RESEARCH REPORT
SWINE HEALTH

Title: Determination of the sites of tissue localization, routes of viral shedding, duration of virus carriage, kinetics of antibody response, and potential of aerosol transmission of Porcine Deltacoronavirus (PDCoV) following inoculation of nursing pigs and their dams – **NPB # 14-182**

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Industry Summary

Porcine deltacoronavirus (PDCoV) has recently emerged in the US. The purpose of this investigation was to determine the sites of tissue localization, routes of viral shedding, duration of virus carriage, kinetics of antibody response, and potential of aerosol transmission of porcine deltacoronavirus (PDCoV) following inoculation of nursing pigs and their dams.

Experimental Animals: Approximately 100 newborn pigs and their dams were obtained from a high health commercial source. All animals were housed in five BSL2 isolation rooms at the Life Science Annex at the University of Nebraska-Lincoln.

Numbering/Grouping: The litters/animals were grouped as indicated in the table below: Group A, (estimated 8 pigs/litter) was PDCoV inoculated. The contact control Group B (2 pigs/litter) were comingled with the inoculated Group A animals. The aerosol transmission Group C (estimated 10 pigs per litter) were not inoculated, but were housed in a separate pen in the common animal room. Negative, source matched controls (Group D) were housed in a separate wing of the animal facility and will serve as proof of freedom of herd source infection since it is not known how prevalent PDCoV is in the field.

Group	Treatment	# of Animals
A	PDCoV oronasal inoculated	47 (6 litters)
B	None—Contact Control	8 (2 from 4 litters)
C	None—aerosol transmission controls	20 (2 litters)
D	Negative Controls	10 (1 litter)
A-Dams	PDCoV oronasal inoculated	6
C-Dams	None—aerosol transmission controls	2
D-Dams	Negative Controls	1

Challenge: The challenge was a pool of gut derived intestinal content that was obtained from a field case and amplified in nursing age pigs to confirm infectivity and purity. The inocula was "titered" for PDCoV nucleic acid content using a real-time PCR assay. Nursing pigs to be challenged and their dams were inoculated at 2-3 days of age via intranasal and oral routes with 5 ml of inocula per route.

Sampling Requirements/Challenge Scheduling: On the days of blood collection, a serum tube was drawn. The sera were used for validation of various antibody assays (IFA, ELISA/Luminex, and SN). Sera, oral fluids, and nasal and fecal swabs were used for the determination of the presence of viral nucleic acid using RT-PCR. At regular time points, inoculated pigs were euthanized and a thorough postmortem examination was conducted. Complete sets of tissues were collected and frozen or fixed in formalin for evaluation of disease severity by histopathology and location of virus within tissues and cell types by PCR and IHC. Tissue samples collected were turbinates, tonsils, esophagus, stomach, duodenum, jejunum (two segments), ileum, cecum, spiral colon, descending colon, trachea, lungs (representative samples from cranial, middle, and caudal lobes), liver, spleen, kidney, thymus, and submandibular, tracheobronchial, mesenteric and inguinal lymph nodes. PDCoV shedding was monitored by performing real-time PCR on fecal and nasal swab samples and oral fluids. Serum samples were collected in order to monitor viremia and antibody response. Fresh and formalized tissues were collected from randomly selected Group A pigs at days 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 21, 28, 35, and 42 post-inoculation in order to monitor tissue tropism of the virus and histopathology.

Study data indicate the following:

Summary of clinical signs:
Clinical signs following infection:

- Inoculated sows developed soft feces on day 2 post-inoculation and diarrhea on day 3 post-inoculation.
- All sows were clinically normal after day 8 post-inoculation.
- Aerosol contact sows developed soft to diarrheic feces on day 5 of the study and returned to normal after day 8.
- Non-infected control sows remained clinically normal throughout the study.
- Inoculated piglets developed soft to diarrheic feces on day 2 post-inoculation, as did the contact controls.
- Morbidity was 100%.
- Mortality was variable among the litters, but was especially high in two of the six inoculated litters. All piglets from those two litters were either euthanized due to severe dehydration or had died by day 8 post-inoculation.
- Aerosol contact litters developed diarrhea on days 3 and 4. All surviving piglets had returned to normal by day 12.
- Non-infected control piglets remained clinically normal.

Virus detection via PCR demonstrated that:

- There was no significant difference between detection rates of inoculated animals versus contact control animals.
- The negative-negative control group remained free of virus throughout the duration of the study.
- Viremia was evaluated in a subset of pigs post inoculation due to IACUC restrictions for blood volume/size of the animal. Viremia was detected at one day post-inoculation in 60% of the pigs tested and remained present in all of the pigs tested until four days post-inoculation, at which time the percent positive fell to 25% at day five and all animals were negative for viremia thereafter.
- Virus detection rates in fecal and nasal samples were generally similar, with fecal samples tending to have a higher virus load than the nasal samples.
- In contrast to viremia, fecal and nasal samples were negative for virus at one day post-inoculation.

- In both sample types, virus was detected at high to moderate levels from 2 to 10 days post-inoculation. Approximately 70% of the samples were positive at day 14, 25% of the samples were positive at day 21, and no samples were positive at 28 days post-inoculation and thereafter.
- Oral fluid samples were positive at the first collection point at 14 and 21 days post-inoculation. The day 28 samples contained low levels of virus in 4 of the 6 infected litters and were negative for virus in all litters at 5 and 6 weeks post-inoculation.
- Virus is clearly detected in the lymph nodes and small intestine of all of the infected animals (sows and their nursing pigs) at 35 and 42 days post-inoculation. In contrast, fecal and nasal samples were negative during those same time points.

Virus detection via immunohistochemistry (IHC) in tissues demonstrated that:

- Rabbit anti-PDCoV nucleocapsid antibody that was kindly provided by Dr. Eric Nelson from SDSU works well as a detection antibody for IHC.
- At 1 day post-inoculation, all PDCoV-inoculated pigs displayed clear positive staining in enterocytes of the jejunum and ileum.
- All inoculated pigs sampled continued to be positive in enterocytes of the jejunum and ileum on days 2 through 6.
- The percentage of individuals with positive staining in the jejunum and ileum quickly declined through days 7 and 8, and no positive staining was observed in samples from day 10 and thereafter.
- On days 2-5 and 8, the majority (71-100%) of the mesenteric lymph nodes were positive. No mesenteric lymph nodes were positive on day 10 or thereafter.
- Other immunopositive tissues included duodenum, cecum, and spiral colon. A lower percentage of pigs had positive staining in these other intestinal segments (typically varying from 0 to 50%), and often the staining affected a low number of scattered enterocytes.
    - Positive staining in the duodenum was observed days 2-6. The spiral colon was only detected positive on days 2 and 3, and cecum samples were variably positive on days 2, 3, 4, and 6.
- No positive immunoreactivity was observed in other tissues examined.
- Pigs from the contact control and aerosol control sacrificed at their selected time points were similar to the inoculated pigs.
- Consistent with the inoculated pigs, no positive immunoreactivity was observed in tissues other than the intestinal tract and mesenteric lymph node.
- The sacrificed negative control pigs remained negative by immunohistochemical analysis throughout the study.

Histologic evaluation demonstrated that:

- Mild to severe atrophic enteritis was detected in sections of jejunum and ileum from days 2-8 post-inoculation. No lesions were observed in sampled pigs on day 10 or thereafter.
- These lesions were characterized by enterocyte vacuolation and attenuation, villous blunting, and occasional villous fusion.
- These lesions correspond to the virus distribution in the jejunum and ileum detected with immunohistochemistry.
- No lesions other than atrophic enteritis were associated with virus infection.

Antibody response via the indirect fluorescent antibody (IFA) and serum neutralization (SN) assays:

- Cell culture adapted virus that was kindly provided by Dr. Sabrina Swenson from the NVSL was used to infect cells that served as the detection substrate for an indirect fluorescent antibody assay and a serum neutralization assay.

- The negative/negative control animals (sows and their pigs) were PCR negative and also negative in the IFA and SN assays throughout the entire study.
- Inoculated animals (sows and their nursing pigs), contact controls, and room aerosol controls all seroconverted and were shown to be IFA and SN positive by 14 days post-inoculation and remained positive through the conclusion of the study at 42 days post-inoculation.

Important finding that may impact transmission of the virus in the field:

- Pigs still harbored detectable virus RNA in the small intestine and mesenteric lymph nodes 42 days post-inoculation.
- Sows still harbored detectable virus RNA in the small intestine and mesenteric lymph nodes 35 days post-inoculation.
- Aerosol transmission readily occurred.

Important gaps in our knowledge of PDCoV—where there is smoke there is usually fire.

