RESEARCH REPORT
SWINE HEALTH

Title: Generation of specimens of precisely known coronavirus infection status for the development and validation of highly-specific porcine coronavirus antibody assays.
- **NPB #15-142**

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Date Submitted: December 8 2016

Industry Summary:

- In the field, pigs are exposed to different coronaviruses that are known to share genetic and antigenic traits that may contribute to false-positive results. Recent evidence suggesting potential antigenic cross-reactivity between PEDV and TGEV and between PEDV and PDCoV has raised concerns about the specificity of PEDV serologic testing based on targets containing the most conserved antigenic regions (e.g., N, M, and WV).
- The major obstacle to the development and assessment of specific and sensitive porcine coronavirus antibody assays is the lack of diagnostic specimens from pigs of precisely known TGEV, PRCV, PEDV, or PDCoV infection status.
- Except under extremely rare circumstances, samples from animals in the field are not very useful in assay development because their true infection status is unknown. For this reason, it is not possible to interpret discordant results.
- The primary purpose of this project was to provide researchers the samples needed to continue the process of porcine coronavirus assay development and improvement. Assay development is not possible in the absence of a sufficient number of samples of precisely known infection status.
- In addition, samples shared among researchers will provide a common basis for comparing the performance of different tests. This common foundation provides for test development and improvement in various, independent laboratories simultaneously.

Keywords: Specimens, coronavirus infection status, porcine coronavirus, antibody assays, oral fluids, feces, serum.

Scientific Abstract:

The development of antibody-based assays is particularly important to detect coronavirus infected animals and to confirm previous virus exposure. As reviewed by Saif and Sestak (2006), the coronaviruses share genetic and antigenic traits in common. Thus, the antibody reaction against PEDV may not be automatic proof that the antibody was produced in response to this virus. In the field, pigs are exposed to different coronaviruses that are known to share genetic and antigenic traits that may contribute to false-positive results. For this reason, antibody cross-reactivity (false positives) among the porcine coronaviruses is a major concern in the development of pathogen-specific assays. This is an area that has not been adequately addressed. The problem addressed in this proposal was the development of antibody assays capable of detecting and differentiating antibodies against PEDV, PEDV variants, TGEV, PRCV, or PDCoV. In this study we successfully generated a bank of specimens (serum, oral fluids, feces) from pigs with precisely known coronavirus (PEDV, TGEV, PRCV, PDCoV) infectious status. The primary outcome of this project will be the development of improved, validated diagnostic assays for porcine coronaviruses.

Introduction:

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the order Nidovirales and the family Coronaviridae. Four genera, Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus, have been described (Woo et al., 2010; Woo et al., 2009; Woo et al., 2012). In pigs, three alphacoronaviruses (PEDV, TGEV, and PRCV), one betacoronavirus (PHEV), and one porcine deltacoronavirus (PDCoV) have been identified. PEDV, TGEV and PDCoV primarily cause enteric infections in pigs. PRCV has a predilection for the respiratory tract, but PRCV is a spike gene deletion mutant of TGEV and remains on the list of enteric coronavirus differentials. In contrast, PHEV infection ("vomiting and wasting disease") produces encephalomyelitis, rather than enteritis, and thus is not often considered when differentiating enteric infections. Ultimately, serology will be a vital tool in the management of the swine coronaviruses. In particular, the ability to identify the status of sows relative to PEDV lactogenic immunity using antibody-based assays will provide a powerful tool for the prevention and control of clinical PED in piglets. To achieve this goal, antibody assays of proven performance are needed. The current situation for swine coronavirus antibody assays is as follows:

- There is a commercial TGEV/PRCV differential blocking ELISA (Boehringer Ingelheim Svanova, Uppsala, Sweden) for the detection of antibodies to TGEV and PRCV in serum and plasma samples.

- Several PEDV ELISAs have been developed recently by U.S. researchers and are offered at veterinary diagnostic laboratories or through commercial entities. Immunofluorescence assays (IFA) and virus neutralization (VN) tests for PEDV antibody detection are also available in some laboratories. Although differences in performance are postulated, at this time we still lack data comparisons of the performance (sensitivity, specificity, accuracy) of these assays.
- No PDCoV antibody assays have been reported in the literature and PHEV antibody assays are limited (Chen et al. 2011).
- Cross-reactivity is an issue that cannot be neglected in the development of coronavirus antibody assays: Common antigens were demonstrated among TGEV, PRCV, canine coronavirus (CCV), feline infectious peritonitis virus (FIPV), and feline enteric coronavirus (FECV) by virus neutralization (VN), immunofluoresence (IFA), and monoclonal antibody reactivity against the S, M, or N structural proteins (Enjuanes 1995; Woods 1982).
- Recently, we found that antibodies against the U.S. PEDV prototype and antibodies against variant strains cross-reacted and cross-neutralized the two strains in vitro (manuscript in preparation). Thus, the IFA, VN, and whole virus-based ELISA tests detect antibodies against both the U.S. PEDV prototype and variant strains.
- Cross-reactivity among the S, M, and N structural proteins of TGEV and PRCV was observed using polyclonal antisera in immunoblotting assays (Callebaut et al. 1988). Some one-way immunoblotting cross-reactivity with the N protein has been reported for PEDV, FIPV, CCV, TGEV, and a putative mink coronavirus (Have et al. 1992; Zhou et al. 1988). Whether porcine coronavirus (PEDV, TGEV, PRCV, PDCoV and PHEV) structural proteins (N, S, M and E) contribute to cross-reactivity is not known.

