CLINICAL DISEASE AND HOST RESPONSE OF NURSERY PIGS FOLLOWING CHALLENGE WITH EMERGING AND RE-EMERGING SWINE VIRUSES

by

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B.S., Northwest Missouri State University, 2005
D.V.M., Kansas State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

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Abstract

Emerging viral diseases cause significant and widespread economic losses to U.S. swine production. Over the last 25 years, porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2) and porcine epidemic diarrhea virus (PEDV) have emerged or re-emerged, costing the industry billions through increased mortality and clinical or subclinical reductions in growth. Nursery pigs are greatly affected by these viruses due to high susceptibility to primary and secondary infections after weaning. However, clinical disease occurs in only a subpopulation of infected pigs and can vary drastically from sudden death to poor growth performance. This thesis documents a series of 4 studies where nursery pigs were challenged with either PRRSV/PCV2 or PEDV; the associations between clinical outcome and several factors affecting viral pathogenesis were investigated.

In the first study, the administration of PRRS modified live virus vaccine prior to co-challenge with PRRSV/PCV2 was shown to protect against PRRS but enhance PCV2 replication and pathogenesis. This study provides insight into the role that PRRS vaccination has in both the control and potentiation of clinical disease. In the second study, microbial populations were compared between pigs with the best and worst clinical outcome following PRRSV/PCV2 co-infection. Increased fecal microbiome diversity was associated with improved clinical outcome; however, worst clinical outcome pigs had prolonged and greater virus replication, highlighting the host response to viral challenge as a primary determinant of clinical outcome. In the third study, 13 clinical phenotypes were compiled for >450 pigs after PRRSV/PCV2 co-infection. Duration of dyspnea and the presence of muscle wasting had the strongest associations with reduced weight gain. This study highlights the opportunity to improve animal welfare and production through improvements in clinical health. In the fourth study, clinical disease was

mild to moderate and occurred within the first week after pigs were challenged with PEDV. However, PEDV was detected weeks after clinical disease had resolved and may implicate nursery pigs as an important source of viral carriage and transmission. Overall, the goal of this thesis was to develop models for understanding the impact of emerging and re-emerging viruses to improve recognition and control of disease.

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Approved by:

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Dedication

This thesis is dedicated to my husband, Jeremy, and our daughter, Harper.

Chapter 1 - Vaccination with a porcine reproductive and respiratory syndrome (PRRS) modified live virus vaccine followed by challenge with PRRSV and porcine circovirus type 2 (PCV2) protects against PRRS but enhances PCV2 replication and pathogenesis when compared to non-vaccinated co-challenged controls

Abstract

Co-infections involving porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) contribute to a group of disease syndromes known as porcine circovirus associated disease (PCVAD). Presumably, PRRSV infection enhances PCV2 replication as a result of modulation of host immunity. The purpose of this study was to evaluate PCV2 replication and pathogenesis in pigs vaccinated with a PRRS modified live virus (MLV) vaccine and subsequently challenged with a combination of PRRSV and PCV2. During the early post-challenge period, PRRSV-associated clinical signs were decreased and average daily gain (ADG) was increased in the vaccinated group, demonstrating the protective effect of PRRS vaccination. However, during the later post-challenge period, the vaccinated group showed greater numbers of pigs with increased PCV2 viremia, decreased ADG, increased PCVAD clinical signs, and increased mortality. In this disease model, the early benefits of PRRSV vaccination were outweighed by the later amplification of PCVAD.

Introduction

Porcine circovirus type 2 (PCV2), a single-stranded DNA virus in the family *Circoviridae*, contributes to a group of syndromes collectively termed porcine circovirus associated disease (PCVAD) (Baekbo et al., 2012). Two important clinical syndromes associated with PCVAD include PCV2-associated pneumonia and postweaning multisystemic wasting syndrome (PMWS) (Baekbo et al., 2012; Ramamoorthy and Meng, 2009). Management of PCV2 through the use of inactivated and subunit vaccines have led to the effective control of PCVAD in North America and Europe. However, the emergence of new PCV2 strains and the lack of PCV2 vaccination programs in other countries create an uncertain future for continued disease control.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus in the family *Arteriveridae* (Benfield et al., 1992; Conzelmann et al., 1993). For the past 20 years, PRRSV has remained the most costly disease affecting swine production worldwide (Chand et al., 2012). PRRSV infection contributes to a number of immunological outcomes that increase the susceptibility of the host to secondary infections by primary and secondary pathogens (Gomez-Laguna et al., 2013; Opriessnig et al., 2011; Renukaradhya et al., 2010). PRRSV is frequently isolated along with PCV2 (Pallares et al., 2002) and is one of the major co-factors linked with increasing PCV2 replication and pathogenesis (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002). Previous work by us and others has shown that a principal contribution of PRRSV is to increase PCV2 viremia (Trible et al., 2012a). Increased PCV2 replication is likely the result of immune stimulation that results in more PCV2-permissive cells combined with PRRSV-induced immunomodulation. The complex etiology of PCVAD, including the role of PRRSV infection, has yet to be fully understood. In an extensive body of

work, we identified the aberrant recognition of a non-neutralizing decoy epitope on the PCV2 capsid protein (CP) as a contributing factor to PCVAD immunopathogenesis. Natural PCV2 infection of a population produces a mixture of pigs that recognize the decoy and neutralizing epitopes, which may explain why only a subpopulation of infected pigs go on to develop PCVAD (Trible et al., 2011; Trible et al., 2012a; Trible et al., 2012b).

In this study, we took advantage of a host genetics study to evaluate clinical and virological outcomes after experimental challenge with PCV2 and PRRSV in pigs with and without prior vaccination with a commercial PRRS MLV. The results demonstrate the protective properties of vaccination; however, the short-term benefit is outweighed by the longer term impact of MLV on PCVAD.

Materials and Methods

Animals and housing. Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use Committees and Institutional Biosafety Committees. The study was conducted as part of the evaluation of a previously described genomic marker, WUR (Boddicker et al., 2012). The population of pigs used in this study was composed of two genotypes, 50% WUR genotype AA and 50% AB or BB. The AB and BB genotypes were predicted to have a beneficial effect in response to PRRSV infection. Both vaccine and non-vaccine groups were balanced according to WUR genotype; therefore, WUR was not a factor when comparing the outcomes of vaccine and non-vaccine groups. Three week-old barrows (n = 226; average age of 19.4 ± 1.8 days) were

obtained from a high-health commercial source negative for PRRSV. While pigs were derived from a sow herd previously vaccinated with a PCV2 capsid subunit vaccine, the piglets were not vaccinated for PCV2, and were obtained after weaning without regards to maternal antibody levels. All pigs were housed in two environmentally controlled rooms at the Kansas State University Large Animal Research Center, and maintained under BSL-2 conditions. Rooms were chemically disinfected, cleaned with a high heat pressure washer and gas decontaminated with vaporized hydrogen peroxide prior to use. Both rooms were empty for at least 19 days prior to the start of the study. Pigs were housed in 20 pens, each 144 sq ft with 11-12 pigs per pen. Pigs were given access to food and water *ad libitum*.

Experimental Design. A total of 226 pigs were randomly allocated into two identical rooms using a random number assignment protocol and housed in groups of 11-12 pigs per pen. After acclimating for four days, one room of 115 pigs was vaccinated with a 2 ml dose of commercial PRRS MLV vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim Animal Health; GenBank Accession #AF159149) administered intramuscularly according to the vaccine label instructions. At 28 days post-vaccination (dpv), all pigs in both rooms were challenged with a combination of PRRSV and PCV2b. Individual body weights were collected on -3, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpv. Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 35, and 42 days post-infection (dpi). Blood was also collected from the vaccinated group on 0, 4, 7, 11, 14, and 21 dpv. At 11 dpi, 10 vaccinated and 10 non-vaccinated pigs were randomly selected for humane euthanasia and complete necropsies were performed. Between 32 and 42 dpi, 11 pigs showing clinical signs of PCVAD and 7 nonclinical pigs were humanely euthanized and complete necropsies were performed. These pigs were selected based on clinical disease without regards to vaccine status.

Challenge inoculum. The PRRSV and PCV2b isolates used to prepare the inoculum were originally derived from the lymph node of a pig with severe PMWS, as described previously (Trible et al., 2011; Trible et al., 2012a). PRRSV (isolate KS62, GenBank Accession #KM035803) was isolated by propagation on MARC-145 cells. The PRRSV component of the challenge inoculum, KS62, shared 88.06% identity with the MLV (GenBank Accession #AF159149) at the peptide sequence level of GP5. Since wild-type PCV2b (Genbank Accession #JQ692110) does not propagate to high levels in cell culture, we took advantage of the heat stability of PCV2 to make a virus preparation from a lymph node suspension enriched for PCV2. The suspension was heat-treated at 55^{o} C for 30 minutes to remove PRRSV, bacteria and other heat-labile agents. The treated homogenate was recombined with the isolated PRRSV to infect cesarean-derived, colostrum-deprived (CD/CD) pigs. A combination lung/lymph node homogenate was prepared from the CD/CD pigs and PRRSV and PCV2 were isolated from the homogenate by the methods described above. Analysis of the heat-treated preparation for common agents showed that the preparation was negative for most heat stable agents, such as parvovirus, but still positive for torque teno sus virus (TTSuV) and porcine oncovirus (PCOV), which are ubiquitous.

PRRSV was tittered on MARC-145 cells. Briefly, virus was serially diluted 1:10 in MEM (Corning) supplemented with 7% FBS (Sigma-Aldrich), Pen Strep (80 Units/ml and 80 μg/ml, respectively; Gibco), 3 ug/ml Fungizone (Gibco), and 25 mM HEPES (Life Technologies). The dilutions were then added in quadruplicate to confluent MARC-145 cells in a 96 well tissue culture plate (BD Falcon). Following a 4 day incubation at 37^{o}C in 5% CO_2, wells were examined for PRRSV induced cytopathic effects, and the 50% tissue culture

infectious dose ($TCID_{50}$/ml) was calculated using the method of Reed and Muench (Reed and Muench, 1938).

The quantity of PCV2 was determined by titration on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of the PCV2 challenge stock were plated in quadruplicate to rapidly dividing ST cells in a 96 well tissue culture plate (BD Falcon). Dilutions were prepared in EMEM (Sigma-Aldrich) supplemented with 7% FBS (Sigma-Aldrich) and 50 µg/ml of gentamycin (Lonza). Following a three day incubation at 37°C in 5% CO_2, cells were fixed and permeabilized with 80% acetone and then stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.). Infected cells were visualized using an inverted fluorescent microscope and the $TCID_{50}$/ml was calculated using the method of Reed and Muench (Reed and Muench, 1938).

The challenge viruses were recombined to yield a 2 ml dose consisting of $10^{3.6}$ $TCID_{50}$ PCV2 and 10^5 $TCID_{50}$ PRRSV in MEM. The 2 ml dose was split with 1 ml administered intranasally and the remaining 1 ml administered intramuscularly.

Clinical Evaluation. Pigs were evaluated daily for the presence of clinical signs associated with PCVAD, including dyspnea, aural cyanosis, coughing, nasal discharge, open mouth breathing, poor body condition, muscle wasting, pallor or jaundice, lameness, joint effusion, depression and lethargy. Each pig was visually examined by a veterinarian or veterinary assistant each day during the study period. Appropriate treatments were initiated for pigs that presented with moderate to severe clinical disease. Examples of clinical presentations where treatment was administered included: 1. Difficult respiration, 2. Mucoid nasal discharge, 3. Lameness with associated joint effusion, 4. Pallor or jaundice associated with muscle wasting, and 5. Lethargy or depression with rectal temperature ≥104°F. For clinically affected pigs,

antibiotic therapy was administered, including ceftiofur hydrochloride for respiratory or systemic disease, oxytetracycline for infectious arthritis, and enrofloxacin for cases unresponsive to the previous two antibiotics. All pigs with overt clinical disease and rectal temperatures ≥104°F were administered flunixin meglumine, a nonsteroidal anti-inflammatory drug (NSAID). Pigs with intractable fevers of greater than 4 days duration were given a 2 day wash-out period and then administered oral meloxicam. All treatments were administered as directed by a veterinarian. Clinical signs and systemic treatments unrelated to PRRSV or PCVAD (e.g. lacerations, dermatitis, hoof wounds, congenital hernias) were documented but were not included in the data analysis related to clinical outcomes. Animals were humanely euthanized with pentobarbital sodium. Pigs that died or were humanely euthanized due to circumstances unrelated to the effects of co-infection were excluded from the mortality analysis. Average daily gain (ADG) was calculated as the change in weight divided by the number of days and was reported as kg per day.

Gross pathology and histopathology. Lungs were removed *in toto* immediately after euthanasia. The scoring of gross lung lesions was performed using two techniques. First, the percentage of lung affected by pneumonia was estimated for both the dorsal and ventral aspects of each lung lobe during gross necropsy. Results were reported as the percentage of whole lung affected by pneumonia (ranging from 0 to 100%) (Halbur et al., 1995). Second, dorsal and ventral aspects of the whole lung were photographed (Olympus Stylus 7010 camera) and digital images were evaluated after gross necropsy using a photo scoring system. Gross anatomical photo scores were determined on a scale of 0 to 4: 0 = no macroscopic lesions; 1 = pneumonia affecting < 25% of gross lung; 2 = pneumonia affecting 25-50% of gross lung; 3 = pneumonia

affecting 50-75% of gross lung; and 4 = pneumonia affecting > 75% of gross lung. The evaluator was blind as to the source of the lung pictures.

For histopathology, tissues collected from lung, tracheobronchial lymph node and inguinal lymph node were immediately placed in 10% neutral buffered formalin and allowed to fix 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 blinded board-certified pathologist. Microscopic lung lesions were estimated based on the following scoring system: 0 = no significant microscopic lesions; 1 = mild interstitial pneumonia with <50% lung lobe involvement; 2 = mild to moderate multifocal interstitial pneumonia with 50-75% lung lobe involvement; 3 = moderate to severe multifocal interstitial pneumonia with 50-75% lung lobe involvement; 4 = severe diffuse interstitial pneumonia with >75% lung lobe involvement. The final scores assigned to each pig was an average from two separate evaluations by the same pathologist, who remained blinded to the source of the lung tissue.

PCV2 immunohistochemical staining. PCV2 antigen staining in paraffin-embedded tissue thin sections was performed by personnel in the Kansas State Veterinary Diagnostic Laboratory. Briefly, deparaffinized slide-mounted thin sections were first treated with proteinase K (1.2 mg/ml diluted in Bond Enzyme Diluent with 0.35% ProClin 950) for 10 minutes at room temperature (Bond Enzyme Pretreatment Kit, Leica Biosystems). Rabbit anti-PCV2 antibody (Iowa State University) was diluted at 1:500 in Bond Primary Antibody Diluent (Leica Biosystems) and applied to the tissue section for 15 minutes at room temperature. Bound antibody was detected by incubation with 25 μg/ml Poly-AP anti-rabbit IgG (Leica Biosystems) in antibody diluent for 25 minutes at room temperature. The complex was visualized using Fast

Red chromogen (Bond Polymer Refine Red Detection Kit, Leica Biosystems) and counterstained with hematoxylin.

Measurement of PRRSV and PCV2 viremia. Viral DNA and RNA were extracted simultaneously from 50 μL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems) in accordance to the manufacturer's instructions. PRRS viral RNA was quantified using EZ-PRRSV MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore) according to the manufacturer's instructions. For consistency, each plate contained Tetracore Quantification Standards and Control Sets for use with EZ-PRRSV MPX 4.0 RT-PCR Reagents. All PCR reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in a 96-well format using the recommended cycling parameters. PCV2 DNA was quantified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively. Primers were used at a final concentration of 300 μM. Nuclease free water was used to bring the mastermix volume to 18 μL per reaction. The addition of 2 μL of template nucleic acid brought the final reaction volume for each sample to 20 μL. Standard curves and positive and negative controls were included on each plate. Plasmid DNA was used for the PCV2 standard curve and positive control template. DNA inserted into the plasmid was obtained from a field strain of PCV2 (PCV2b 321/393). Plasmid DNA was isolated using the PureYield Plasmid Miniprep System (Promega) according to the manufacturer's instructions. The DNA for the standard curve was quantified using a NanoDrop 8000 Spectrophotometer. The standard curve was produced by diluting the purified plasmid DNA 1:1000 in nuclease free water followed by five serial 1:10 dilutions in nuclease free water. The final standard curve contained 6 points ranging from 10^7 to 10^2 logs of template DNA which

produced threshold crossing values between 15 and 33 cycles. Standard curves were run in duplicate with nuclease free water as a negative control. The PCV2 PCR was carried out on a CFX96 Touch Real-Time PCR Detection System using the following settings: activation at 98°C for 2 minutes, followed by 40 cycles of denaturing at 98°C for 5 seconds and annealing/extension at 60°C for 10 seconds. The melting curve was performed between 65-95°C using 0.5°C increments. The PCR assay results were reported as $\log_{10}$ PRRSV RNA starting quantity (copy number) per 50 µL reaction volume or $\log_{10}$ PCV2 DNA starting quantity per 20 µL reaction volume.

Microsphere immunoassay for detection of PCV2 antibodies. A PCV2b capsid protein (CP) polypeptide fragments CP(43-233) and CP(160-233) were cloned and expressed in the *E.coli* vector, pHUE, as previously described (Trible et al., 2011). For protein expression, bacteria were grown in Luria-Bertani (LB) broth plus ampicillin (0.01 mg/ml) and incubated at 37^{o}C with shaking. When the OD^{600} reached 0.4-0.6, protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM/ml final concentration) and bacteria harvested 4 hours later. Protein was purified using the USB PrepEase Histidine-tagged Protein Purification Kit (Affymetrix) under non-denaturing conditions, according to the manufacturer's directions. Purity was assessed by SDS-PAGE and total protein measured using the Bio-Rad Protein Assay.

Proteins were coupled to carboxylated Luminex MagPlex® polystyrene microspheres according to the manufacturer's directions. For the assay, approximately 2500 antigen-coated beads, suspended in 50 µL PBS with 10% goat serum (PBS-GS), were placed in each well of a 96-well polystyrene round bottom plate (Costar). Sera were diluted 1:400 in PBS-GS and 50 µL was added to each well. The plate was wrapped in foil and incubated for 30 min at room

temperature with gentle shaking. The plate was placed on a magnet and beads were washed three times with 190 µL of PBS-GS. For the detection of IgG, 50 µL of biotin-SP-conjugated affinity purified goat anti-swine secondary antibody (IgG, Jackson ImmunoResearch) was diluted to 2 µg/mL in PBS-GS and 100 µl was added to each well. The plate was incubated at room temperature for 30 min and washed three times followed by the addition of 50 µL of streptavidin-conjugated phycoerythrin (2 ug/ml in PBS-GS; SAPE). After 30 min, the plate was washed and microspheres resuspended in 100 µL of PBS-GS. Microspheres were analyzed using a MAGPIX instrument (Luminex) and Luminex® xPONENT 4.2 software. A minimum of 100 microspheres was used for the calculation of mean fluorescence intensity (MFI). The sample to positive (S/P) ratio was calculated as the MFI of sample minus MFI of negative control divided by MFI of standard positive control minus MFI of negative control.

Statistical analyses. A logistic mixed model was used to evaluate the effect of vaccination on the binary traits of blue ear, veterinary treatment for PCVAD, and mortality. The model included the design effects of vaccination, WUR, the interaction between vaccination and WUR, and initial body weight as a covariate. Random effects included sire, dam and pen. Odds ratios for the effect of vaccination were estimated as the vaccinated group over the non-vaccinated group (treatment and mortality) and the non-vaccinated group over the vaccinated group (blue ear). Odds ratios included Wald's confidence intervals (CI).

Data on percent lung lobe involvement was analyzed as a normal quantitative response variable, whereas gross anatomical photo scores and microscopic lung lesion scores were analyzed as ordinal categorical variables in a mixed multinomial regression. The model included the fixed effects of PRRS vaccination, WUR, their interaction, and clinical signs (presence/absence). Sire and dam were included as random effects. All analyses were

performed using the GLIMMIX procedure of SAS 9.3 (Statistical Analysis System Institute, Inc.). Average daily gain, viremia, and antibody response comparisons between groups were performed using GraphPad Prism 5.00 software using the unpaired t-test.

Results

PRRSV viremia is decreased after PRRS vaccination but PCV2 viremia is increased. PRRS MLV replication in the vaccine group prior to challenge was assessed by RT-PCR on serum samples collected at 11 dpv. The results showed that 78 of the 84 pig sera tested (or 93%) possessed detectable levels of vaccine virus, confirming that the pigs supported active MLV replication (Figure 1). Mean viremia at 11 dpv was 2.7 ± 1.7 $\log_{10}$ templates per PCR reaction. Prior to challenge, the non-vaccine group was negative for PRRSV nucleic acid in serum (data not shown).

The results for PRRSV and PCV2 viremia after challenge are shown in Figure 2. PRRSV infection in the non-vaccine group followed the typical course of viremia, peaking between 7 and 11 dpi, followed by a decay and eventual disappearance of virus from the blood by 42 dpi. In the vaccine group, 90% of pigs (102/113) possessed detectable levels of virus nucleic acid in serum at 28 days after vaccination or at the time of challenge. The PRRSV viremia in the vaccine group peaked at about seven days after challenge and then decayed. Except for the day of challenge and day 42, the mean PRRSV level was significantly lower on all days in the vaccine group (see Figure 1A). Compared to the non-vaccinated pigs, peak viremia at days 7 and 11 for the vaccine group was reduced by more than one log. The results demonstrated that vaccination was effective in reducing PRRS viremia in a heterologous challenge model.

Mean PCV2 viremia for vaccine and non-vaccine groups is presented in Figure 2B. In the non-vaccine group, mean PCV2 viremia peaked at about 21 days after challenge and remained elevated for the remainder of the study. In contrast, mean PCV2 viremia for the vaccine group peaked at 14 dpi when the mean virus level was approximately 1.5 logs greater for the vaccine group compared to the non-vaccine group ($p < 0.0001$). Thus, PCV2 viremia in the vaccine group peaked much earlier.

PCV2 immunization of dams provides temporary protection of piglets from PCV2 infection. However, by 35 days after weaning, passive immunity decays to the point that pigs become susceptible to PCV2, a virus that is normally present in the environment. At the time of challenge, low but detectable levels of PCV2 nucleic acid were present in 7 of 115 vaccinated pigs (6%) and 25 of 111 non-vaccinated pigs (22%). Mean PCV2 viremia prior to challenge was 2.3 and 2.9 $\log_{10}$ templates per PCR reaction for PCR-positive vaccine and non-vaccine pigs, respectively. The increased proportion of non-vaccinated pigs with evidence of PCV2 exposure prior to challenge may account for the differences in the outcomes for the two groups. However, the exclusion of these 32 pigs did not alter the conclusions of the study.

PRRS vaccination results in reduced clinical signs and pathology during the first 21 days after co-infection. Prior to virus challenge, clinical signs were not apparent in either the vaccinated or the non-vaccine group. After infection, two clinical syndromes emerged. The first was a PRRSV-associated syndrome, aural cyanosis, commonly known as "blue ear" (Done and Paton, 1995; Paton et al., 1991), which was easily identified in pigs by the presence of red, cyanotic, or blue discoloration of the ear tissue. Even though blue ear is not pathognomonic for PRRS, it often coincides with acute infection. A representative example of a pig with "blue ear" is shown in Figure 3. No "blue ear" was observed prior to challenge in either the vaccinated or

the non-vaccinated pigs. However, during the post-challenge period, 64 of the 226 pigs or 28.3% of all pigs were documented as having blue ear on one or more days. As shown in Figure 4A, the percentage of pigs with blue ear peaked between 8 and 17 dpi, which corresponded to the peak in PRRSV viremia (compare Figure 2A). Overall, 19% of vaccinated pigs (22/115) and 38% of non-vaccinated pigs (42/111) were documented with blue ear. A non-vaccinated pig was 3.02 times (95% CI [1.7, 5.9]) more likely to develop blue ear than a vaccinated pig ($p = 0.001$). The total number of days with blue ear was 65 and 201 for the vaccine and non-vaccine groups, respectively. The reduction in the number of pigs with blue ear in the vaccine group is consistent with a beneficial effect of the PRRS MLV.

The primary clinical sign associated with acute PRRSV infection is respiratory disease resulting from interstitial pneumonia. Lungs were removed from 20 euthanized pigs (10 pigs from each group), which were randomly selected at 11 dpi. Figure 5 shows a summary of the gross and microscopic lung scores. Mean scores for the percent gross lung lobe involvement and microscopic scoring for interstitial pneumonia were higher in the non-vaccine group; however, the differences were not statistically significant ($p = 0.62$ for percent lung involvement and $p = 0.30$ for histopathology score). Together, the results showed that PRRS vaccination with MLV had an overall protective effect by reducing PRRSV viremia and decreasing PRRS-associated clinical signs.

PRRS vaccination results in increased clinical signs and pathology at 22-42 days after co-infection. Beyond the acute period of infection, a second clinical syndrome appeared, which first became apparent by an increase in the number of pigs receiving systemic veterinary treatment due to clinical signs associated which PCVAD, such as tachypnea, dyspnea, pyrexia, loss of condition, muscle wasting, mucoid nasal discharge, lethargy, and pallor or jaundice (see

Figure 4B). Lesions typical of PCVAD were found by gross anatomical and microscopic examinations of lung and lymph nodes from pigs that died or were euthanized. Representative pictures and photomicrographs showing the lesions associated with clinically affected pigs are presented in Figure 6. Lungs showed multifocal to diffuse interstitial pneumonia with mottling of lung tissue, hemorrhage, and consolidation (Figure 6A). At the microscopic level, multifocal to diffuse interstitial pneumonia with lymphohistiocytic infiltration into the alveolar septa and peribronchiolar areas was easily visible (see Figure 6B). Lymph nodes of affected pigs showed depletion of lymphocytes (see Figure 6C). Positive staining for PCV2 antigen was observed in lymph nodes and lungs of affected pigs (see Figure 6D). The analysis of gross and microscopic lesions combined with the accumulation of PCV2 antigen in target organs confirmed the presence of PCVAD. The number of pigs undergoing treatment as a result of PCVAD-associated clinical signs peaked between 22 and 35 dpi (see Figure 4B). During this time, there were 39 pigs that received at least 1 day of veterinary treatment, including 12 non-vaccinated pigs (12/101; 12%) versus 27 pigs in the vaccine group (27/105; 26%). A vaccinated pig was 2.67 times (95% CI [1.23, 5.80]) more likely to receive veterinary treatment during peak PCVAD than a non-vaccinated pig ($p = 0.01$). High amounts of PCV2 in serum were associated with the 39 pigs that went on to develop PCVAD. At 14 dpi, significantly higher levels of circulating PCV2 were present in the 39 PCVAD pigs (mean = 5.8 $\log_{10}$ templates/PCR reaction) compared to the 163 nonclinical pigs (mean = 4.8 $\log_{10}$ templates/PCR reaction; $p = 0.004$). Different treatments administered to the 39 clinical pigs included a single antibiotic and a NSAID (16/39; 41%), multiple antibiotics and a NSAID (7/39; 18%), a single antibiotic (6/39; 15%), multiple antibiotics (3/39; 8%), and a NSAID alone (3/39; 8%). Four of the 39 pigs (10%) were humanely euthanized after the initial treatment due to the severity of the clinical

presentations. The decline in the percentage of pigs with PCVAD clinical signs was largely the result of increased mortality or the euthanization of pigs that were moribund or nonresponsive to treatment (compare Figures 4B and C). Over the entire study period, there were 49 pigs that received at least 1 day of systemic veterinary treatment; 16% in the non-vaccine group (18/111) versus 27% in the vaccine group (31/115). A vaccinated pig was 1.79 times (90% CI [0.99, 3.25]) more likely to receive veterinary treatment during the entire study period than a non-vaccinated pig ($p = 0.11$).