- Understand transmission.
  - Biological and physical routes.
- Define age differences following PDCoV infection.
  - Duration of shedding.
  - Persistence.
- Develop a good understanding of virus stability
- Investigate national and regional sero prevalence
- Develop efficacious vaccines.
  - Lactogenic immunity

Keywords: novel coronaviruses, porcine deltacoronavirus, porcine diarrhea, porcine enteric coronavirus

Scientific Abstract:

Porcine deltacoronavirus (PDCoV) is a newly identified virus which has been detected in swine herds of North America associated with enteric disease. The aim of this study was to demonstrate the pathogenicity, time course of infection, virus kinetics, and aerosol transmission of PDCoV using 87 conventional piglets and their 9 dams, including aerosol and contact controls to emulate field conditions. Piglets 2-4 days of age and their sows were administered an oronasal PDCoV inoculum with a quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR) Ct of 22 generated from a field sample having 100% nucleotide identity to USA/Illinois121/2014 and testing negative for other enteric disease agents using standard diagnostic assays including metagenomic sequencing. Serial samples of blood, serum, oral fluids, nasal and fecal swabs, and tissues from sequential necropsy, conducted daily from days 1-8 and regular intervals thereafter, were collected throughout the 42-day study for qRT-PCR, histopathology, and immunohistochemistry (IHC). Diarrhea developed in all inoculated and contact control pigs, including sows, by 2 days post-inoculation (DPI) and aerosol control pigs and sows by 3-4 DPI, with resolution occurring by 12 DPI. Mild to severe atrophic enteritis with PDCoV antigen staining was observed in the small intestine of affected piglets from 2 to 8 DPI. Mesenteric lymph node and small intestine were the primary sites of antigen detection by IHC, and virus RNA was detected in these tissue samples to the end of the study. Virus RNA was detectable in piglet fecal and nasal swabs to 21 DPI, and sows to 14-35 DPI.

Introduction

Coronaviruses (family *Coronaviridae*) are large enveloped viruses that contain a helical nucleocapsid and a single-stranded, positive sense RNA genome, which can vary in size from 25.4 to 31.7 kb.[13,24] There are currently four recognized genera of coronaviruses, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and, the most recently described, *Deltacoronavirus*.[1] The genus *Alphacoronavirus* contains two important porcine enteric pathogens: transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV). Transmissible gastroenteritis virus has been recognized worldwide for decades as a cause of atrophic enteritis in infected piglets, leading to severe diarrhea, vomiting, inappetence, and subsequent dehydration and death. High mortality rates (often 90 to 100%) are observed in infected naïve nursing piglets, while infections in growing pigs and adults may go undiagnosed due to the much milder clinical course.[16,17] Porcine epidemic diarrhea virus also causes atrophic enteritis and severe diarrhea, and has been documented to occur in all ages of swine.[25] In April of 2013, PEDV was detected for the first time in the United States in outbreaks of diarrhea, and led to widespread, severe morbidity and mortality in suckling piglets, of which the effects on the swine industry continue to linger more than a year after the outbreak.[21]

A novel virus in the *Deltacoronavirus* genus has also been recently associated with swine. Porcine coronavirus HKU15, more commonly known as porcine deltacoronavirus (PDCoV), was first detected in 2012 during a retrospective study conducted in Hong Kong using PCR surveillance to examine rectal swabs from animals including domestic pigs.[24] The virus was detected on 5 Ohio farms experiencing outbreaks of diarrhea in sows and piglets in 2014. These outbreaks were unique in that the majority of affected animals had detectable PDCoV in the absence of other pathogens known to cause enteric diseases in swine.[23] Since this report, PDCoV has been detected in many herds in the United States and Canada.[14] Since this study was conducted, PDCoV has been isolated and propagated in swine testicular and LLC porcine kidney cell cultures.[7] Few studies have reproduced clinical diarrheal disease secondary to experimental PDCoV infection in gnotobiotic and conventional pigs, but these studies have been limited in piglet numbers and study time course.[3,9,11] The objective of the current study was to demonstrate the primary pathogenicity of PDCoV, evaluate for aerosol transmission of virus, and study the clinical course of infection using multiple diagnostic modalities including histopathology, quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR), and PDCoV specific immunohistochemistry (IHC).

Materials and Methods

Challenge inoculum preparation

A pilot study was conducted to generate sufficient quantity of infectious inoculum for the primary experiment. Preliminary virus inoculums were prepared from 2 field specimens found to be positive for PDCoV by qRT-PCR assay (SDSU Diagnostic Lab), inoculum A from a sow with a history of diarrhea (PDCoV Cycle threshold (Ct) value 18.66) and inoculum B from a neonatal pig (PDCoV Ct value 19.59). The pilot study was conducted using 3 groups of 3-one-day-old piglets, with one group receiving inoculum A, one group receiving inoculum B, and one serving as the source-matched negative control. The PDCoV preliminary inoculum material tested negative for other common enteric viruses (TGEV, PEDV, Rotaviruses A, B, C) by PCR, enterotoxigenic *E. coli* by PCR, and bacterial pathogens by culture. Inoculums were prepared by dilution of fecal or gut contents in physiologic phosphate buffered saline (PBS) and clarified by stepwise centrifugation at 400 x g for 20 min and again at 4,000 x g for 20 min. The supernatant was aliquoted into PBS, and gentamicin

was added to a final concentration of 50 µg/mL inoculum. Control inoculum from normal piglets was prepared in an identical manner.

Piglets were sourced from a closed commercial farrowing facility that was negative for PEDV by serology and PCR testing, and negative for other enteric viruses by PCR. The piglets were separated into three biosecure animal holding rooms (UNL Life Science Annex), maintained in stainless steel cages with heat lamps and fed Similac supplemented with gentamicin at a maintenance nutrition level. After one day of acclimation, piglets were gavaged with 5.0 mL clarified inoculum using a gavage needle and syringe. Twenty-four hours after inoculation the animals receiving inoculum A were vomiting, passing large amounts of liquid feces, and significantly dehydrated. All of the piglets that received inoculum A were euthanized and intestinal contents collected for qRT-PCR. Tissues were collected for histologic examination and qRT-PCR. The intestinal contents were stored at 4ºC for use in the primary experiment. The piglets administered inoculum B did not generate a productive infection, as determined with negative fecal qRT-PCR results and a lack of clinical disease.

The primary experiment inoculum was prepared by pooling intestinal contents with low PDCoV qRT-PCR Ct values from those inoculated animals in the preliminary study exhibiting clinical disease. Each animal sample tested qRT-PCR negative for TGEV, PEDV, Torovirus, and Rotavirus (A, B, and C). The pooled sample was clarified by centrifugation and prepared in a manner similar to the previous inoculum with the addition of gentamicin for a final concentration of 50 µg/mL. The inoculum was increased in volume to 650 mL with high antibiotic Minimal Essential Media (EMEM base media, 1.25 µg/mL Amphotericin B, 50 units/mL penicillin, 50 µg/mL streptomycin, 0.02 mg/mL ciprofloxacin), and divided into aliquots with a final qRT-PCR Ct of 22. Pooled challenge inoculum was also cultured and found negative for *Salmonella* sp., Enterotoxigenic *E. coli*, and *Clostridium perfringens*.

Metagenomic sequencing was performed on the primary challenge inoculum and a clinical sample from an experimental pig post-inoculation to determine both the PDCoV genome sequence and identify other extraneous viruses present in the sample. The library was prepared using a method previously described.[18] Approximately 2.5 million reads (challenge material) and 3.2 million reads (clinical sample) were generated on an Illumina MiSeq instrument using paired end 150 bp reads. Reads mapping to host DNA were subtracted and the remaining sequences were assembled *de novo* using CLC Genomics Workbench[a] into 135 contigs and classified based on BLASTN (Basic Local Alignment Search Tool Nucleotide) expectation (*E*) scores. Besides the expected PDCoV genome sequences, the only eukaryotic virus identified was IAS virus (challenge material) and porcine kobuvirus (clinical sample). A total of 20,516 reads mapped from the challenge material to a reference PDCoV genome yielded only 9% genome coverage; however, the clinical sample identified 81,361 reads spanning 99% of the PDCoV genome. The consensus sequence had 99% nucleic acid identity to all other PDCoV genomes in GenBank, and 100% nucleic acid identity to other U.S. Midwestern strains including USA/Illinois121/2014 and OhioCVM1/2014.

Animal infection

Nine near-term gestating sows were obtained from the same source as the pilot study, and were housed in the same facility and maintained in separate standard farrowing stalls. The sows were farrowed in a window of two days following a standard induction protocol. The sows and piglets of their subsequent litters were randomly divided into four groups: group A, PDCoV inoculated (47 piglets, 6 sows); group B, contact control

(8 piglets originating from 4 group A sows); group C, aerosol control (20 piglets, 2 sows); and group D, negative control (12 piglets, 1 sow). All litters were kept intact and there was no cross fostering. The pigs in group A were inoculated with the developed challenge inoculum at the start of the study (day 0), when piglets were approximately 2-4 days of age. The pigs in group B (approximately 2 per litter) served as a contact control which were not inoculated and were littermates of the pigs of the inoculated group A animals. The group C aerosol control pigs were not inoculated, housed in a separate pen in the common animal room as a group A sow, and separated by a distance of 0.6 m and a floor to ceiling length solid plastic curtain. Air movement was possible around the periphery of the curtain, but particles could not directly pass between farrowing stalls of the sows. Manipulations were performed on aerosol control (group C) animals first by individuals wearing clean disposable coveralls, gloves, masks, headcovers, and disinfected boots which were left in their respective rooms. The curtain and floor space between farrowing stalls was disinfected after each manipulation. Source-matched negative control animals were designated as group D, and were housed in a separate designated room. Animal care staff was assigned to exclusively collect samples in each room to minimize the possibility of cross contamination.

