RESEARCH REPORT
SWINE HEALTH

Title: Tissue localization, shedding, virus carriage, antibody response, and aerosol transmission of porcine epidemic diarrhea virus (PEDV) following inoculation of 4 week old feeder pigs
NPB # 13-228

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Industry Summary

Porcine epidemic diarrhea virus (PEDV) has recently emerged in the US. The purpose of this investigation was to determine tissue localization, shedding pattern, virus carriage, antibody response, and aerosol transmission of PEDV following oral/nasal inoculation of 4-week-old feeder pigs.
Experimental Animals: Thirty-three PEDV naive 3-week-old feeder pigs obtained from a high health commercial source were allowed to acclimate for one week prior to inoculation. The study was conducted under BSL2 containment at the Biosecurity Research Institute at Kansas State University. Twenty-three Group A pigs were inoculated with the PEDV challenge material. Five Group B pigs were not inoculated, but were comingled with inoculated Group A animals approximately 6 hours post inoculation (PI). Five aerosol transmission Group C pigs were not inoculated, but were housed in a separate pen in the same common animal room as Groups A and B.
The challenge material used in this study was a pool of gut-derived intestinal contents that has been used as "feedback" inocula for controlled exposure of a sow herd in a commercial swine production unit. This material tested negative for PRRS and PCV and produced a PEDV nucleic acid "CT titer" of 22. Challenged animals (Group A) were inoculated at 4 weeks of age via the oral and intranasal routes with 5 ml of inocula per route. Following inoculation, the animals were observed daily for clinical symptoms. Nasal and fecal swabs as well as serum samples were collected prior to challenge and at days 0-7, 9, 14, 21, 28, 35, and 42 post inoculation (PI). Pen oral fluid samples were also collected at the same time points for Groups A/B and the aerosol control Group C.

PEDV 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, 2,4,7,9,14,21,28, 35, and 42 PI in order to monitor tissue tropism of the virus and histopathology.

Experimental data indicate the following:
Mild clinical signs appeared 2 days post inoculation and resolved by 8 days post inoculation.
Fecal and nasal swabs were PCR positive in the inoculated group at 48 hours post inoculation.
Peak fecal shedding occurred 5 to 6 days post challenge and was significantly higher than that in the nasal swabs.
Most group A and B animals were negative by fecal and/or nasal swab testing at 21 days post inoculation; however, some animals shed virus as long as 35 days PI The inoculated piglets were qRT-PCR positive in fecal and nasal swabs to 21 DPI.
Productive transmission did not appear to occur in the aerosol control group in spite of the fact that PEDV nucleic acid could be detected in the nares of some of those animals and oral fluids.
Room environmental samples were collected at 14 days post inoculation and the data demonstrate that viral nucleic acid was abundant on the walls, pens, and food bins on both the inoculated and aerosol control areas in the challenge room. Due to the possibility of a false positive PCR reaction, questionable samples were retested and the reaction products were sequenced to determine if the product was PEDV specific. All questionable reactions demonstrated the presence of PEDV viral nucleic acid. PEDV viremia was clearly detected in 3 of the 5 contact controls and 9 of the 22 inoculated animals. No detectable viremia was detected in any of the aerosol control animals. The raw data suggest that there seems to be a correlation with viremia and extended duration of shedding either fecal or nasal. Serological data (IFA) show that pre-inoculation samples were negative and that there was significant seroconversion in all of the inoculated and contact control animals. There is no evidence of seroconversion in the aerosol control group in spite of the clear demonstration of PEDV nucleic acid in nasal and oral fluid samples.

Atrophic enteritis was observed in the jejunum and ileum of affected piglets from 2 to 8 DPI, and corresponded to positive antigen detection by IHC. Mesenteric lymph node and small intestine were the primary sites of antigen detection by IHC and tissue qRT-PCR, and most inoculated group A piglets in were qRT-PCR positive in the intestinal tissue samples out to the end of the study. Tissue blocks were sent to Dr. Madson at ISU for PEDV preliminary immunohistochemistry (IHC) evaluation. IHC results were subsequently confirmed and results expanded at KSVDL. The only samples that tested positive for the presence of viral antigen were tissues from the GI tract. Turbinates, trachea, lung, bronchial lymph nodes, spleen, and other visceral tissues were all negative for PEDV as evaluated by IHC.
A complete set of serum samples has been provided to 5 laboratories (~1,200 samples) for assay development/standardization. In addition, 3 complete sets of oral fluid samples and tissues have been provided to other laboratories. These samples have been used for assay development and standardization across the different diagnostic laboratories.

The experimental results demonstrate that aerosol transmission did not occur in this study. These results seem to be in conflict with reports from the field that implicate aerosol transmission, but lack confirmation via bioassay. Factors like disinfectant and ultraviolet inactivation of PEDV, sensitivity of the indicator animal (nursing pigs vs. weaned pigs) and infectious dose as a function of route of exposure need to be investigated in order to gain insight into modes of transmission of PEDV.

The tissue PCR positivity for PED nucleic acid at day 43 post inoculation was an unexpected finding which provides insight into virus carriage and potential transmission of the virus long after the clinical disease has abated. In view of these findings, additional animal co-mingle studies will need to be conducted to determine the actual duration of horizontal transmission between infected and naïve pigs.

Keywords:
novel coronaviruses, porcine epidemic diarrhea virus, porcine diarrhea, porcine enteric coronavirus

Scientific Abstract:

The purpose of this investigation was to determine tissue localization, shedding pattern, virus carriage, antibody response, and aerosol transmission of PEDV following oral/nasal inoculation of 4-week-old feeder pigs. Thirty-three PEDV naive 3-week-old feeder pigs obtained from a high health commercial source were allowed to acclimate for one week prior to inoculation. The study was conducted under BSL3Ag containment at the Biosecurity Research Institute at Kansas State University. Twenty-three 4-week-old pigs (Group A) were inoculated with a "feedback" inocula used for controlled exposure of a sow herd via the oral and intranasal routes with 5 ml of inocula per route. Five pigs were not inoculated, but were comingled (Group B) with inoculated animals approximately 6 hours post inoculation. Five aerosol transmission pigs (Group C) were not inoculated, but were housed in a separate pen in the common animal room. Nasal and fecal swabs, serum, and oral fluid samples were collected prior to challenge and subsequently at days 0-7, 9, 14, 21, 28, 35, and 42 days post inoculation (DPI). PEDV shedding was monitored by real-time PCR of fecal and nasal swab samples and oral fluids. Serum samples were collected in order to monitor viremia and antibody response. Fresh and formalin-fixed tissues were collected from randomly selected Group A pigs at days 0, 2, 4, 7, 9, 14, 21, 28, 35, and 42 DPI in order to monitor tissue tropism of the virus and histopathology. Following inoculation, the animals were also observed daily for clinical signs of disease. Experimental data indicate the following: mild clinical signs appeared on DPI 2 and resolved by DPI 8 in Group A and B pigs. Fecal and nasal swabs were PCR positive in the inoculated group by DPI 2. Peak fecal shedding occurred on DPI 5 and was significantly higher than nasal swabs. Most group A and B animals were PCR negative by fecal or nasal swab testing at 21 DPI; however, some animals shed virus as long as 35 DPI.

Atrophic enteritis was observed in the jejunum and ileum of affected piglets from 2 to 8 DPI, and corresponded to positive antigen detection by IHC. Mesenteric lymph node and small intestine were the primary sites of antigen detection by IHC and tissue qRT-PCR, and most inoculated group A piglets were qRT-PCR positive in the intestinal tissue samples out to the end of the study.

Tissue blocks were sent to Dr. Madson at ISU for PEDV preliminary immunohistochemistry (IHC) evaluation. IHC results were subsequently confirmed and results expanded at KSVDL. The only samples that tested positive for the presence of viral antigen were tissues from the GI tract. Turbinates, trachea, lung, bronchial lymph nodes, spleen, and other visceral tissues were all negative for PEDV as evaluated by IHC.

A complete set of serum samples has been provided to 5 laboratories (~1,200 samples) for assay development/standardization. In addition, 3 complete sets of oral fluid samples and tissues have been provided to other laboratories. These samples have been used for assay development and standardization across the different diagnostic laboratories.