Macroscopic and microscopic changes in organs and tissues were evaluated between 32 and 42 dpi in 11 clinically affected pigs, which were humanely euthanized as a result of failure to respond to treatment. For the purpose of comparison, 7 nonclinical pigs were also necropsied. As summarized in Table 1, all 11 clinical pigs showed some form of macroscopic lung involvement, as determined by the photographic score. The macroscopic scores for the nonclinical group were significantly lower ($p = 0.04$). A similar trend between clinically affected and non-affected pigs appeared at the microscopic level; however, the difference was not statistically significant ($p = 0.16$). Mild to severe lymphoid depletion was observed in 8 of the 11 clinical pigs (73%) compared to only 3 of the 7 (43%) nonclinical pigs. Even though the nonclinical pigs appeared normal, almost all showed some form of pathology related to PCVAD, such as mild to moderate pneumonia and/or mild lymphoid depletion.

The effect of PRRS MLV vaccination on mortality. As illustrated in Figure 4C, of the 101 non-vaccinated pigs, 9 died, resulting in an overall survival rate of 91.1%. Of the 105 vaccinated pigs, 14 died, for an overall survival rate of 86.7% (see Figure 4C). A vaccinated pig was 1.7 times (95% CI [0.89, 3.72]) more likely to die during the overall study period than a non-vaccinated pig ($p = 0.35$). Increased mortality became apparent after 20 days and was

associated with the appearance of PCVAD. Of the 39 pigs that developed clinical signs of PCVAD, 21 died prior to the end of the study (14 vaccinated and 7 non-vaccinated), resulting in a mortality rate of approximately 54% in pigs exhibiting clinical signs. Between 22 and 35 dpi, a vaccinated pig was 2.1 times (90% CI [1.03, 5.87]) more likely to die than a non-vaccinated pig ($p = 0.09$). Even though mortality was higher in the vaccine group, the differences between vaccine and no vaccine groups were not significantly different.

Vaccination increases the appearance of antibodies against a PCV2 decoy epitope. In previous work we identified the presence of antibodies against a decoy epitope in the capsid protein of PCV2, CP(160-180), correlated with PCVAD. The presence of an anti-CP(160-180) response is associated with the absence of PCV2 neutralizing activity in serum. Pigs vaccinated for PCV2 and protected from disease produce little anti-CP(160-180) activity and preferentially recognize a larger epitope, CP(43-233). Pigs that are naturally infected with PCV2 show a mixture of the two antibody responses. As a means to standardize results across plates, the anti-PCV2 antibody response for the vaccine and non-vaccine groups was presented as a CP(160-233)/CP(43-233) ratio. The higher the ratio, the more the immune response is skewed towards the recognition of the decoy epitope. The results, presented in Figure 7, showed a significant ($p = 0.0006$) difference in the CP(160-233)/CP(43-233) mean ratios; 0.85 for the non-vaccinated pigs versus 0.97 for the vaccinated group. The exclusion of the 32 pigs which showed the presence of PCV2 nucleic acid at the time of challenge changed the ratios, but did not affect the conclusion; CP(160-233)/CP(43-233) mean ratios of 0.92 for the non-vaccinated pigs ($n = 61$) and 0.98 for the vaccinated group ($n = 72$; $p = 0.03$).

As discussed above, previous work showed that pigs with low CP(160-233)/CP(43-233) ratios are protected from disease. Therefore, non-vaccinated pigs with ratios less than 0.5 ($n =$

13) were compared to non-vaccinated pigs with PCV2 antibody ratios greater than 0.5 (n = 68). Pigs with antibody ratios of less than 0.5 had significantly lower levels of circulating PCV2 in the serum on 21 ($p = 0.0001$), 28 ($p = 0.0008$), 35 ($p = 0.007$), and 42 ($p = 0.03$) dpi compared to the pigs with the higher ratios. The results confirm earlier findings describing the non-protective effect of anti-CP(160-233) antibodies.

The effect of PRRS MLV vaccination on average daily gain. Over the entire 70 day study period, the mean average daily gain (ADG) for the vaccine group (n = 91) was 0.65 ± 0.11 kg compared to 0.68 ± 0.10 kg for the no vaccine pigs (n = 92). The means between the two groups were significantly different ($p = 0.029$). Decreased mean ADG was also observed in the vaccine group during the 42 day post-virus challenge period. However, the difference between the vaccine (0.82 ± 0.14 kg, n = 91) and no vaccine (0.86 ± 0.14 kg, n = 92) groups was not statistically significant ($p = 0.061$). In addition, ADG differences between the two groups were no longer significant during the 70 day study period after excluding the 32 pigs with PCV2 detected prior to challenge.

Therefore, a more detailed analysis was conducted by calculating ADG on a weekly basis (see Figure 8). The results showed that ADG differences between no vaccine and vaccine groups could be divided into three distinct phases. The first phase, covering the pre-challenge period, showed that mean ADG after vaccination was reduced, with a significant difference between vaccinated and non-vaccinated groups appearing at three weeks after vaccination. The second phase covered the period during acute PRRSV infection. At one week after virus challenge, mean ADG was significantly increased for the vaccine group. ADG remained higher in the second week post-challenge, but the difference was not significant. Improved ADG in vaccinated pigs was likely the result of the positive effect of vaccination on reducing PRRS-

associated clinical signs and virus load (see Figures 2A and 4A). The third phase covered the period from the onset of PCVAD, beginning at about three weeks after virus challenge, until the end of the study. During this phase, ADG was lower in the vaccinated group at every time point, with significantly lower mean ADG values on week six after virus challenge. A significant decrease in ADG was also initially detected for vaccinated pigs on week three post-challenge; however, this difference was no longer significant after the 32 PCV2 positive pigs had been excluded ($p = 0.08$). The lower mean ADG values are consistent with the effect of PCVAD, characterized by poor growth performance and muscle wasting.

The negative effect of vaccination on ADG could have been the result of increased numbers of pigs with clinically apparent PCVAD. Therefore, a separate analysis was performed after the 26 clinical pigs that survived the length of the study had been excluded from the vaccine and no vaccine groups. Removing clinical pigs increased ADG of the two groups; 0.69 ± 0.09 kg in nonclinical non-vaccinated pigs ($n = 83$) and 0.66 ± 0.10 kg in nonclinical vaccinated pigs ($n = 74$; $p = 0.047$). However, even in the absence of overt clinical signs, PRRS vaccination possessed a negative effect on weight gain.

Discussion

Enhanced PCV2 infection leading to PCVAD is typically associated with immune stimulation (Krakowka et al., 2001; Opriessnig and Halbur, 2012). PCV2 replication is located in the nucleus of permissive cells and is dependent on cellular enzymes expressed during the S phase of the cell cycle (Tischer et al., 1987). It is presumed that actively dividing lymphocytes, in response to an immune stimulus, provide the cellular environment ideal for supporting PCV2 replication. PRRSV, porcine parvovirus and *Mycoplasma hyopneumoniae* are common co-

pathogens linked with lymphoproliferation and increased PCV2 pathogenesis (Allan et al., 1999; Allan et al., 2000a; Ellis et al., 1999; Fan et al., 2013; Harms et al., 2001; Kennedy et al., 2000; Krakowka et al., 2000; Opriessnig et al., 2004; Rovira et al., 2002; Sinha et al., 2011; Trible et al., 2012a). Examples of non-infectious immunostimulators include immunization with keyhole limpet hemocyanin in incomplete Freund's adjuvant (Krakowka et al., 2007; Krakowka et al., 2001) and inactivated vaccines, such as *Mycoplasma hyopneumoniae* (Allan et al., 2000b; Krakowka et al., 2007; Opriessnig et al., 2003) and *Actinobacillus pleuropneumoniae* (Allan et al., 2000b; Opriessnig et al., 2003). The results from this study showed that PRRS MLV initially had a beneficial effect in reducing PRRS-associated clinical signs and PRRS viremia; however, PRRS-vaccinated pigs showed increased PCV2 replication, reduced average daily gain and increased clinical signs associated with PCVAD.

Previous experimental studies documenting interactions between PRRS MLV and PCV2 infection have yielded conflicting results. Allan et al. (Allan et al., 2007) found that colostrum-deprived, specific-pathogen-free (SPF) pigs infected with PCV2 at five weeks of age and administered PRRS MLV one week later had greater amounts of PCV2 antigen in tissues and more severe histologic lesions, characteristic of PMWS, compared to pigs infected with PCV2 alone. However, pigs failed to exhibit clinical signs or gross lesions typical of PCVAD (Allan et al., 2007). In contrast, Opriessnig et al. (Opriessnig et al., 2006) evaluated the effects of PCV2 infection on the efficacy of PRRS MLV in groups of 10 early weaned SPF pigs. Pigs were inoculated with PCV2 at six weeks of age, vaccinated with PRRS MLV two weeks later, and then challenged with PRRSV at twelve weeks of age. The results showed that the group with PCV2 and MLV exhibited lower ADG and more severe lung lesions after PRRSV challenge compared to those pigs that were vaccinated and received PRRSV challenge without PCV2.

Because PCV2 was not detected by IHC in affected lungs, the authors attributed the lesions to PRRSV infection and further concluded that the effect of PCV2 was to reduce the efficacy of PRRS MLV (Opriessnig et al., 2006). Park et al. (Park et al., 2013) investigated the potential for PRRS MLV to reduce PRRSV-associated amplification of PCV2 pathogenesis after co-infection with PRRSV and PCV2. Groups of 8 conventional pigs were subjected to a variety of treatments involving different combinations of PRRS MLV, wild-type PRRSV, PCV2 vaccine, and PCV2. Pigs were vaccinated with PRRS MLV, PCV2 subunit vaccine or both and then four weeks later, challenged with PRRSV, PCV2 or both. The group that received PRRS MLV followed by co-infection showed no differences in PCV2 viremia, PCV2 associated pathology, or the number of PCV2-positive cells in lymph node and lung when compared to the co-infected group that was not vaccinated with PRRS MLV (Park et al., 2013). In contrast to our study in which vaccination enhanced PCVAD, these authors found that co-infected pigs, with or without previous PRRS MLV vaccination, had similar PCV2 replication and pathogenesis.

Field studies have also yielded conflicting results. A survey conducted on 70 pig farms in the Netherlands included questions regarding PRRS MLV use on farms with and without PMWS or PCVAD. Results showed that PRRS MLV is a significant risk factor for PMWS outbreaks (De Jong et al., 2003). In contrast, an analysis of the effect of PRRS MLV on farms affected by PCVAD in the U.S. found that farms incorporating PRRS MLV have significantly lower PCV2 viremia compared to non-vaccinated farms during peak wasting disease (Genzow et al., 2009). In this study, qPCR was used to measure PCV2 in serum samples collected at different time points from 6 herds using PRRS MLV and 12 non-vaccinated herds. These results suggest that PRRS MLV can reduce PCV2 viremia (Genzow et al., 2009).

The failure of experimental studies to find a consistent link between PRRS MLV and PCVAD was likely in part due to the length of the observation period. For example, the studies described above were terminated on 25 dpi with PCV2 (Allan et al., 2007) and 21 days after challenge with PRRSV/PCV2 (Park et al., 2013). In the current study, peak PCVAD occurred between 22-35 days after challenge with PRRSV/PCV2. Another important difference is the size of the experimental groups. Typically, the prevalence of clinically ill pigs on farms affected by PCVAD is only 2-25% (Kyriakis et al., 2002; Quintana et al., 2001; Sorden et al., 1998). Pigs with clinical disease may not be apparent or present in small groups of pigs. The current study utilized over 200 pigs, mimicking the environment found in the field and providing the depth of data to observe and quantify low-percentage outcomes. PCVAD morbidity was 11.9% and 25.7% in non-vaccinated and vaccinated pigs, respectively. Therefore, PCVAD should be assessed by comparing mortality rates, clinical disease presentation, viremia, and weight gain in relatively large groups evaluated for several weeks after infection.

Because large groups of pigs were required to ensure clinical disease expression, some control groups, including pigs challenged with PCV2 or PRRSV alone, were not incorporated into the study design. Therefore, conclusions are based on vaccinated and non-vaccinated co-challenged pigs and generalizing results to single infections (i.e. PRRS MLV effect on PCV2 challenged pigs) should be considered with caution.

PCV2 is ubiquitous in swine populations and elimination of the virus from the environment is extremely difficult. As demonstrated in this study, 32 of 226 pigs had detectable PCV2 in the serum at the time of challenge. Although these pigs were the minority (14%) and group titers were relatively low (2.3 and 2.9 $\log_{10}$ templates per PCR reaction), the presence of PCV2 prior to challenge had to be considered as a possible factor in post-challenge response.

This was especially true due to the difference between proportions of PCV2-positive pigs in each group, despite randomized allocation of pigs and balanced genotypes. This difference was likely due to the failure of randomization to equally distribute PCV2-positive pigs across the two groups. Therefore, analyses were also completed after the 32 pigs were excluded and all conclusions of the study were confirmed. Regardless, this highlights the difficulty in eliminating PCV2 from the environment and further, suggests that pigs should be balanced according to PCV2 status prior to challenge.

Significantly increased PCV2 viremia in PRRS MLV treated pigs was observed at 11 and 14 days after challenge, but not at later time points (see Figure 2B). This effect of PRRS MLV on PCV2 infection is similar to that seen in a previous study by us, which showed a significant increase in PCV2 viremia at 23 days after co-infection with PRRSV and PCV2 (Trible et al., 2012a). In the current study, it is interesting to note that PCV2 viremia levels were not significantly different between the vaccine and no vaccine groups during peak PCVAD. However, the 39 clinical pigs did maintain significantly higher levels of circulating PCV2 during these later time points (21 and 35 dpi, $p < 0.02$, data not shown). Increased incidence of PCVAD in vaccinated pigs between 22-35 dpi may also be the result of greater levels of localized PCV2 in tissues. Future studies are needed to assess differences in quantity and tissue distribution of PCV2 between vaccinated and non-vaccinated pigs.

Average daily gain (ADG) is used in swine production as an objective measure of overall health and performance. The negative effect of PRRS MLV on growth performance is well documented. For example, Opriessnig et al. reported that pigs vaccinated with PRRS MLV at two weeks of age exhibited a significant reduction in ADG compared to non-vaccinated pigs (Opriessnig et al., 2005). Pretzer et al. found that PRRS MLV vaccinated weaned pigs had lower

ADG between 0-14 dpv compared to non-vaccinated pigs (Pretzer et al., 1996). While this effect on ADG was no longer apparent between 21 and 42 dpv, the vaccinated pigs maintained lighter weights overall (Pretzer et al., 1996). We confirmed the negative effect of PRRS MLV on ADG during the 28 day period prior to co-infection; significantly reduced ADG was observed during the third week after vaccination (see Figure 8). The benefit of PRRS vaccination was documented during the first two weeks after PCV2/PRRSV challenge, when MLV had a positive effect on ADG. Although vaccinated pigs in this study had increased ADG in the first two weeks post-challenge, this effect was quickly outweighed when ADG was decreased in the presence of PCVAD. Vaccination decreased ADG in both clinically-affected and nonclinical pigs during the study period, demonstrating that poor growth performance may be a subclinical manifestation of PCVAD in apparently healthy pigs.

At least three mechanisms may be involved in the enhancement of PCVAD following PRRS vaccination. PRRS MLV may function to stimulate the immune system and increase the number of PCV2 permissive cells. Similar to wild-type viruses, lymphocytes undergo mitosis in response to vaccination with PRRS MLV, thereby increasing the population of cells with the ability to support PCV2 replication. In addition, the vaccine likely stimulates PRRSV-specific lymphocyte populations that are re-stimulated after challenge with a wild-type PRRSV. Secondly, similar to wild-type PRRS viruses, PRRS MLV may suppress innate immunity, thereby blocking anti-PCV2 responses. For example, PRRSV non-structural proteins, such as nsp1 and nsp2, block the induction and response of cells to interferon (Chand et al., 2012). Viral proteins, such as nsp1α and nsp1β, antagonize the type I interferon response by degrading key components needed for interferon gene expression and inhibiting interferon signaling pathways (Chen et al., 2010; Han and Yoo, 2014). And finally, the third mechanism is based on the

possibility that PRRS MLV may skew the immune response towards the production of non-neutralizing PCV2-specific antibodies.

PCV2 has circulated in the swine population for at least 25 years. In 2005, the emergence of PCV2b in North America was attributed to outbreaks of PCVAD (Carman et al., 2008; Cheung et al., 2007; Horlen et al., 2007). Since then, the disease has been effectively managed through the use of PCV2 vaccines (Horlen et al., 2008; Kixmoller et al., 2008; Velasova et al., 2013). Therefore, the negative effect of MLV on PCV2 infection may not be relevant. However, there remain several countries in which PRRS MLV vaccination is in wide use, but in the absence of a comprehensive PCVAD vaccination program. Further, there is the potential for new and emerging PCV2 strains to escape current vaccine protection. Emerging PCV2 mutant strains have been documented in China (Guo et al., 2010) and more recently were associated with PCVAD outbreaks in vaccinated herds in the U.S. and Korea (Opriessnig et al., 2013; Seo et al., 2014). Overall, this study supports the notion that maintaining a successful PCV2 control program and assessing the risk of virulent PRRSV exposure is critical to weighing the benefits of PRRS MLV.

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Table 1:1 Lung and lymph node lesions of pigs with clinical signs associated with PCVAD and nonclinical pigs between 32 and 42 dpi[a]

Macroscopic Lung Lesion Score[b]*	0	1	2	3	4
Clinical Pigs	0 (0)	4 (36)	2 (18)	1 (9)	4 (36)
Nonclinical Pigs	1 (14)	4 (57)	1 (14)	1 (14)	0 (0)
Microscopic Lung Lesion Score[c]	0	1	2	3	4
Clinical Pigs	0 (0)	1 (9)	3 (27)	7 (64)	0 (0)
Nonclinical Pigs	0 (0)	1 (14)	4 (57)	2 (29)	0 (0)
Amount of Lymphoid Depletion[d]	None	Mild	Moderate	Severe	
Clinical Pigs	3 (27)	4 (36)	2 (18)	2 (18)	
Nonclinical Pigs	4 (57)	3 (43)	0 (0)	0 (0)	

[a] 18 pigs were necropsied including pigs showing clinical signs associated with PCVAD (n = 11) and apparently healthy pigs (n = 7). Data are shown as the number and percentage of pigs in parentheses.
[b] Scores were determined by evaluation of ventral and dorsal photographs of lungs: 0 = no macroscopic lesions; 1 = pneumonia affecting < 25% of gross lung; 2 = pneumonia affecting 25-50% of gross lung; 3 = pneumonia affecting 50-75% of gross lung; and 4 = pneumonia affecting > 75% of gross lung.
[c] Scores were determined by evaluation of tissue sections stained with hematoxylin and eosin: 0 = no significant microscopic lesions; 1 = mild interstitial pneumonia with <50% lung lobe involvement; 2 = mild to moderate multifocal interstitial pneumonia with 50-75% lung lobe involvement; 3 = moderate to severe multifocal interstitial pneumonia with 50-75% lung lobe involvement; 4 = severe diffuse interstitial pneumonia with >75% lung lobe involvement.
[d] Amount of lymphoid depletion was determined by evaluation of lymph node and tonsil tissue sections stained with hematoxylin and eosin: Mild = small amount of lymphocyte depletion with replacement by histiocytes; Moderate = intermediate amount of lymphocyte depletion with replacement by histiocytes; Severe = large amount of lymphocyte depletion with replacement by histiocytes.
* Data for Clinical versus Nonclinical pigs was statistically different ($p = 0.0426$), based on ordinal logistic regression.

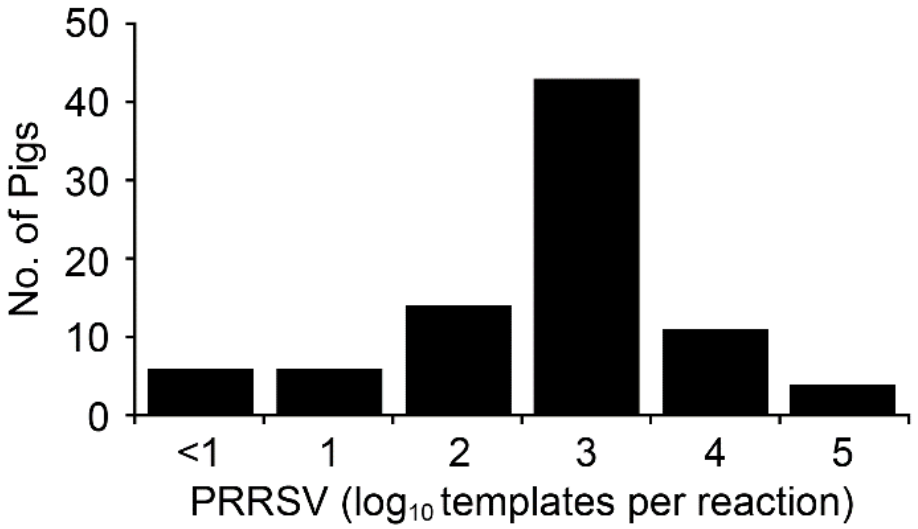


Figure 1:1 Distribution of viremia at 11 days after vaccination with PRRS MLV.

The figure represents PRRSV RT-PCR results for 84 pigs in the vaccine group.

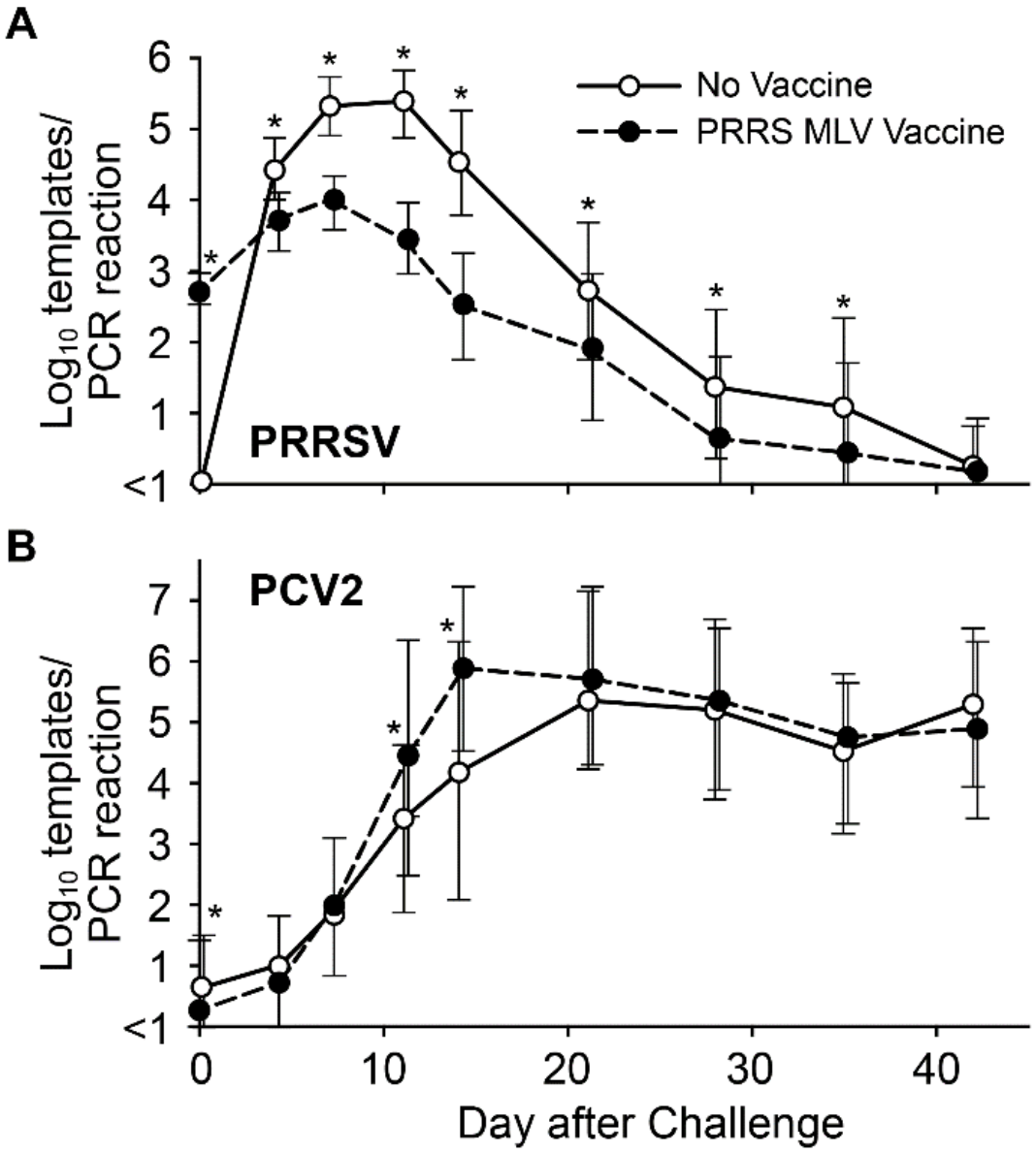


Figure 1:2 PRRSV and PCV2 viremia in vaccinated and non-vaccinated pigs.

The figure shows PCR values as mean ± 1 standard deviation. Asterisks identify statistically significant differences between groups ($p < 0.015$, Student's t-test).