Objectives:

The objective of this project is to create a bank of serum, oral fluid, and fecal specimens collected from pigs infected with PEDV, PDCoV, TGEV, or PRCV under experimental conditions and sampled over time post-inoculation. These specimens will be shared among project collaborators for use in the development and validation of diagnostic assays for PEDV, PDCoV and other porcine coronaviruses.

Materials & Methods:

The study was conducted under the approval of the Iowa State University Office for Responsible Research. Eighty-four 7-week-old pigs were purchased from a conventional wean-to-finish farm with no previous history of porcine coronavirus infections. The pigs were prescreened at the ISU-VDL for evidence of infection with PEDV, TGEV, PRCV, PDCoV, and PHEV. Pig fecal swabs

were tested for PEDV N gene-based rRT-PCR, PHEV N gene-based rRT-PCR, and PDCoV M gene-based rRT-PCR, while pig fecal and nasal swabs were tested for TGEV (S gene)/PRCV (N gene)-based differential rRT-PCR. The pigs' serum samples were tested with the PEDV immunofluorescence assay (ISU-VDL), PEDV WV ELISA (ISU-VDL), TGEV/PRCV differential ELISA (Svanova, Sweden), and PDCoV IFA (ISU-VDL). Selected porcine coronavirus-negative animals (n = 84) were randomized into six groups. Each group consisted of 12 pigs in one room, with 6 pens per room and 2 pigs per pen. Details related to virus strains and the routes used for experimental inoculation are presented below in the result section.

The pigs were closely observed twice daily for clinical signs throughout the study. Serum samples (n = 924) were collected from each group on days post-infection (DPI) –7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42, where pen-based feces (n = 2058) and oral fluid samples (n = 2058) were colleted daily throughout the study (from DPI -7 to 42). Virus shedding within groups and absence of cross-contamination between groups during the observation period (–7 to 42 DPI) was confirmed by rRT-PCR. At 42 DPI, all pigs were humanely euthanized by penetrating captive bolt (Accles and Shelvoke, Ltd., Sutton Coldfield, UK), followed by exsanguination. Specimens will be aliquoted and archived with the necessary descriptive information (animal number, specimen, DPI) immediately following collection.

Results:

1. **Screening herds to source naïve pigs:**

- Several commercial swine herds were screened for identification potential source of porcine coronavirus-free animals, i.e., Choice Genetics, PIC North America, AMVC, Spring Point, and Midwest Research Swine.
- Pigs were prescreened for evidence of infection with PEDV, PDCoV, TGEV, and PRCV. Only pigs virologically and serologically negative for PEDV, PDCoV, TGEV, and PRCV were used. Fecal swabs from pigs were tested for PEDV, PDCoV, and TGEV and nasal swabs were tested for PRCV by virus-specific RT-PCRs. Serum samples from individual pigs were tested by ISU PEDV IFA, ISU PEDV whole virus-based ELISA, TGEV/PRCV differential ELISA (Svanova), ISU S1-PDCoV ELISA, and ISU S1-PHEV ELISA.
- Midwest Research farm was selected as source of animals for this study.
- A second pre-screening was performed among animals within the selected farm all tagged for individual identification, two weeks before project starts.
- Selected animals were again tested at arrival (ISU animal facilities) by PCR and antibody-based methods.

2. Generation of bank of specimens:

- 84 pigs (~6 weeks of age) will be assigned into 5 treatment groups (n=12 per group) inoculated with different porcine coronavirus (i.e., PEDV, TGEV Purdue, TGEV Miller, PRCV, PDCoV, and PHEV) and a negative control group (n= 12) divided in two subgroups of 6 animals. Groups will be housed separately by room at the ISU Livestock Infectious Disease Isolation Facility. Each room will contain 6 pens. The 12 pigs in each treatment group will be distributed 2 pigs per pen. This design will allow collection of 6 replicates of pen-based specimens (oral fluid and fecal samples) per treatment group.
- To minimize the risk of potential cross-contamination between rooms, the animal study will be carried out in two different rounds (42 pigs/round; 4 groups/round) as show in Table 1 below:

Table 1. Porcine coronaviruses strains, inoculum dose and route used during experimental inoculations.