Productive transmission did not appear to occur in the aerosol control group in spite of the PEDV nucleic acid detected in the nares of some of those animals and in the oral fluids. Room environmental samples collected at DPI 14 demonstrated that viral nucleic acid was abundant on the walls, pens, and food bins in the challenge room. PEDV viremia was clearly detected in 3 of the 5 contact controls and 9 of the 22 inoculated animals. No detectable viremia was detected in any of the aerosol control animals. Serological data (IFA) proves that pre-inoculation samples were negative and that there was significant seroconversion in all of the inoculated and contact control animals. There was no evidence of seroconversion in the aerosol control group. These results seem to be in conflict with reports from the field that implicate aerosol transmission, but lack confirmation via bioassay. Factors like disinfectant and ultraviolet inactivation of PEDV, sensitivity of the indicator animal (nursing pigs vs. weaned pigs) and infectious dose as a function of route of exposure need to be investigated in order to gain insight into modes of transmission of PEDV.

The tissue PCR positivity for PED nucleic acid at day 43 post inoculation was an unexpected finding which provides insight into virus carriage and potential transmission of the virus long after the clinical disease has abated. In view of these findings, additional animal co-mingle studies will need to be conducted to determine the actual duration of horizontal transmission between infected and naïve pigs.

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a new swine enteric coronavirus in North America. PEDV was first observed in the United States in April of 2013 and has spread across the western hemisphere. The peak disease period for this virus is in the late fall and early winter months in temperate climates and clinical disease typically abates in the late spring, summer, and early fall. Following the introduction of PEDV in the US, an estimated 8-10 million pigs have died which has resulted in a shortage of pork for consumption. The clinical presentation of PED is very similar to that caused by another coronavirus, transmissible gastroenteritis virus (TGEV), which is genetically related to (alphacoronaviruses), but serologically distinct from, PEDV. Severity of clinical disease caused by PEDV tends to be age related. Infection of nursing pigs usually results in extremely high mortality (approaching 100%) due to malabsorption diarrhea as a result of enterocyte destruction in the small intestine. Infection of grow/finish animals results in high morbidity and low mortality with vomiting and mild to moderate diarrhea as the clinical presentation. Infection of mature swine is often overlooked due to minimal or no clinical disease. An experimental animal inoculation study in 4-week-old pigs or nursing pigs has recently been conducted for PEDV. Data from this study is presented below.

MATERIALS AND METHODS

Experimental Animals. Thirty-three PEDV naïve 3-week-old feeder pigs obtained from a high health commercial source were used in this investigation. The animals were allowed to acclimate for one week prior to inoculation. The study was conducted under BSL3-Ag containment at the Biosecurity Research Institute at Kansas State University.

Numbers/Grouping. Experimental groups are summarized in Table 1. Group A pigs were inoculated with the PEDV challenge material. Group B pigs were not inoculated, but were comingled with inoculated Group A animals approximately 6 hours after challenge. The aerosol transmission Group C pigs were not inoculated, but were housed in a separate pen in the same animal room as Groups A and B. A large tarp separated Groups A and B from Group C.

Table 1. Experimental groups

Group	Treatment	Number of Animals
A	PEDV oronasal inoculated	23
B	None: Contact Controls	5
C	None: Aerosol Transmission Controls	5

PEDV Challenge. The challenge material was a pool of gut-derived intestinal contents that had been used as "feedback" inocula for controlled exposure of a sow herd in a commercial swine production unit. The challenge material was kindly provided by Dr. Matt Ackerman of Swine Veterinary Services. The inocula had a PEDV nucleic acid "CT titer" of 22 as determined by the Kansas State Veterinary Diagnostic Laboratory (KSVDL) real-time PCR assay. Challenged animals (Group A) were inoculated at 4 weeks of age via intranasal and oral routes with 5 ml of inocula per route.

Sample Collection Schedule. Sample collection and sacrifice schedule for all three groups is summarized in Table 2. Nasal swabs, fecal swabs, oral fluids, and serum samples were collected prior to challenge on day -3 and days 0-7, 9, 14, 21, 28, 35, and 42 post-challenge. PEDV shedding was monitored by real-time PCR testing of fecal swabs, nasal swabs, and oral fluids. Serum samples were collected to monitor viremia and antibody response. To assess the presence of virus in the environment, swabs were collected from the V-troughs, walls, pens, and food bins from both the inoculated and aerosol control sides of the room on day 14 post-challenge.

Table 2. Sample collection and sequential sacrifice schedule

Day Post Challenge	Blood	Nasal Swab	Fecal Swab	Sacrifice Group (Number)	Oral Fluids
-3	A, B,C	A, B, C	A, B, C		A, B, C
0	A, B,C	A, B, C	A, B, C	A (1)	A, B, C
1	A, B,C	A, B, C	A, B, C		A, B, C
2	A, B,C	A, B, C	A, B, C	A (1)	A, B, C
3	A, B,C	A, B, C	A, B, C		A, B, C
4	A, B,C	A, B, C	A, B, C	A (1)	A, B, C
5	A, B,C	A, B, C	A, B, C		A, B, C
6	A, B,C	A, B, C	A, B, C		A, B, C
7	A, B,C	A, B, C	A, B, C	A (2)	A, B, C
9	A, B,C	A, B, C	A, B, C	A (2)	A, B, C
14	A, B,C	A, B, C	A, B, C	A (2)	A, B, C
21	A, B,C	A, B, C	A, B, C	A (2)	A, B, C
28	A, B,C	A, B, C	A, B, C	A (2)	A, B, C
35	A, B,C	A, B, C	A, B, C		A, B, C
42	A, B,C	A, B, C	A, B, C		A, B, C

Clinical Evaluation. All pigs were evaluated daily by a veterinarian on day -3 and days 0 through 11 post-challenge. Physical examinations were performed on individual animals in all three experimental groups. Relevant health parameters were assessed and documented in daily medical records for each pig. Attitude and response to stimuli were assessed and each pig was recorded as bright, quiet, slightly depressed, depressed, or moribund. Body condition scores were assessed using a 5 point scale adapted from Patience and Thacker, 1989.

Dehydration was assessed through multiple examination parameters including bilateral enophthalmos and third eyelid protrusion, nasal planum moisture and coloration, mucus membrane moisture and coloration, and skin turgor. Skin turgor was assessed by grasping the skin dorsal to the scapula for one second and then releasing. Time for the skin to return to its normal position was designated as normal or prolonged.

Individual animals as well as pens were assessed for evidence of emesis and diarrhea. Fecal consistency scores were determined for each pig using a 5 point scale: 0 = no feces; 1 = normal feces; 2 = soft but formed feces; 3 = brown diarrhea with particulate fecal material; 4 = brown diarrhea without particulate fecal material; 5 = clear, watery diarrhea. The perineum and caudomedial aspects of the hind limbs were also assessed for evidence of diarrhea.

Gross Necropsy and Histopathology. Pigs were sequentially sacrificed through the study to evaluate for gross and microscopic lesions as well as monitor tissue tropism of the virus. The timeline for sequential sacrifice is summarized in Table 2. Randomly selected Group A pigs were sacrificed at days 0, 2, 4, 7, 9, 14, 21, 28, 35, and 42 post-challenge. Pigs were euthanized by intravenous injection of pentobarbital and gross necropsies were performed immediately after euthanasia. A complete set of tissues was collected from each pig including inguinal lymph node, submandibular lymph node, tonsil, thymus, thyroid glands, esophagus, trachea, lung (1 section from each lobe), tracheobronchial lymph node, heart, liver, adrenal glands, kidneys, spleen, stomach, mesenteric lymph node, duodenum with pancreas, jejunum (2 locations), ileum, cecum, spiral colon (2 locations), descending colon, nasal turbinates, bone marrow, and brain. Tissues were frozen or allowed to fix in 10% neutral buffered formalin for at least 7 days. Fixed tissues were processed in an automated tissue processor and embedded in paraffin. Slide-mounted tissue sections were stained with hematoxylin and eosin (H&E stain) and evaluated by a board-certified pathologist.

Immunohistochemistry. Immunohistochemical staining was performed on formalin fixed paraffin embedded tissues that were sectioned at 4μm thickness onto positively charged slides. Slides were stained using the Leica Bond-Max autostainer with the Polymer Refine Detection kit. The PEDV primary antibody was diluted with Bond Primary Antibody Diluent (Leica Biosystems, Tris-buffered saline) to 1:100,000. Heat mediated epitope retrieval was done using citrate pH 6.0 for 20 minutes at 100°C. Tissue sections were incubated with the primary antibody for 15 minutes at ambient temperature. Polymerization was performed with Powervision Poly-HRP α-Mouse Polymer (Leica Biosystems) for 25 minutes at ambient temperature. Visualization was done with DAB and counterstained with hematoxylin.