Clinical evaluation

The animals were observed daily for the following clinical signs: diarrhea, dehydration, wasting, coughing, sneezing, lethargy, ocular discharge, and any other unexpected clinical manifestation of disease. Fecal scoring was recorded using the system: 0 = no feces noted, 1 = normal feces, 2 = soft but formed feces, and 3 = diarrhea.

Necropsy and sample collection

A small subset of pigs from group A were sequentially euthanized on predetermined DPI, and a thorough postmortem examination was conducted. Pigs from group B, C, and D were more variably sampled during select study days (Fig. 1).

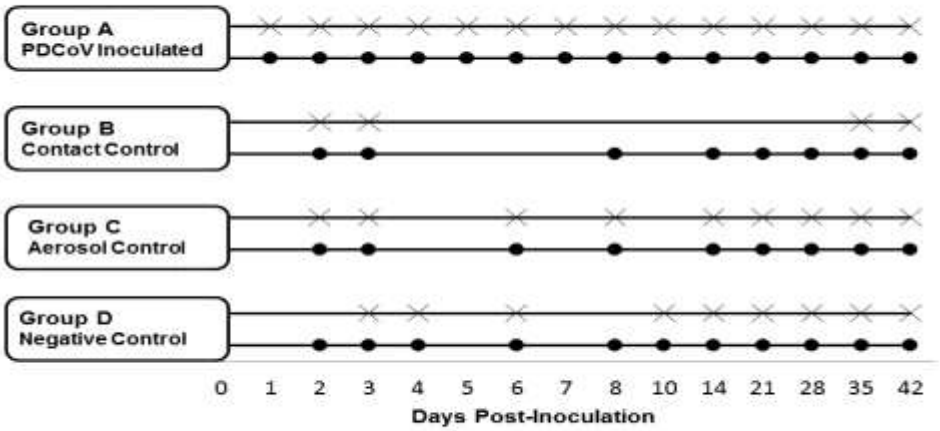

Figure 1. Experimental design indicating piglet blood sample collection and sequential sacrifice. Within each experimental group, circles denote collection of blood samples and the crosses indicate necropsy was performed on a representative sampling of the group.

Pigs severely affected by dehydration or if moribund were selectively chosen for euthanasia and necropsy. A complete set of tissues, including tonsil, nasal turbinate, esophagus, stomach, trachea, cranial lung lobe, middle lung lobe, caudal lung lobe, submandibular lymph node, proximal jejunum, distal jejunum, thymus, ileum, tracheobronchial lymph node, cecum, spleen, liver, spiral colon, mesenteric lymph node, kidney, descending colon, inguinal lymph node, and duodenum, was collected from each pig, including fresh-frozen and formalin-fixed specimens. Serum and whole blood was also collected from each necropsied pig. Nasal and fecal swabs were collected from all available piglets and sows at each sampling time point post-inoculation, and whole blood and serum were collected more variably (Fig. 1). Oral fluids were collected from each litter at 14, 21, 28, 35, and 39 DPI. Oral fluid collection ropes from a commercial kit[b] were applied to each litter's farrowing stall and collected and processed according to the manufacturer's directions. Blood and serum was collected from sows at 8, 14, 21, 28, and 35 DPI, and all sows were sacrificed and examined by necropsy at 35 DPI. Mortality data was analyzed using Kaplan-Meier survival curve analysis generated with commercially available software[b]. Pigs noted as moribund and euthanized or dead were considered as deaths in analysis.

Polymerase chain reaction

Serum, fecal swabs, nasal swabs, fresh-frozen tissues, and oral fluids collected from the experiment were subjected to qRT-PCR analysis. For virus RNA extraction, 50 µL of each sample were loaded into a deep well plate and extracted using a Kingfisher 96 magnetic particle processor[d] and a viral RNA isolation kit[e] according to the manufacturer's instructions with one modification, reducing the final elution volume to 60 µL. At least one negative extraction control consisting of all reagents except sample was included in each extraction. The extracted RNA was frozen at -20°C until assayed by qRT-PCR.

A duplex qRT-PCR was designed for the dual purpose of detecting PDCoV in samples by targeting the virus polymerase (RdRp) and monitoring extraction efficiency by targeting the 18S ribosomal RNA subunit as an internal control. Primers and probes for porcine deltacoronavirus (PDCoV-F: 5'-GTGCATGC TCTTTGTGGAT-3', PDCoV-R: 5'-TAGGGTCAACCTTGGTGAGG-3', PDCoV probe: 5'-FAM-TTCAGA ACCTTGAACGTTACATCTCA-BHQ1-3') and 18S (18S-F: 5'-GGAGTATGGTTGCAAAGCTGA 3', 18S-R: 5'GGTGAGGTTTCCCGTGTTG-3', 18S probe: 5'-Cy5AAG GAATTGACGGAAGGGCA-BHQ2-3') were used in conjunction with the One-Step RT-PCR kit[d] in a 20 µL reaction volume. The qRT-PCR reaction mix consisted of 3.5 µL nuclease-free water, 10 µL 2x Reaction Buffer, 1.0 µL PDCoV forward and reverse primers (10 µM each), 1.0 µL 18S forward and reverse primers (10 µM each), 0.5 µL PDCoV FAM-labeled probe (10 µM), 1.0 µL 18S Cy5-labeled probe (10 µM), 1 µL One-Step RT-PCR enzyme mix, and 2.0 µL extracted RNA. Each qRT-PCR plate was run on a Real-Time PCR Detection System[f] under the following conditions: 48°C for 10 min; 95°C for 10 min; followed by 45 cycles of 95°C for 20 sec, 58°C for 20 sec and 72°C for 30 sec. Positive controls consisting of synthetic PDCoV template and negative extraction and negative amplification controls were included in each run. Real time cycle threshold bar was set to the middle of the linear range of the amplification curves under log view. If a sample crossed the Ct before 38 cycles, the sample was considered positive. Based on quantification data estimating RNA copy numbers, this Ct value represents approximately 1-10 copies of virus RNA/reaction.

PDCoV antibody

An anti-PDCoV antibody was generated for use in IHC and other serologic assays. Briefly, the antibody was developed cloning the full-length nucleoprotein (NP) of PDCoV and expressing it 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. Purified recombinant NP was used as an antigen to hyperimmunize rabbits. Hyperimmune serum was collected and processed for use in assays. This serum antibody was shown to recognize the NP of purified PDCoV by Western blotting and fluorescent antibody staining of infected cell cultures, but did not react with PEDV or TGEV under similar conditions.

Histopathology and immunohistochemistry

For all pigs, representative formalin-fixed samples from the complete set of tissues collected at necropsy were routinely processed and embedded in paraffin blocks within 10 days after collection. Tissues were sectioned at 4 µm, stained with hematoxylin and eosin, and examined with light microscopy at the Veterinary Diagnostic Center, University of Nebraska-Lincoln.

The formalin-fixed paraffin-embedded tissues examined histologically were also stained for IHC. One section was evaluated for each tissue. There were some non-uniform sample sizes based on the pig age and available tissue from collection. The sections were cut at 4 µm and applied to slides, which were deparaffinized and stained using an automated immunohistochemical stainer[g]. The primary antibody consisted of the anti-PDCoV polyclonal rabbit serum previously developed. Positive and negative controls for PDCoV staining consisted of a slide containing known positive tissue collected in the pilot study along with slides of test samples using an irrelevant antibody, normal rabbit polyclonal immunoglobulin[g]. The positive control tissue was examined with standard laboratory methods by IHC for PEDV and TGEV prior to use and was immunonegative for either agent. After deparaffinization, the slides were incubated with a cell conditioning solution[g] for 36 min. Before application of the primary antibody (optimally diluted at 1:10,000), a blocking step using normal goat serum[h] diluted at 1:10 was applied for 15 min. Primary antibody incubation was for 40 min at 36ºC. Secondary antibody incubation and staining were conducted with commercial reagents using manufacturer's recommended protocols[g]. Tissues were counterstained with hematoxylin for four minutes and coverslipped with glass coverslips. The slides were examined with light microscopy for positive immunoreactivity. Intestinal enterocyte immunoreactivity was semi-quantitatively scored based on the estimated percent of villous enterocytes with positive intracellular staining across the section examined, using a scale of 0-4 (Table 1).

Score	Criteria
0	No immunopositive cells
1	<10% enterocytes strongly positive in section
2	10-50% enterocytes strongly positive in section
3	50-75% enterocytes strongly positive in section
4	75-100% enterocytes strongly positive in section

Table 1. Immunohistochemistry scoring system applied to intestinal segments.

Clostridium difficile antigen and toxin testing

Fecal samples from six pigs (four group A and two group D) with gross lesions of mesocolonic edema were examined for *Clostridium difficile* antigen and toxin using a commercially available rapid membrane enzyme immunoassay kit[i] according the manufacturer's directions.