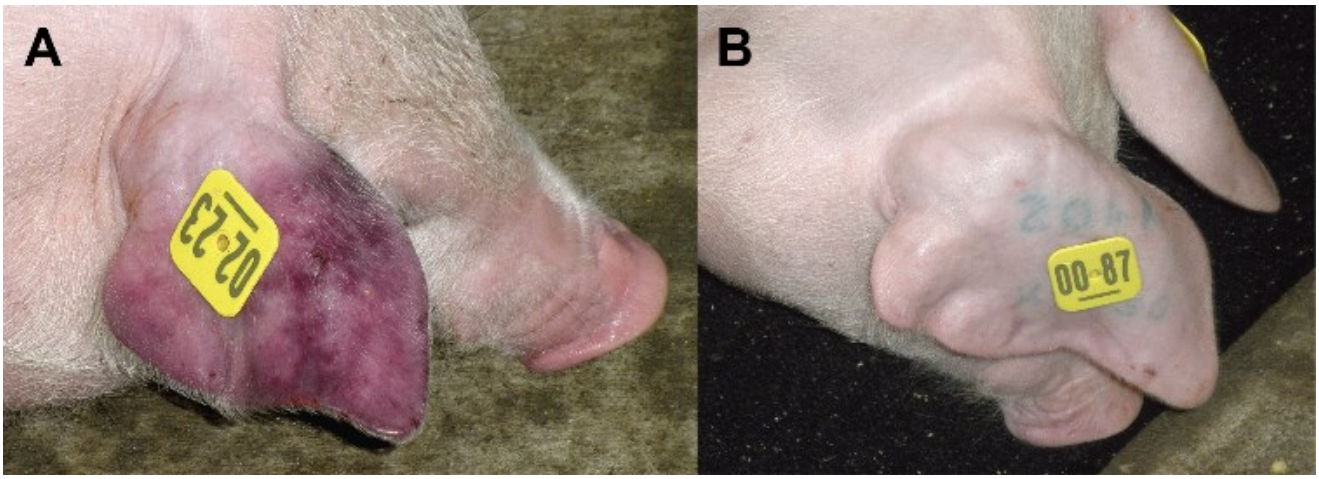


Figure 1:3 Aural cyanosis or "blue ear".

The photograph in panel A is representative of the ear discoloration associated with aural cyanosis during PRRSV infection. The photograph was taken at 11 days after virus challenge. For the purpose of comparison, a normal ear is shown (B).

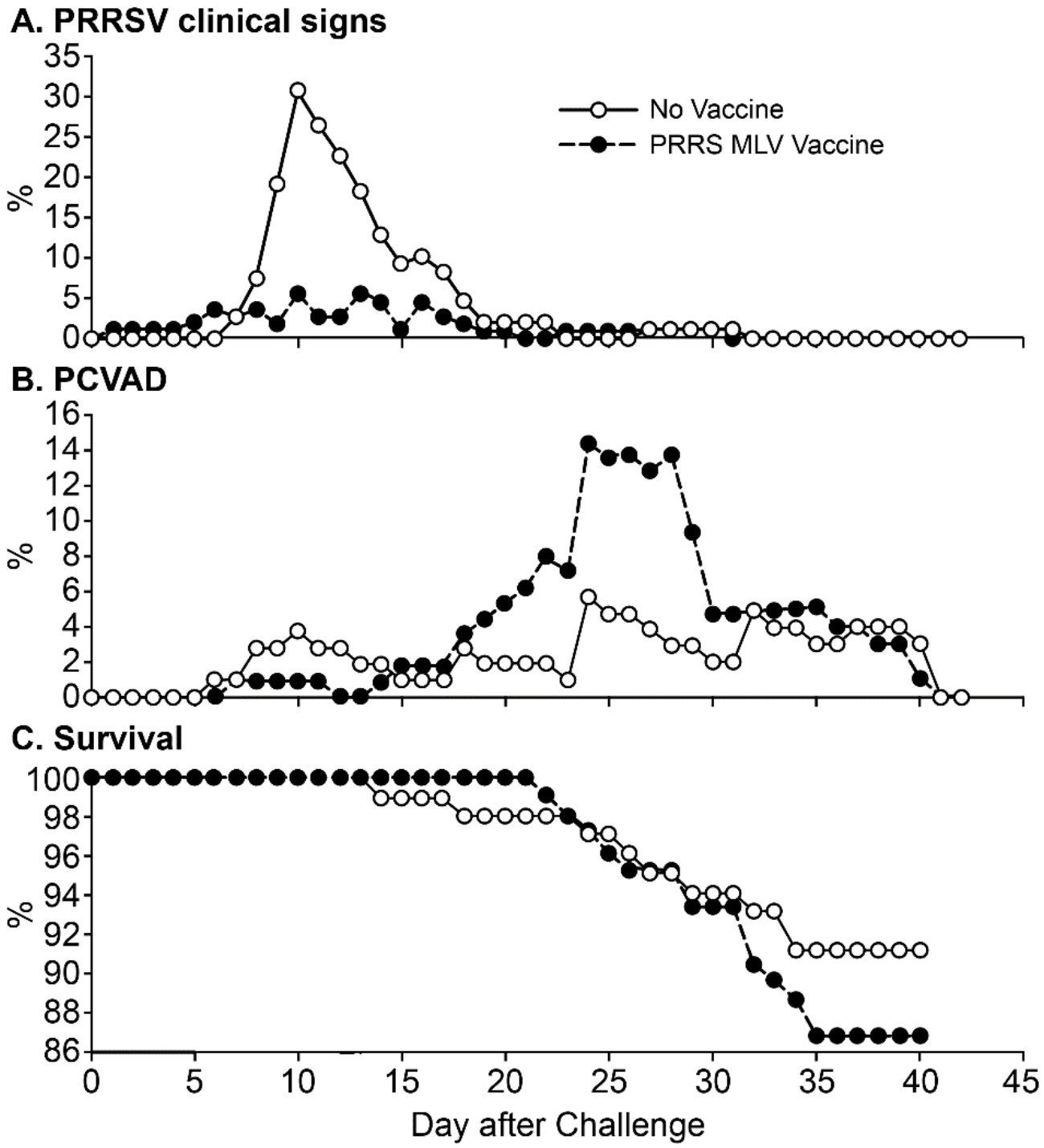


Figure 1:4 Clinical outcomes in vaccinated and non-vaccinated groups after dual challenge with PRRSV and PCV2.

Clinical signs were assessed as described in the materials and methods.

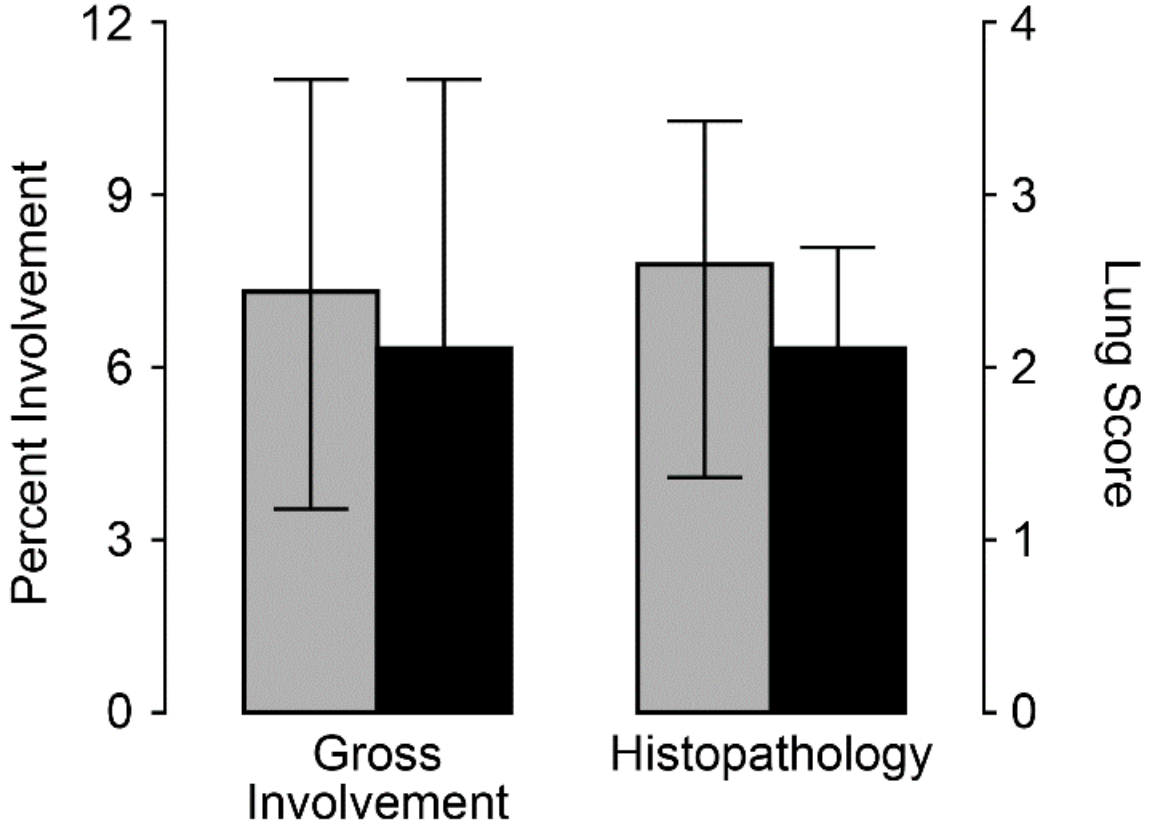


Figure 1:5 Assessment of lung pathology at 11 days after PRRSV/PCV2 challenge.

The results are from 10 vaccine (black bars) and 10 non-vaccine (open bars) pigs randomly removed from the study at 11 days. The results show the assessment of pneumonia as percent of gross lung involvement and histopathology score. Results are shown as mean ± standard deviation. The differences between vaccine and non-vaccine groups were not significant ($p > 0.05$).

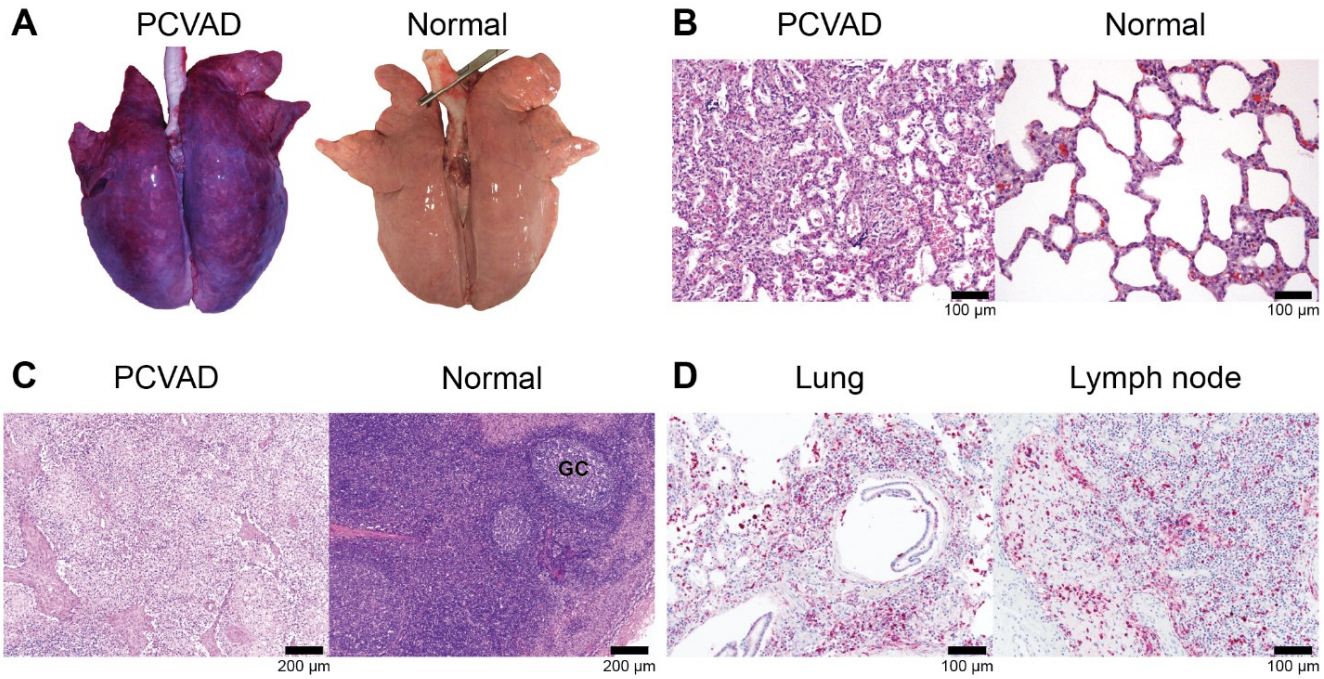


Figure 1:6 Gross and microscopic lesions associated with PCVAD.

The results are representative of PCVAD-affected pigs necropsied between 32-42 days after combined PRRSV and PCV2 challenge. (A) Shows a set of lungs from a challenged pig with pneumonia, mottling and consolidation. A normal lung from an age-matched pig is shown for comparison. (B) H&E-stained lung from a challenged pig showing moderate to severe multifocal interstitial pneumonia with lymphohistiocytic infiltration of alveolar septa. A normal lung is shown for the purpose of comparison. (C) Lymphoid depletion in lymph node from a challenged pig. A normal lymph node with prominent germinal centers (GC) is shown for the purpose of comparison. (D) Immunohistochemical staining showing the accumulation of PCV2 antigen in lung and lymph node from a challenged pig.

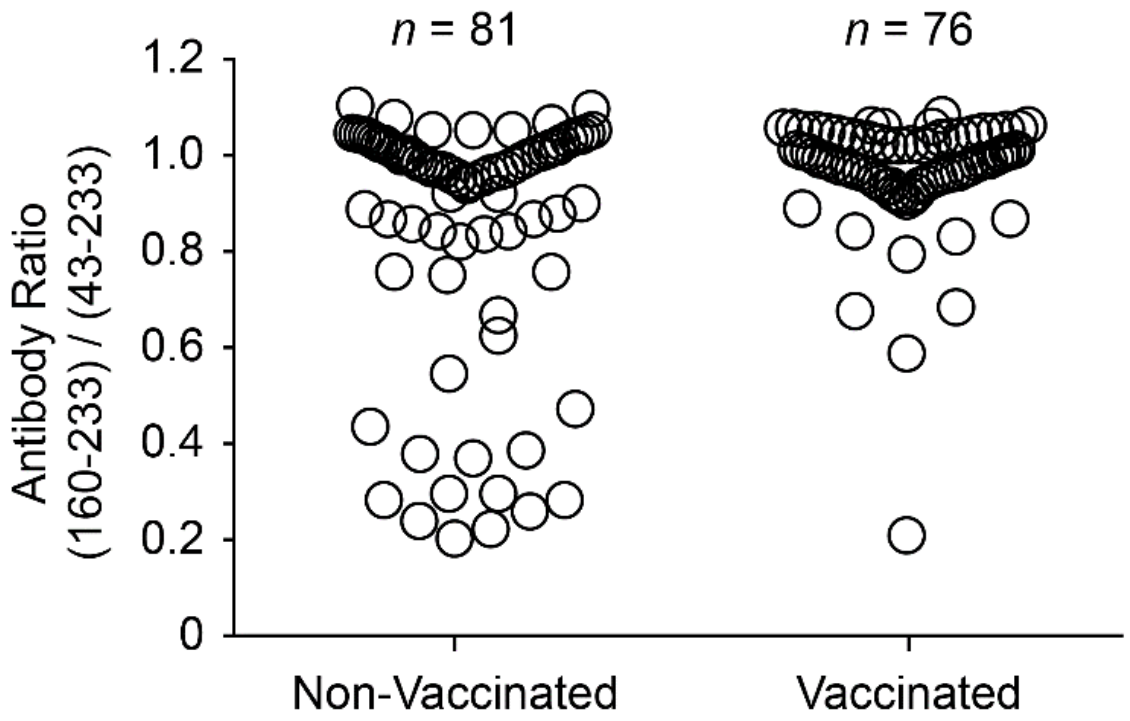
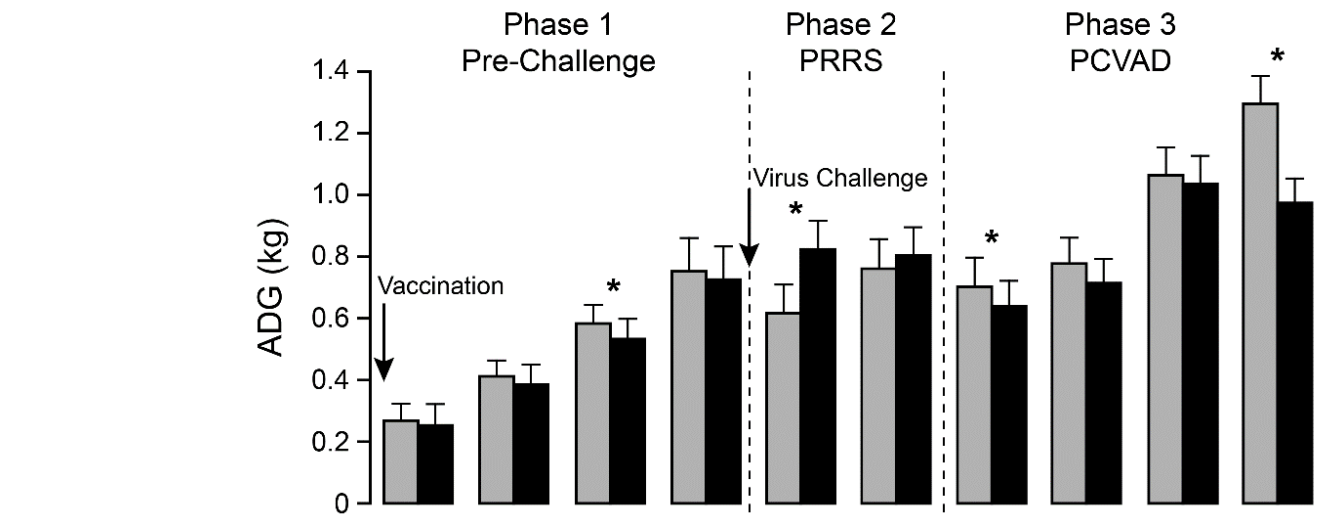


Figure 1:7 PCV2 CP(160-233)/CP(43-233) ratio at 42 days after co-infection.

Sera were analyzed for anti-PCV2 CP antibodies. The serum antibody ratio was calculated as MFI for reactivity with the CP(160-233) decoy epitope divided by the MFI for the CP(43-233) conformational antigen. The difference between the means for the PRRS vaccinated and non-vaccinated groups was significant ($p = 0.0006$, Student's t-test).



Week Relative to Challenge	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
No. Vaccinated Pigs	115	115	115	115	115	105	105	100	93	91
No. Non-Vaccinated Pigs	113	111	111	111	111	101	99	96	94	92

Figure 1:8 Weekly average daily gain (ADG) before and after virus challenge.

The mean ADG values for no vaccine (open bars) and vaccine (black bars) groups were calculated on a weekly basis. The results show mean and standard deviation. The numbers below the figure are the number of pigs used in the analysis. Asterisks identify statistically significant differences in ADG between groups, $p < 0.03$, Student's t-test.

Chapter 2 - Microbiome associations in pigs with the best and worst clinical outcomes following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)

Abstract

On a world-wide basis, co-infections involving porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are common and contribute to a range of polymicrobial disease syndromes in swine. Both viruses compromise host defenses, resulting in increased susceptibility to infections by primary and secondary pathogens that can affect growth performance as well as increase morbidity and mortality. An experimental population of 95 pigs was co-infected with PRRSV and PCV2. At 70 days post-infection (dpi), 20 representative pigs were selected as having the best or worst clinical outcome based on average daily gain (ADG) and the presence of clinical disease. Worst clinical outcome pigs had prolonged and greater levels of viremia as measured by qPCR. Serum, lung and fecal samples collected at 70 dpi were analyzed using DNA microarray technology to detect over 8,000 microbes. An increased number of pathogens were detected in serum from pigs in the worst performing group. At the level of the fecal microbiome, the overall microbial diversity was lower in the worst clinical outcome group. The results reinforce the importance of pathogen load in determining clinical outcome and suggest an important role of microbial diversity as a contributing factor in disease.

Introduction

On a global basis, co-infections involving porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) are common in growing pigs and contribute to a range of polymicrobial disease syndromes. PCV2 is a single-stranded DNA virus in the family *Circoviridae* which contributes to a group of disease syndromes collectively termed porcine circovirus associated disease or PCVAD (Baekbo et al., 2012). Although PCV2 is essential for PCVAD, PCV2 infection alone rarely results in clinical signs and co-pathogens are often necessary for disease expression (Opriessnig and Halbur, 2012). Three important syndromes associated with PCVAD include PCV2-associated respiratory disease, poor growth performance, and postweaning multisystemic wasting syndrome (PMWS) (Baekbo et al., 2012; Ramamoorthy and Meng, 2009; Segales, 2012). The hallmark lesion of PCVAD is lymphoid depletion (Opriessnig and Langohr, 2013), which reduces the ability of the host to respond to primary and secondary pathogens. Since 2006, the application of PCV2 vaccines has largely controlled PCVAD in North America and Europe. However, the disease still maintains a global impact, especially in countries without vaccination programs.

PRRSV is a single-stranded RNA virus in the family *Arteriveridae* (Benfield et al., 1992; Conzelmann et al., 1993) and causes the single most costly disease affecting swine production worldwide (Chand et al., 2012). Losses due to PRRSV infection are associated with increased mortality and reduced growth rates due to respiratory disease in weaned pigs (Holtkamp et al., 2013). PRRSV infection also contributes to a number of immunological outcomes that increase the susceptibility of the host to secondary infections by primary and secondary pathogens (Gomez-Laguna et al., 2013; Opriessnig et al., 2011; Renukaradhya et al., 2010). PRRSV is frequently isolated along with PCV2 in the field (Pallares et al., 2002) and is one of the major co-

factors linked with increasing PCV2 replication and pathogenesis (Allan et al., 2000; Harms et al., 2001; Niederwerder et al., 2015; Rovira et al., 2002; Trible et al., 2012). Clinical outcome following co-infection with PRRSV and PCV2 is often subclinical, with overt clinical disease occurring in only a subpopulation of pigs. Morbidity varies drastically, from subclinical infections and reduced growth performance to increased mortality. Host genetics, immunity, environmental stressors and secondary pathogens are all factors which can impact clinical outcome following PRRSV or PCV2 infections (Boddicker et al., 2012; Chand et al., 2012; Opriessnig and Halbur, 2012; Patterson et al., 2015; Rowland et al., 2012).

An emerging area of study is understanding the role of the microbiome in health and disease. Recent studies have demonstrated that microbiome plays a role in the response of swine to experimental inoculation with the bacterial pathogens *Mycoplasma hyopneumoniae*, *Salmonella enterica* and *Brachyspira hampsonii* (Bearson et al., 2013; Costa et al., 2014; Schachtschneider et al., 2013). Shifts in the abundance of bacterial populations or changes in the microbiome diversity were found to have associations with systemic immune response, pathogen shedding, and the development and severity of clinical disease. In the current study, we took advantage of a population of experimentally infected pigs to evaluate differences between the best and worst clinical outcomes following co-infection with PRRSV and PCV2.

Materials and Methods

Animals and housing. Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and

Use Committees and Institutional Biosafety Committees. The study was conducted as part of a project to evaluate the role of host genetics in determining the outcome following co-infection with PRRSV and PCV2. Four week-old barrows (n = 95; average age 26.8 ± 2.0 days) were obtained from a high-health commercial source negative for PRRSV. While pigs were derived from a sow herd previously vaccinated with a PCV2 capsid subunit vaccine, the piglets were not vaccinated for PCV2 and were utilized in the study without regards to the presence of maternal antibody. All pigs were housed in one environmentally controlled room at the Kansas State University Large Animal Research Center, and maintained under BSL-2 conditions. The room was chemically disinfected, cleaned with a high heat pressure washer and gas decontaminated with vaporized hydrogen peroxide prior to use. The room was empty for approximately 9 weeks prior to the start of the study. Pigs were housed in 10 pens, each 144 sq ft with 9-10 pigs per pen. Pigs were given access to food and water *ad libitum*.

Challenge inoculum. The PRRSV and PCV2b isolates used to prepare the inoculum were originally derived from the lymph node of a pig with severe PMWS, as described previously (Trible et al., 2011; Trible et al., 2012). PRRSV (isolate KS62, GenBank Accession #KM035803) was isolated by propagation on MARC-145 cells. Since wild-type PCV2b (Genbank Accession #JQ692110) does not propagate to high levels in cell culture, we took advantage of the heat stability of PCV2 to make a virus preparation from a lymph node suspension enriched for PCV2. The suspension was heat-treated at 55^{o} C for 30 minutes to remove PRRSV, bacteria and other heat-labile agents. The treated homogenate was recombined with the isolated PRRSV to infect cesarean-derived, colostrum-deprived (CD/CD) pigs. A combination lung/lymph node homogenate was prepared from the CD/CD pigs and PRRSV and PCV2 were isolated from the homogenate by the methods described above. Analysis of the heat-

treated preparation for common agents showed that the preparation was negative for most heat stable agents, such as parvovirus, but still positive for torque teno sus viruses (TTSuV) and porcine endogenous retroviruses (PERVs) on DNA microarray (Jaing et al., 2015). Both TTSuV and PERV are ubiquitous to swine.

PRRSV was titered on MARC-145 cells. Briefly, virus was serially diluted 1:10 in MEM (Corning) supplemented with 7% FBS (Sigma-Aldrich), Pen Strep (80 Units/ml and 80 μg/ml, respectively; Gibco), 3 ug/ml Fungizone (Gibco), and 25 mM HEPES (Life Technologies). The dilutions were then added in quadruplicate to confluent MARC-145 cells in a 96 well tissue culture plate (BD Falcon). Following a 4 day incubation at 37^{o}C in 5% CO_2, wells were examined for PRRSV induced cytopathic effects, and the 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated using the method of Reed and Muench (Reed and Muench, 1938).

The quantity of PCV2 was determined by titration on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of the PCV2 challenge stock were plated in quadruplicate to rapidly dividing ST cells in a 96 well tissue culture plate (BD Falcon). Dilutions were prepared in EMEM (Sigma-Aldrich) supplemented with 7% FBS (Sigma-Aldrich) and 50 μg/ml of gentamycin (Lonza). Following a three day incubation at 37^{o}C in 5% CO_2, cells were fixed and permeabilized with 80% acetone and then stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.). Infected cells were visualized using an inverted fluorescent microscope and the $TCID_{50}$/ml was calculated using the method of Reed and Muench (Reed and Muench, 1938).

