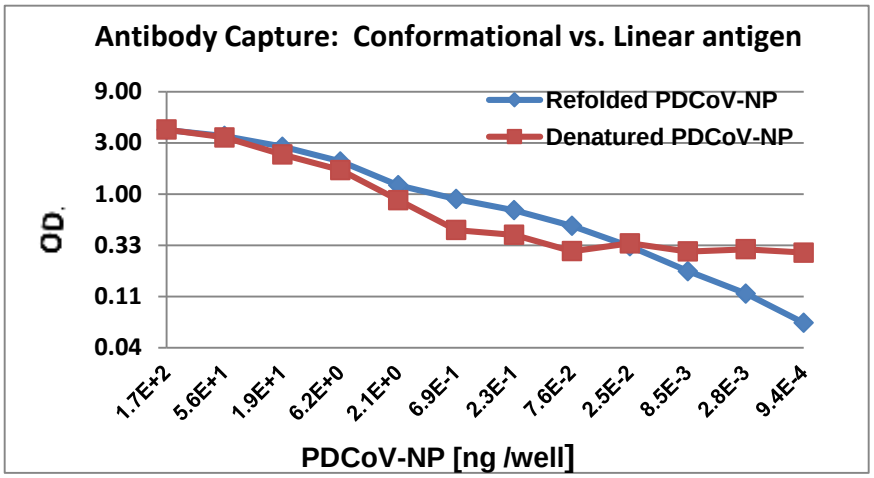


Figure 6: Comparison of denatured, linear NP antigen vs. refolded antigen.

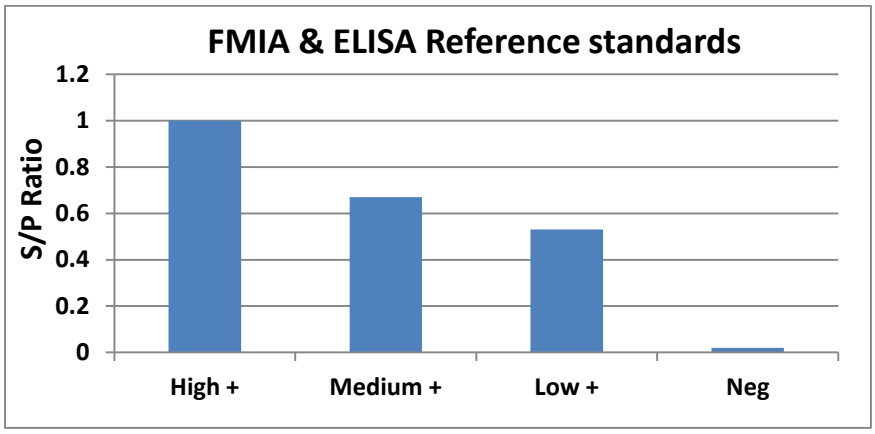
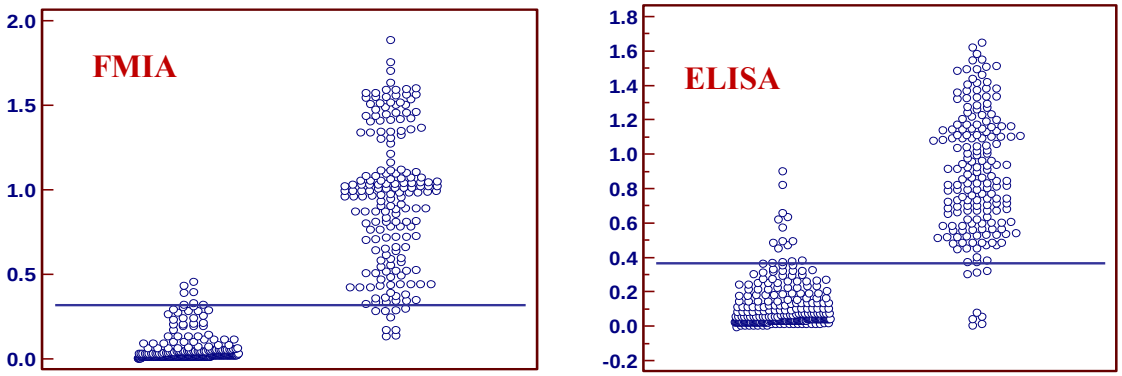


Figure 7. Serological reference standards and internal quality control standards for both ELISA and FMIA tests.

Both prototype serological assays were evaluated by receiver operator characteristic (ROC) analysis using samples of known status to determine optimum positive to negative cutoff values and assay sensitivity and specificity (Figure 8). Assays were then evaluated using serum sample sets collected soon after exposure to PDCoV and 28 days later (Figure 9), and sequential sample sets from an experimental challenge study (Figure 10). Statistical testing agreement is summarized in Table 1.



Negative N = 272 Positive N = 183
Sens. = 95.1%, Spec.= 98.1%
Cutoff = 0.32

Negative N = 260 Positive N = 191
Sens.= 95.6%, Spec.= 93.9%
Cutoff = 0.36

Figure 8. Initial receiver operator characteristic (ROC) validation and determination of diagnostic sensitivity and specificity of the PDCoV-NP FMIA and ELISA assays. Sensitivity and specificity were calculated using serum samples from expected PDCoV-infected and PDCoV-uninfected populations. 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 population and the plot on the right represents the positive population. The horizontal line represents the tentative cutoff value that gives the optimal diagnostic sensitivity and specificity.