PCR. The MagMAX-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY) was used together with a Kingfisher 96 magnetic particle processor (Fisher Scientific, Pittsburgh, PA) for all sample types. Tissue samples were homogenized using a Stomacher® 80 Biomaster (ThermoScientific, Swedesboro, NJ) while serum samples were used untreated for RNA extraction. 1 ml of 1X PBS buffer was added to approximately 0.5 g of the fecal samples as well as the nasal swab tubes, vortexed briefly, and allowed to sit for 2-3 min. The supernatant was then used for RNA extraction. For all sample types, 70 µl of liquid was used for RNA extraction. The extracted RNA was frozen at -20°C until analysis by real-time reverse-transcription PCR (qRT-PCR) was performed as described below.

A duplex qRT-PCR was designed for the dual purpose of detecting porcine epidemic diarrhea virus (PEDV) in samples by targeting the nucleocapsid protein-encoding gene, the N gene, and by targeting the host 18S ribosomal RNA subunit to monitor extraction efficiency. Primers and probe sequences for PEDV are: PEDVn-F2: GCT ATG CTC AGA TCG CCA GT, PEDVn-R2: TCT CGT AAG AGT CCG CTA GCT C, PEDVn-Pr2 probe: FAM-TGC TCT TTG GTG GTA ATG TGG C-BHQ1; Primers and probe sequences for 18S are: 18S-F: GGA GTA TGG TTG CAA AGC TGA, 18S-R: GGT GAG GTT TCC CGT GTT G, 18S-Pr probe: Cy5-AAG GAA TTG ACG GAA GGG CA-BHQ2. Life Technologies (Grand Island, NY) Path-ID™ Multiplex One-Step Kit was used for all real-time PCR reactions. qRT-PCR reactions in 20 µL consisted of 1.5 µL nuclease-free water, 10 µL 2x Reaction Buffer, 1 µL 10 µM PEDVn forward and reverse primers, 1 µL 10 µM 18S forward and reverse primers, 1 µL 10 µM 18S probe, 0.5 µL PEDV probe (10 µM), 1 µL Path-ID™ Multiplex One-Step Kit enzyme mix and 4 µL extracted RNA. Each qRT-PCR reaction plate was run on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) under the following conditions: 48°C for 10 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 sec and 60°C for 40 sec. Positive and negative PCR amplification controls were included in each run.

Antibody Response

Throughout the course of the study, all of the animals were bled at the time indicated in the experimental design table listed previously. Sera were processed and collected using normal procedures and stored at -70ºC for future antibody and virus testing.

Serially diluted serum samples were assayed for PEDV antibodies using an indirect fluorescent antibody assay (IFA) in a 96-well format. The IFA antigen was obtained by infecting ST cells with a standardized stock of PEDV virus. IFA assay endpoints were calculated as the reciprocal of the last serum dilution that gave a positive IFA response when viewed with a fluorescent microscope.

PEDV neutralizing antibody levels were determined using a 96-well microtiter system with VERO cells as the substrate and a standardized trypsin independent stock of a PEDV as the indicator virus. Serial dilutions of serum were mixed with a constant quantity of PEDV virus (50-300 $TCID_{50}$), incubated for 1 hour at 37ºC and inoculated into 4 replicate wells of 3 day old VERO cells in 96 well plates. Cultures were incubated 3 days at 37ºC and the presence of virus was determined by the presence of the cytopathic effect of the virus on the cells. Serum neutralization titers were based on 50% inhibition of the indicator virus and 50% endpoints were then determined by the method of Spearman and Karber.

RESULTS AND DISCUSSION

Clinical Evaluation. A timeline of clinical signs documented in PEDV inoculated pigs are summarized in Figure 1. The peak in clinical signs correlates well with the time course of peak fecal and nasal PEDV shedding between days 4 and 7 post-challenge (compare Figure 1 with Figures 3 and 4).

In the inoculated pigs, clinical signs of lethargy were most prominent on days 5 and 6 post-challenge. Over this 2-day period, 45% of inoculated pigs were documented with some degree of lethargy. Clinical signs of lethargy were moderate and resulted in pigs having lowered heads, droopy ears, decreased resistance to restraint, decreased responsiveness, slow movements, and reluctance to rise and ambulate. No pigs were considered moribund during the study. After day 6 post-infection, clinical signs of lethargy gradually improved until the conclusion of the evaluation period.

The average body condition score of the inoculated pigs was lowest (approximately 2.5/5) on days 4 through 7 post-challenge. By day 11 post-challenge, the average body condition score of the inoculated pigs had increased to ideal (approximately 3/5). The average body condition score was higher in the aerosol control group on every day of the evaluation period when compared to the inoculated group (see Figure 2).

Evidence of clinical dehydration was most prominent in the inoculated pigs between days 4 and 8 post-challenge. Over this 5-day period, 70% of the inoculated pigs were documented with some degree of dehydration. Dehydration was considered relevant when 3 or more clinical signs of dehydration were present. Dehydration was overall considered mild to moderate and resulted in pigs having enophthalmos with third eyelid protrusion, dry nasal planum, tacky mucus membranes, and decreased skin turgor.

Emesis was the first clinical sign of disease documented on day 3 post-challenge. Vomitus was noted on the floor of the inoculated pen and active emesis was documented in one inoculated pig. Vomitus was yellow liquid, contained partially digested food particulate, and did not contain blood or mucus.

In the inoculated pigs, the most severe diarrhea (score 4 = brown diarrhea without particulate fecal material) was noted between days 4 and 7 post-challenge. Over this 4-day period, 30% of the inoculated pigs were documented with this diarrhea score. Diarrhea was light brown liquid, contained minimal particulate fecal

material, and did not contain blood or mucus. A diarrhea score of 4 was initially noted on day 4 post-challenge, affected the most pigs on day 5 post-challenge, and resolved on day 8 post-challenge.

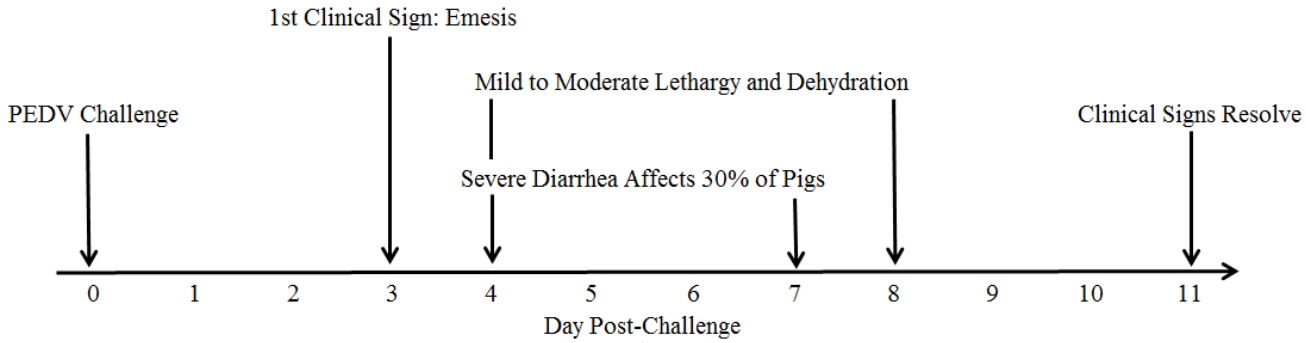


Figure 1. Timeline of clinical signs documented in 4 week-old pigs on days 0-11 post-challenge with PEDV.