Animal model and experimental design. A total of 95 pigs were randomly allocated into 10 identical pens using a random number assignment protocol and housed in groups of 9-10 pigs per pen. After acclimating for two weeks, all pigs were challenged with a combination of

PRRSV and PCV2b. The challenge viruses were recombined to yield a 2 ml dose consisting of $10^{3.6}$ $TCID_{50}$ PCV2 and 10^{5} $TCID_{50}$ PRRSV in MEM. The 2 ml dose was split with 1 ml administered intranasally and the remaining 1 ml administered intramuscularly. Individual body weights were collected on -14, -7, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days post-infection (dpi). Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 35, 42, 56 and 70 dpi. At 63 dpi, 20 pigs were selected from the surviving 78 pigs and categorized as having the best or worst clinical outcomes. The 10 best clinical outcome pigs were selected due to having the highest average daily gain (ADG) from 0-63 dpi and due to a complete lack of overt clinical disease during the post-challenge period. The 10 worst clinical outcome pigs were selected due to having the lowest ADG from 0-63 dpi and due to having at least 10 days of moderate to severe clinical disease during the post-challenge period. All 20 pigs were humanely euthanized on 70 dpi and complete necropsies were performed.

Clinical Disease and Pathology. Pigs were evaluated daily for the presence of clinical signs, including dyspnea, aural cyanosis, coughing, nasal discharge, open mouth breathing, poor body condition, muscle wasting, pallor or jaundice, lameness, joint effusion, depression and lethargy. Each pig was visually examined by a veterinarian or veterinary assistant each day during the study period. Appropriate treatments were initiated for pigs that presented with moderate to severe clinical disease. Examples of clinical presentations where treatment was administered included: 1. Difficult respiration, 2. Mucoid nasal discharge, 3. Lameness with associated joint effusion, 4. Pallor or jaundice associated with muscle wasting, and 5. Lethargy or depression with a rectal temperature ≥104°F. For clinically affected pigs, antibiotic therapy was administered, including ceftiofur hydrochloride for respiratory or systemic disease and oxytetracycline for infectious arthritis. All pigs with overt clinical disease and rectal

temperatures ≥104°F were administered flunixin meglumine, a nonsteroidal anti-inflammatory drug (NSAID). Pigs with intractable fevers of greater than 4 days duration were given a 2 day wash-out period and then administered oral meloxicam. Clinical signs and systemic treatments unrelated to PRRSV/PCV2 co-infection (e.g. lacerations, dermatitis, hoof wounds, congenital hernias) were documented but were not considered as clinical disease in selecting the best and worst outcome groups. Animals were humanely euthanized with pentobarbital sodium. Complete necropsies were performed by a board certified pathologist who was blinded as to the source of the pigs. Pigs that died or were humanely euthanized due to pathology unrelated to PCV2/PRRSV co-infection (e.g. gastric torsion, non-infectious lameness, etc.) were excluded from the study. Average daily gain (ADG) was calculated as the change in weight divided by the number of days and was reported in kg.

Measurement of PRRSV and PCV2 viremia. Viral DNA and RNA were extracted simultaneously from 50 μL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems) in accordance to the manufacturer's instructions. PRRS viral RNA was quantified using EZ-PRRSV MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore) according to the manufacturer's instructions. For consistency, each plate contained Tetracore Quantification Standards and Control Sets for use with EZ-PRRSV MPX 4.0 RT-PCR Reagents. All PCR reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in a 96-well format using the recommended cycling parameters. PCV2 DNA was quantified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively. Primers were used at a final concentration of 300 μM. Nuclease free water was used to bring the mastermix volume to 18 μL per reaction.

The addition of 2 µL of template nucleic acid brought the final reaction volume for each sample to 20 µL. Standard curves and positive and negative controls were included on each plate. Plasmid DNA was used for the PCV2 standard curve and positive control template. DNA inserted into the plasmid was obtained from a field strain of PCV2 (PCV2b 321/393). Plasmid DNA was isolated using the PureYield Plasmid Miniprep System (Promega) according to the manufacturer's instructions. The DNA for the standard curve was quantified using a NanoDrop 8000 Spectrophotometer. The standard curve was produced by diluting the purified plasmid DNA 1:1000 in nuclease free water followed by five serial 1:10 dilutions in nuclease free water. The final standard curve contained 6 points ranging from 10^7 to 10^2 logs of template DNA which produced threshold crossing values between 15 and 33 cycles. Standard curves were run in duplicate with nuclease free water as a negative control. The PCV2 PCR was carried out on a CFX96 Touch Real-Time PCR Detection System using the following settings: activation at 98°C for 2 minutes, followed by 40 cycles of denaturing at 98°C for 5 seconds and annealing/extension at 60°C for 10 seconds. The melting curve was performed between 65-95°C using 0.5°C increments. The PCR assay results were reported as $\log_{10}$ PRRSV RNA starting quantity (copy number) per 50 µL reaction volume or $\log_{10}$ PCV2 DNA starting quantity per 20 µL reaction volume.

DNA Microarray analysis. Each 1 mL sample for extraction consisted of 250 µL sample and 750 µL of Trizol LS reagent (Life Technologies). The purpose of adding Trizol was to inactivate all viruses and bacteria in tissue, fecal and serum samples so they could be shipped to Lawrence Livermore National Laboratory for molecular analysis. Nucleic acid purification could also be performed using commercially available kits, but only the Trizol method was used in this study. Each sample was brought to room temperature, 200 µL of chloroform was added,

and the tube was shaken vigorously for 15 seconds. Samples were incubated at room temperature for 15 min and then centrifuged at 12,000xg for 15 min at 4°C. The upper aqueous layer was removed by pipetting and placed in a new tube for RNA extraction. The lower phases were saved for DNA extraction**.** For RNA extraction, 10 µg of glycogen was added to the aqueous phase along with 500 µL of 100% isopropanol. Following 10 min incubation, samples were centrifuged for 10 minutes at 12,000xg and 4°C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol. The sample was vortexed and centrifuged at 7500xg for 5 minutes at 4°C. Following centrifugation, the supernatant was removed and the RNA was air dried for 10 min. RNA pellets were re-suspended in DEPC water and RNA concentration was determined by the Qubit fluorometer (Life Technologies).

For DNA isolation, 300 µL of 100% ethanol was added to the interphase/organic phase, the tube was inverted several times, and incubated for 3 min at room temperature. Samples were centrifuged at 2000xg for 5 min at 4°C and the supernatant was discarded. The DNA pellet was washed with 1 mL sodium citrate/ethanol solution (0.1M sodium citrate in 10% ethanol, pH 8.5), incubated for 30 min at room temperature, centrifuged at 2000xg for 5 min at 4°C, and the supernatant was removed. The sodium citrate/ethanol wash procedure was repeated once more. Following the wash procedures, 2 mL of 75% ethanol was added to the pellet and incubated at room temperature for 20 min and centrifuged at 2000xg for 5 min at 4°C. The supernatant was removed and the samples were air dried for 10 min. DNA pellets were resuspended in 8 mM NaOH solution and the DNA concentration was determined by the Qubit fluorometer.

For each lung sample, 10 µL (approximately 7.0-12.0 µg) of extracted RNA was used as input to generate double stranded cDNA following the standard Roche NimbleGen protocols. Briefly, first strand cDNA was generated followed by second strand synthesis. Next, the cDNA

was treated with RNase A and precipitated. After cDNA generation, 500 ng each of cDNA and DNA was mixed and labeled as detailed below.

Since less extracted nucleic acid was obtained from the fecal and serum samples, a random amplification was performed on these samples as described previously (Rosenstierne et al., 2014). For each amplification reaction, 5µL each of extracted DNA and RNA was input into the reaction. The amplified DNA was purified using the Qiaquick PCR purification columns (Qiagen) and the yield was determined by the Qubit fluorometer.

Approximately 400-500 ng of amplified cDNA and DNA were mixed together and labeled using a one-color labeling kit (One-Color Labeling kit, Roche NimbleGen) following the standard manufacturer's protocols. Following the labeling reaction, a hybridization reaction was prepared with 10µg of each labeled DNA and Roche NimbleGen hybridization reagents following the standard manufacturer's protocols. The MDAv5 12x135K microarray (Roche NimbleGen) was utilized for this work and samples were loaded onto the array and allowed to hybridize for 65 hr at 42°C in a Roche NimbleGen Hybridization System set to mix mode B. After hybridization, the microarrays were washed following standard manufacturer protocols with Roche NimbleGen wash buffers. Each array was washed for 2 minutes and 15 seconds in Wash 1 at 42°C followed by 1 min in Wash 2 at room temperature and 15 seconds in Wash 3 at room temperature. After washing, the microarrays were exposed to a stream of nitrogen gas to remove any particulates from the array surface. Microarrays were scanned on a microarray scanner (MS200 microarray scanner, Roche NimbleGen) at a resolution of 2 µM.

Microarray data were analyzed using the composite likelihood maximization method (CLiMax) developed at Lawrence Livermore National Laboratory (Gardner et al., 2010). The log likelihood for each of the possible targets is estimated from the BLAST similarity scores of

the array feature and target sequences, together with the feature sequence complexity and other covariates derived from the BLAST results (Gardner et al., 2010). Presented are the data for microbe sequences that were equal to or greater than the 99% threshold.

Statistical analyses. All statistical analyses were performed using GraphPad Prism 6.00 software. Body weights, ADG, viremia and area under the curve were analyzed and compared between groups using the unpaired t-test. Microbial families detected in feces were compared between groups using the Mann-Whitney U-test. Correlation analyses were performed using Pearson correlation coefficients and linear regression. Proportions of individual microbe species detected in each group were compared using Fisher's exact test, and the expected false discovery rate (FDR) was calculated using the Benjamini-Hochberg method to correct for multiple testing.

Results

Selection of pigs with the best and worst outcome. Clinical disease and weight gain were used as the criteria for the selection of the two groups of pigs used in the study. The time course of clinical disease after co-infection of the 95 pigs with PRRSV and PCV2 is summarized in Figure 1. The pattern of clinical signs over time reflected the acute disease associated with PRRS followed by the later onset of PCVAD. Throughout the study period, 33 pigs (33/95; 35%) exhibited clinical signs severe enough to receive at least one day of systemic veterinary treatment. Supportive therapy was administered in the form of antibiotics and/or NSAIDS to reduce secondary bacterial infections, inflammation, and pyrexia. Seventeen of the 33 treated pigs (51.5%) died or were euthanized prior to the end of the 70 day study. Euthanasia was performed as a result of being moribund or nonresponsive to treatment. Of the 16 remaining treated pigs, 10 pigs were selected based on having the lowest ADG. The clinical signs in the 10

worst outcome pigs are summarized in Table 1. Overall, the clinical outcomes were representative of the disease syndromes associated with PRRSV and PCV2 co-infection. Clinical disease progression in the 10 worst outcome pigs is shown in Figure 1C and on the whole, clinical disease in this group mirrored the clinical disease that appeared in the entire population (n = 95, see Figure 1A). Two clinical disease phases are evident post-infection. First, peaking between 10-20 dpi were clinical signs consistent with acute PRRSV infection, such as respiratory distress and aural cyanosis. Second, peaking between 32-42 dpi were clinical signs consistent with PCVAD, such as pallor or jaundice and decreased body condition. All pigs in the worst clinical outcome group showed signs of dyspnea and rhinorrhea. The majority of the worst outcome pigs showed signs of lethargy or depression, decreased body condition, pyrexia, coughing, and diarrhea. All 10 pigs received between 2-4 doses of ceftiofur and 4 pigs received between 2-6 doses of oxytetracycline. The best clinical outcome pigs did not receive antibiotics or NSAIDs during the pre and post-infection period and showed no evidence of clinical disease.

The ADG values for the 10 best and 10 worst clinical outcome pigs over the 70 day post-infection period are shown in Table 2. The mean ADG for the 10 worst clinical outcome group was 0.475 ± 0.15 kg compared to 0.837 ± 0.04 kg for the 10 best clinical outcome pigs. The difference between the means was highly significant ($p < 0.0001$, unpaired t-test).

At the end of the 70 day study period, necropsies were performed on all 20 pigs. All 10 of the worst outcome pigs had gross lesions consistent with PRRS, PCVAD, or virus associated immunosuppression, such as polyserositis. Specifically, gross lesions in the worst outcome group included moderate to diffuse fibrous adhesions between the lungs and thoracic cavity and/or the pericardium (6 pigs), moderate interstitial pneumonia (4 pigs), multifocal fibrous adhesions in the abdomen (4 pigs), and granulomatous nephritis (1 pig). In the best clinical

outcome pigs, 8 of the 10 pigs showed no significant gross lesions. In the remaining two pigs, both had multifocal fibrous adhesions in the abdomen, one had a splenic infarction, and one had diffuse fibrous adhesions on the pericardium.

Mean weights for the two groups are show in Figure 2. By the end of the study, the mean weights for the best and worst outcome groups were 72.3 ± 3.8 kg and 43.0 ± 11.3 kg, respectively. The 29 kg difference was highly statistically significant ($p < 0.0001$, unpaired t-test). Even though the best and worst performing pigs were selected at the end of the study, the means of the two groups were significantly different at all other time points, including prior to infection. For example, the means for the best and worst outcome pigs at two weeks before infection were 6.9 ± 1.0 kg and 5.5 ± 1.1 kg, respectively ($p = 0.008$, unpaired t-test). As shown by the standard deviation bars, the mean weights in the best clinical outcome group were more uniform throughout the study period compared to the worst clinical outcome group. A regression line was fit to the mean weights for both groups over time. The greatest deviation from the estimated line appeared at between weeks 5 and 6 for the worst outcome group, which correlated with peak PCVAD (see Figure 1C).

PRRSV and PCV2 viremia. The results for PRRSV and PCV2 viremia are shown in Figure 3. PRRSV infection in the best outcome pigs followed the typical course, peaking at between 7 and 11 dpi then followed by a decay to less than 1 $\log_{10}$ copies/PCR reaction after 21 dpi (see Figure 3). The worst clinical outcome pigs had a slight delay in viremia, peaking at around 14 dpi, but with prolonged and increased viremia throughout the remainder of the study. A significant difference between the two groups was shown on 28 dpi, when mean PRRSV viremia was 2.46 and 0.86 $\log_{10}$ copies/PCR reaction for the worst and best outcome groups, respectively ($p = 0.015$, unpaired t-test). After the initial decay, rebound peaks were observed in

several pigs, a phenomenon initially described in 2010 (Reiner et al., 2010) and later by us (Boddicker et al., 2012). Another way to present infection is through the calculation of virus load or the area under the viremia curve (Boddicker et al., 2012). The total viral load for each of the 20 pigs was quantified as the area under the curve (AUC) from 0 to 70 dpi (data not shown). The range for the best and worst outcome groups was 88.0 – 151.7 and 95.5 – 245.9, respectively. The means for the best and worst outcome groups were significantly different (116.2 and 153.5, respectively; $p = 0.04$, unpaired t-test), further demonstrating that PRRS virus replication was higher in the worst outcome group.

The results for PCV2 viremia are presented in Figure 3B. Unlike PRRSV, PCV2 viremia shows a delay in peak replication, but is maintained at fairly high levels in the blood for an extended period of time (>3 $\log_{10}$ copies/PCR reaction between 14-70 dpi for the worst clinical outcome group). Both groups showed a peak in PCV2 viremia between about 21 and 42 dpi. Even though not significantly different, the worst clinical outcome group maintained a higher peak virus level. A significant increase in PCV2 viremia for the worst outcome group was only observed on 14 dpi when mean virus levels were 3.44 and 1.74 $\log_{10}$ copies/PCR reaction for the worst and best outcome groups, respectively ($p = 0.03$, unpaired t-test). Comparing the virus loads or AUC for the two groups showed a significant increase in total PCV2 replication in the worst outcome group; means were 210.9 and 260.7 for the best and worst outcome groups, respectively ($p = 0.02$, unpaired t-test). The range AUC values for the best and worst outcome groups were 154.8 – 305.5 and 187.9 – 350.1, respectively. Together, these data show that the amount of virus is increased in the worst outcome pigs.

DNA microarray results for serum. DNA microarray results for microbial species detected in serum, feces and lung are presented in Figures 4 through 7. The results for each

bacterium or virus were ranked according the highest log likelihood score and the first species in each microbe family with the greatest percentage of target-specific probes is shown. The results for serum at day 70 are shown in Figure 4. The results for bacteria identified *Bacillus cereus* as being detected at a significantly higher rate in the worst outcome pigs (7 of 10 pigs) compared to only one positive pig for the best outcome group ($p = 0.02$, Fisher's exact test; B-H FDR = 0.36). Some strains of *Bacillus cereus* are considered nonpathogenic but others have been implicated in cases of human foodborne illness (Berthold-Pluta et al., 2015). For viruses, all pigs showed the presence of torque teno sus virus (TTSuV) and porcine endogenous retrovirus (PERV), which are ubiquitous and were also present in the virus inoculum used for challenge.

In general, the PCR results for PRRSV and PCV2 were similar to the microarray. For example, at 70 dpi, mean PCV2 viremia was 2.3 and 3.3 $\log_{10}$ copies/PCR reaction for the best clinical outcome and worst clinical outcome groups, respectively. The microarray detected PCV2 in the serum of 8 best outcome pigs and all 10 worst outcome pigs. The two best clinical outcome pigs negative for PCV2 on the microarray had 0 and 1.8 $\log_{10}$ copies/PCR reaction, which are levels under the threshold of detection by microarray (Jaing et al., 2015).

With regards to PRRSV, by 70 dpi, the majority of pigs showed undetectable levels of PRRSV in the blood. PRRSV was detected by microarray in only one best outcome pig and one worst outcome pig. The corresponding PCR values for those two pigs were 2.4 and 2.0 $\log_{10}$ copies/PCR reaction, respectively, which is near the threshold for detection of PRRSV by microarray (Jaing et al., 2015). The values for all other pigs were lower.

DNA microarray results for lung. Viral and bacterial species detected by microarray in lung samples are shown in Figure 5. Only 6 species were detected with no significant differences between the two groups. The microarray detected PCV2 in all worst outcome pigs

and in 8 of the best outcome pigs. By 70 dpi, PRRSV was undetectable in the lungs of all pigs. Porcine endogenous retrovirus A was detected in all pigs and torque teno sus virus was detected in 5 best outcome and 2 worst outcome pigs. Bacterial species were rare; 3 of the worst outcome pigs and 1 of the best outcome pigs had bacteria detected in lung samples.

DNA microarray results for feces. A total of 28 bacterial species were detected by the array. Those bacteria that were only found at a low prevalence (10% or less) included *Mannheimia haemolytica*, *Dechlorosoma suillum*, *Campylobacter lari*, *Erwinia amylovora*, *Brevibacillus brevis*, *Enterococcus faecalis*, *Dorea formicigenerans*, *Solibacillus silvestris* and *Bacteroides fragilis*. The remaining bacteria are listed in Figure 6. Several bacteria, such as *Prevotella copri*, *Treponema succinifaciens*, *Phascolarctobacterium* sp., *Megasphaera elsdenii*, *Faecalibacterium prausnitzii*, and *Lactobacillus johnsonii*, were present at a relatively high prevalence; i.e. > 50% of pigs. The most interesting finding was the presence of *Escherichia coli* in 5 best outcome pigs but not detected in any member of the worst outcome group ($p = 0.03$, Fisher's exact test; B-H FDR = 0.98). Although some strains of *E. coli* are enteropathogenic and cause diarrhea in piglets, there were no toxin genes detected. In serum, *Bacillus cereus* was detected at a relatively high prevalence in the worst outcome pigs (see Figure 4); however, in the feces, the higher prevalence was in the best performing group.

Viral species detected in the feces of both groups are also shown in Figure 6. Although these pigs were from a herd with a recent PED outbreak, PEDV was not detected by microarray in any of the pigs in the two groups. PCV2 was detected in the feces, with a higher prevalence in the worst outcome group. PRRSV was not detected. Several of the ubiquitous viruses were detected, including torque teno sus virus 1b and porcine type C retrovirus. A variety of other

viruses were detected at different frequencies, including porcine kobuvirus, porcine bocavirus, porcine astrovirus, po-circo-like virus, porcine teschovirus and porcine enterovirus.

Microbiome diversity was calculated by quantifying the number of microbial families detected in each fecal sample. The results are presented in Figure 7. The range of microbial families detected for the best outcome and worst outcome groups were 10 – 16 and 7 – 12, respectively. The best clinical outcome group had significantly greater family diversity than the worst clinical outcome group ($p = 0.017$, Mann-Whitney U-test).

Discussion

Co-infections involving PCV2 and PRRSV contribute to PCVAD, associated with significant losses through increased mortality and poor growth performance. In acute outbreaks of PCVAD in the field, the prevalence of clinically ill pigs is typically only 2-25% (Kyriakis et al., 2002; Quintana et al., 2001; Sorden et al., 1998). In a previous study by us, co-infection with PRRSV and PCV2 resulted in 12-26% of pigs showing clinical signs of PCVAD (Niederwerder et al., 2015). Thus, the majority of pigs are able to support virus replication without overt clinical disease. Understanding the factors that influence how a pig responds to virus challenge, through subclinical or clinical infections, was a primary goal of this study. Specifically, we sought to compare microbiome and pathogen load between those pigs which were classified as having the best or worst clinical outcome following co-infection with PRRSV and PCV2 in a controlled experimental trial.

The best and worst clinical outcome groups were selected based on ADG and clinical disease after virus challenge. As such, it is interesting to note that there were significant differences between the mean weights of the two groups in the weeks prior to challenge.

Presumably, factors unrelated to co-infection were affecting weight gain in the worst outcome group during these two weeks. These factors may include host genetics, pathogen exposure and environmental conditions prior to arrival, or immunity. However, differences between the growth rates of the two groups become most prominent during peak PCVAD. Between 28-35 dpi, mean weight gain for the worst performing group was 0.36 kg compared to 6.91 kg for the best performing group. This highlights how the development of clinical disease significantly impacts growth. Further, it is clear that some pigs can thrive while maintaining subclinical infections, even during peak virus replication and clinical disease.

When comparing PRRSV and PCV2 viremia between the two groups, it should be considered that primary differences were detected outside of either peak in virus replication. For example, a significant difference was found in PRRSV viremia at 28 dpi, when virus load typically decays. A significant difference was noted for PCV2 at 14 dpi, a time point prior to peak virus replication. This demonstrates that replication patterns may have a more rapid incline (PCV2) or a more gradual decline (PRRSV) in poor performing pigs. However, comparing the total virus loads allowed us to demonstrate that overall, virus replication was higher in the worst outcome group throughout the entire course of the study. This data emphasizes the underlying importance of viral pathogen load in the determination of outcome after challenge.

The Lawrence Livermore Microbial Detection Array (LLMDA) technology utilized in this study provides the opportunity to test for the presence of over 8000 microbes including viruses, bacteria, fungi, protozoa and archaea species. In our previous work, we confirmed the applicability of the microarray in detecting known and unknown pathogens in various diagnostic samples from pigs (Jaing et al., 2015). In the current report, we applied the microarray to serum, lung, and fecal samples to investigate differences between groups of pigs with either the best or

worst clinical outcome after co-infection. Overall, the DNA microarray detected the microbial signatures of 18 species in serum, 6 species in lung and 39 species in feces.

In serum, DNA microarray detected *Bacillus cereus* in a significantly greater proportion of worst outcome pigs compared to best outcome pigs. Because *B. cereus* is typically present in the gastrointestinal tract (phylum Firmicutes), this finding likely represents a generalized increase in gastrointestinal permeability of poor performing pigs, allowing bacterial microbes to invade the bloodstream. Our previous microarray work also reported evidence of bacteremia, where approximately one third of pigs tested (6/18; 33%) had bacterial signatures detected in the serum (Jaing et al., 2015).

Although pulmonary tissues were long thought of as sterile, the lung microbiome is now a well-recognized factor impacting human health and disease of the respiratory tract (Cui et al., 2014; Dickson et al., 2013). In infectious diseases such as HIV and non-infectious diseases such as chronic obstructive pulmonary disease, lung microbiome profiles have been associated with treatment success or clinical outcome (Cui et al., 2014). The lung is a primary site for both PRRSV and PCV2 replication, and modulation of the host immune response to primary and secondary pathogens in the lung is known to increase viral pathogenesis (Gomez-Laguna et al., 2013; Opriessnig and Halbur, 2012). However, studies to investigate how the lung microbiome impacts these viral infections in swine are lacking. In the current study, very few microbes, including only two bacterial species, were detected by DNA microarray in lung. No significant difference was detected that could help explain clinical outcome. Similarly, Schachtschneider et al. (2013) found that the microbiome present in lower respiratory tract samples did not affect outcome after challenge with *Mycoplasma hyopneumoniae*, another common respiratory pathogen in swine (Schachtschneider et al., 2013). The lack of pathogenic and non-pathogenic

microbes detected in the lungs from our study may be due to several factors. First, the DNA microarray is less sensitive than PCR and may not detect microbes that are present in low numbers. Therefore, the microarray may not be the best tool for interrogating the porcine lung microbiome, where non-pathogenic microbes are likely present in limited quantities. Second, the timing of collection may have impacted microbe detection. At 70 days post-infection, all 20 pigs had cleared PRRSV and although many pigs still had PCV2 detected, all pigs had resolved overt clinical disease (compare Figure 1 and Table 5). Therefore, finding clinically relevant secondary bacterial populations in the lung would have been less likely at this time point.

Although much is still unknown, significant advances have been made in understanding the impact of the gastrointestinal microbiome on systemic and respiratory diseases in humans (Fujimura and Lynch, 2015; Redinbo, 2014). Investigating the effects of the microbiome on food animal diseases is relatively new and previous studies have primarily focused on bacterial pathogens. For example, a recent study examined the fecal microbiome differences between those pigs which did and did not develop mucohemorrhagic diarrhea after inoculation with *Brachyspira hampsonii*. Pigs which developed mucohemorrhagic diarrhea were associated with lower bacterial counts in the feces and a decreased Bacteroidetes:Firmicutes ratio (Costa et al., 2014). Schachtschneider et al. (2013) evaluated the effects of orally administering the gastrointestinal microbiota from a healthy adult boar to nursery pigs prior to challenge with *Mycoplasma hyopneumonia*. Oral microbial inoculation increased fecal microbiome diversity, resulted in earlier seroconversion, decreased gross lung lesions and significantly reduced coughing levels after challenge. However, there was no significant difference in weight gain between the two groups (Schachtschneider et al., 2013). Bearson et al. (2013) investigated the differences in fecal microbiome between those pigs which were classified as high or low

Salmonella-shedders after experimental challenge with *Salmonella enterica* serovar Typhimurium. Low *Salmonella*-shedders had significantly lower levels of diarrhea, increased presence of *Ruminococcaceae* bacteria in the feces prior to inoculation, and increased *Prevotella* species 2 days after inoculation (Bearson et al., 2013). Although similarities are present between these studies and our study, direct comparisons are difficult as the above studies used quantitative PCR in which abundance, and not just detection, can be evaluated.