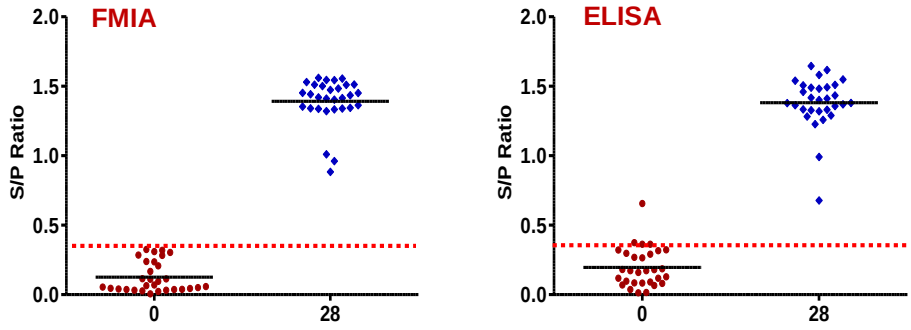


Figure 9. Prototype PDCoV FMIA and ELISA results from a group of 30 pigs sampled near the time of initial exposure and 28 days later. Both assays show clear seroconversion to PDCoV.

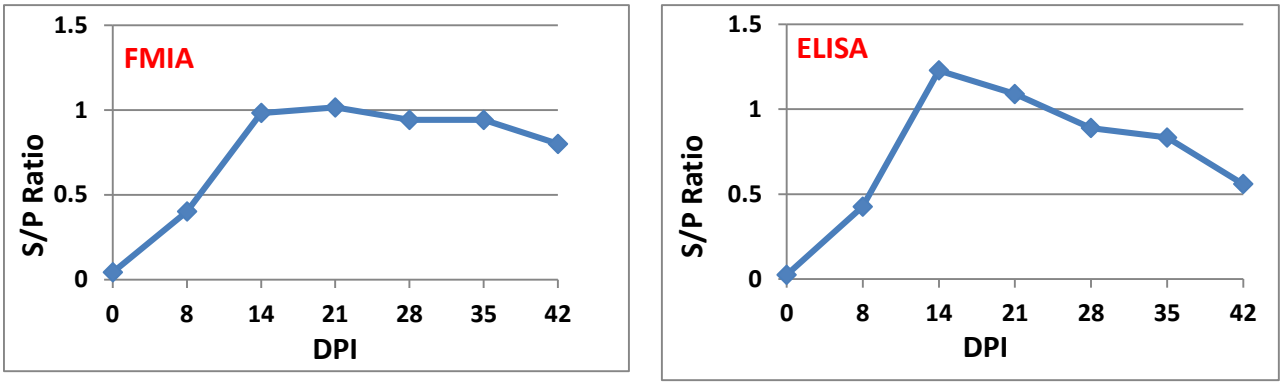


Figure 10. Antibody kinetic time course using serum from experimentally infected pigs associated with NPB Project 14-182 (Hesse, et al.). Serological responses detected by PDCoV ELISA and FMIA following challenge. Challenged animals show seroconversion between days 8 and 14.

	FMIA	Indirect ELISA	IFA
IFA	0.875	0.844	1
Indirect ELISA	0.863	1	0.844
FMIA	1	0.863	0.875
Positive	183	191	181
Negative	272	260	271
Total	455	451	455

Table 1. Statistical testing agreement. Multiple comparison, inter-rater agreement (kappa association) was calculated among all three tests. Kappa values shown represent a statistical measure of test agreement that demonstrates a significant agreement among all tests.

Discussion:

As a recently identified virus, the full, long-term impact of PDCoV on the swine industry is not yet fully understood. Field observations and recent research studies have suggested that the virus can cause substantial morbidity and mortality in nursing piglets (Vitosh-Sillman, et al., 2014). Therefore, the primary objective of this NPB funded project was to develop an initial generation of antibody-based diagnostic reagents and serological assays for the further study of PDCoV.

The expressed proteins and rabbit hyperimmune serum produced in this study have already proven to be valuable reagents for research and diagnostic applications. The PDCoV antisera provided an essential reagent for IHC studies associated with NPB Project 14-182 led by Dr. Dick Hesse. That project provided substantial information on the pathogenesis of PDCoV. The rabbit hyperimmune serum has been distributed to many research and diagnostic laboratories as a tool for the identification of PDCoV antigen. In separate but related work in our laboratory, hybridoma cell lines producing monoclonal antibodies against the PDCoV-NP were also developed. These monoclonal antibodies should provide greater specificity than the initial rabbit hyperimmune sera developed as part of this project. The monoclonal antibodies can also provide a long-term supply of reagents after the existing stocks of rabbit antisera are depleted. In addition to their application in the production of antibody-based reagents, the expressed proteins have been utilized in development of the serological assays discussed below.