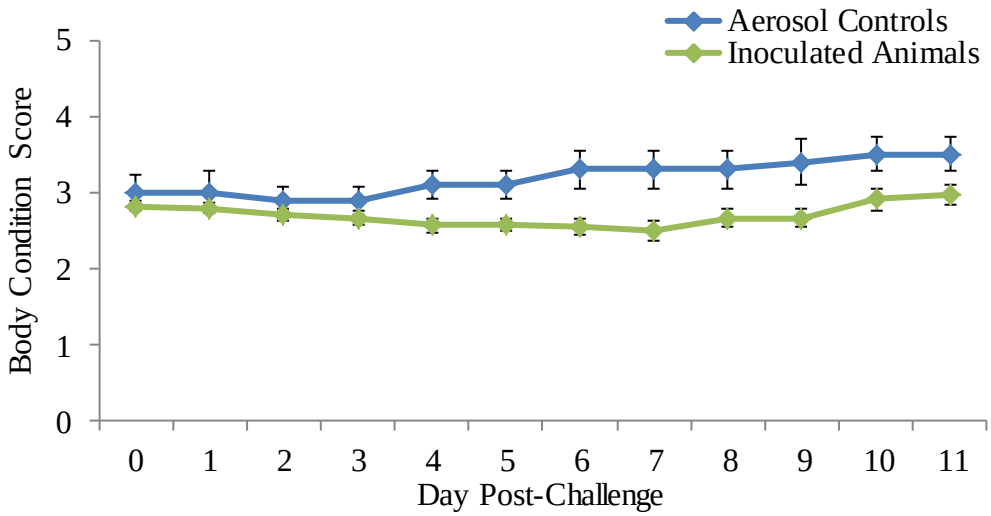


Figure 2. Average body condition scores of the aerosol control and inoculated groups on days 0-11 post-challenge with PEDV. Data is shown as mean body condition score ± 1 standard error.

Gross Necropsy and Histopathology. No gross lesions were observed in any of the sacrificed pigs. Microscopic lesions were noted in the small intestines of 3 of the 13 sacrificed pigs. Moderate to moderately severe villous atrophy and fusion was noted in the jejunum and ileum of a pig sacrificed on day 4 post-challenge. Mild to moderate subacute diffuse villous atrophy was noted in the small intestine of a pig sacrificed on day 7 post-challenge. Mild subacute villous atrophy was noted in the small intestine of a pig sacrificed on day 28 post-challenge.

Fecal and Nasal PEDV Shedding. Real time PCR group average CT values for fecal and nasal shedding are shown in Figures 3 and 4. Surprisingly, all samples were negative for the virus at 24 hours post-inoculation. Fecal and nasal shedding of the inoculated group (A) was first observed at 48 hours post-inoculation. Nasal shedding was detected in the contact control group (B) at 48 hours post-inoculation and fecal shedding occurred 24 hours later. Peak fecal shedding occurred between 5 to 6 days post-challenge and was significantly higher than nasal shedding. In Groups A and B, the majority of the animals were negative for fecal shedding at 21 days post-inoculation. However, 3 of 11 animals in the inoculated group and 1 of 5 animals in the contact control group were still shedding virus at 21 days post-inoculation and 1 of 11 was positive at 28 days post-

inoculation. Most inoculated (A) and contact control (B) animals were not shedding intranasal virus at 21 days post-inoculation.

Fecal and nasal shedding patters are presented in figures 5 and 6. There appears to be considerable variation in fecal shedding patterns among inoculated pigs. Fecal shedding patterns among contact controls appear to be more consistent. Fecal shedding appeared to be absent in each of the aerosol control animals. Nasal shedding patterns were less variable than fecal shedding patterns. Based on a CT cut off value of 38, two of the five aerosol control animals appeared to have viral nucleic acid in their nares. These same animals showed no evidence of fecal shedding.

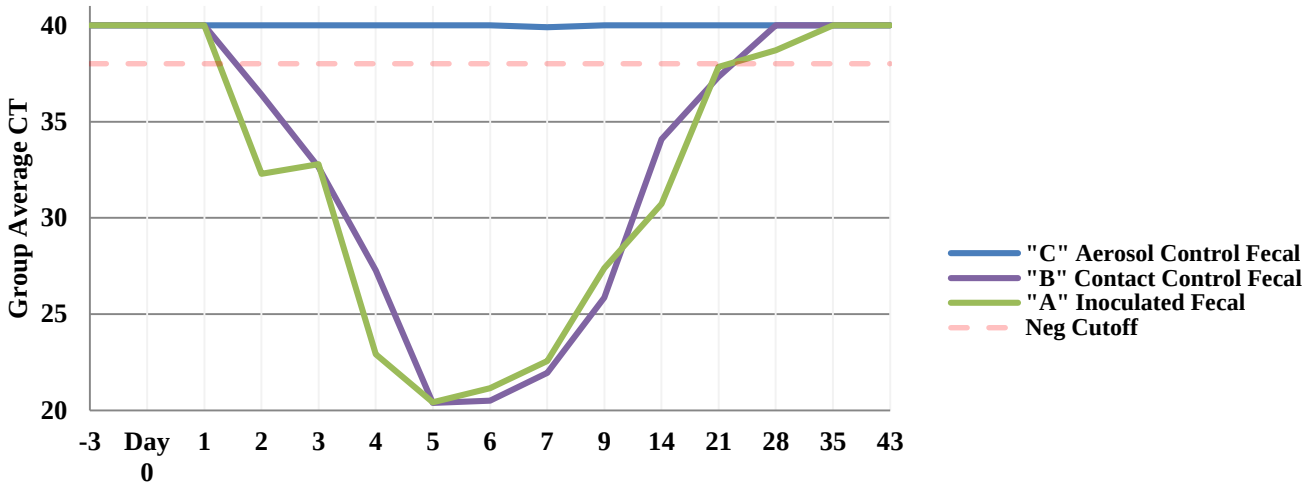


Figure 3. Fecal PEDV shedding of experimental groups. Data is represented by real-time PCR group average cycle thresholds (CT). A CT value of 38 or greater is considered negative.

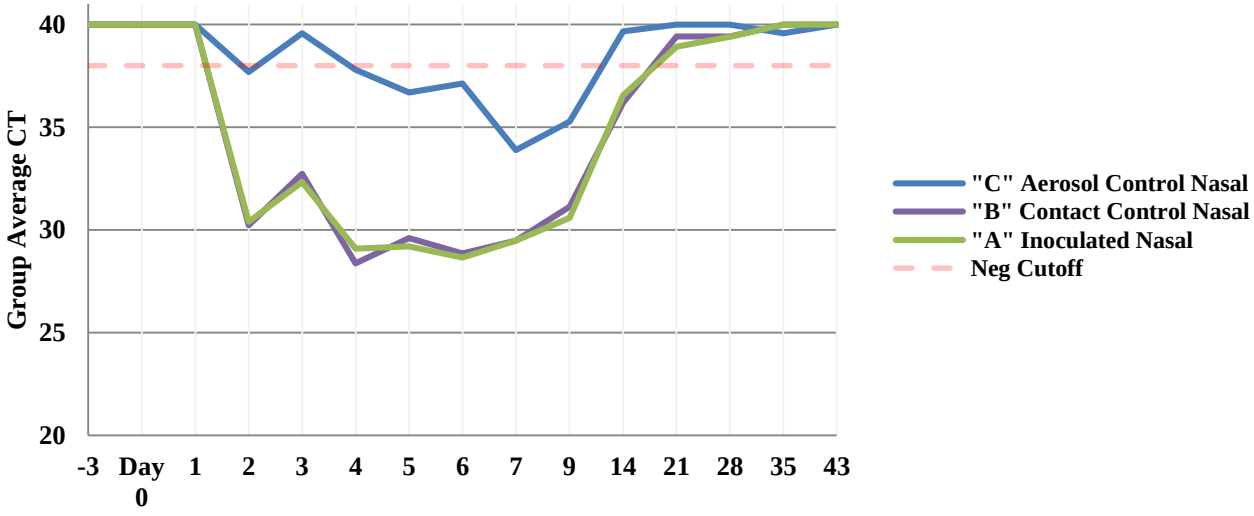


Figure 4. Nasal PEDV shedding of experimental groups. Data is represented by real-time PCR group average cycle thresholds (CT). A CT value of 38 or greater is considered negative.