An overall trend described above and continued in our study is the association between increased fecal microbe diversity and density with improved outcome. In the current study, the total number of microbial families detected in feces was significantly higher in the best clinical outcome group. Reduced family diversity in the worst clinical outcome pigs may have been due to antibiotic treatment for clinical signs during the study period. Therefore, linear regression analysis was performed on the number of antibiotic doses received (independent variable) and the number of microbial families detected in feces (dependent variable) for each of the worst outcome pigs; no significant associations were detected (data not shown). Although there does not appear to be a significant correlation between antibiotics received and fecal microbial diversity, the overall effect of antibiotic administration cannot be eliminated. However, it would be expected that parenteral antibiotic therapy would affect serum and lung samples in a similar manner to feces, and reductions were not uniformly observed across all samples collected from worst outcome pigs.

Consistent with previous reports (Holman et al., 2014; Kim et al., 2011), the majority of bacterial species detected in feces by the microarray fall within the Firmicutes phylum (18/28 species). Although the ratio of Bacteroidetes:Firmicutes has been linked with both weight gain and clinical outcome (Costa et al., 2014; Guo et al., 2008), no significant ratio difference was

detected between the two clinical outcome groups in this study. However, these ratios are typically calculated by quantifying relative abundance, an analysis we are unable to perform using the DNA microarray. All pigs except one had *Lactobacillus sp*. and *Faecalibacterium prausnitzii* detected in the feces. *F. prausnitzii* is a common anaerobic bacterium, considered anti-inflammatory (Sokol et al., 2008) and makes up approximately 5% of all bacteria found in human feces (Arumugam et al., 2011). In contrast, *Bacteroides sp.* was only detected in one pig, whereas it is the most prevalent bacterial genus detected in human feces (Arumugam et al., 2011). This is consistent with a previous report showing *Bacteroides sp.* are detected at a higher rate in humans than in swine (Lamendella et al., 2011). *Prevotella sp.*, the most abundant bacterial genus reported in swine feces (Lamendella et al., 2011), was detected at a higher rate in best clinical outcome pigs (10/10; 100%) compared to worst clinical outcome pigs (7/9; 78%); however, this difference was not statistically significant ($p = 0.21$; Fisher's exact test).

Overall, members of the Proteobacteria phylum, including *Escherichia coli*, *Erwinia amylovora*, *Campylobacter lari*, *Dechlorosoma suillum,* and *Mannheimia haemolytica,* were only detected in the best clinical outcome group feces. Specifically, *E. coli* was detected at a significantly higher rate in best outcome pigs. It should be considered that members of this phylum potentially contribute to improved clinical outcome and weight gain after co-infection. Other studies have found similar associations. For example, a significant increase in the proportion of Proteobacteria was detected in the pre-inoculation feces of pigs which remained nonclinical after *B. hampsonii* challenge (Costa et al., 2014). In another study, pigs administered a medicated diet known to improve feed efficiency had significant increases in bacterial species, primarily *E. coli*, of the Proteobacteria phylum compared to non-medicated pigs (Looft et al., 2012). *E. coli* has also been associated with excessive weight gain in pregnant women

(Santacruz et al., 2010) as well as increased body weight and fat deposition in rats (Karlsson et al., 2011). Although this may be an undesirable outcome in studies of obesity, increased weight gain associated with *E. coli* colonization could be extremely valuable within a production system. Whether members of this bacterial phylum truly play a protective role in host response and increasing growth performance remains to be investigated.

The relationship between PCV2 and PRRSV is complex and co-infection with both viruses has been shown to increase pathogenicity compared to either virus infection alone (Harms et al., 2001). In the current study, the presence of high serum titers of both PRRSV and PCV2 were associated with poor clinical outcome. Although both viruses contribute to modulation of the host immune response and increase susceptibility to primary and secondary pathogens, this study highlights how host response to primary virus challenge is a major determinant of clinical outcome. In addition, this study provides valuable insight into how microbiome may contribute to outcome following systemic viral infection.

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Table 2:1 Summary of clinical signs in 10 pigs with worst clinical outcome

	Pigs Affected	Duration Range (days)	Mean Duration ± SD (days)
Treatment for Clinical Signs	100%	10-29	17.5 ± 7.3
Dyspnea	100%	3-17	8.9 ± 3.7
Rhinorrhea	100%	1-15	5.4 ± 5.1
Lethargy	90%	0-22	6.3 ± 7.8
Decreased Body Condition	80%	0-37	10.8 ± 14.0
Pyrexia	80%	0-23	8.3 ± 8.0
Coughing	80%	0-11	2.5 ± 3.3
Diarrhea	60%	0-14	2.4 ± 4.3
Pallor or Jaundice	50%	0-5	1.3 ± 1.8
Muscle Wasting	30%	0-4	0.9 ± 1.5
Open Mouth Breathing	30%	0-1	0.3 ± 0.5
Lameness	20%	0-22	2.3 ± 6.9
Joint Effusion	20%	0-7	1.1 ± 2.4
Aural Cyanosis	10%	0-2	0.2 ± 0.6

Table 2:2 Average daily gain between 0-70 dpi

Worst Performing		Best Performing	
Pig	ADG	Pig	ADG
1	0.537	6	0.883
3	0.492	15	0.831
12	0.149	29	0.827
16	0.506	30	0.779
24	0.555	43	0.805
28	0.542	54	0.903
47	0.493	55	0.797
50	0.280	62	0.848
61	0.495	63	0.889
88	0.698	98	0.808
*Mean	0.475	Mean	0.837
SD	0.153	SD	0.042

* Significant difference between means, $p < 0.0001$, unpaired t-test

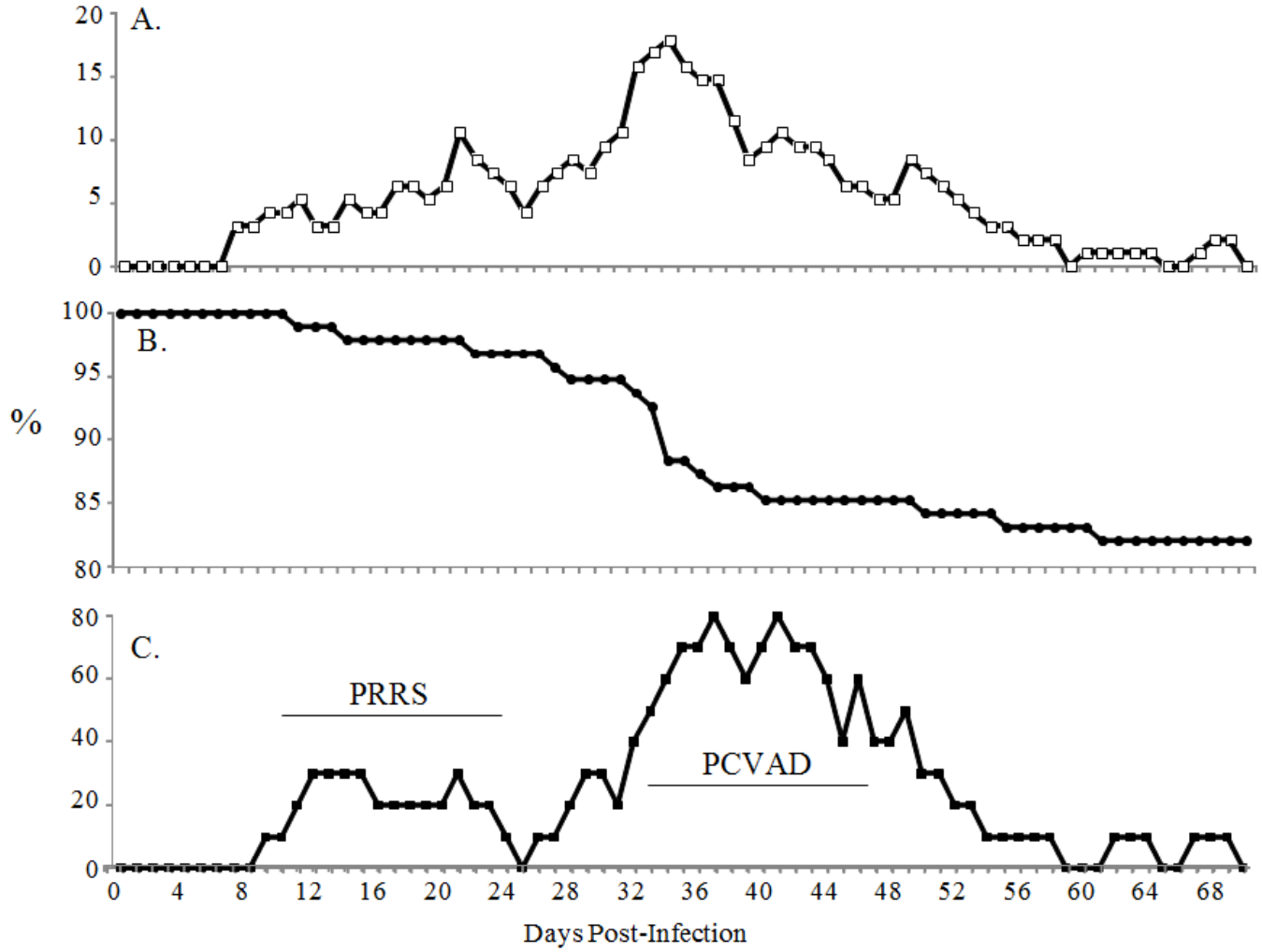

Figure 2:1 Clinical disease following co-infection with PRRSV and PCV2.

(A) Percent of pigs receiving treatment as a result of clinical signs (n = 95). (B) Percent survival over time (n = 95). (C) Percent of worst outcome pigs receiving treatment as a result of clinical signs (n = 10).

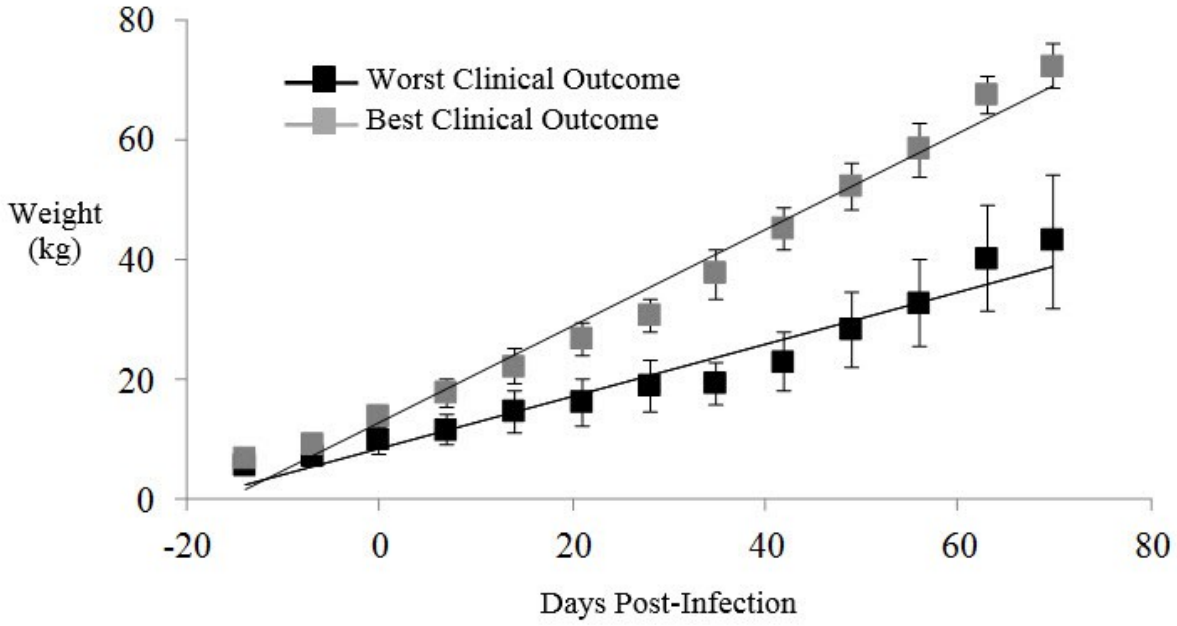

Figure 2:2 Mean weights over time for pigs with the best and worst clinical outcomes.

Data is shown as mean weight ± one standard deviation with regression lines. Mean weights were significantly different between the two groups on every week shown ($p < 0.05$, unpaired t-test).

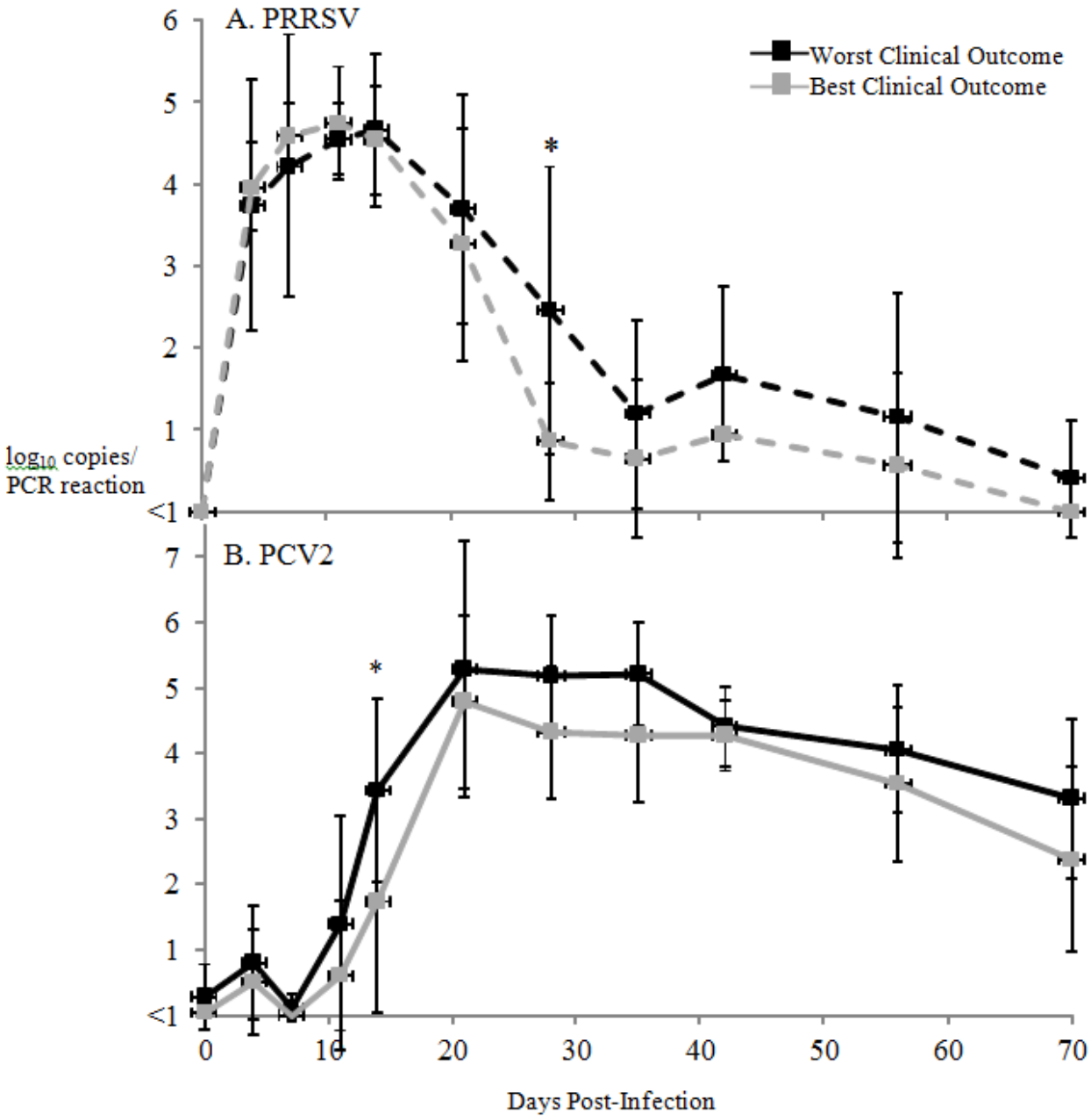

Figure 2:3 PRRSV and PCV2 viremia in pigs with best and worst clinical outcomes.

The figure shows mean PCR values ± 1 standard deviation after challenge with PRRSV and PCV2. Asterisks identify statistically significant differences between groups ($p < 0.05$, unpaired t-test).

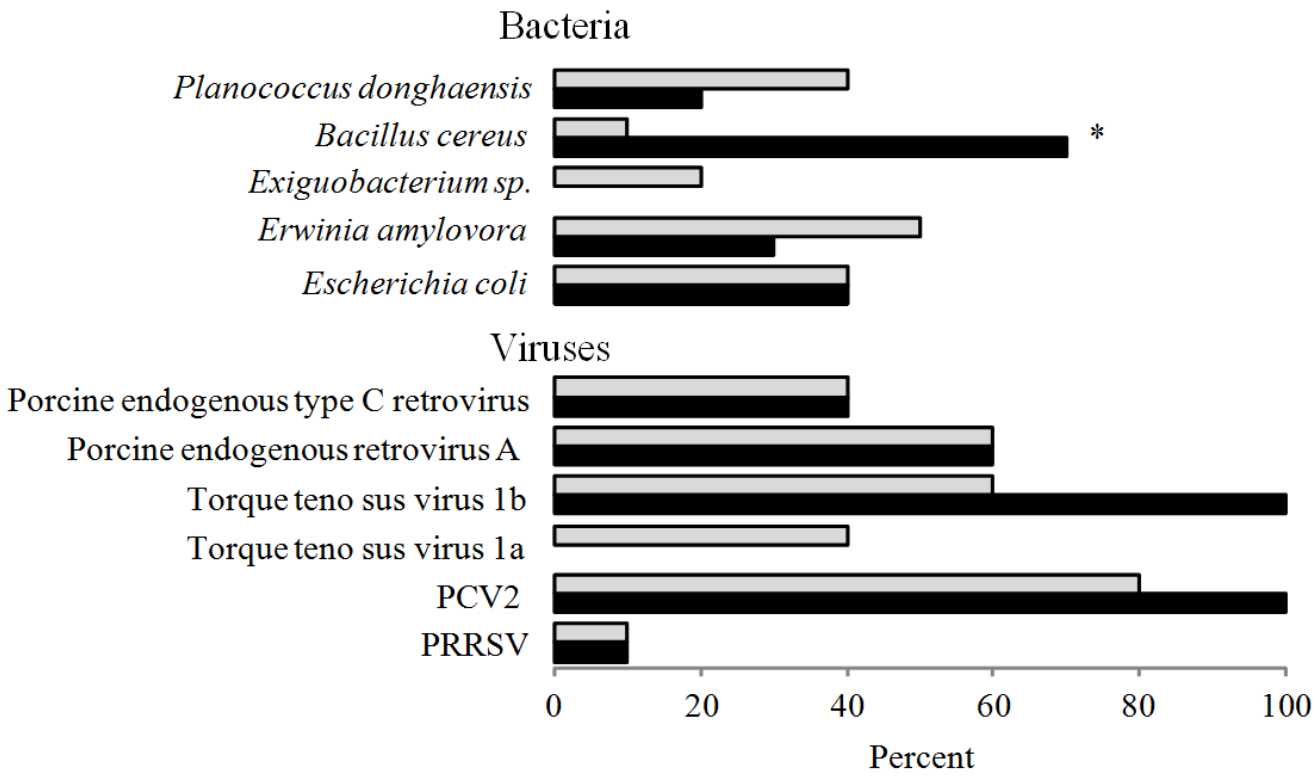

Figure 2:4 Microarray detection of microbes in serum 70 days after co-infection with PCV2 and PRRSV.

Percent of best clinical outcome pigs (n = 10, open bars) and worst clinical outcome pigs (n = 10, black bars) are shown for each microbe detected on the array. Asterisks identify statistically significant differences between groups (p = 0.02, Fisher's exact test).

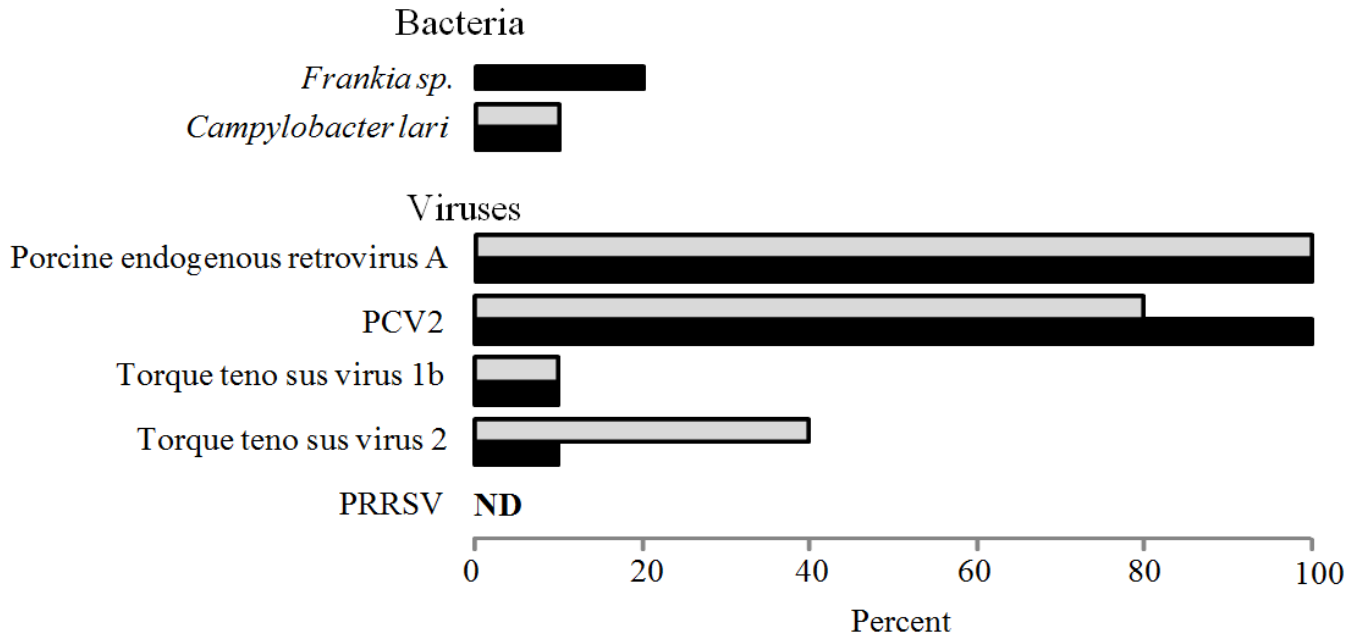

Figure 2:5 Microarray detection of microbes in lungs 70 days after co-infection with PCV2 and PRRSV.

Percent of best clinical outcome pigs (n = 10, open bars) and worst clinical outcome pigs (n = 10, black bars) are shown for each microbe detected on the array. No significant differences were detected between groups. ND = not detected.

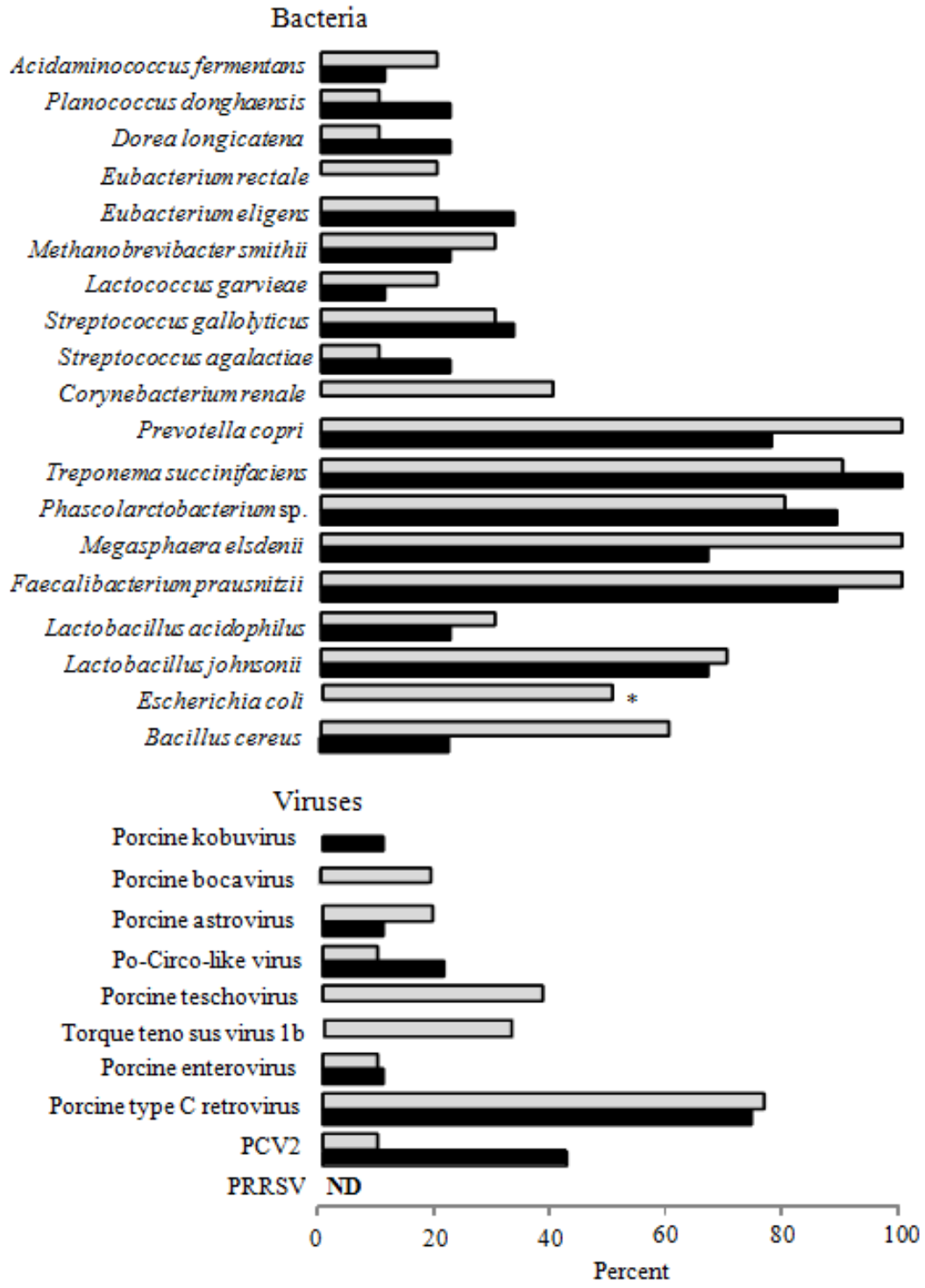

Figure 2:6 Microarray detection of microbes in feces 70 days after co-infection with PCV2 and PRRSV.

Percent of best clinical outcome pigs (n = 10, open bars) and worst clinical outcome pigs (n = 9, black bars) are shown for each microbe detected on the array. Asterisks identify statistically significant differences between groups (p = 0.03, Fisher's exact test). ND = not detected.