Results

Clinical data

Group A and B pigs developed soft to diarrheic feces by 2 DPI, with many affected pigs having notable liquid fecal staining of the perineum. From 2-4 DPI, widespread severe diarrhea was evident in these pigs, with average fecal scores of 2.58-2.83.

Groups A and B transitioned to soft, formed stools and then to normal stools by day 12 post-inoculation. Emesis was observed in four pigs from group A on day 2, but no emesis was observed in the other experimental groups or on subsequent days. Group C pigs developed diarrhea with perineal fecal staining on days 3 and 4. The litter that developed diarrhea at day 3 was exposed by direct contact with the inoculated sow of the room that escaped her farrowing stall overnight on during day 1 post-inoculation. Corresponding to the onset of clinical disease, several individuals in the exposed litters (groups A, B, and C) developed moderate to severe dehydration at 2 DPI. Lethargy was noted and peaked in groups A and B on days 2 and 3, with 8 (out of 55) and 8 (out of 46) pigs affected, respectively. Clinical status improved with the resolution of soft to diarrheic feces among the litters over the next 9 days. All surviving exposed pigs had normal feces and were otherwise clinically normal by 12 DPI.

Group A sows developed soft feces 2 DPI, which progressed to diarrhea on day 3. Group C sows developed soft to diarrheic feces on day 5. During the first few days of diarrheal disease, sows were inappetent. Emesis was not observed in the sows, and no significant dehydration or lethargy was detected in sows during the study. All sows were clinically normal by 9 DPI. The negative control sows and piglets remained clinically normal throughout the study.

Morbidity was 100% among the exposed pigs. Mortality was variable among the litters, but particularly impacted two of the six inoculated litters (including group A and B pigs), as evidenced by the lowest documented litter survival at 63.5% and 50%. Two other inoculated litters had higher survival percentages of 88.9%, with 3 to 4 pigs surviving to the end of the study. Survival of the negative control pigs was 90.9% to the end of the study, while overall survival of PDCoV exposed pigs (groups A, B, and C) was 72.9%. Group A and B pig survival was roughly similar at 69.6% and 62.5%, respectively. Group C pigs had a survival of 78%. Mortality in all exposed groups was distributed over the clinical phase of infection, as demonstrated in Fig. 2, with the highest mortality occurring on day 3. Survival was also analyzed by the age of pigs at inoculation (Group A and B). Pigs approximately 2 days old at inoculation had a survival of 49.9%, and pigs which were 3 days or older at inoculation had a survival of 78.5%. All sows survived to their planned sacrifice and necropsy near the termination of the study.

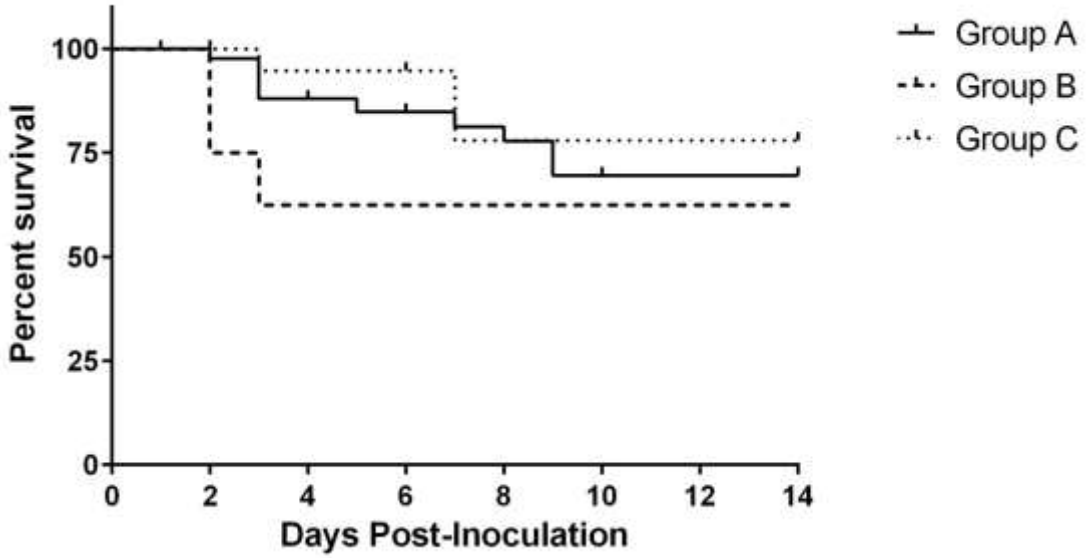

Figure 2. Survivability of pigs in each porcine deltacoronavirus (PDCoV) exposed experimental group. Sham challenged (negative) animals are not indicated, as survival excluding sacrifice was 90.9%.

Polymerase chain reaction

Fecal swabs from all group A and B pigs were qRT-PCR positive by 2 DPI. In group C, half of the pigs were fecal qRT-PCR positive 2 DPI (one litter of two), and all of the group C pigs were positive by day 3. The duration of fecal virus shedding as detected by qRT-PCR varied among individual pigs. Fecal swabs from all pigs of groups A and B had undetectable virus RNA by 28 DPI and after. One group C pig remained weakly fecal positive (Ct 35.2, weak positive 35-38) at 28 DPI, but was negative by the next testing period at day 35 (Fig. 3B).

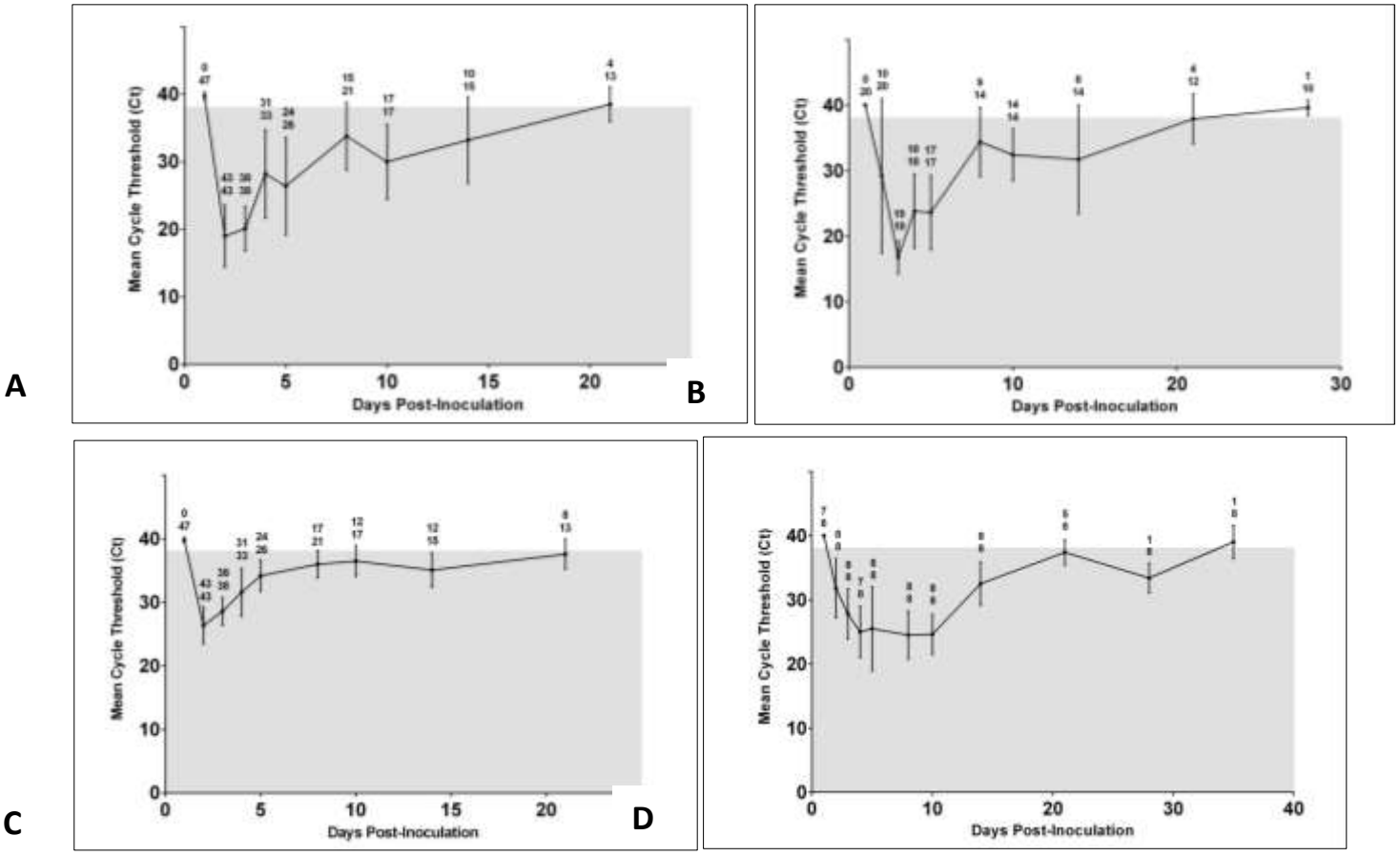

Figure 3. Mean cycle threshold values and standard deviation of reverse transcription polymerase chain reaction (RT-PCR) for experimental groups. Values above the linear data points indicate the number of individual animals that were RT-PCR positive over the total animals tested at that time sampling point on the x-axis; A, mean cycle threshold (Ct) of group A piglet fecal swabs; B, mean Ct of group C piglet fecal swabs; C, mean Ct of group A piglet nasal swabs; D, mean Ct of PDCoV exposed sows.