Together, these new reagents should be of substantial value in the detection of PDCoV antigen in a variety of applications including: early verification of virus isolation attempts and virus titrations; immunohistochemistry staining of fixed tissues; fluorescent antibody staining of fresh tissues; development of field-based antigen capture assays such as lateral flow devices; and ELISA applications (competitive ELISA and antigen capture). Due to our limited understanding of PDCoV and other deltacoronaviruses, additional work needs to be done to better characterize the specificity of these reagents. Since many described deltacoronaviruses of other species have not yet been adapted to cell culture replication or fully characterized, we do not know if these reagents may cross-react with other members of the genus *Deltacoronavirus*. However, we were not able to demonstrate any cross-reactivity with other major swine coronaviruses including PEDV and TGEV.

Several new serological assays for detection of antibody responses to PDCoV were developed during the course of this study. The IgG ELISA and FMIA tests were based on a recombinant nucleoprotein antigen since this protein was expected to be highly conserved among PDCoV isolates. Both assays provide the capability of high-throughput testing with reasonable diagnostic sensitivity and specificity. The assays can clearly identify seroconversion of animals by 8 to 14 days post-exposure as shown in Figure 10 and show no cross-reactivity with PEDV. These results indicate that expressed and purified nucleoprotein is a useful antigen for the serodiagnosis of PDCoV in indirect ELISA and FMIA platforms. A refolded version of the protein was used as an antigen in both assays as it was shown to impart higher antibody capture efficacy. This may indicate that using antigen in its more native conformational state may present epitopes that are able to capture a larger

percentage of PDCoV-NP antibodies. Preliminary ROC analysis was performed and diagnostic sensitivities for both tests were greater than 95%. The FMIA demonstrated good specificity of 98.1% while the ELISA showed a lower specificity of 93.9%. Inter-rater (kappa) agreement, a statistical measure of test agreement, was calculated to be 0.875 between FMIA and IFA, 0.844 between ELISA and IFA and 0.863 between ELISA and FMIA, which demonstrated a significant level of agreement among tests. Full validation of a serological assay is a multi-step process including 5 stages defined by Jacobson. The stage involving final determination of accuracy parameters should include at least 700 known uninfected animals and 300 infected animals (Jacobson, 1998). Full validation of these assays will be completed in the coming months as additional samples of absolute known status become available.

The PDCoV IFA test was developed as soon as cell culture adapted PDCoV became available courtesy of Drs. Melinda Jenkins-Moore and Sabrina Swenson at the National Veterinary Services Laboratories, Ames, IA. This test allowed further characterization of samples used in the validation of the ELISA and FMIA and provides an alternative assay format for resolution of questionable test results. A virus neutralization assay for detection of antibodies that neutralize PDCoV *in-vitro* was also developed. This prototype assay is based on the fluorescent focus neutralization (FFN) assay we previously developed for PEDV and measures the ability of sample antibody to reduce the infection of host cells in a cell culture system. Additional well-characterized samples from animals exposed to PDCoV will be needed for the further validation of all these tests and to determine the duration of detectable antibody by each assay.

Although preliminary sensitivity and specificity determinations for the first generation serological assays described here were slightly less than ideal, these new assays should provide valuable tools for assessment of PDCoV exposure on a herd level. One explanation for the approximately 95% sensitivity values determined to date may be related to selection of the presumed positive validation populations. These sample sets were collected at approximately 3 weeks after initial diagnosis of PDCoV by PCR. It is possible that PDCoV may not move through a herd at the very rapid rate seen with PEDV. Therefore, some animals in some herds may not have been infected until a week or more after initial detection in the population, resulting in delayed seroconversion in a percentage of the presumed positive population. Likewise, since the initial origination and distribution of PDCoV in the U.S. is not fully understood at this time, it is possible that a small percentage of our presumed seronegative population may have been subject to prior exposure. Many of the samples in our presumed negative population were archived samples collected prior to 2009. However, some originated from recent field submissions from high health, biosecure herds with no clinical or PCR evidence of prior PDCoV or PEDV exposure. The observed specificity values of approximately 98% for FMIA and 94% for indirect ELISA are within the expected range for first generation assays using these test formats. Apparent false positive reactions could also be due to an epitope on the expressed antigen having some commonality with another agent or contaminants in the antigen preparations.

In summary, the rabbit antisera reagents developed here provide important research and diagnostic tools for the industry. They will continue to have value where protein-specific polyclonal antibody reagents are needed due to their ability to recognize multiple protein epitopes rather than a single specific epitope as is the case with monoclonal antibodies. The serological assays developed here allow the detection of antibodies developed in response to PDCoV infection. The PDCoV IgG ELISA and FMIA will allow high-throughput screening of swine serum samples. These tests should be adequately optimized and validated for sero-surveillance on a herd level, but further improvement is needed for full confidence on an individual animal basis. The IFA or other tests may be required for confirmation of individual unexpected results. Further work to better validate these assays is in progress and future research to adapt the assays to different sample matrices such as milk or oral fluids is needed. Since lactogenic immunity is likely critical for protection of nursing piglets, these assays will be modified for detection of IgA as well.

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