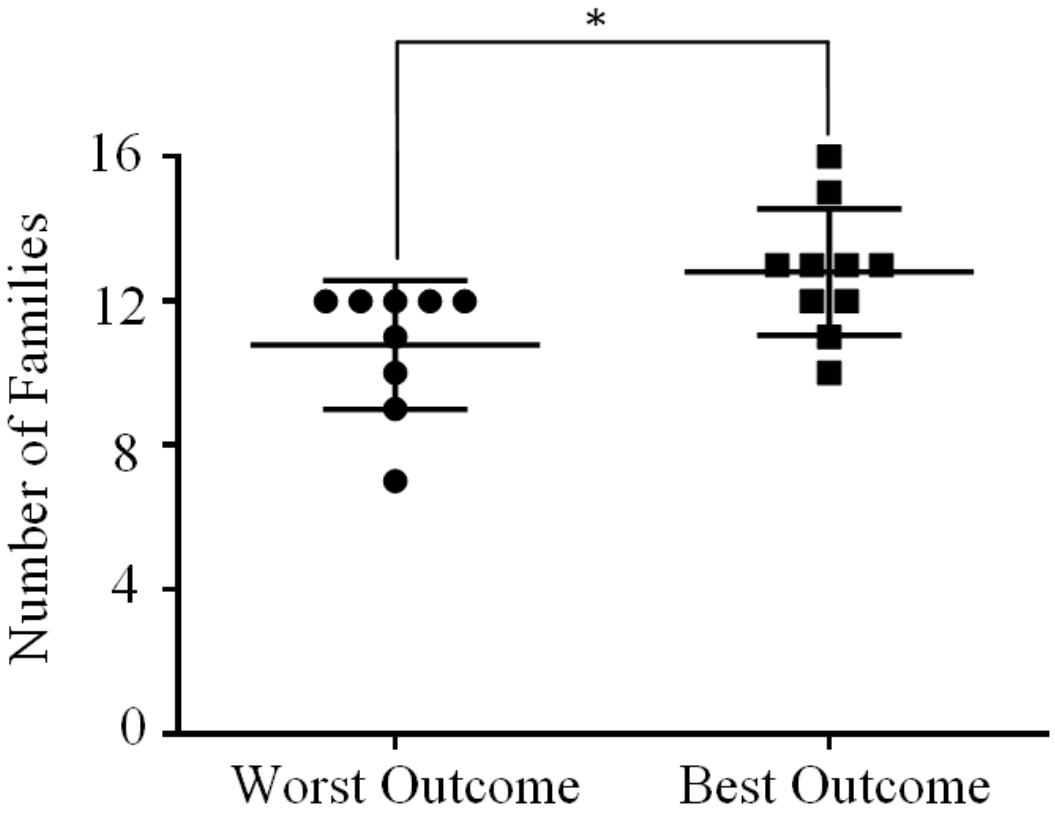

Figure 2:7 Fecal microbiome diversity in pigs with the best and worst clinical outcomes.

Data is shown as the total number of microbial families detected by DNA microarray 70 days after co-infection with PRRSV and PCV2. Group means and standard deviations are represented by horizontal lines. The number of microbial families detected in feces were significantly different between the best and worst outcome groups (*p = 0.017, Mann-Whitney U-test).

Chapter 3 - Time course of clinical disease and effects on growth performance in nursery pigs following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)

Abstract

Co-infections involving porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are common and contribute to several disease syndromes that result in a broad range of clinical signs. The purpose of this study was to standardize collection of clinical data in co-infected nursery pigs and investigate the impact of clinical disease on animal welfare and growth. Clinical phenotypes were documented for 476 nursery pigs (average age 53.3 ± 6.9 days) after co-infection with PRRSV and PCV2 for 42 days post-infection (dpi) or until death. Clinical phenotypes included pallor or jaundice, lethargy or depression, muscle wasting, decreased body condition, dyspnea, open mouth breathing, rhinorrhea, diarrhea, aural cyanosis, lameness, joint effusion, coughing, and veterinary treatment. Other than aural cyanosis, most clinical phenotypes peaked in the second half of the post-challenge period (21-42 dpi) and corresponded to increased PCV2 replication. The presence of muscle wasting was associated with the greatest reduction (62%) in average daily gain (ADG) whereas the duration of dyspnea had the strongest negative association (r = -0.51) with ADG. Large reductions in ADG were associated with each day of open mouth breathing (0.227 kg). Lameness and aural cyanosis were the only two clinical phenotypes where no significant association was detected with ADG for either clinical presence or duration. In this experimental model, respiratory distress and muscle wasting were the clinical phenotypes associated with the

greatest impact on growth. This study provides insight into how detailed clinical observations can be used to improve animal welfare and productivity.

Introduction

Porcine circovirus type 2 (PCV2), a single-stranded DNA virus in the family *Circoviridae*, contributes to a group of disease syndromes collectively termed porcine circovirus associated disease or PCVAD (Baekbo et al., 2012). Syndromes associated with PCVAD include subclinical reductions in growth or clinical respiratory, enteric, and systemic infections (Segales, 2012). Clinical signs of PCVAD are diverse and may include paleness of the skin, icterus, pyrexia, wasting or weight loss, reduced growth rate, ill thrift, diarrhea, cutaneous macules and papules, sudden death, dyspnea and other signs of respiratory illness such as coughing, sneezing and nasal discharge (Baekbo et al., 2012; Harding, 1997; Opriessnig and Langohr, 2013; Ramamoorthy and Meng, 2009; Segales, 2012). Since inactivated and subunit vaccines have been applied to the management of PCV2, PCVAD has been largely controlled in North America and Europe (Horlen et al., 2008; Kixmoller et al., 2008; Velasova et al., 2013). However, due to the recent emergence of PCV2 strains that escape vaccine protection (Opriessnig et al., 2013; Seo et al., 2014) and the lack of consistent vaccination programs in some countries, PCVAD remains a global threat to swine production.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus in the family *Arteriveridae* (Benfield et al., 1992; Conzelmann et al., 1993). Syndromes associated with PRRSV include subclinical reductions in growth or clinical respiratory and systemic infections by primary and secondary pathogens due to PRRSV induced immunosuppression (Gomez-Laguna et al., 2013; Opriessnig et al., 2011; Renukaradhya et al.,

2010). Clinical signs of PRRS may include cyanosis of the ears and skin, lameness, joint effusion, sudden death, pyrexia, inappetence, poor growth performance and signs of respiratory illness such as dyspnea, coughing, sneezing, conjunctivitis and nasal discharge (Done and Paton, 1995; Done et al., 1996; Paton et al., 1991). Modified live virus vaccines reduce losses associated with PRRSV infections. However, due to several disadvantages to the current vaccines, such as incomplete protection against emerging heterologous strains (Wang et al., 2015) and potentiation of other pathogens (Niederwerder et al., 2015), PRRS continues to be the most costly disease to the swine industry (Chand et al., 2012).

Evaluations of health in a large swine production system are typically nonspecific, such as detailing percent mortalities and days in the nursery as key performance indicators (Stalder, 2013). Field research studies investigating the clinical signs of PRRSV have reported the presence or absence of generalized respiratory disease and mortalities (Rosendal et al., 2014; Young et al., 2010). Detailed information regarding what percent of pigs develop distinct clinical signs, such as jaundice or open mouth breathing per month of production, and how these numbers are altered during disease outbreaks are widely unknown. However, detailed clinical data collection provides an opportunity for recognizing subtle changes in herd health for early intervention and improved outcomes. Further, defining which clinical signs have the greatest impact on growth and welfare may provide guidelines for targeted veterinary treatment and the judicious use of antibiotics.

In this study, we utilized a polymicrobial disease model through co-infection of nursery pigs with PRRSV and PCV2 to: 1) Standardize collection of detailed clinical data in a population over the time course of infection and 2) Investigate the relationships between specific clinical phenotypes with growth performance and animal welfare.

Materials and Methods

Animals and housing. Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use Committees and Institutional Biosafety Committees. Data for the current study was compiled from 3 separate trials performed over an 18 month period under similar conditions. All studies were conducted as part of a project to evaluate the role of host genetics in determining the outcome following co-infection with PRRSV and PCV2. Weaned barrows (total $n = 476$; average age of 26.9 ± 3.8 days) were obtained from high-health commercial sources negative for PRRSV. While pigs were derived from sow herds previously vaccinated with a PCV2 capsid subunit vaccine, the piglets were not vaccinated for PCV2 and were utilized in the study without regards to the presence of maternal antibody. All pigs were housed in two environmentally controlled rooms at the Kansas State University Large Animal Research Center, and maintained under BSL-2 conditions. Pigs were housed in 144 sq ft pens and were given access to food and water *ad libitum*.

Challenge inoculum. The PRRSV and PCV2b isolates used to prepare the inoculum were originally derived from the lymph node of a pig with severe PMWS, as described previously (Trible et al., 2011; Trible et al., 2012). PRRSV (isolate KS62, GenBank Accession #KM035803) was isolated by propagation on MARC-145 cells. The PRRSV component of the challenge inoculum, KS62, shared 88.06% identity with the MLV (GenBank Accession #AF159149) at the peptide sequence level of GP5. Since wild-type PCV2b (Genbank Accession #JQ692110) does not propagate to high levels in cell culture, we took advantage of the heat

stability of PCV2 to make a virus preparation from a lymph node suspension enriched for PCV2. The suspension was heat-treated at 55^{o} C for 30 minutes to remove PRRSV, bacteria and other heat-labile agents. The treated homogenate was recombined with the isolated PRRSV to infect cesarean-derived, colostrum-deprived (CD/CD) pigs. A combination lung/lymph node homogenate was prepared from the CD/CD pigs and PRRSV and PCV2 were isolated from the homogenate by the methods described above. Analysis of the heat-treated preparation for common agents showed that the preparation was negative for most heat stable agents, such as parvovirus, but still positive for torque teno sus virus (TTSuV) and porcine oncovirus (PCOV) (Jaing et al., 2015), which are ubiquitous.

PRRSV was titered on MARC-145 cells. Briefly, virus was serially diluted 1:10 in MEM (Corning) supplemented with 7% FBS (Sigma-Aldrich), Pen Strep (80 Units/ml and 80 μg/ml, respectively; Gibco), 3 ug/ml Fungizone (Gibco), and 25 mM HEPES (Life Technologies). The dilutions were then added in quadruplicate to confluent MARC-145 cells in a 96 well tissue culture plate (BD Falcon). Following a 4 day incubation at 37^{o}C in 5% CO_2, wells were examined for PRRSV induced cytopathic effects, and the 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated using the method of Reed and Muench (Reed and Muench, 1938).

The quantity of PCV2 was determined by titration on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of the PCV2 challenge stock were plated in quadruplicate to rapidly dividing ST cells in a 96 well tissue culture plate (BD Falcon). Dilutions were prepared in EMEM (Sigma-Aldrich) supplemented with 7% FBS (Sigma-Aldrich) and 50 μg/ml of gentamycin (Lonza). Following a three day incubation at 37^{o}C in 5% CO_2, cells were fixed and permeabilized with 80% acetone and then stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.). Infected cells were

visualized using an inverted fluorescent microscope and the $TCID_{50}$/ml was calculated using the method of Reed and Muench (Reed and Muench, 1938).

Experimental Design. Data from a total of 476 nursery pigs were compiled for this report from 3 studies (study 1, $n = 96$; study 2, $n = 189$; study 3, $n = 191$). Pigs in each study were randomly allocated into one of two identical rooms using a random number assignment protocol and housed in groups of 8-10 pigs per pen. At approximately 7.5 weeks of age (average 53.3 ± 6.9 days), all pigs were challenged with a combination of PRRSV and PCV2b. The challenge viruses were recombined to yield a 2 ml dose consisting of $10^{3.6}$ $TCID_{50}$ PCV2 and 10^5 $TCID_{50}$ PRRSV in MEM. The 2 ml dose was split with 1 ml administered intranasally and the remaining 1 ml administered intramuscularly. Twenty-eight days prior to challenge, 39.7% of pigs (189/476) were vaccinated with a 2 ml dose of commercial PRRS MLV vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim Animal Health; GenBank Accession #AF159149) administered intramuscularly according to the vaccine label instructions. For the purpose of this report, medical records and clinical data have been combined for vaccinated and non-vaccinated pigs. All pigs were followed for a total of 42 days post-infection (dpi). Individual body weights were collected weekly on 0, 7, 14, 21, 28, 35, and 42 dpi. Blood samples were collected on 0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi and clinical data was collected on a daily basis.

Clinical Evaluation. Pigs were evaluated daily for the presence of clinical signs associated with PRRSV and PCV2 infections. Every pig was visually examined by a veterinarian or veterinary assistant each day of the study period by walking through each pen to ensure animals were responsive and ambulatory. Any pig showing clinical signs of disease was restrained for a detailed examination. A standardized health evaluation questionnaire (see Table 1) guided evaluators through a series of questions for any pig showing clinical signs of disease.

Each clinical phenotype was documented as either yes for presence, no for absence or scored based on severity. Documented clinical phenotypes included dyspnea, aural cyanosis, coughing, rhinorrhea, open mouth breathing, pyrexia, decreased body condition, diarrhea, muscle wasting, pallor or jaundice, lameness, joint effusion, and depression or lethargy.

Dyspnea scores were determined based on a 6 point scale as previously described (Halbur et al., 1995). Briefly, 0 = no dyspnea; 1 = mild dyspnea upon restraint; 2 = mild dyspnea at rest; 3 = moderate dyspnea upon restraint; 4 = moderate dyspnea at rest; 5 = severe dyspnea upon restraint; 6 = severe dyspnea at rest. Lethargy and depression were assessed through response to stimuli and interaction with pen mates using a 4 point scale: 0 = bright, alert and responsive; 1 = quiet, alert and responsive; 2 = slightly depressed; 3 = depressed; 4 = moribund. Body condition scores (BCS) were assigned using an adapted 5 point scale (Patience and Thacker, 1989). Briefly, 1 = emaciated; 2 = thin; 3 = ideal; 4 = fat; 5 = obese. Lameness was assessed using a 4 point scale: 0 = no lameness; 1 = stiffness in some joints; 2 = mild lameness, slight limp visible; 3 = moderate lameness, obvious limp that includes toe-touching; 4 = severe lameness, non-weight bearing, needs encouragement to ambulate. Fecal consistency scores were assigned 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. Open mouth breathing, coughing, rhinorrhea, aural cyanosis, pallor or jaundice, muscle wasting, and joint effusion were described as: yes = present or no = absent. Rectal temperature was only measured in pigs showing other visual signs of disease; as such, this parameter cannot be considered a stand-alone phenotype and has been excluded from the current report.

Appropriate veterinary treatments were initiated for pigs that presented with moderate to severe clinical disease. Examples of clinical presentations where treatment was administered included: 1. Difficult respiration, 2. Mucoid nasal discharge, 3. Lameness with associated joint effusion, 4. Pallor or jaundice associated with muscle wasting, and 5. Lethargy or depression with rectal temperature ≥104°F. For clinically affected pigs, antibiotic therapy was administered, including ceftiofur hydrochloride for respiratory or systemic disease, oxytetracycline for infectious arthritis, and enrofloxacin for cases refractory to the previous two antibiotics. All pigs with overt clinical disease and rectal temperatures ≥104°F were administered flunixin meglumine, a nonsteroidal anti-inflammatory drug (NSAID). Pigs with intractable fevers of greater than 4 days duration were given a 2 day wash-out period and then administered oral meloxicam. All treatments were administered as directed by a veterinarian. For the purpose of recording veterinary treatment as a clinical phenotype, each day was described as: yes = treatment was administered or no = treatment was not administered.

Clinical signs and systemic treatments unrelated to PRRSV or PCV2 (e.g. lacerations, dermatitis, hoof wounds, congenital hernias) were documented but were not included in the data analysis related to clinical phenotypes. Animals were humanely euthanized with pentobarbital sodium if moribund or unresponsive to treatment. Pigs that died or were humanely euthanized due to circumstances unrelated to the effects of co-infection (e.g. gastric torsion, musculoskeletal injuries, etc.) were excluded from the mortality analysis. Average daily gain (ADG) was calculated as the change in weight divided by the number of days and was reported in kg.

Measurement of PRRSV and PCV2 viremia. Viral DNA and RNA were extracted simultaneously from 50 µL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems) in accordance to the manufacturer's instructions. PRRS viral RNA was quantified

using EZ-PRRSV MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore) according to the manufacturer's instructions. For consistency, each plate contained Tetracore Quantification Standards and Control Sets for use with EZ-PRRSV MPX 4.0 RT-PCR Reagents. All PCR reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in a 96-well format using the recommended cycling parameters. PCV2 DNA was quantified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively. Primers were used at a final concentration of 300 μM. Nuclease free water was used to bring the mastermix volume to 18 μL per reaction. The addition of 2 μL of template nucleic acid brought the final reaction volume for each sample to 20 μL. Standard curves and positive and negative controls were included on each plate. Plasmid DNA was used for the PCV2 standard curve and positive control template. DNA inserted into the plasmid was obtained from a field strain of PCV2 (PCV2b 321/393). Plasmid DNA was isolated using the PureYield Plasmid Miniprep System (Promega) according to the manufacturer's instructions. The DNA for the standard curve was quantified using a NanoDrop 8000 Spectrophotometer. The standard curve was produced by diluting the purified plasmid DNA 1:1000 in nuclease free water followed by five serial 1:10 dilutions in nuclease free water. The final standard curve contained 6 points ranging from 10^7 to 10^2 logs of template DNA which produced threshold crossing values between 15 and 33 cycles. Standard curves were run in duplicate with nuclease free water as a negative control. The PCV2 PCR was carried out on a CFX96 Touch Real-Time PCR Detection System using the following settings: activation at 98°C for 2 minutes, followed by 40 cycles of denaturing at 98°C for 5 seconds and annealing/extension at 60°C for 10 seconds. The melting curve was performed between 65-95°C

using 0.5°C increments. The PCR assay results were reported as $\log_{10}$ PRRSV RNA starting quantity (copy number) per 50 μL reaction volume or $\log_{10}$ PCV2 DNA starting quantity per 20 μL reaction volume.

Statistical analyses. Mortality was compared in the first half (0-20 dpi) and second half (21-42 dpi) of the post-challenge period using Fisher's exact test with odds ratio reported. Clinical phenotypes in pigs that died compared to pigs that survived were also compared using Fisher's exact test. Mortalities were excluded from statistical analyses involving ADG and only those pigs surviving the 42 day post-challenge period were analyzed. ADG comparisons for each clinical phenotype (presence or absence) were analyzed using the unpaired t-test. Linear regression analysis was completed using days with each clinical phenotype as the predictor variable (x axis) and the ADG over 0-42 dpi as the response variable (y axis). Linear regression was used to determine slope and significance of the relationship between the two variables. Pearson correlation coefficients (r) are reported for each linear regression analysis. All analyses were performed using the GraphPad Prism 6.00 software.

Results

PRRSV and PCV2 Viremia. The results for PRRSV and PCV2 viremia are shown for 285 pigs in Figure 1. On the day of challenge, 28% of pigs (77/276) had detectable PRRSV in the serum, consistent with the PRRS MLV vaccine administered 28 days prior to challenge. At this time, low but detectable levels of the vaccine virus were detectable (mean = 0.67 $\log_{10}$ templates per PCR reaction). Throughout the post-challenge period, PRRSV infection followed the typical course, with replication peaking at between 4 and 11 dpi, followed by rapid decay and

virtual disappearance by 35 dpi, when 72.5% of pigs (177/244) had no detectable PRRSV in the serum.

On the day of challenge, only 3% of pigs (9/276) had detectable levels of PCV2, a virus typically found in the environment. Throughout the post-challenge period, PCV2 replication gradually increased until peak viremia at 21 dpi. Between 21-42 dpi, large amounts of PCV2 were maintained in the serum of most pigs. At the conclusion of the study, PCV2 was still detected at an average of 3.0 $\log_{10}$ templates per PCR reaction and 27.5% of pigs (68/247) had greater than 4 $\log_{10}$ templates per PCR reaction. Taken together, this data shows two different virus replication patterns, with the first half of the study characterized by PRRSV replication and the second half of the study characterized by PCV2 replication.

Clinical Phenotypes. For the purpose of reporting clinical phenotypes in the current study, all of the scored clinical parameters were simplified into: yes = present or no = absent. For body condition, a score of < 3 was considered decreased (yes) and a score of ≥ 3 was considered normal (no). For dyspnea, a score of ≥ 1 was considered present. For lameness, a score of ≥ 1 was considered present. For lethargy or depression, a score of ≥ 1 was considered present. For diarrhea, a score of ≥ 2 was considered present.

Table 2 summarizes the 13 clinical phenotypes measured in the 433 pigs that survived the 42 day post-challenge period. Clinical signs consistent with PRRSV or PCVAD were not present prior to challenge. Phenotypes are listed in order of prevalence, with veterinary treatment administration, used as an overall measurement of morbidity, recorded in the greatest percentage of pigs (16.6%). The remaining phenotypes provide more detailed information on clinical disease and were categorized into high, moderate or low prevalence. High prevalence included dyspnea (12.0%), aural cyanosis (11.3%) and lethargy or depression (9.5%). Moderate

prevalence included rhinorrhea (8.5%), coughing (8.1%), lameness (7.6%) and decreased body condition (5.8%). Low prevalence phenotypes included clinical signs that occurred in less than 5% of pigs. These included jaundice or pallor (3.9%), diarrhea (3.5%), joint effusion (3.0%), open mouth breathing (2.5%) and muscle wasting (2.3%).

The time course of clinical phenotypes over the 42 day post-challenge period is summarized in Figure 2. Each day post-challenge was considered a separate data point; thus, the clinical phenotypes documented are representative of all pigs surviving on that day. Therefore, it is important to consider that the reduction in clinical signs at approximately 34 dpi was due to the increase in mortality or euthanization of pigs that were moribund or nonresponsive to treatment (see Figure 4). Clinical phenotypes were organized into four categories: A. nonspecific clinical phenotypes; B. porcine respiratory disease complex; C. phenotypes associated with PRRSV; and D. phenotypes associated with PCV2.

Other than aural cyanosis, all clinical phenotypes primarily occurred in the second half of the post-infection period, which corresponded to high levels of PCV2 replication as well as an increase in mortality (see Figures 1 and 4). Aural cyanosis is commonly known as “blue ear” (Done and Paton, 1995; Paton et al., 1991) and a representative image is shown in Figure 3A. The peak in aural cyanosis occurred between 9 and 14 dpi, corresponding to the peak in PRRSV viremia (see Figure 1). Veterinary treatment, dyspnea and rhinorrhea have similar biphasic peaks, occurring between 20-24 dpi and 30-38 dpi. Several phenotypes, such as coughing, open mouth breathing and diarrhea, failed to peak during the course of infection; these phenotypes never affected more than 2% of the population at any one time point. Decreased body condition, lethargy or depression, muscle wasting and pallor or jaundice all peaked late in the post-infection period on either 33 or 34 dpi. This peak is during high levels of PCV2 replication (see Figure 1)

and these clinical phenotypes are consistent with PCVAD. A representative image of pallor is shown in Figure 3B. Lameness and joint effusion peaked simultaneously between 34-42 dpi. Although lameness and joint effusion are not directly caused by PRRSV, these clinical signs are typically associated with PRRSV-induced immunosuppression.

M**ortality.** During the 42 day study, 43 pigs died or were euthanized (43/476; 9.0%) due to clinical disease associated with PRRSV or PCV2 infections. As illustrated in Figure 4, the majority of deaths occurred in the second half of the post-challenge period; 79% (34/43) between 21-42 dpi compared to 21% (9/43) between 0-20 dpi. Pigs were 4.0 times (95% CI [1.9, 8.4]) more likely to die between 21-42 dpi than 0-20 dpi ($p = 0.0001$, Fisher's exact test). This increase in mortality rate correlated with high levels of PCV2 and the peak in PCVAD.

As expected, clinical phenotypes in the mortalities occurred at a much higher rate than the overall population. For example, almost all mortalities were treated by a veterinarian (41/43; 95.3%) and were documented to have lethargy or depression (37/43; 86.0%) and dyspnea (33/43; 76.7%). Other clinical phenotypes documented in the mortalities included decreased body condition (29/43; 67.4%), rhinorrhea (24/43; 55.8%), pallor or jaundice (19/43; 44.2%), coughing (15/43; 34.9%), open mouth breathing (14/43; 32.6%) and muscle wasting (11/43; 25.6%). The clinical phenotypes that occurred in the lowest proportion of mortalities were also shown to have the weakest associations with ADG (see below); aural cyanosis (7/43; 16.3%), lameness (8/43; 18.6%), joint effusion (3/43; 7.0%) and diarrhea (10/43; 23.3%).

Mortality rates for pigs that were documented with each clinical phenotype are shown in Figure 5. The highest mortality rates were documented in pigs with open mouth breathing (56.0%), decreased body condition (53.7%), jaundice or pallor (52.8%), and muscle wasting (52.4%). The lowest mortality rates were documented in pigs with aural cyanosis (12.5%), joint

effusion (18.8%) and lameness (19.5%). Almost all clinical phenotypes were more likely to be present in a pig that died compared to a pig that survived the 42 day post-infection period ($p < 0.0001$, Fisher's exact test). For example, a pig that died was 18.5 times (95% CI [7.7, 44.4]) more likely to develop open mouth breathing than a pig that survived. Lameness was more prevalent in the mortalities but at a lower statistical significance ($p = 0.02$, Fisher's exact test). Two clinical phenotypes did not have significantly higher rates in the pigs that died; joint effusion and aural cyanosis ($p = 0.17$ and $p = 0.32$, respectively; Fisher's exact test).

Effect of Clinical Disease on Growth. The distribution of average daily gain (ADG) from 0-42 dpi for all surviving pigs ($n = 433$) is shown in Figure 6. Mean ADG for the whole group was 0.80 ± 0.20 kg and values ranged from 0.14 kg to 1.18 kg. The ADG of pigs with clinical signs of PCVAD, including pallor or jaundice and/or muscle wasting, are highlighted in black on the distribution plot (see Figure 6). Mean ADG for these pigs was 0.38 ± 0.15 kg and values ranged from 0.14 kg to 0.69 kg. This data demonstrates how ADG is skewed to the left for pigs with these PCVAD phenotypes.

ADG comparisons between pigs with and without each clinical phenotype are shown in Figure 7 and listed in Table 2. The presence of muscle wasting, decreased body condition, and jaundice or pallor was associated with the greatest reductions in ADG; 62%, 56%, and 54% reductions, respectively ($p < 0.0001$; unpaired t-test). Respiratory phenotypes were also associated with significant reductions in ADG, including coughing (43%), rhinorrhea (43%), open mouth breathing (40%) and dyspnea (34%). Only the presence of two clinical phenotypes did not have significant associations with reducing ADG; lameness and aural cyanosis ($p = 0.66$ and $p = 0.18$, respectively; unpaired t-test).