The lowest mean fecal Ct value of positive inoculated pigs, indicative of peak fecal virus shedding, occurred at 2 DPI (Fig. 3A). For nasal shedding, all inoculated and contact control pigs were qRT-PCR positive by nasal swab on day 2 and all samples had undetectable virus on day 28 and thereafter. Generally, fecal swab qRT-PCR results had lower Ct values (Fig. 3C). The aerosol control pigs were positive on nasal and fecal swab qRT-PCR on the same day, except for 7 pigs from one litter who were nasal swab qRT-PCR positive at day two and fecal qRT-PCR positive at day 3. The aerosol control pigs were all negative on nasal swabs by 35 DPI. Throughout the duration of the study, the negative control pigs had undetectable PDCoV RNA in fecal and nasal swabs by qRT-PCR.

The majority of exposed sows (groups A and C) became qRT-PCR positive on fecal swabs synchronously with her litter, except sow 4 from group A was fecal negative on day 2 when her piglets were all strongly fecal positive and sow 8 of group C became weakly positive (Ct 37) at day 2 prior to her litter becoming positive on day 3. Typically the sows had notably higher Ct values on fecal qRT-PCR (suggestive of lower virus genome copies) than the majority of their piglets the first day or two of infection, but then the Ct values appeared similar to the piglets thereafter (Fig. 3D). The range of days that sows were detected shedding

was 11 to 35 days, with an average of 21 days. One inoculated sow demonstrated the longest duration of PCR positive feces from day 2 to 35 DPI, at which time the sows were removed from the study and sacrificed.

Blood and/or serum samples collected were subjected to qRT-PCR analysis. Day 1 post-inoculation, viremia was detected in 50% of the sampled group A pigs (Ct 31.1-34.4). Viremia was detectable in most group A pigs from days 2-5 post inoculation (Ct 27.4-37.7), where the prevalence of viremia is demonstrated graphically (Fig. 4). At day 6, all group A pigs sampled had undetectable levels of virus. All sampled group A pigs (ranging from seven to fourteen each sample time point) remained PCR negative on blood/serum analysis for the rest of the study. Viremia was not detected in any blood/serum samples from the sows.

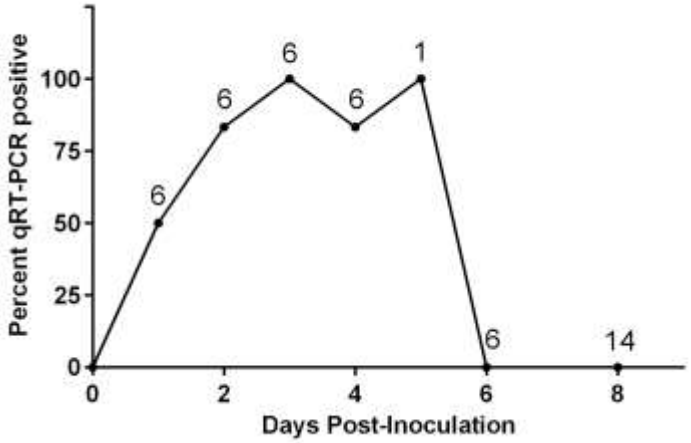

Figure 4. Percent of positive serum and/or whole blood samples from individual sample inoculated pigs from days 1-8 post-inoculation. Values above the linear data points indicate the number of animals sampled and the y-axis position indicates the percent of those samples positive.

In group B, 100% (2/2) sampled were qRT-PCR positive on serum on day 2 (Ct 28.1 and 33.7). In Group C, a pig sampled on day 2 was qRT-PCR positive on serum (Ct 33), and one on day 3 had undetectable levels of virus. The group B and C pigs were all negative by serum qRT-PCR on day 8 and thereafter for the remainder of the study. All negative control pigs had undetectable levels of virus in serum and/or whole blood throughout the study.

Quantitative real time reverse-transcription PCR was completed on tissues collected at necropsy including: trachea, turbinate, tonsil, lung, liver, kidney, spleen, lymph node, thymus, esophagus, stomach, small intestine, cecum, spiral colon, and descending colon. Nearly all tissues collected during the first two days of the study were variably positive. On day one (only group A pigs selected), the small intestine was consistently strongly positive ($Ct \leq 22$), while the spiral colon, cecum, and lymph node samples were strongly to moderately positive ($22 < Ct > 35$) (refer to supplemental image 1). Samples from the descending colon on day one were moderately to weakly positive ($Ct \geq 35$). On day two, the large and small intestine and lymph node were consistently strongly positive and continued to be strongly to moderately positive to day 6 post-inoculation. Following day 6, lymph node and small intestine samples were mostly moderately positive out to the end of the study at day 42. Some random individual samples of descending colon, spiral colon, and cecum from group C pigs only were weakly to moderately positive from day 28 to termination (refer to supplemental image 3). The negative control pigs sacrificed and sampled remained tissue qRT-PCR negative throughout the study.

A corresponding set of tissue samples collected from sows 35 DPI were subjected to qRT-PCR analysis. All exposed sows were qRT-PCR positive in lymph node, with Ct values ranging from 27.3 to 34.6 (refer to supplemental image 5). Sow 1 (group A) was positive in the descending colon (Ct 36.1) and spiral colon (Ct 34.6). Three sows from group A were detected as qRT-PCR positive in the small intestine (Ct 35.4-37.9), and both group C sows were qRT-PCR positive in the small intestine (Ct 31.2 and 32.7). All other tissue samples from the sows were qRT-PCR negative.

Oral fluids were subjected to qRT-PCR analysis. Two of the group A litters were not sampled as there were no remaining piglets in either litter at day 14. All exposed litters sampled (groups A, B, and C) were qRT-PCR positive on oral fluids taken on day 14 and 21 (Ct 28.1-36.9). On day 28, four of six litters were PCR positive on oral fluids. Two of these were group C litters (Ct 34.5 and 36.6), and the other two positive litters were comprised of group A and B pigs (Ct 30.3 and 37.0). Oral fluids from all litters were negative by qRT-PCR on 35 DPI, and one group C litter was weakly positive 39 DPI with a Ct of 37.9.

Histopathology

By 2 DPI, all group A pigs exhibited lesions of mild to severe atrophic enteritis within the jejunum and ileum. These lesions included villous enterocyte swelling with intracytoplasmic vacuolation, enterocyte attenuation and loss, mild to severe villous blunting and fusion, and submucosal edema with an occasional infiltrate of polymorphonuclear cells (Fig. 5).

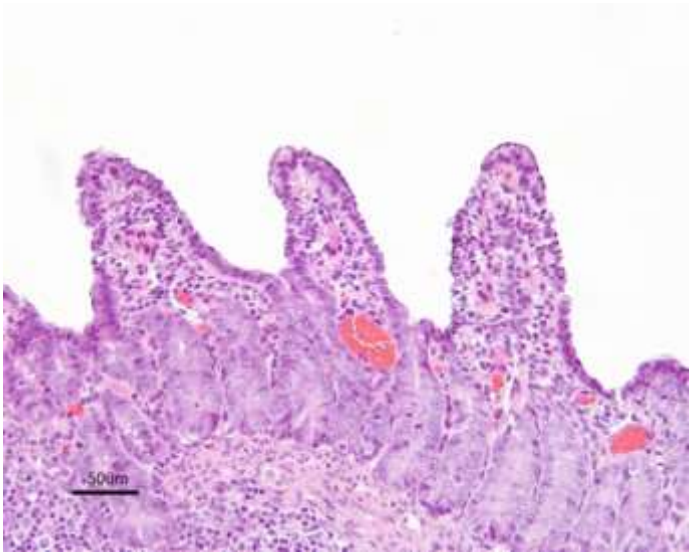

Figure 5. Section of ileum of PDCoV infected piglet 2 days post-inoculation with diffuse intestinal villous blunting, fusion, and enterocyte attenuation. Hematoxylin and eosin. Bar = 300 µm.

Not all samples of small intestine from each exposed pig were affected, and the severity of lesions often varied between the small intestinal segments examined in individual pigs. On days 3 through 6, all group A pigs examined had lesions of mild to moderate atrophic enteritis in the jejunum and ileum, although 6 of 49 samples were too autolyzed to determine the full extent of the lesions. Of the three group A pigs examined on day 7, all were too autolyzed to examine in detail. Two of the four group A pigs examined on day 8 had lesions of mild to moderate atrophic enteritis in the jejunum and ileum. No significant enteric lesions were observed in group A pigs at 10 DPI or thereafter.