Fecal Swab	Day -3	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 14	Day 21	Day 28	Day 35	Day 43
Pig 1	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Pig 2	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Pig 3	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Pig 4	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Pig 5	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Aero Con X	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Aero Con SD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pig 10	40	40	40	35	37	23	20	21	24	32	37	40	40	40	40
Pig 13	40	40	40	37	34	27	21	21	20	24	28	40	40	40	40
Pig 18	40	40	40	39	36	28	19	22	22	24	40	37	40	40	40
Pig 19	40	40	40	35	36	22	20	20	21	27	33	40	40	40	40
Pig 32	40	40	40	36	20	35	23	19	22	23	32	30	40	40	40
Contact Con X	40	40	40	36	33	27	20	21	22	26	34	37	40	40	40
Contact Con SD	0	0	0	2	7	5	1	1	2	4	4	5	0	0	0
Pig 6	40	40	40	36	37	21	22	26	26	30	24	40	40	40	40
Pig 9	40	40	40	31	21	18	20	28	25	25	36	40	40	40	40
Pig 14	40	40	40	38	37	25	22	23	22	33	27	40	40	40	40
Pig 20	40	40	40	34	32	26	19	18	18	24	33	40	40	40	40
Pig 23	40	40	40	37	35	20	20	19	19	26	35	40	40	40	40
Pig 24	40	40	40	23	29	21	19	19	22	26	24	30	40	40	40
Pig 25	40	40	40	35	35	25	20	17	17	22	40	40	40	40	40
Pig 26	40	40	40	35	35	30	22	23	27	25	28	32	40	40	40
Pig 27	40	40	40	34	36	26	19	22	21	33	28	40	40	40	40
Pig 33	40	40	40	34	35	29	22	19	23	27	40	40	40	40	40
Inoc Non-Sac X	40	40	40	34	33	24	20	21	22	27	31	38	40	40	40
Inoc Non-Sac SD	0	0	0	4	5	4	1	4	3	4	6	4	0	0	0
Pig 15	40	40													
Pig 11	40	40	40	35											
Pig 17	40	40	40	33	40	21									
Pig 21	40	40	40	32	32	25	21	20	22						
Pig 31	40	40	40	29	40	19	20	19	22						
Pig 28	40	40	40	25	31	22	21	21	25	23					
Pig 30	40	40	40	17	23	25	23	24	27	30					
Pig 7	40	40	40	21	22	19	20	24	26	32	26				
Pig 12	40	40	40	37	37	22	20	20	22	27	33				
Pig 8	40	40	40	34	20	19	21	21	23	28	28	28			
Pig 29	40	40	40	34	36	26	19	20	22	29	30	40			
Pig 16	40	40	40	34	34	20	18	19	20	23	34	40	40		
Pig 22	40	40	40	40	40	22	21	21	21	29	26	40	25		
Inoc Sac X	40	40	40	31	32	22	20	21	23	28	30	37	32		
Inoc Sac SD	0	0	0	7	7	3	1	2	2	3	3	6	11		

Figure 5. Fecal PEDV shedding in individual pigs. Data is represented by real-time PCR cycle thresholds (CT) between days -3 to 43 post-challenge or until day of sacrifice.

Nasal Swab	Day -3	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 14	Day 21	Day 28	Day 35	Day 43
Pig 1	40	40	40	40	40	40	36	38	33	34	38	40	40	40	40
Pig 2	40	40	40	37	40	37	37	37	36	40	40	40	40	40	40
Pig 3	40	40	40	36	40	37	37	37	34	36	40	40	40	40	40
Pig 4	40	40	40	35	38	40	37	37	33	35	40	40	40	38	40
Pig 5	40	40	40	40	40	36	36	37	33	31	40	40	40	40	40
Aero Con X	40	40	40	38	40	38	37	37	34	35	40	40	40	40	40
Aero Con SD	0	0	0	2	1	2	1	1	2	3	1	0	0	1	0
Pig 10	40	40	40	30	33	30	30	27	28	31	35	40	40	40	40
Pig 13	40	40	40	29	37	30	30	29	27	31	37	40	40	40	40
Pig 18	40	40	40	29	32	30	30	30	32	33	37	40	40	40	40
Pig 19	40	40	40	30	30	29	29	30	33	31	34	40	37	40	40
Pig 32	40	40	40	33	31	22	30	28	28	30	38	37	40	40	40
Contact Con X	40	40	40	30	33	28	30	29	29	31	36	39	39	40	40
Contact Con SD	0	0	0	2	3	3	0	1	2	1	2	1	1	0	0
Pig 6	40	40	40	30	34	28	29	29	29	33	40	40	40	40	40
Pig 9	40	40	40	27	31	28	29	27	29	31	35	37	40	40	40
Pig 14	40	40	40	32	33	32	30	29	31	33	40	40	40	40	40
Pig 20	40	40	40	29	30	25	27	26	28	30	35	40	40	40	40
Pig 23	40	40	40	31	32	28	28	25	31	31	38	40	40	40	40
Pig 24	40	40	40	33	31	28	30	28	31	29	36	38	40	40	40
Pig 25	40	40	40	29	33	28	29	30	21	29	37	40	40	40	40
Pig 26	40	40	40	29	32	29	28	30	31	30		40	40	40	40
Pig 27	40	40	40	30	33	30	29	27	32	28	36	40	40	40	40
Pig 33	40	40	40	31	31	29	29	30	31	29	35	40	40	40	40
Inoc Non-Sac X	40	40	40	30	32	29	29	28	29	30	37	39	40	40	40
Inoc Non-Sac SD	0	0	0	2	1	2	1	2	3	2	2	1	0	0	0
Pig 15	40	40													
Pig 11	40	40	40	32											
Pig 17	40	40	40	29	33	31									
Pig 21	40	40	40	31	34	29	29	30	29						
Pig 31	40	40	40	30	32	30	29	32	30						
Pig 28	40	40	40	29	31	30	29	28	29	30					
Pig 30	40	40	40	28	31	29	29	28	31	31					
Pig 7	40	40	40	30	37	31	32	29	29	33	37				
Pig 12	40	40	40	32	32	29	30	28	27	32	37				
Pig 8	40	40	40	31	34	31	30	30	30	30	36	36			
Pig 29	40	40	40	30	32	30	30	31	30	33	35	40			
Pig 16	40	40	40	32	31	29	30	29	29	30	36	38	40		
Pig 22	40	40	40	32	29	27	29	28	31	29	36	36	33		
Inoc Sac X	40	40	40	31	32	30	30	29	30	31	36	38	36		
Inoc Sac SD	0	0	0	2	2	1	1	1	1	1	1	2	5		

Figure 6. Nasal PEDV shedding in individual pigs. Data is represented by real-time PCR cycle thresholds (CT) between days -3 to 43 post-challenge or until day of sacrifice.

Oral Fluids Shedding and Environmental Samples. Real time PCR group average CT values for oral fluids are shown in Figure 7. Oral Fluids from the pen housing inoculated animals (Group A) and contact controls (Group B) were PCR positive at 48 hours post-inoculation and remained positive until day 28 post-inoculation. Oral fluids from the aerosol control group appeared to be positive at the time of the first successful collection point (D-4) and they remained positive through day 28 post-inoculation. A weak CT (>37) was observed in the aerosol group at days 21 and 28 post-inoculation.

Room environmental samples were collected at 14 days post-inoculation and the real time PCR values are summarized in Figure 8. The data demonstrates that viral nucleic acid was abundant on the walls, pens and food bins in both the inoculated and aerosol control areas of the challenge room. Due to the possibility of a false positive PCR reaction, questionable samples were retested and the reaction products were sequenced to

determine if the product was PEDV specific. All questionable reactions demonstrated the presence of PEDV viral nucleic acid.