Group	No Pigs	Virus strain	Titer	Inoculation route	Status	Date
1	12	PEDV USA/NC/2013/35140	10^6 TCID50/ml	Orogastric	Terminated	Oct-Dec 2015
2	12	PRCV ISU-1 (propagated from PRCV 1998)	4^5 TCID50ml	Nasal	Terminated	Oct-Dec 2015
3	12	TGEV Purdue strain (ATCC VR763)	2.4^8 TCID50/ml	Orogastric	Terminated	Oct-Dec 2015
4A	6	Negative control	-	Oronasal	Terminated	Oct-Dec 2015
BREAK FOR CLEANING, DESINFECTION AND PLANNING 2nd ROUND						
5	12	PHEV Mengling strain; lot# 001-PDV; NVSL	HA titer 1:128	Oronasal	Terminated	Mar-May 2016
6	12	PDCoV USA/IL/2014 (NVSL)	1.5^6 TCID50/ml	Orogastric	Terminated	Mar-May 2016
7	12	Miller strain (ATCC VR1740)	1.8^6 TCID50/ml	Orogastric	Terminated	Mar-May 2016
4B	6	Negative control (culture medium)	-	Oronasal	Terminated	Mar-May 2016

1st round (TERMINATED):

- Group 1 (PEDV; n=12), group 2 (PRCV; n=12), group 3 (TGEV Purdue; n=12) and the negative subgroup 4A (n=6). Pigs within each room were housed in pens of 3 pigs/pen.
- Following inoculation, pigs were monitored for vomiting, diarrhea, dehydration throughout the observation period. The bank of specimens consisted of pen-based oral fluid and feces (2 pigs per pen) collected daily from -7 to 42 DPI. Individual serum samples will be collected at -7, -3, 0, 3, 7, 10, 14, 21, 28, 35, and 42 DPI. All samples were aliquoted within each room to avoid cross-contamination and stored at -80C thereafter.

2nd round (TERMINATED):

- Group 4 (PHEV; n=12), group 5 (PDCoV; n=12), group 6 (TGEV Miller; n=12) and the negative subgroup 4B (n=6). Pigs were housed and samples collected and aliquoted as described above for round 1.

Table 2. Summary of sample collection by specimen type

Group	Fecal swabs (individual pig)	Nasal swabs (individual pig)	Oral fluid samples (pen-based)	Fecal samples (pen-based)	Serum samples (individual pig)
G1 PEDV	216		294	294	132
G2 PRCV		216	294	294	132
G3 TGEV Purdue	216		294	294	132
G4 Negative	216	216	294	294	132
G5 PHEV	216	216	294	294	132
G6 PDCoV	216		294	294	132
G7 TGEV Miller	216		294	294	132

Discussion:

As a transboundary and emerging disease, porcine epidemic diarrhea was first detected in the U.S. in April 2013 and has resulted in severe economic losses to the US swine industry. In February 2014, PDCoV was detected in US swine. Two other porcine coronaviruses TGEV and PRCV are already endemic in US swine. A number of assays have been developed for PEDV antibody detection, but the performance of these assays has not been compared "head-to-head". So far there have been no serological assays commercially available for detection of PDCoV antibodies. As we more progress in our understanding of swine coronaviruses, we must determine whether we need to account for serological cross-reactions among porcine coronaviruses, e.g. PEDV, PDCoV, TGEV, and PRCV.

The creation of an extensive coronavirus specimen bank will provide the resource needed to address this question and will also accelerate the evaluation of the various coronavirus diagnostic assays that are currently under development. This study is a key step in providing the information swine veterinarians, producers, and diagnostic laboratories need to select tests that will provide the most accurate diagnostic results for PEDV, PDCoV and other porcine coronaviruses.

Specifically, the problem addressed in this proposal is the development of antibody assays capable of detecting and differentiating antibodies against PEDV, PEDV variants, TGEV, PRCV, or PDCoV. These specimens generated in this proposal are being shared among project

collaborators (i.e., UMN, SDSU, KSU, USDA) for use in the development and validation of diagnostic assays for PEDV, PDCoV and other porcine coronaviruses. Therefore, the primary purpose of this proposal is to provide researchers the samples of precisely known infectious status needed to continue the process of porcine coronavirus assay development and improvement. These samples will enable researchers to evaluate the specificity of the assays they are developing and/or performing. In addition, samples shared among researchers provide a common basis for comparing the performance of different tests. This common foundation provides for test development and improvement in various, independent laboratories simultaneously. In addition, the process of monitoring groups of pigs inoculated with PEDV, PDCoV, TGEV, or PRCV will provide further information on the dynamics of viral shedding and antibody responses against these viruses, as measured using contemporary assays.

The development of oral fluid and fecal antibody assays will provide for easier population monitoring and may provide a meaningful indicator of protection against porcine coronaviruses. That is, the development and validation of assays capable of detecting oral fluid and fecal antibody may provide the means of monitoring sow herds for levels of protective immunity. Although oral fluid antibody assays are old news, fecal antibody assays are still a novelty. Fecal antibody ELISAs have been reported for classical swine fever virus (Seo et al. 2012) and African swine fever virus (Gimenez-Lirola et al.2016).