Pigs from group B and C were examined on select days. The two group B pigs examined at day 2 had lesions of atrophic enteritis in the jejunum. Only one of these pigs had detectable lesions within the ileum. No lesions were observed in the group C pig examined at 2 DPI. The group B pig examined at day 3 had mild lesions of atrophic enteritis in the jejunum and ileum. One group C pig examined on each day 3 and 6 displayed atrophic enteritis in the jejunum and ileum. Three group C pigs were examined on day 8, but autolysis obscured identification of any lesions of atrophic enteritis. No enteric lesions were observed in group B pigs examined at 28 and 42 DPI, and no enteric lesions were observed in group C pigs examined at 14 DPI and each sampling day thereafter.

Enteric lesions other than characteristic atrophic enteritis were infrequently noted. In the duodenum from 3 to 8 DPI, eight pigs from group A and one from group C had scattered foci of mild to moderate, and rarely severe, necrosuppurative lesions in the tips of the villi with occasional fibrin thrombi within small vessels of the villous and local enterocyte attenuation. Four of these duodenal samples had minimal IHC staining (score 1), and the others were IHC negative. Five group A pigs and one group C pig had small to large necrosuppurative lesions multifocally scattered across the superficial cecal mucosa, accompanied by moderate to marked submucosal edema in the cecum and/or spiral colon. These lesions were only noted up to 10 DPI.

No lesions of atrophic enteritis were observed in the negative control pigs throughout the study. Moderate cecal submucosal edema and mesenteric edema were observed in a control pig examined at day 4 post-inoculation. Ulcerative and suppurative colitis with mesenteric edema of the cecum and spiral colon was observed in a negative control pig examined at 6 DPI. No other significant lesions were noted in other organ systems that were examined of the negative control pigs.

No significant enteric lesions were observed by microscopic examination in the sows. One sow had a grossly and histologically evident chronic infarct affecting 75% of the spleen.

Immunohistochemistry

At 1 DPI, all three sacrificed group A pigs displayed strong positive intracytoplasmic staining in villous enterocytes of the jejunum and ileum (Table 2, Fig. 6).

	Percent IHC positive pigs and mean IHC score on indicated experimental day sample:													
	Group A								**Group B**		**Group C**			
Tissues	1	2	3	4	5	6	7	8	2	3	2	3	6	8
Duodenum	0 (0)	25 (0.25)	43 (0.43)	60 (0.6)	100 (1)	50 (0.5)	0 (0)	0 (0)	100 (3)	100 (2)	100 (2)	0 (0)	100 (1)	0 (0)
Jejunum 1	67 (1)	75 (2)	100 (2.29)	100 (1.2)	100 (2)	100 (1.5)	33 (0.3)	0 (0)	100 (3)	--	100 (1)	100 (2)	100 (2)	0 (0)
Jejunum 2	100 (2)	100 (3.25)	100 (2.83)	100 (2.6)	100 (2.5)	100 (3)	33 (0.3)	25 (0.5)	100 (3)	100 (2)	100 (2)	100 (4)	100 (2)	67 (0.67)
Ileum	100 (2)	100 (3)	100 (2.29)	100 (2.4)	100 (2)	100 (2)	33 (0.3)	25 (0.5)	100 (3)	100 (3)	--	100 (2)	100 (2)	67 (0.67)
Cecum	0 (0)	75 (0.75)	43 (0.43)	20 (0.2)	0 (0)	50 (0.5)	0 (0)	0 (0)	50 (0.5)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Spiral Colon	0 (0)	25 (0.25)	29 (0.43)	29 (0.29)	0 (0)	0 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mesenteric LN	0	100	71	100	100	100	0	75	100	100	0	100	100	33
# pigs sampled	3	4	7	5	2	2	3	4	2	1	1	1	1	3

Table 2. Percent immunohistochemistry (IHC) positive pigs and mean IHC score (out of 4). The columns are organized by experimental group and day post-inoculation when tissues were collected. The mean IHC score for the intestinal tissue of pigs sampled in each group is given within parenthesis and the percent positive is given with the left-handed number in the column. The number of pigs sampled on a given day is present at the bottom row of the table. Any tissue or collection day not represented on the chart indicates tissues were not found to be IHC positive.

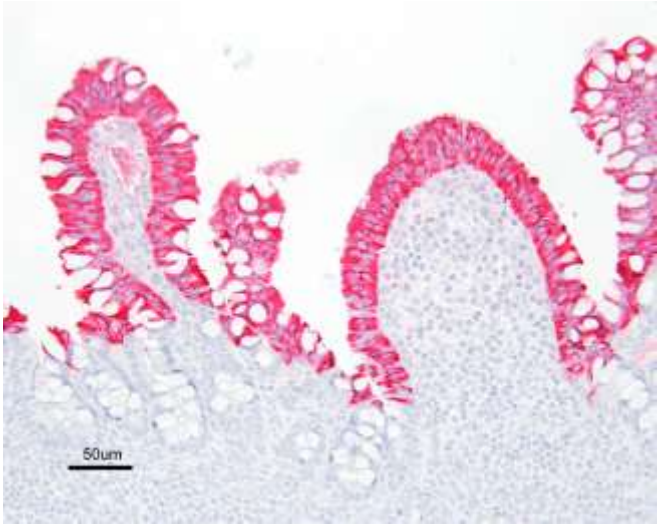

Figure 6. Section of ileum of PDCoV infected piglet 2 days post-inoculation with strong diffuse immunostaining of the villous enterocytes. Sections stained with rabbit anti-porcine deltacoronavirus hyperimmune serum (red chromogen) with hematoxylin counter stain. Bar = 50 µm.

All group A pigs sampled continued to be IHC positive in villous enterocytes of the jejunum and ileum on days 2 through 6. Occasional scattered interdigitating cells within the Peyer's patches were also positive after day 2. The percentage of group A individuals with positive staining in the jejunum and ileum quickly declined through days 7 and 8, and no positive staining was observed in samples from 10 DPI and thereafter. No mesenteric lymph node samples from group A pigs were positive at day 1 or 7. On days 2-5 and 8, the majority (71-100%) of the mesenteric lymph nodes sampled were positive, characterized by scattered interdigitating cells with strong intracytoplasmic staining (Fig. 7).

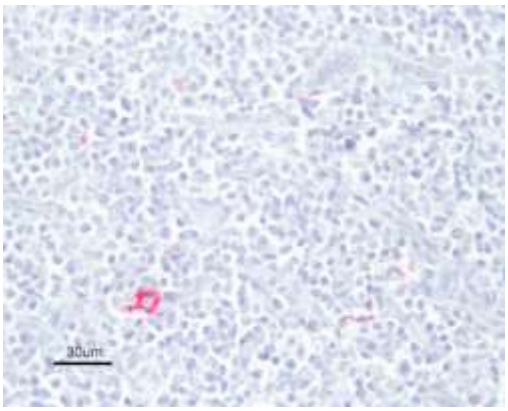

Figure 7. Section of mesenteric lymph node of PDCoV infected piglet 8 days post-inoculation with scattered individual immunopositive cells. Sections stained with rabbit anti-porcine deltacoronavirus hyperimmune serum (red chromogen) with hematoxylin counter stain. Bar = 100 µm.

No mesenteric lymph nodes were positive on day 10 or thereafter. Other immunopositive tissues included duodenum, cecum, and spiral colon. A lower percentage of pigs had positive staining in these other intestinal segments (Table 2), and often the staining affected a low number of scattered enterocytes. Positive staining in the duodenum was observed 2-6 DPI. The spiral colon was only detected positive on days 2 and 3, and cecum samples were variably positive on days 2, 3, 4, and 6. No positive immunoreactivity was observed in

other tissues examined. Pigs from group B and C were also sacrificed and sampled at their selected time points (Fig. 1). Similar to group A, the duodenum, jejunum, ileum, and mesenteric lymph node samples were consistently positive at 2-8 DPI (Table 2). No immunopositive tissues were observed after day 8 in group B and C pigs. Consistent with group A, no positive immunoreactivity was observed in tissues other than the intestinal tract and mesenteric lymph node. The sacrificed negative control pigs remained negative by immunohistochemical analysis throughout the study.

A mean IHC score was calculated from fixed tissue samples collected from the groups at each DPI (Table 2). The highest mean IHC scores in group A pigs were observed 1-6 DPI in the jejunum and ileum. A low mean IHC score (≤0.5) was observed in the jejunum and ileum of group A pigs on day 8. A low mean IHC score was observed in the duodenum, spiral colon, and cecum 2-6 DPI. A similar trend was observed for the data set obtained for groups B and C, although the sample of pigs from these groups was notably smaller.

Staining with IHC was also completed on tonsil, liver, spleen, lymph node, stomach, descending colon, spiral colon, small intestine, and cecum from each sow collected at 35 DPI. PDCoV was not detected by IHC in any tissue sample from the sows.

Clostridium difficile antigen and toxin testing

Three group A pigs tested positive for *C. difficile* antigen and toxin, while one group A pig was positive for antigen but for negative toxin. Two group D pigs were positive for antigen but negative for toxin.