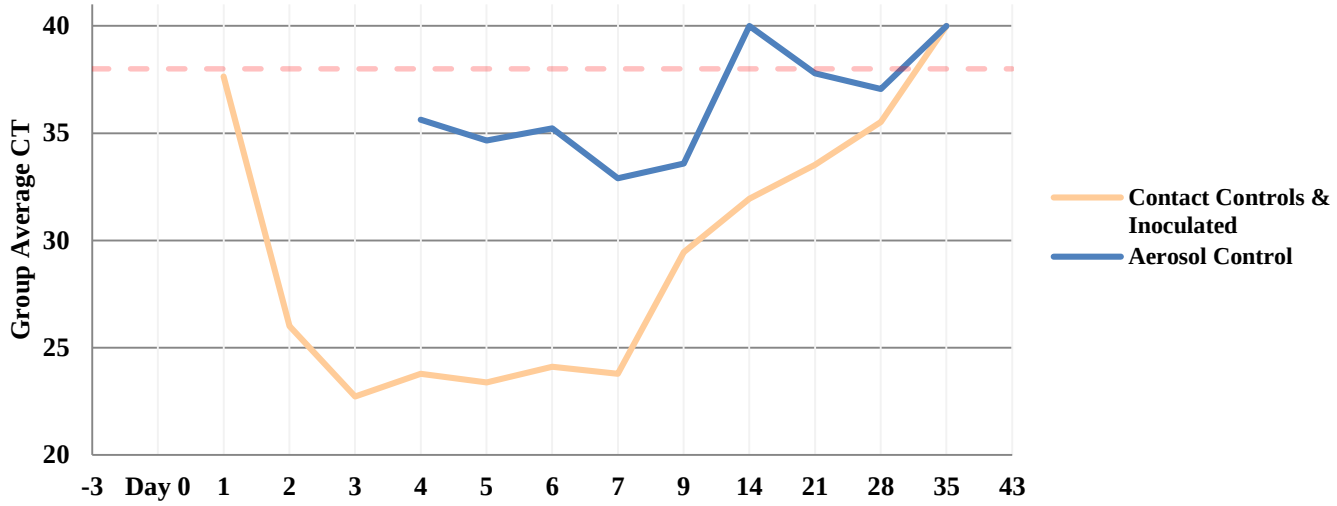


Figure 7. Oral fluids PEDV shedding of experimental groups. Data is represented by real time PCR group average cycle thresholds (CT). A CT value of 38 or greater is considered negative.

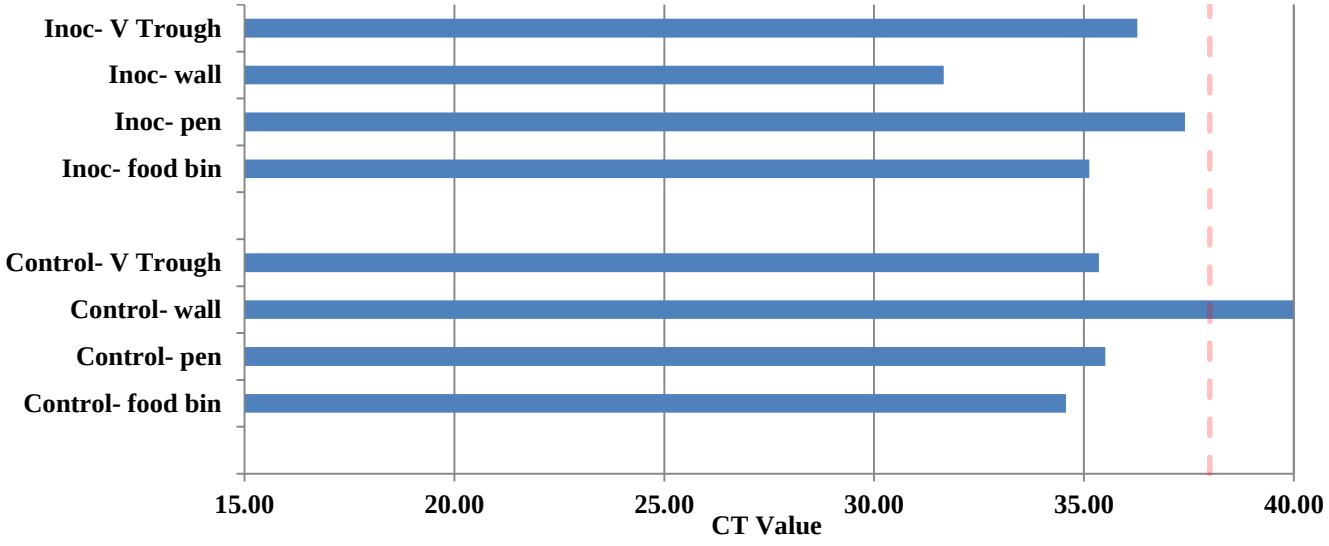


Figure 8. Real time PCR cycle thresholds (CT) of environmental samples collected on day 14 post-challenge. Data is shown for both the inoculated and aerosol control sides of the room.

Viremia. Average PEDV viremia post-challenge is summarized below in the three experimental groups (Figure 9) and in individual animals (Figure 10). PEDV viremia was clearly detected in 3 of the 5 contact controls and 9 of the 22 inoculated animals. No detectable viremia was detected in any of the aerosol control animals. The raw data suggests that there seems to be a correlation with viremia and extended duration of shedding in either feces or nasal secretions.

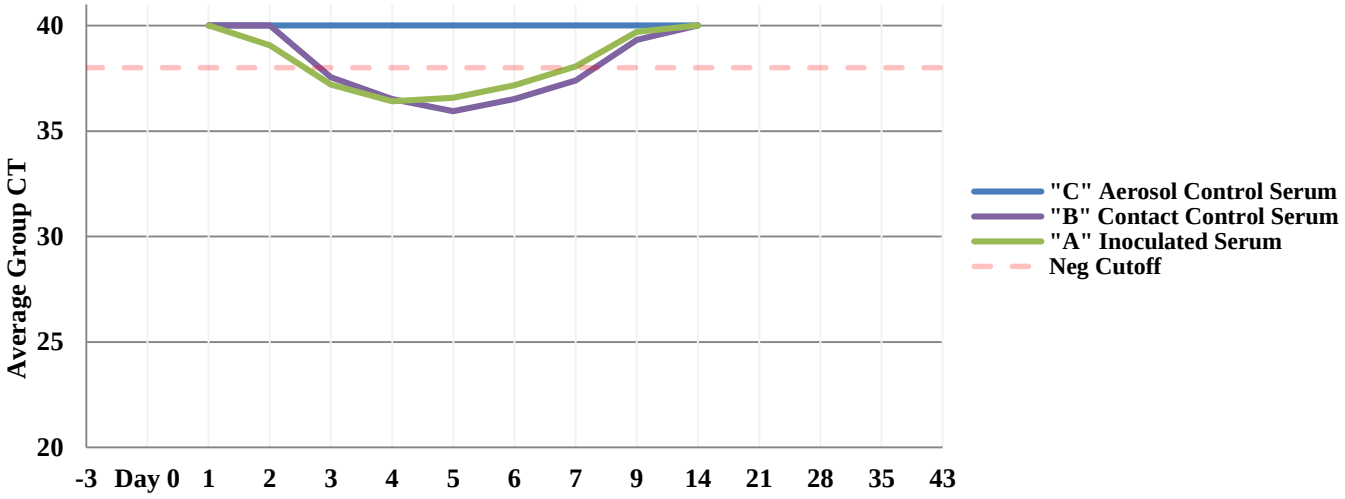


Figure 9. PEDV viremia of experimental groups. Data is represented by real-time PCR group average cycle thresholds (CT). A CT value of 38 or greater is considered negative.

Serum	Day -3	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 14	Day 21	Day 28	Day 35	Day 43
Pig 1			40	40	40	40	40	40	40	40	40				40
Pig 2			40	40	40	40	40	40	40	40	40				40
Pig 3			40	40	40	40	40	40	40	40	40				40
Pig 4			40	40	40	40	40	40	40	40	40				40
Pig 5			40	40	40	40	40	40	40	40	40				40
Aero Con X			40	40	40	40	40	40	40	40	40				40
Aero Con SD			0	0	0	0	0	0	0	0	0				0
Pig 10			40	40	35	31	32	33	33	39	40				40
Pig 13			40	40	40	40	40	40	40	40	40				40
Pig 18			40	40	32	34	35	37	37	40	40				40
Pig 19			40	40	40	37	36	36	40	40	40				40
Pig 32			40	40	40	40	37	36	37	37	40				40
Contact Con X			40	40	38	37	36	37	37	39	40				40
Contact Con SD			0	0	4	4	3	2	3	1	0				0
Pig 6			40	40	40	40	36	36	40	40	40				40
Pig 9			40	33	37	36	35	35	40	40	40				40
Pig 14			40	40	34	34	40	40	40	40	40				40
Pig 20			40	40	40	34	40	40	40	40	40				40
Pig 23			40	40	35	32	30	29	30	40	40				40
Pig 24			40	40	40	37	40	40	40	40	40				40
Pig 25			40	40	35	36	40	40	40	40	40				40
Pig 26			40	40	40	40	36	40	37	40	40				40
Pig 27			40	40	40	36	34	38	40	40	40				40
Pig 33			40	40	40	36	33	34	35	37	40				40
Inoc Non-Sac X			40	39	38	36	36	37	38	40	40				40
Inoc Non-Sac SD			0	2	3	3	4	4	3	1	0				0
Pig 15															
Pig 11			40	40											
Pig 17			40	40	37	37									
Pig 21			40	40	31	38	37	36	40						
Pig 31			40	40	38	40	40	40	40						
Pig 28			40	34	30	36	40	40	40	40					
Pig 30			40	33	34	32	32	34	37	40					
Pig 7			40	40	40	40	40	40	40	40	40				
Pig 12			40	40	40	37	37	38	37	40	40				
Pig 8			40	40	30	31	30	30	32	37	40				
Pig 29			40	40	40	37	40	40	40	40	40				
Pig 16			40	40	40	40	33	33	32	40	40				
Pig 22			40	40	40	37	37	40	40	40	40				
Inoc Sac X			40	39	36	37	37	37	38	40	40				
Inoc Sac SD			0	3	4	3	4	4	3	1	0				

Figure 10. PEDV viremia in individual pigs. Data is represented by real-time PCR cycle thresholds (CT) between days -3 to 43 post-challenge or until day of sacrifice.