Linear regression analysis was performed between the number of days with each clinical phenotype and ADG from 0-42 dpi. Table 2 summarizes the data from the linear regression analyses on all 13 clinical phenotypes and eight representative plots are shown in Figure 8. The duration of veterinary treatment had a moderate to moderately low association with reducing ADG (r = -0.36) due to high levels of variability around the regression line (see Figure 8A). As veterinary treatment is administered for a broad range of clinical signs, the remaining clinical phenotypes provide more detailed insight into the effects of clinical disease duration on ADG. For example, the duration of dyspnea had the strongest negative association with ADG (r = -0.51), as demonstrated by a consistent decrease in ADG as duration of dyspnea progressed (see Figure 8B). Open mouth breathing and muscle wasting have steep regression lines, with slopes equaling -0.227 kg and -0.193 kg, respectively. This relationship is visually apparent in Figures 8C and 8D, where the regression line estimates the ADG to equal zero at approximately 4 days duration. However, the correlation coefficients are -0.25 and -0.32 for open mouth breathing and muscle wasting, respectively. This moderately low association is likely due to the small number of pigs that had these clinical phenotypes (n = 11 and n = 10, respectively). Jaundice or pallor and rhinorrhea had moderate associations with reducing ADG (r = -0.38 for both) and moderately steep regression lines (-0.063 kg and -0.059 kg, respectively; see Figures 8E and 8F). Similar associations were found for lethargy or depression, coughing, and decreased body condition (plots not shown). The duration of diarrhea had a weak association with reducing ADG (r = -0.16) and a gradual regression line (-0.022 kg, plot not shown). The duration of joint effusion, lameness and aural cyanosis had no significant associations with reducing ADG; this is demonstrated for the latter two phenotypes by the horizontal regression lines and evidence of prolonged clinical duration coupled with high ADG (see Figures 8G and 8H).

Discussion

Early identification of nursery pigs that have a high likelihood of poor growth performance or mortality is important to maintaining productivity and long-term economic viability in the swine industry. Clinical disease can be used for early recognition of these poor performing pigs through keen observations and well-trained staff. The prevalence of clinical disease is affected by several factors, including but not limited to host genetics, pathogen load, environmental conditions, vaccination, and immunity. Co-infection of nursery pigs with two common swine pathogens, PCV2 and PRRSV, was used in these trials to model a polymicrobial disease syndrome and investigate the role of host genetics in determining outcome. In each trial, large populations of co-infected pigs provided a unique opportunity to detail the broad range of clinical outcomes that occur after co-infection and investigate how clinical disease impacts growth and animal welfare.

Over the course of 3 trials involving close to 500 pigs, thirteen clinical phenotypes were documented. Historically, clinical signs have been difficult to measure in challenge experiments involving PRRSV or PCV2; precluding the collecting of significant data are low numbers of infected pigs combined with only a subpopulation of these pigs developing clinical disease. Large populations were necessary to mimic field conditions, where only 2-25% of pigs develop clinical disease during acute outbreaks of PCVAD (Kyriakis et al., 2002; Quintana et al., 2001; Sorden et al., 1998). In the current study, only 1 out of every 4 pigs showed significant clinical disease (115/476; 24.2%), demonstrating the depth of data necessary to observe and quantify low-percentage clinical outcomes.

Of 13 clinical phenotypes, aural cyanosis was the only one to peak in the first half of the post infection period. Aural cyanosis is not pathopneumonic for PRRS; however, it often

coincides with acute PRRSV infection (Done and Paton, 1995; Paton et al., 1991) and is prevented by PRRS vaccination (Niederwerder et al., 2015). In this study, the peak in aural cyanosis corresponded to the peak in PRRSV viremia at approximately 11 dpi. Aural cyanosis was one of only two clinical phenotypes to lack a significant association with growth performance. In early descriptions of PRRS, this lack of correlation between skin discoloration and other clinical signs was also recognized (Done and Paton, 1995). However, this finding is in opposition to more recent reports on highly pathogenic PRRSV infections in China, where aural cyanosis coincides with severe disease (Zhou et al., 2008). Thus, it is important to consider differences in strain virulence when determining the severity of aural cyanosis as a clinical phenotype. Aural cyanosis is an easily identifiable clinical sign with low evaluator subjectivity. Due to its close relationship with the peak in PRRSV replication shown here and previously (Niederwerder et al., 2015), aural cyanosis may be useful in early recognition of a PRRS outbreak. For example, an increased prevalence of aural cyanosis may indicate a PRRSV introduction or an emerging PRRSV isolate. Although the presence and duration of aural cyanosis did not correlate with growth, monitoring changes in aural cyanosis over time may provide valuable information on the epidemiological trends of PRRS in a herd.

Both PRRSV and PCV2 are common contributors to porcine respiratory disease complex or PRDC (Brockmeier, 2002). Several phenotypes consistent with PRDC were documented in this study, including dyspnea, open mouth breathing, rhinorrhea, and coughing. Dyspnea and rhinorrhea had similar biphasic peaks, while coughing and open mouth breathing failed to peak at any time point. Similarly, two primary peaks in clinical disease were seen in field conditions, where weaned pigs were naturally exposed to both PCV2 and PRRSV (Martelli et al., 2013). This time course is likely due to associations with both PRRSV and PCV2 replication patterns

(see Figure 1). Dyspnea leading to open mouth breathing had a strong association with reducing ADG. Open mouth breathing is a severe sign of acute distress and compromised welfare in swine. Measuring this clinical phenotype has been used to assess welfare during gas euthanasia (Sadler et al., 2014) and is commonly used to assess stress associated with transportation (Kephart et al., 2010; Pilcher et al., 2011; Ritter et al., 2007; Ritter et al., 2008). In the current study, open mouth breathing was relatively rare but was associated with the highest mortality rate; of the 25 pigs that developed open mouth breathing, 14 pigs died (56% mortality). With regards to recognition, open mouth breathing is arguably the least subjective clinical phenotype; therefore, it may be a model parameter to measure and implement into population monitoring records. For a single day of open mouth breathing, there was an average reduction in ADG by 0.227 kg. When taken over the course of the 42 day study, this equates to 9.5 kg or 21.0 lbs in lost weight gain. This would be a devastating loss to nursery pigs, in which the average exit weight in 2012 was 50.7 ± 8.4 lbs (Stalder, 2013). Reducing the prevalence of open mouth breathing in nursery pig populations would decrease mortality, improve animal welfare, and increase growth.

Although lameness and joint effusion are not directly caused by PRRSV infection, septic polyarthritis can be indirectly associated with PRRS-induced immunosuppression, increasing susceptibility to bacterial infections known to affect the joints, such as *Mycoplasma hyosynoviae* and *Streptococcus suis* (Galina et al., 1994; McOrist, 2014; Thanawongnuwech et al., 2000). For example, pigs co-challenged with PRRSV and *S. suis* had a significantly higher incidence of arthritis compared to those pigs challenged with PRRSV or *S. suis* alone (Thanawongnuwech et al., 2000). In the current study, lameness and joint effusion peaked late in the post-challenge period and were not associated with the peak in PRRSV replication. This may be due to the

long-term effects of PRRS-induced immunosuppression. Evidence of polyarthritis was seen at a similar rate to that reported for natural PRRSV infections; 8.8% in the current study (42/476) and 15% in the field (Done et al., 1996). Interestingly, the duration of lameness and joint effusion were not associated with significant reductions in ADG, and thus may not represent a significant factor in productivity losses due to clinical disease.

Phenotypes associated with PCVAD, such as decreased body condition, lethargy or depression, muscle wasting and pallor or jaundice, all peaked late in the post-infection period during levels of high PCV2 replication. Although diarrhea can be a sign of PCV2 enteritis (Segales, 2012), diarrhea was likely not associated with PCVAD in this study due to a lack of increased prevalence during PCV2 replication. Diarrhea occurred in a low but relatively consistent percentage of pigs throughout the study period, failing to peak at any time point. Muscle wasting and reduced body condition are typical of PCV2-systemic infections, where pigs can lose extensive muscle mass and become emaciated. However, growth retardation can also occur in pigs persistently infected with PRRSV, which likely contributed to decreased body condition scores late in the post-infection period. Jaundice or pallor are also commonly reported in cases of PCVAD and are associated with gastric ulceration or granulomatous hepatitis resulting in hepatocellular necrosis (Opriessnig and Langohr, 2013; Quintana et al., 2001).

Because of the ability for several diseases to cause decreased body condition and lethargy, jaundice or pallor and muscle wasting may be more specific for monitoring outbreaks of PCVAD. Jaundice or pallor and muscle wasting were associated with significant reductions in ADG (see Figure 6) and high rates of mortality; 52.8% and 52.4%, respectively. However, these mortality rates are lower than reports in the field during outbreaks of PCVAD, where case fatality rates can reach approximately 80% (Harding, 1997; Quintana et al., 2001). Large

amounts of PCV2 were circulating in the serum of pigs that developed jaundice or pallor and/or muscle wasting. On 7, 11, 14, 21, 28, 35 and42 dpi, PCV2 viremia levels were significantly greater in these pigs compared to the rest of the population ($p < 0.02$, unpaired t-test). The greatest difference between the two groups was evident on 14 dpi, where mean PCV2 levels were 4.6 $\log_{10}$ templates/PCR reaction and 2.5 $\log_{10}$ templates/PCR reaction for pigs with and without these two clinical phenotypes, respectively ($p < 0.0001$, unpaired t-test). As new isolates of PCV2 emerge, potentially resulting in outbreaks due to incomplete vaccine protection, integrating a herd monitoring plan for jaundice or pallor and muscle wasting may serve to improve early detection and response to PCVAD.

Key performance indicators in swine production include measurements such as percent post-weaning mortality and time to market weight. Ultimately, these indices are somewhat limited in their ability to detect disease early and prevent future loss. Although it is widely accepted that clinical disease reduces growth, detailed clinical observations for individual pigs are typically considered impractical due to time and financial considerations as well as sheer volume in a production setting. However, recently developed programs, such as Individual Pig Care (IPC; Zoetis), train swine husbandry personnel on early detection of clinical disease and administration of parenteral antimicrobial treatment to individual pigs. This program emphasizes the individual, including electronic detailed daily observations and prompt treatment responses prior to progression of clinical disease (Azlor et al., 2013; Pantoja et al., 2013; Pineiro et al., 2014). In the current study, veterinary protocols were also directed at individualized evaluations and treatment; no group antimicrobials were administered in the water or feed. Legal regulations limit the use of group administered antimicrobials, creating a need for early recognition of disease and targeted antibiotic administration. Recognizing what clinical signs are most

predictive of poor growth and mortality provides an opportunity to eliminate antibiotic treatment for some clinical signs (i.e. aural cyanosis) and aggressively utilize antibiotics to treat others (i.e. open mouth breathing and muscle wasting). Detailing clinical disease parameters as key performance indicators may help limit broad-scale antibiotic usage while demonstrating a commitment to improving animal welfare through improvements in clinical health.

In conclusion, this report details clinical signs that occurred in a population of pigs after co-infection with PCV2 and PRRSV, two globally distributed viruses associated with severe economic losses to the swine industry. Clinical phenotypes were diverse due to several polymicrobial syndromes and were shown to have associations with distress and compromised welfare, growth performance, and peak virus replication.

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Table 3:1 Standardized health evaluation questionnaire*

Attitude	0	1	2	3	4		
Body Condition	1	2	3	4	5		
Muscle Wasting	No	Yes					
Pallor or Jaundice	No	Yes					
Aural Cyanosis	No	Yes					
Rectal Temperature							
Lameness	0	1	2	3	4		
⁞Limb Affected	LH	RH	LF	RF			
Joint Effusion	No	Yes					
Dyspnea	0	1	2	3	4	5	6
Open Mouth Breathing	No	Yes					
Rhinorrhea	No	Yes					
Coughing	No	Yes					
Diarrhea	0	1	2	3	4	5	
‡Treatment Administration	No	Yes					

*Clinical phenotypes associated with PRRSV/PCV2 co-infection assessed for presence and/or scored as described in the materials and methods
‡Systemic treatment administered under the direction of a veterinarian; treatment protocols described in the materials and methods
⁞Key: LH, Left hind; RH, Right hind; LF, Left front; RF, Right front

Table 3:2 Effect of presence and duration of clinical phenotype on average daily gain (ADG) from 0 to 42 days post-infection with PRRSV and PCV2

Clinical Phenotype	ADG‡	ADG Reduction†	r value‖	Slope⁞	Observations≡
Treatment	0.65 ± 0.27	22%*	-0.36	-0.017*	72
Dyspnea	0.55 ± 0.25	34%*	-0.51	-0.042*	52
Aural Cyanosis	0.76 ± 0.23	5%	-0.04	-0.006	49
Lethargy or Depression	0.50 ± 0.24	40%*	-0.44	-0.043*	41
Rhinorrhea	0.47 ± 0.20	43%*	-0.38	-0.059*	37
Coughing	0.47 ± 0.20	43%*	-0.35	-0.072*	35
Lameness	0.81 ± 0.23	0%	+0.04	+0.003	33
Decreased Body Condition	0.36 ± 0.13	56%*	-0.41	-0.026*	25
Jaundice or Pallor	0.37 ± 0.15	54%*	-0.38	-0.063*	17
Diarrhea	0.66 ± 0.31	17%*	-0.16	-0.022*	15
Joint Effusion	0.68 ± 0.31	15%*	-0.02	-0.003	13
Open Mouth Breathing	0.48 ± 0.20	40%*	-0.25	-0.227*	11
Muscle Wasting	0.31 ± 0.11	62%*	-0.32	-0.193*	10

‡ ADG shown in kg ± 1 standard deviation for pigs with each clinical phenotype

† ADG reduction calculated for pigs with phenotype compared to pigs without phenotype; unpaired t-test

‖ Pearson correlation coefficient

⁞ Slope calculated by linear regression analysis

≡ Number of pigs with each clinical phenotype out of 433 surviving pigs

* Statistical significance $p < 0.05$

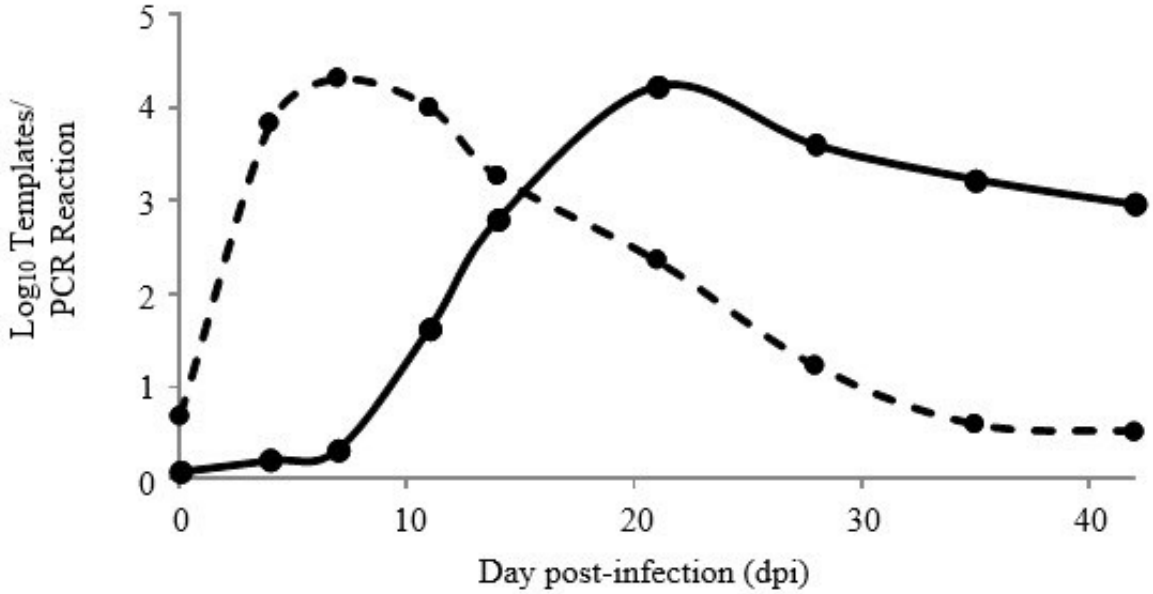

Figure 3:1 Time course of viremia post-challenge with PRRSV and PCV2.

Mean viremia is shown for PRRSV (dashed line) and PCV2 (solid line). Data is shown for 285 pigs.

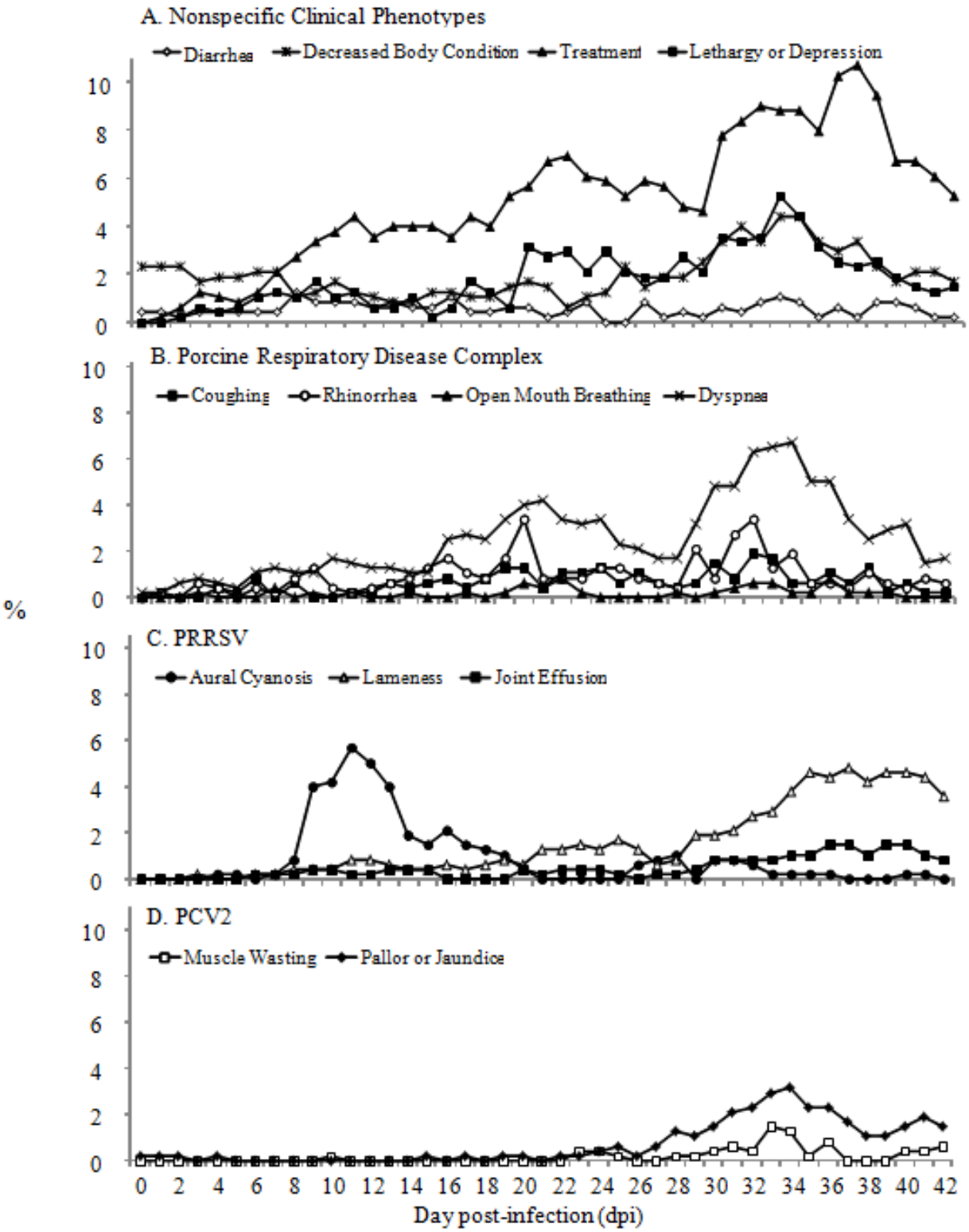

Figure 3:2 Time course of clinical phenotypes after PRRSV/PCV2 challenge.

Percent of pigs with each clinical phenotype is shown over the 42 day post-infection period for 476 challenged pigs. Phenotypes are categorized based on association with nonspecific disease (A.), porcine respiratory disease complex (B.), PRRSV infection (C.), or PCV2 infection (D.).

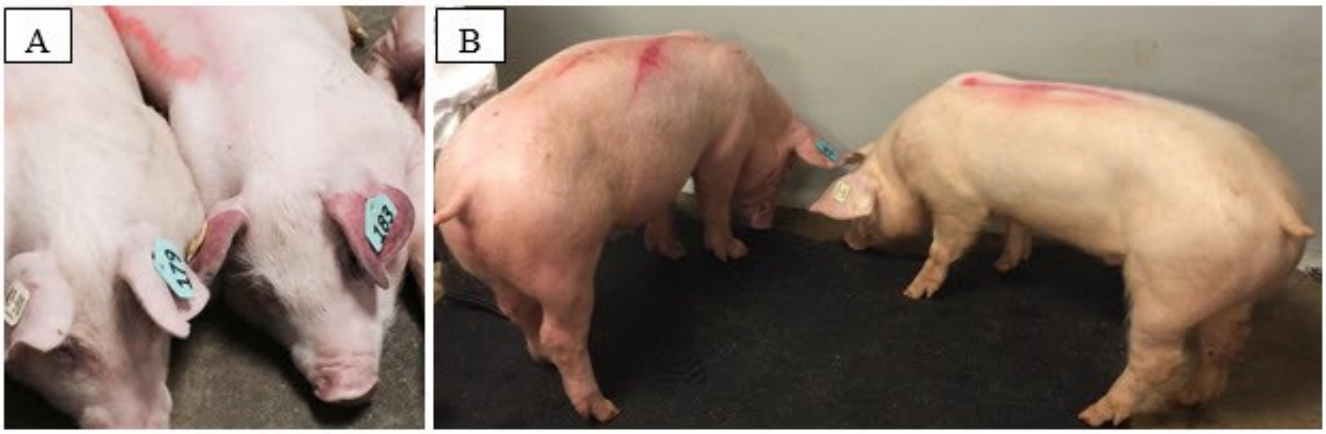

Figure 3:3 Examples of clinical phenotypes after PRRSV/PCV2 co-infection

Aural cyanosis (A) and pallor (B) are shown. A normal pig is included in the left side of each photo for the purposes of comparison.

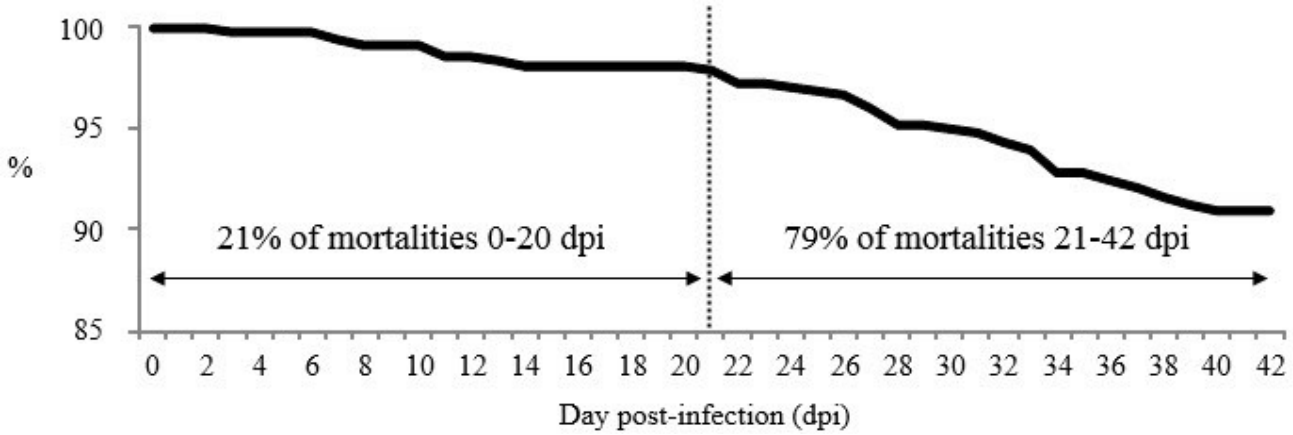

Figure 3:4 Survival curve after challenge with PRRSV and PCV2.

Percent of surviving pigs is shown for each day post-challenge. Overall survival rate post challenge was 91% (433/476). Approximately 21% (9/43) of the mortalities occurred in the first 0-21 dpi; the remaining 79% (34/43) of the mortalities occurred in the second half of the post-challenge period (21-42 dpi).

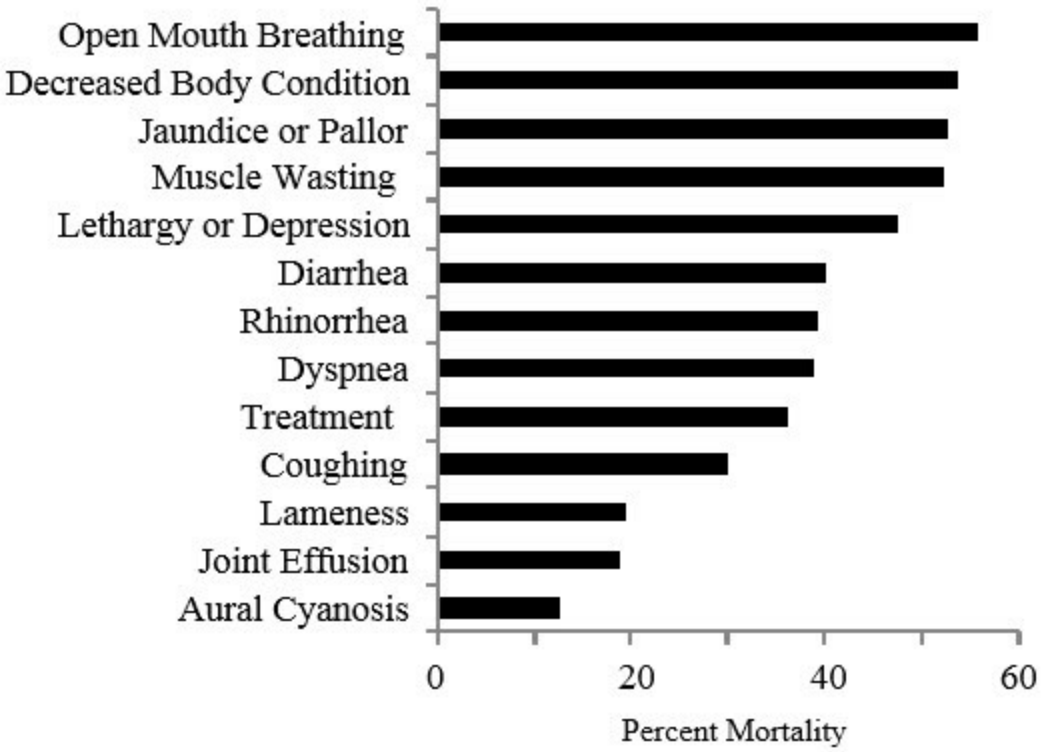

Figure 3:5 Mortality rates for pigs documented with each clinical phenotype after co-infection with PRRSV and PCV2.