Discussion

Experimental inoculation demonstrated that PDCoV infection in the absence of other enteric pathogens is capable of producing clinical diarrhea, emesis, and dehydration, as well as appreciable mortality in conventional neonatal pigs. Clinical signs were much less severe in infected sows than piglet infections. Disease in the sows was characterized by transient soft to diarrheic feces and inappetence in the absence of clinically significant dehydration or mortality. The use of this challenge model of conventional piglets and sows in lieu of gnotobiotic pigs or conventional weaned pigs may provide altered host-pathogen interaction and different pathological observations. Work with human noroviruses has shown that the microbiota of the gut can have a critical effect on virus pathogenesis.[8] The lack of a cell culture-adapted PDCoV at the time of trial necessitated the generation of an infectious inoculum from a field sample, which was demonstrated to be free of other etiologic agents of porcine enteric disease by all available modalities including bacterial culture, PCR, and metagenomic sequencing. Importantly, no significant extraneous or confounding viruses were detected in the challenge inoculum, although partial genome coverage of porcine kobuvirus was demonstrated in a fecal swab from a PDCoV-infected animal. As porcine kobuvirus has been isolated from healthy pigs and those with diarrhea, the virus has no distinct role as a gastrointestinal pathogen of pigs and is considered an insignificant finding in this study.[22] The use of a sizable experimental sampling of conventional pigs and a non-cell culture adapted virus inoculum in this study closely mimics natural field conditions.

The PDCoV clinical disease course of diarrhea, emesis, and inappetence observed in piglets and sows in this study is similar to TGEV and PEDV.[25] The incubation period prior to the onset of clinical diarrhea in the current study also resembles experimental and natural infections with TGEV and PEDV in neonatal pigs, where the onset of clinical signs can vary from 24-48 hours and may depend on pig and virus strain characteristics.[4,6,10] Other experimental inoculations with PDCoV in 19 and 5 day-old conventional and gnotobiotic piglets,

respectively, demonstrate the onset of clinical diarrheal disease can vary from 24 hours to 2-4 days post-infection. [3,9,11] Emesis, an inconsistent feature of both PEDV and TGEV infections, was documented to occur in a few PDCoV inoculated pigs of this study only on day 2 post-inoculation, and may be an early transient feature of PDCoV infection. The contact control pigs in this study developed diarrhea synchronously with the inoculated pigs, and the aerosol control pigs developed disease one to two days after, indicating rapid and efficient horizontal transfer of PDCoV in pigs. It is possible that virus inoculum from the group A pigs contaminated the contact control pigs, rather than spread of fecal virus, resulting in this rapid development of disease. One aerosol control litter had no direct contact with inoculated animals, while the other aerosol control litter did have incidental direct contact with an inoculated sow. These data indicate PDCoV infection would likely spread rapidly throughout modern farrowing and gestation barns, despite minor barriers such as farrowing stalls and physical barriers. PEDV has been demonstrated to have the potential for airborne transmission of infectious virus, and thus PDCoV may also have similar characteristics.[2]

Porcine deltacoronavirus inoculated piglets had diarrheic to soft feces for several days, until day 12 post-inoculation in a few individuals, and the clinical course of diarrhea was shorter in sows, ending at day 9. Given that the demonstrated small intestinal villous epithelium replacement time is generally about 7-10 days in one day old pigs, it is expected that diarrhea due to uncomplicated neonatal TGEV and PEDV infections, as well as the currently described PDCoV, would resolve in approximately this interval post-infection.[15] As older pigs have a faster regenerative capacity of the villous epithelium, it is also expected that sows should recover more quickly.[15] Other models of PDCoV conventional neonatal piglet infection have demonstrated resolution of diarrhea at 7 to 10 days post-infection, and this is generally consistent with the overall findings of this study.[11]

Despite many similarities between the demonstrated PDCoV infection and TGEV and PEDV, some differences may exist in the severity of clinical disease and mortality during infection of neonatal pigs. Mortality due to epidemic TGEV and PEDV in neonatal pigs of naïve herds is high, often approaching 90-100% in the youngest pigs and decreasing with age.[6,21] The first field reports of PDCoV associated with diarrheal disease in pigs noted that the mortality in affected piglets was approximately 30-40%, much lower than typically observed with PEDV infection.[23] In the current study, it was not possible to calculate a true mortality rate due to the rigorous serial sequential sacrifice of pigs and selection bias of severely diseased pigs, thus survival analysis was conducted using dead and moribund pig numbers. The survival of all PDCoV exposed groups was less than the negative control pigs with mortality distributed over the clinical phase of diarrheal disease, demonstrating an increase in mortality of clinically ill PDCoV infected neonates. The one pig that died in the negative control group was runted with evidence of septicemia, and appears to represent normal neonatal mortality. A notable survival effect was demonstrated by comparing the different ages of pigs at inoculation. Pigs approximately 2 days of age at inoculation had lower survival rates than those 3 days or older at inoculation. This indicates that disease severity and mortality of infected pigs likely decreases with age, similar to TGEV and PEDV. This is important to note as recent experimental studies for comparison have all utilized older conventional pigs (5-10 days of age) for challenge.[3,11]

Histologic examination revealed lesions characteristic of atrophic enteritis, which appeared to be confined to the jejunum and ileum for 7 experimental days during the clinical disease course. Overall, these lesions were clearly impactful but did not appear to demonstrate the severity seen in PEDV infections in naïve neonatal piglets. The onset of histologic lesions corresponded to the onset of diarrhea, and was associated with the lowest qRT-PCR Ct values in PDCoV in blood, tissues, and feces, and IHC detection of PDCoV in the small

intestine. Intestinal lesions resolved 2-3 days prior to the complete resolution of diarrheal disease at day 12 post-inoculation. Other studies in 5 day-old conventional piglets demonstrated a more gradual onset of diarrhea and fecal virus shedding by PCR from 2-5 days post-infection.[3] Virus RNA was detected in serum and whole blood samples in animals of this study, in contrast to other studies which found none.[9] By IHC, the jejunum and ileum were the primary sites of PDCoV replication, as these were the first tissues to be positive and were the most consistently positive throughout the experiment which is consistent with other experimental PDCoV infections.[3] Virus antigen was less frequently detected by IHC in the duodenum, cecum, and spiral colon, and was not associated with any significant lesions in these areas of the gastrointestinal tract. In contrast to these findings, other studies have not demonstrated PDCoV IHC staining in sections of cecum and colon.[3] The mild to moderate necrosuppurative lesions detected in some duodenal samples were inconsistent with viral atrophic enteritis, and no significant IHC detection of virus was associated with these lesions. These lesions appeared to be limited to duodenum, and were not significantly associated with mortality. Therefore, the duodenal lesions are considered to be likely a result of intestinal dyshomeostasis. The mesenteric lymph nodes were the only non-intestinal tissue found to be positive by IHC, and no specific lesions were associated with IHC staining. One PDCoV study has demonstrated epithelial degeneration and necrosis and occasional syncytial cell formation in the stomach, however, no significant lesions or IHC positive staining of the stomach was observed in the current study.[11] This study utilized gnotobiotic pigs which may have resulted in infection differences. Overall, the lesions and virus distribution by IHC observed in this experiment resembles that observed in TGEV and PEDV.[12,20] Additional studies are needed to further investigate potential mechanisms underlying the strong tropism of PDCoV for the jejunum and ileum, and lesser tropism of other intestinal segments.

While the onset and resolution of lesions correlate with detection of virus by IHC, PDCoV was detected in tissues and feces by qRT-PCR for many days after the clinical course of diarrhea ended. In general, most PDCoV exposed pigs and sows were qRT-PCR positive in the small intestine and lymph node out to termination. In comparison, PEDV has been demonstrated to persist in lymph node and small intestine tissues such that animals are PCR positive for at least 28 days post-infection (personal communication Richard Hesse). The positive fecal swabs from PDCoV inoculated pigs up to day 21 post-inoculation is comparable to similar studies of three week-old pigs experimentally challenged with PEDV, where fecal shedding of virus was demonstrated extending out to 24 days post-infection.[12] In the current study, one sow demonstrated fecal shedding out to day 35 post-inoculation, and one aerosol control pig demonstrated fecal shedding to day 28 post-inoculation. This suggests that fecal shedding of PDCoV could extend longer, particularly in adults, which is important as they may serve as reservoirs infecting naïve sows and piglets in farrowing facilities. However, it should be noted that PCR detection of virus does not necessarily indicate the presence of viable virus or indicate that there is viral replication in the tissues. Detection of PDCoV RNA concurrently with negative IHC may relate to the larger sampling and greater sensitivity of the PCR method or persistence of detectable nucleic acids vs replicating virus in the samples. In a natural disease outbreak, IHC may be useful to determine if an affected pig with acute onset of diarrhea has an active PDCoV infection or detect PDCoV when only fixed tissues are available for examination. Currently, a number of PEDV infected farms also have detectable PDCoV in the herd, and IHC could be used to determine the contribution of PDCoV to active enteric disease. This is clearly important as, demonstrated in this study, fecal shedding and tissue PCR detection can be prolonged weeks beyond the clinical phase of disease. However, the PCR results obtained from a positive herd may be important in order to monitor the reservoir of infected animals shedding fecal-borne virus into the environment and screen for recently infected animals.