Immunohistochemistry. PEDV immunohistochemistry in small intestinal sections of sacrificed pigs is summarized in Table 3 with microscopic images shown in Figure 3. PEDV antigen was not evenly distributed throughout the entire small intestine nor was it consistently detected in pigs sacrificed at the same time points. Based on an evaluation of one intestinal section, it would be highly possible to incorrectly classify a positive pig as negative. This is especially true on days 9 or greater post-challenge (see Table 2).

PEDV antigen was also detected in the mesenteric lymph nodes of pigs sacrificed on days 4, 7, and 21 post-challenge with PEDV. A representative image of positive IHC staining in a mesenteric lymph node is shown in Figure 12.

Table 3. Immunohistochemistry summary of small intestinal sections collected sequentially from pigs after challenge with PEDV.

Day Post Challenge	Number of Positive Pigs (Total Evaluated)	Positive Sections (5 Collected)
0	0 (1)	0
2	0 (1)	0
4	1 (1)	5
7	1 (2)	5
9	0 (2)	0
14	1 (2)	1
21	1 (2)	2
28	1 (2)	2

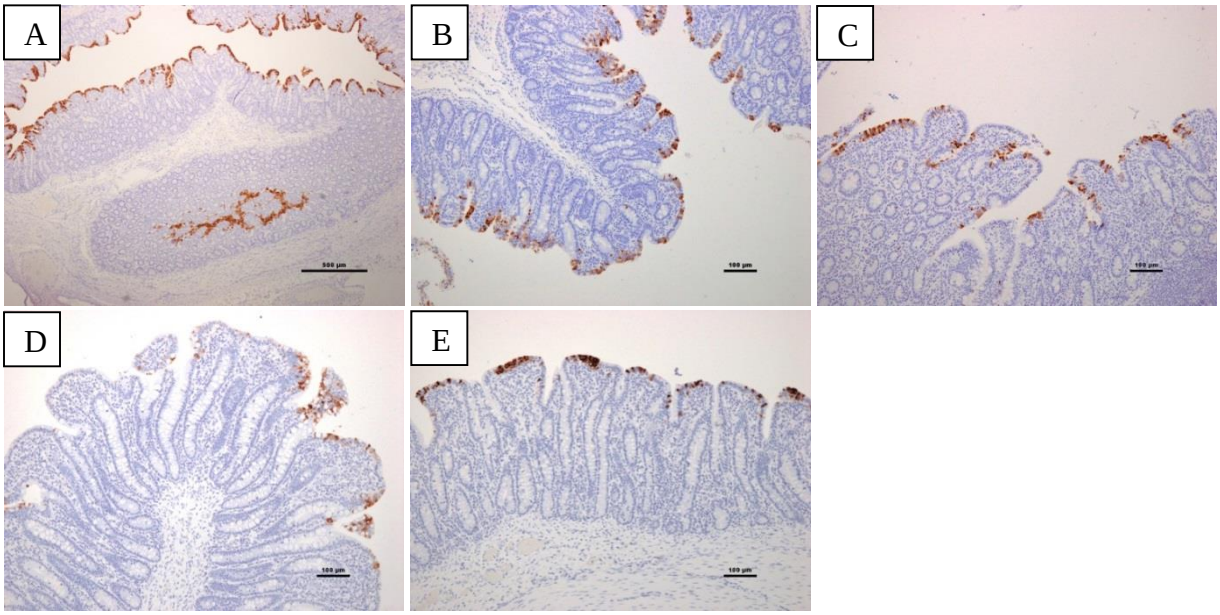


Figure 11. Sequential immunohistochemistry of small intestinal sections from five pigs on days 4 (A), 7 (B), 14 (C), 21 (D), and 28 (E) post-challenge with PEDV.

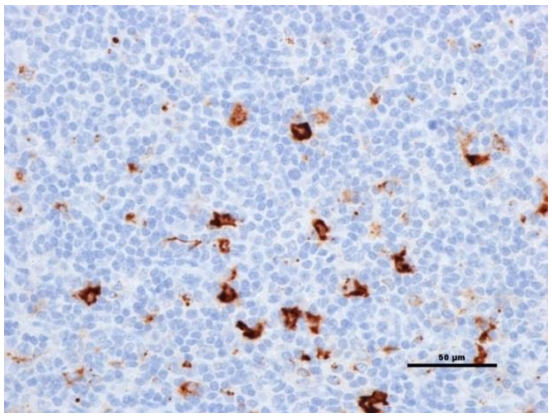
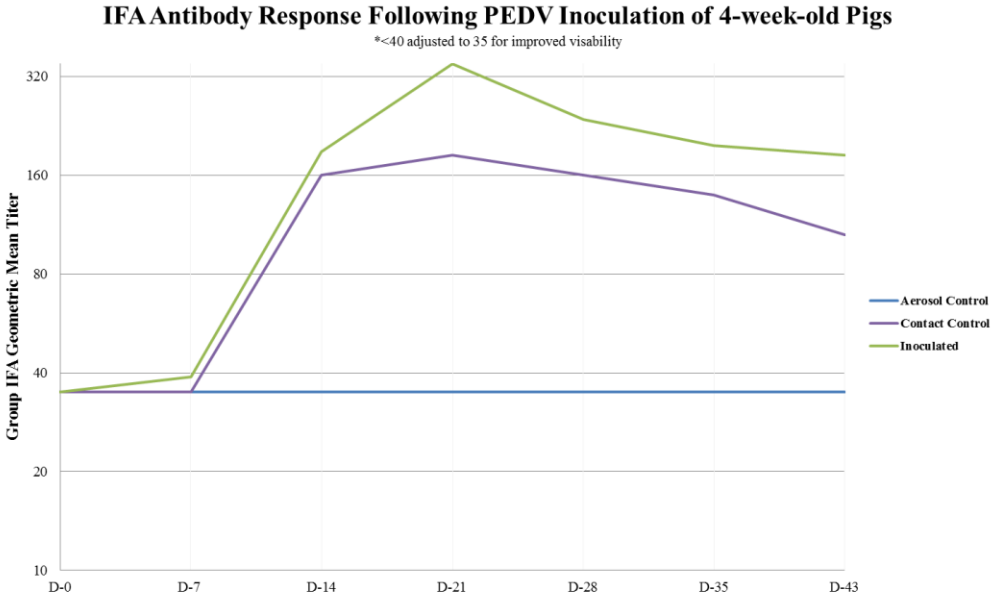


Figure 12. Immunohistochemistry showing large numbers of positive PEDV cells in the mesenteric lymph node of a pig 4 days after challenge with PEDV.

		Serum	Turbinate	Tonsil	Trachea	Lung	Lymph Node	Thymus	Spleen	Small Intestine
Sac'd Pigs	Pig 15 (D0)	N/A	40.00	40.00	40.00	40.00	40.00	40.00	40.00	39.93
	Pig 11 (D2)	40.00	36.21	38.42	40.00	40.00	40.00	36.31	40.00	36.78
	Pig 17 (D4)	40.00	40.00	35.31	40.00	39.10	27.65	40.00	40.00	13.62
	Pig 21 (D7)	34.49	39.65	36.57	40.00	40.00	23.34	39.88	36.69	14.53
	Pig 31 (D7)	40.00	38.73	35.48	40.00	40.00	26.74	34.47	40.00	24.95
	Pig 28 (D9)	40.00	40.00	35.23	40.00	40.00	31.67	40.00	29.19	26.30
	Pig 30 (D9)	40.00	40.00	40.00	40.00	40.00	33.54	38.27	33.45	27.83
	Pig 7 (D14)	40.00	40.00	38.93	40.00	40.00	30.73	40.00	38.23	21.95
	Pig 12 (D14)	40.00	40.00	40.00	40.00	40.00	31.88	40.00	35.52	29.69
	Pig 8 (D21)	33.43	38.55	38.55	40.00	40.00	32.90	40.00	35.14	19.93
	Pig 29 (D21)	40.00	40.00	40.00	40.00	40.00	35.32	40.00	37.27	28.22
	Pig 16 (D28)	40.00	40.00	40.00	40.00	40.00	30.10	40.00	38.34	27.46
	Pig 22 (D28)	40.00	40.00	36.46	40.00	40.00	34.72	35.59	40.00	18.92
Non-Sac'd Pigs	Pig 1						40.00			40.00
	Pig 2						40.00			40.00
	Pig 3						40.00			40.00
	Pig 4						40.00			40.00
	Pig 5						40.00			40.00
	Pig 10						40.00			40.00
	Pig 13						40.00			40.00
	Pig 18						40.00			40.00
	Pig 19						40.00			40.00
	Pig 32						40.00			40.00
	Pig 6						40.00			32.64
	Pig 9						40.00			40.00
	Pig 14						40.00			40.00
	Pig 20						40.00			32.14
	Pig 23						40.00			31.96
	Pig 24						40.00			35.85
	Pig 25						38.79			38.43
	Pig 26						40.00			32.38
	Pig 27						40.00			34.34
	Pig 33						39.03			35.32

Figure 13. PEDV nucleic acid in tissues collected from sacrificed individual pigs throughout the study. Data is represented by real-time PCR cycle thresholds (CT) between days 0 to 43 post-challenge or until day of sacrifice.

Antibody Response. Serological testing of serum samples from all three groups was performed using indirect fluorescent antibody assay and the serum neutralization assay. Geometric mean titers for each group for both assay systems is shown in Figure 14. The data show pre-inoculation samples are negative and that there was significant seroconversion in the inoculated and contact control animals. There is no evidence of seroconversion in the aerosol control group despite clear demonstration of PEDV nucleic acid in nasal and oral fluid samples. Interestingly, the contact control animals reached lower peak antibody titers and appear to have more rapid antibody decay in the IFA antibody response.



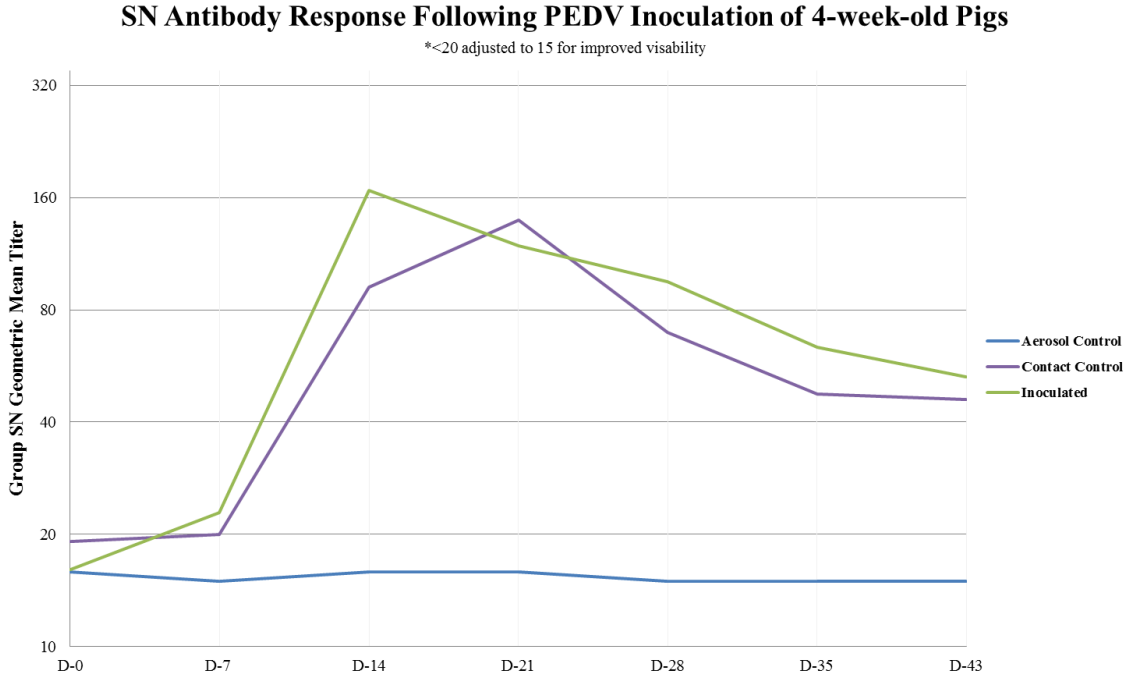


Figure 14. Antibody response of all three experimental groups. Data is shown as geometric mean indirect fluorescent antibody titers and serum neutralization titers on days 0, 7, 14, 21, 28, 35, and 43 post-challenge with PEDV.

Summary and Conclusions.

Fecal, nasal, and oral fluid viral nucleic acid detection data via real time PCR clearly demonstrate productive PEDV infection in the inoculated and contact control groups in this study. Pigs that were positive for nasal shedding were also positive for fecal shedding; fecal shedding was typically detected at a 10 fold or greater level than that observed in the nares. Viral nucleic acid levels in oral fluids were in between those observed in both fecal and nasal samples. In contrast, fecal shedding was not demonstrated in the aerosol control group but samples did test positive for the presence of PEDV viral nucleic acid in nasal and oral fluid samples. Experimental data indicate the following: mild clinical signs appeared on DPI 2 and resolved by DPI 8 in Group A and B pigs. Fecal and nasal swabs were PCR positive in the inoculated group by DPI 2. Peak fecal shedding occurred on DPI 5 and was significantly higher than nasal swabs. Most group A and B animals were PCR negative by fecal or nasal swab testing at 21 DPI; however, some animals shed virus as long as 35 DPI.

Atrophic enteritis was observed in the jejunum and ileum of affected piglets from 2 to 8 DPI, and corresponded to positive antigen detection by IHC. Mesenteric lymph node and small intestine were the primary sites of antigen detection by IHC and tissue qRT-PCR, and most inoculated group A piglets were qRT-PCR positive in the intestinal tissue samples out to the end of the study.

PEDV immunohistochemistry evaluation demonstrated that the only samples that tested positive for the presence of viral antigen were tissues from the GI tract and mesenteric lymph nodes. Turbinates, trachea, lung, bronchial lymph nodes, spleen, and other visceral tissues were all negative for PEDV antigen.

A complete set of serum samples has been provided to 5 laboratories (~1,200 samples) for assay development/standardization. In addition, 3 complete sets of oral fluid samples and tissues have been provided to other laboratories. These samples have been used for assay development and standardization across the different diagnostic laboratories.

Productive transmission did not appear to occur in the aerosol control group in spite of the PEDV nucleic acid detected in the nares of some of those animals and in the oral fluids. Room environmental samples collected at DPI 14 demonstrated that viral nucleic acid was abundant on the walls, pens, and food bins in the challenge room. PEDV viremia was clearly detected in 3 of the 5 contact controls and 9 of the 22 inoculated animals. No detectable viremia was detected in any of the aerosol control animals. Serological data (IFA) proves that pre-inoculation samples were negative and that there was significant seroconversion in all of the inoculated and contact control animals. There was no evidence of seroconversion in the aerosol control group. These results seem to be in conflict with reports from the field that implicate aerosol transmission, but lack confirmation via bioassay. Factors like disinfectant and ultraviolet inactivation of PEDV, sensitivity of the indicator animal (nursing pigs vs. weaned pigs) and infectious dose as a function of route of exposure need to be investigated in order to gain insight into modes of transmission of PEDV.

The tissue PCR positivity for PED nucleic acid at day 43 post inoculation was an unexpected finding which provides insight into virus carriage and potential transmission of the virus long after the clinical disease has abated. In view of these findings, additional animal co-mingle studies will need to be conducted to determine the actual duration of horizontal transmission between infected and naïve pigs.