Oronasal virus detection was successful during the study. Nasal swabs and oral fluids were qRT-PCR positive and correlated with fecal qRT-PCR results temporally. Average Ct values indicated that nasal swabs contained less viral RNA than fecal swabs. No virus was demonstrated within the nasal turbinate, trachea, or lung by IHC and no respiratory tract lesions were observed. These observations indicate that experimental PDCoV infection in this study did not yield a detectable respiratory infection even though they were exposed to virus via an intranasal route. Other recent studies also did not observe lesions or detect antigen by immunostaining reactions in respiratory tissue of PDCoV-challenged 11-14 day-old gnotobiotic piglets or 5 day-old conventional piglets.[3,9] In contrast, related viruses PRCV and TGEV have been shown capable of replication in alveolar cells and epithelial cells of the respiratory tract from the nasal mucosa to bronchioles, as well as in the ileum.[5] PEDV has also been described to replicate in alveolar macrophages.[19] The current findings also contrast a PDCoV experimental inoculation of gnotobiotic pigs in which interstitial pneumonia was noted along with PDCoV antigen detection in bronchial mucosal epithelial cells using IHC.[11] However, the investigators utilized hyperimmune sow serum for the primary antibody, and this may result in non-specific or background staining. In the current study, the positive PCR detection on nasal swabs is interpreted to be environmental contamination of external nares. However, nasal swab and oral fluid PCR may be complementary diagnostic tests to monitor PDCoV infection and virus shedding in infected pigs on a herd level.

Select pigs from group A and group D had gross and histologic evidence of *Clostridium difficile* enteritis. Due to the fact that both PDCoV inoculated and negative control animals were affected by *C. difficile* lesions, it appears that it was likely an endogenous agent that was circulating in the pigs upon arrival and it did not appear to be associated with the severity of viral lesions, severity of clinical disease, or any mortality. Therefore, it is not considered to have impacted the evaluation of PDCoV pathogenicity.

In the current study, PDCoV has been demonstrated as a pathogenic agent causing atrophic enteritis and diarrheal disease in conventional neonatal piglets and sows, confirming other PDCoV experimental inoculations and providing a comprehensive pathogenesis model to enable comparisons to the other important enteric coronaviruses of swine, PEDV and TGEV. PDCoV should be considered as a differential diagnosis in swine diarrheal disease in neonatal as well as adult pigs. The presented pathogenesis described in this study using conventional pigs serves as model for the expected acute disease course in a typical naïve swine production system infected with PDCoV. It is important to note that while disease was reproduced successfully in this study from a PDCoV field case, a second inoculum generated from a separate field case failed to cause disease in the primary piglet challenge. This discrepancy may highlight an important feature of PDCoV in that pathogenicity may depend on uncharacterized viral virulence properties or other host factors. This study also demonstrated rapid transmission to aerosol control animals, suggesting that infective virus can readily spread under confinement conditions and may have the capacity to spread in an airborne manner as demonstrated with PEDV.[2] The data generated in the present study regarding virus detection with IHC and qRT-PCR enables development of guidelines for PDCoV diagnostic testing, particularly when investigating outbreaks of diarrheal disease in pigs and monitoring closed herds.

Sources and manufacturers

a) CLC Genomics Workbench version 7.5, CLC bio, Qiagen USA, Valencia, CA.

b) TEGO Swine Oral Fluids kit, ITL Biomedical Animal Healthcare, Reston, VA.

c) GraphPad Prism version 6.05 for Windows, GraphPad Software, La Jolla, CA.

d) Kingfisher 96 magnetic particle processor and AgPath-ID One-Step RT-PCR kit, Fisher Scientific, Pittsburgh, PA.

e) MagMAX-96 Viral RNA Isolation kit, Life Technologies, Grand Island, NY.

f) Bio-Rad CFX96 Touch Real-Time PCR Detection System, Bio-Rad, Hercules, CA.

g) Ventana Benchmark ULTRA IHC/ISH slide staining platform, CONFIRM Negative Rabbit Ig, Cell Conditioner 1, and proprietary reagents, Ventana Medical Systems, Inc., Tucson, AZ.

h) Goat serum, Colorado Serum Company, Denver, CO.

i) C. DIFF QUIK CHEK COMPLETE, TechLab, Blacksburk, VA.

Acknowledgements

The authors thank Travis Clement for coordination of PCR testing and inoculum preparation, as well as Steve Lawson, Aaron Singrey, and Faten Okda for preparation of anti-PDCoV antisera. The authors also thank Dr. Kelly Heath, Vicky Samek, Jon Kolman, Sheryl Hemmer, Brandon Stewart, Kevin Brodersen, Taylor Engle, and the UNL Veterinary Diagnostic Center staff and animal care staff for all their efforts in helping to complete many tasks of sample collection and processing.

Funding

This study was supported by the National Pork Board (#14-182).

References cited

1 2012, Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses Elsevier Academic Press, San Diego.

2 Alonso C, Goede DP, Morrison RB, et al.: 2014, Evidence of infectivity of airborne porcine epidemic diarrhea virus and detection of airborne viral RNA at long distances from infected herds. Vet Res 45:73.

3 Chen Q, Gauger P, Stafne M, et al.: 2015, Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. Virology 482:51-59.

4 Coussement W, Ducatelle R, Debouck P, Hoorens J: 1982, Pathology of experimental CV777 coronavirus enteritis in piglets. I. Histological and histochemical study. Vet Pathol 19:46-56.

5 Cox E, Hooyberghs J, Pensaert MB: 1990, Sites of replication of a porcine respiratory coronavirus related to transmissible gastroenteritis virus. Res Vet Sci 48:165-169.

6 Djurickovic S, Thorsen J, Duncan JR, Roe CK: 1969, Transmissible gastroenteritis of swine in Ontario. Can J Comp Med 33:59-63.

7 Hu H, Jung K, Vlasova AN, et al.: 2015, Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. J Clin Microbiol 53:1537-1548.

8 Jones MK, Watanabe M, Zhu S, et al.: 2014, Enteric bacteria promote human and mouse norovirus infection of B cells. Science 346:755-759.

9 Jung K, Hu H, Eyerly B, et al.: 2015, Pathogenicity of 2 porcine deltacoronavirus strains in gnotobiotic pigs. Emerg Infect Dis 21:650-654.

10 Kim B, Chae C: 2002, Experimental infection of piglets with transmissible gastroenteritis virus: a comparison of three strains (Korean, Purdue and Miller). J Comp Pathol 126:30-37.

11 Ma Y, Zhang Y, Liang X, et al.: 2015, Origin, evolution, and virulence of porcine deltacoronaviruses in the United States. MBio 6.

12 Madson DM, Magstadt DR, Arruda PH, et al.: 2014, Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol 174:60-68.

13 Markey BK: 2013, Clinical veterinary microbiology, 2nd ed., p. p. Elsevier, Edinburgh.

14 Marthaler D, Raymond L, Jiang Y, et al.: 2014, Rapid detection, complete genome sequencing, and phylogenetic analysis of porcine deltacoronavirus. Emerg Infect Dis 20:1347-1350.

15 Moon HW: 1971, Epithelial cell migration in the alimentary mucosa of the suckling pig. Proc Soc Exp Biol Med 137:151-154.

16 Morin M, Morehouse LG, Solorzano RF, Olson LD: 1973, Transmissible gastroenteritis in feeder swine: clinical, immunofluorescence and histopathological obervations. Can J Comp Med 37:239-248.

17 Moxley RA, Olson LR: 1989, Lesions of transmissible gastroenteritis virus infection in experimentally inoculated pigs suckling immunized sows. Am J Vet Res 50:708-716.

18 Neill JD, Bayles DO, Ridpath JF: 2014, Simultaneous rapid sequencing of multiple RNA virus genomes. J Virol Methods 201:68-72.

19 Park JE, Shin HJ: 2014, Porcine epidemic diarrhea virus infects and replicates in porcine alveolar macrophages. Virus Res 191:143-152.

20 Shoup DI, Swayne DE, Jackwood DJ, Saif LJ: 1996, Immunohistochemistry of transmissible gastroenteritis virus antigens in fixed paraffin-embedded tissues. J Vet Diagn Invest 8:161-167.

21 Stevenson GW, Hoang H, Schwartz KJ, et al.: 2013, Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest 25:649-654.

22 Verma H, Mor SK, Abdel-Glil MY, Goyal SM: 2013, Identification and molecular characterization of porcine kobuvirus in U. S. swine. Virus Genes 46:551-553.

23 Wang L, Byrum B, Zhang Y: 2014, Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. Emerg Infect Dis 20:1227-1230.

24 Woo PC, Lau SK, Lam CS, et al.: 2012, Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J Virol 86:3995-4008.

25 Zimmerman JJ: 2012, Diseases of swine, 10th ed., pp. xxiii, 983 p., 982 p. of plates. Wiley-Blackwell, Chichester, West Sussex ; Ames, Iowa.