Percent of pigs that died prior to the conclusion of the 42 day study period are shown for each clinical phenotype in descending order.

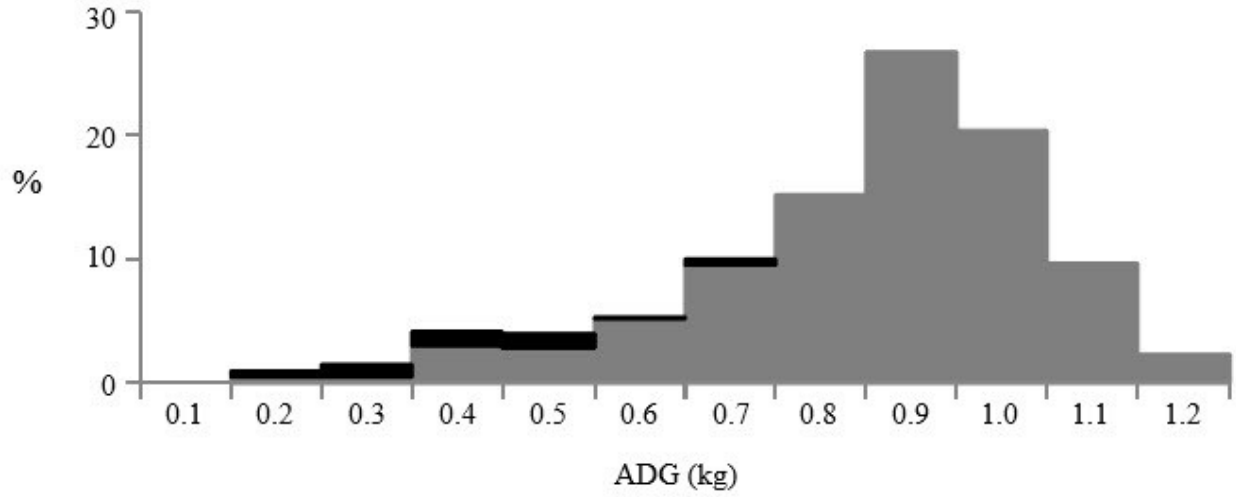

Figure 3:6 Distribution of average daily gain (ADG) from 0 to 42 days post-infection with PRRSV and PCV2.

The ADG is shown for all surviving pigs (n = 433). Pigs with clinical signs of porcine circovirus associated disease (PCVAD), including pallor or jaundice and/or muscle wasting, are shown in black (19/433; 4.4%). Significant reductions in ADG were seen for each of these clinical phenotypes.

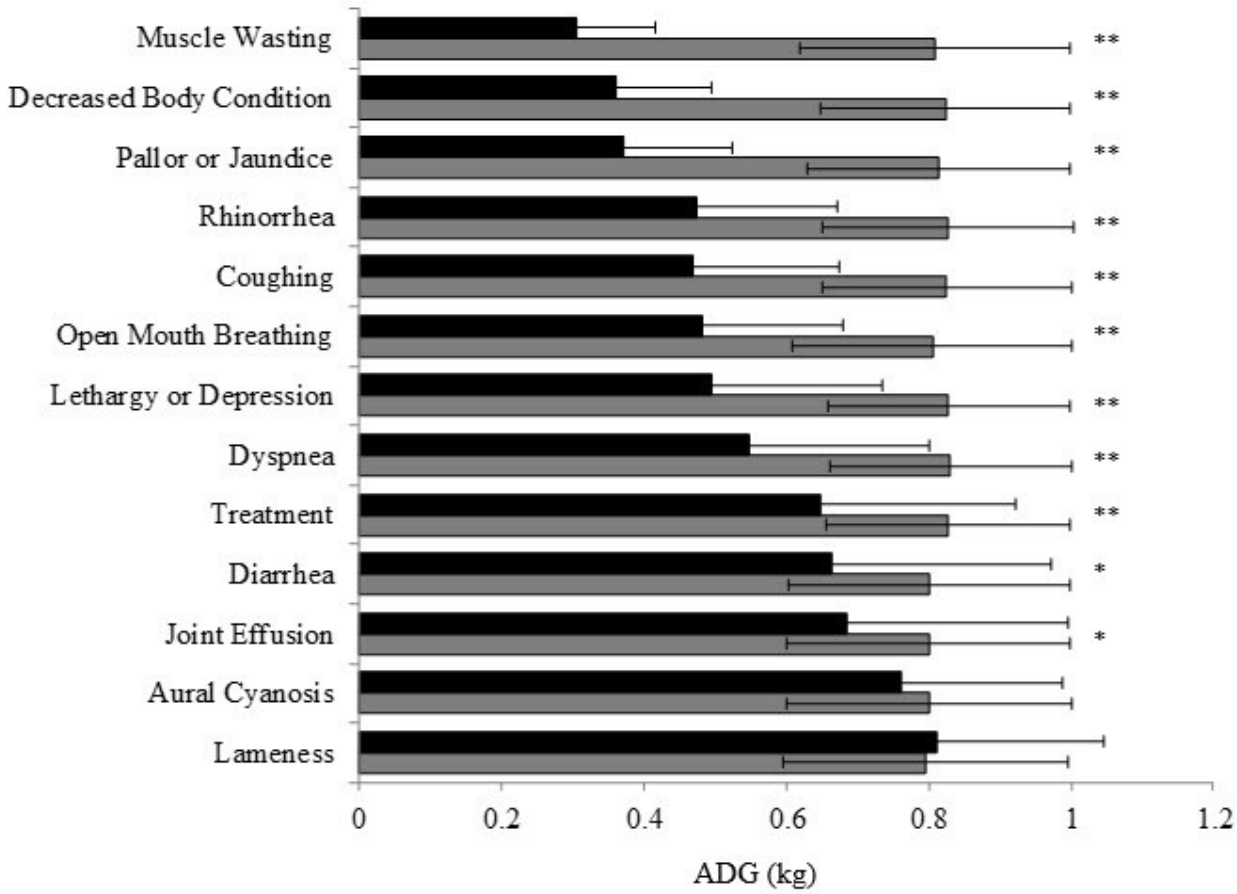

Figure 3:7 Reduction of ADG associated with the presence of each clinical phenotype.

Data is shown as mean ADG in kg ± 1 standard deviation for pigs with one or more days of the clinical phenotype (black bars) and pigs without the clinical phenotype (grey bars). Asterisks identify statistically significant differences between groups (**$p < 0.0001$, *$p < 0.05$; unpaired t-test).

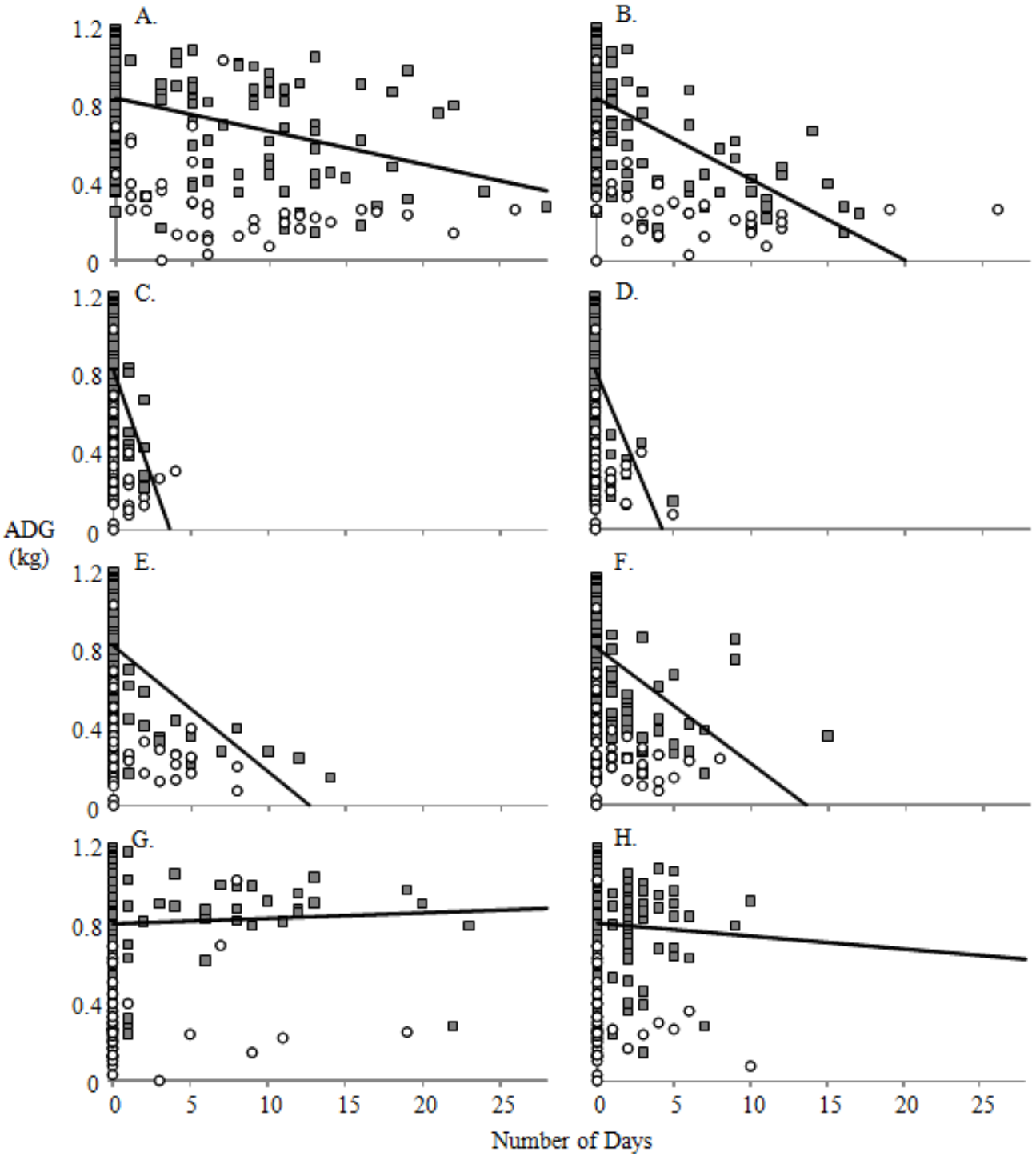

Figure 3:8 Linear regression plots showing association between days with each clinical phenotype and average daily gain (ADG).

ADG is shown for each pig from 0-42 dpi (■) or until death (○). Linear regression line calculated for surviving pigs only (■). Clinical phenotypes include A. Treatment, B. Dyspnea, C. Open Mouth Breathing, D. Muscle wasting, E. Jaundice or Pallor, F. Rhinorrhea, G. Lameness, and H. Aural Cyanosis.

Chapter 4 - Tissue localization, shedding, virus carriage, antibody response, and aerosol transmission of porcine epidemic diarrhea virus (PEDV) following inoculation of 4 week-old feeder pigs

Abstract

Porcine epidemic diarrhea virus (PEDV) emerged in the U.S. in April 2013 and caused significant losses to the swine industry. The purpose of this investigation was to determine tissue localization, shedding patterns, virus carriage, antibody response, and aerosol transmission of PEDV following inoculation of 4 week-old feeder pigs. Thirty-three pigs were randomly assigned to one of three groups for the 42 day study; challenge (Group A; *n*=23), contact transmission (Group B; *n*=5) and aerosol transmission (Group C; *n*=5). Contact transmission occurred rapidly to Group B pigs whereas productive aerosol transmission failed to occur to Group C pigs. Emesis was the first clinical sign noted at 3 days post-inoculation (DPI) followed by mild to moderate diarrhea lasting for an additional 5 days. Real-time PCR detected PEDV in fecal and nasal swabs, oral fluids, serum, gastrointestinal and lymphoid tissues. Shedding primarily occurred during the first 2 weeks post-challenge, peaking at between 5 and 6 DPI; however, some pigs had PEDV nucleic acid detected in swabs collected at 21 and 28 DPI. Antibody titers were measurable between 14 and 42 DPI. Although feces and intestines collected at 42 DPI were PEDV negative by PCR and immunohistochemistry, respectively, small intestines from 70% of Group A pigs were PCR positive. Although disease was relatively mild and transient in this age group, the results demonstrate that 4 week-old pigs are productively

infected and can sustain virus replication for several weeks. Long-term shedding of PEDV in subclinical pigs should be considered an important source for PEDV transmission.

Introduction

Porcine epidemic diarrhea (PED) emerged as a new swine enteric disease in North America in the spring of 2013 (Stevenson et al., 2013). PEDV is an enveloped, positive-sense RNA virus in the family *Coronaviridae* (Jung and Saif, 2015). Since initial detection, PEDV has spread rapidly across the U.S. and has now been reported in 34 states (American Association of Swine Veterinarians, http://www.aasv.org). The mechanisms for introduction and rapid dissemination throughout pig farms in the U.S. and North America are still poorly understood; although, transportation, feed, and aerosols have all been implicated as potential contributors. 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. Infection of neonatal pigs usually results in extremely high mortality (approaching 100%), due to malabsorptive diarrhea and dehydration as a result of enterocyte necrosis in the small intestine (Madson et al., 2015). Infection of grow/finish animals results in high morbidity but low mortality with vomiting and mild to moderate diarrhea as the typical clinical presentation. Infection of adult swine is often overlooked due to minimal gastrointestinal signs or a complete lack of clinical disease. Following the introduction of PEDV in the U.S., an estimated 7 million pigs died within the first year, placing pressure on domestic and foreign markets (Jung and Saif, 2015). Even though PEDV is very similar to the disease presentation caused by another coronavirus, transmissible gastroenteritis virus (TGEV), both viruses are genetically and immunologically distinct (Lin et al., 2015).

For the PEDV challenge in this study, 4 week-old pigs were selected as an intermediate age group, where clinical disease was expected to be mild to moderate and low mortality would allow the analysis of viral shedding, tissue distribution, aerosol transmission and antibody response over the 42-day period. The results showed that clinical disease and associated pathology occur primarily within the first week after PEDV challenge, consistent with the outcome observed for similar-aged pigs in the field. However, PEDV was still detected weeks after clinical signs had disappeared and may implicate pigs in this age group as an important source of viral carriage and transmission.

Materials and Methods

Animals. Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use Committees and Institutional Biosafety Committees. Thirty-three 3 week-old pigs were obtained from a high-health commercial source negative for PEDV. All pigs were housed in one environmentally controlled room at the Biosecurity Research Institute at Kansas State University, and maintained under BSL3-Ag containment conditions. The room was decontaminated with chlorine gas prior to the start of the study and was approximately 66 sq meters in size. Pigs were housed in two raised stainless steel pens with slotted fiberglass flooring. Pens were 6.9 and 3.5 sq meters in size for Groups A/B and Group C, respectively. The two pens were approximately 3 meters apart and were separated by a nonpermeable plastic tarp to avoid overt cross contamination while cleaning. The tarp was placed directly between the

two pens and covered approximately 60% of the room length. Pens were cleaned daily using a high heat and high pressure water hose. Floors of the room were chemically disinfected daily with Virkon (DuPont). Pigs were given access to food and water *ad libitum*. Complete exchange of air within the room occurred 14.5 times/hour. Room temperature ranged from 73°F to 82°F and humidity ranged from 32% to 45% during the study.

Experimental Design. Pigs were randomly assigned into one of three groups including a challenge group (Group A; $n = 23$), contact transmission group (Group B; $n = 5$) and aerosol transmission group (Group C; $n = 5$). Experimental groups are summarized in Table 1. The pigs were given one week to acclimate upon arrival. At 4 weeks of age, Group A was inoculated with PEDV "feedback" inoculum via the oral and intranasal routes, with 5 ml of inoculum per route. The challenge material was a pool of gut-derived intestinal contents that had been used as "feedback" inoculum 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 PEDV inoculum (isolate USA/KS/2013, GenBank Accession #KJ184549.1) had a Ct of 22 as determined by the Kansas State Veterinary Diagnostic Laboratory (KSVDL) real-time PCR assay. This Ct value is equivalent to 1.42×10^5 $TCID_{50}$. Routine diagnostic PCR testing by KSVDL showed the challenge inoculum was negative for porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus (PCV), and group A, B and C rotaviruses.

To inoculate Group A, pigs were individually removed from the group pen and administered the challenge material. The Group B pigs were not inoculated and were housed separately from the Group A pigs during inoculation. Approximately 6 hours post-inoculation of the Group A pigs, Groups A and B were allowed to co-mingle and were housed together for the

remainder of the study. The Group C pigs were not inoculated and were housed in a separate pen in the same animal room as Groups A and B (described above). Nasal swabs, fecal swabs, oral fluids, and serum samples were collected from all pigs prior to challenge on -3 and 0, 1, 2, 3, 4, 5, 6, 7, 9, 14, 21, 28, 35, and 42 days post-inoculation (DPI). One pig on 0, 2, and 4 DPI and two pigs on 7, 9, 14, 21, and 28 DPI were randomly selected for euthanasia and necropsy. All remaining pigs were euthanized on 42 DPI. To assess the presence of virus in the environment, swabs were collected from the V-troughs used for blood collection, walls, pens, and food bins from both the Group A/B and Group C sides of the room on 14 DPI.

Clinical Evaluation. Pigs were evaluated daily throughout the study period for the presence of clinical signs associated with PED. During the acute period of infection from 0-11 DPI, each pig was physically examined by a veterinarian for clinical signs such as decreased body condition, dehydration, lethargy, and evidence of diarrhea or vomiting. Lethargy was assessed through response to stimuli and interaction with pen mates. Body condition scores (BCS) were assigned using an adapted 5 point scale (Patience and Thacker, 1989). Dehydration was assessed through evaluation of enophthalmos and third eyelid protrusion, nasal planum moisture and coloration, mucus membrane moisture and coloration, and skin turgor. Fecal consistency scores were assigned 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.

Gross Anatomical and Histological Examination. Pigs were sequentially euthanized throughout the study to assess gross and microscopic changes during infection. Pigs were euthanized with pentobarbital sodium, necropsied immediately and the following tissues were collected from each pig: inguinal lymph node, submandibular lymph node, tonsil, thymus,

thyroid gland, esophagus, trachea, lung (one section from each lobe), tracheobronchial lymph node, heart, liver, adrenal gland, kidney, spleen, stomach, mesenteric lymph node, duodenum with pancreas, jejunum (three locations), ileum, cecum, spiral colon (two locations), descending colon, nasal turbinates, bone marrow, and brain. Tissues were frozen at -70°C and fixed in 10% neutral buffered formalin. After 7 days, formalin-fixed tissues were routinely processed in an automated tissue processor, embedded in paraffin, tissue sections cut at 4 µm, mounted on glass slides, and stained with hematoxylin and eosin (H&E stain). Necropsies and histologic evaluations of tissues were by a board-certified pathologist.

PCR. The MagMAX-96 Viral RNA Isolation Kit (Life Technologies) was used together with a Kingfisher 96 magnetic particle processor (Fisher Scientific) for all sample types. Tissue samples were homogenized using a Stomacher® 80 Biomaster (ThermoScientific). One milliliter of 1X PBS buffer was added to approximately 0.5 g of the feces or a nasal swab tube, vortexed briefly, and allowed to sit for 2-3 minutes. 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).

A duplex qRT-PCR was designed for the dual purpose of detecting PEDV nucleocapsid (N) protein gene and the host 18S ribosomal RNA subunit to monitor extraction efficiency. Primers and probe sequences for PEDV were: 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 were: 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. The Path-ID™ Multiplex One-Step Kit (Life Technologies) was used for all real-time PCR reactions. The 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) 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 and a negative extraction control were included in each run.

Immunohistochemical (IHC) staining. A primary goal for IHC staining was to determine if virus replication occurred in the respiratory tract as well as in the small intestine. Therefore, tissues from the gastrointestinal and respiratory tracts were prioritized for IHC staining after collection from pigs euthanized between 0 and 28 DPI. Preliminary IHC examination of tissues for PEDV antigen was performed by Dr. Darin Madson at the Iowa State University Veterinary Diagnostic Laboratory. Subsequently, Dr. Jerome Nietfeld performed IHC on these same tissues at the Kansas State Veterinary Diagnostic Laboratory (KSVDL), where positive and negative results were independently confirmed.

Immunohistochemical staining at the KSVDL was performed on formalin-fixed paraffin-embedded tissues that were sectioned at 4 µm thickness onto positively charged slides. Slides were stained using the Bond-Max autostainer (Leica Biosystems Inc.) with the Polymer Refine Detection kit (Leica Biosystems Inc.). The PEDV primary antibody (SD 6-29, kindly provided by Dr. Eric Nelson) was diluted with Bond Primary Antibody Diluent (Leica Biosystems Inc.) to 1:100,000. Epitope retrieval was performed by incubating slides in 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 Inc.) for 25 minutes at ambient temperature. Visualization was done with DAB and counterstained with hematoxylin. Positive and negative controls were included with each run.

Antibody Response. Serum samples were collected throughout the study and stored at -80^{o}C for antibody testing using two methods. First, 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 swine testicular (ST) cells with a standardized stock of PEDV virus (Colorado 2013 Isolate, kindly provided by Dr. Sabrina Swenson). 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 African green monkey kidney (Vero) cells as the substrate and a standardized trypsin independent stock of 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 37^{o}C and inoculated into four replicate wells of 3-day old Vero cells in 96 well plates. Cultures were incubated for 3 days at 37^{o}C and the presence of virus was determined by the presence of virus associated cytopathic effect. 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 (Finney, 1964).

Results

Transmission. Group B pigs became rapidly infected with PEDV after co-mingling with Group A pigs. In contrast, aerosol transmission did not occur as Group C pigs failed to develop a productive PEDV infection during the 42-day study period.

Clinical Evaluation. Clinical signs associated with PEDV infection were documented in Groups A and B between 3 and 8 DPI. Figure 1 summarizes the time course of clinical disease in Group A pigs with the duration of PEDV detection in serum, fecal and nasal swabs, and small intestine. Clinical disease correlated well with the time course of peak fecal and nasal PEDV shedding between 4 and 7 DPI (compare Figure 1 with Figures 3A and 3B). The first clinical sign associated with PED was emesis noted on 3 DPI. Vomitus contained partially digested food particulate and had no blood or mucus present. The most severe diarrhea observed was assigned a score of 4/5 and included brown liquid feces without particulate fecal material, blood or mucus. This diarrheal score was recorded in 30% of the Group A and B pigs between 4 and 7 DPI. Evidence of clinical dehydration was documented in approximately 70% of Group A and B pigs between 4 and 8 DPI. Dehydration was mild to moderate and resulted in pigs having enophthalmos with third eyelid protrusion, dry nasal planums, tacky mucus membranes, and decreased skin turgor. During this time, most pigs also demonstrated evidence of mild to moderate lethargy, including a reluctance to rise and ambulate, decreased resistance to handling and restraint, and reduced responsiveness. Starting on 4 DPI, the majority of pigs in Groups A and B (16/26; 61.5%) had body condition scores that were less than ideal (< 3/5). However, body condition scores gradually improved after resolution of clinical disease and by 11 DPI, the majority of Group A and B pigs (13/21; 61.9%) had ideal body condition scores ($\geq$ 3/5). No pigs were considered moribund during the study and there were no mortalities.

Gross Anatomical, Histological and IHC Examination. Except for the mild to moderate dehydration observed clinically, gross lesions were not observed in any of the euthanized pigs. All tissues collected from Group A pigs necropsied from 0 to 28 DPI were examined histologically and significant microscopic changes were present in the small intestines of 3 of 13 (23%) pigs. There was diffuse, moderate to severe atrophy and fusion of villi in the jejunum and ileum of the pig necropsied on 4 DPI and in one of the two pigs necropsied on 7 DPI. Mild to moderate villous atrophy and fusion was present in the jejunum and ileum in one of the two pigs necropsied on 28 DPI.

Immunohistochemical staining results from Iowa State University and the KSVDL were identical. PEDV was detected by IHC staining in the small intestines of six pigs (Table 2) and in the mesenteric lymph nodes of three pigs. All five small intestinal samples from the pig euthanized at 4 DPI and in one of the two pigs euthanized at 7 DPI were positive. One of five sections from both pigs euthanized at 14 DPI, and two of five sections from one of the two pigs euthanized at 21 and 28 DPI were positive. In the pigs where all small intestinal sections were positive, PEDV antigen was multifocal in the villous epithelium of the duodenum and diffuse in the villous epithelium of the jejunum and ileum. In pigs where only a portion of the sections were positive, viral antigen was limited to the jejunum and/or ileum and was absent from the duodenum. In positive sections with Peyer's patches, positive staining was often present within lymphoid tissue as well as the villous epithelium.

Representative microscopic images of PEDV IHC in jejunal sections from Group A pigs are shown in Figure 2. On 4 DPI, PEDV antigen was uniformly detected along the epithelial border, associated with villous atrophy and fusion (Figure 2A). Between 7 and 28 DPI, PEDV antigen became less consistent along the epithelial border (Figures 2B-2E). Taken together, this

data show a high degree of variability in IHC detection of PEDV antigen, particularly after 7 DPI.

PEDV Shedding and Viremia. Real-time PCR results for PEDV in fecal and nasal swabs collected from all three groups are summarized in Figures 3A and 3B. Individual animal results are shown in Tables 3 and 4. In general, fecal and nasal shedding patterns were very similar between Group A and Group B pigs. Surprisingly, all samples were negative for the virus at 24 hours post-inoculation. Fecal and nasal shedding of Group A was first observed at 48 hours post-inoculation. Nasal shedding was detected in the Group B pigs at 48 hours post-inoculation and fecal shedding was present in all five Group B pigs by 72 hours post-inoculation. Peak fecal shedding occurred between 5 and 6 DPI and was significantly greater than nasal shedding in both Groups A and B (compare Figures 3A and 3B). Fecal shedding was typically detected at a 10 fold or greater level than that observed in the nares. In Groups A and B, the majority of animals were negative for fecal and nasal shedding by 21 DPI. However, three pigs in Group A (3/14; 21%) and two pigs in Group B (2/5; 40%) were still shedding virus in feces at 21 DPI. In addition, one pig from Group A (1/12; 8%) was shedding PEDV in feces and nasal secretions at 28 DPI. Although low levels of PEDV nucleic acid were detected in all five Group C nasal swabs (5/5; 100%) during the early part of the study, the absence of fecal shedding and lack of antibodies suggested that the PEDV detected in nares was non-infectious.

Mean PEDV viremia is shown in Figure 3C, and results for individual animals are listed in Table 5. In general, the amount of PEDV nucleic acid was lower in serum compared to fecal and nasal samples. Viremia peaked around 5 DPI, similar to fecal and nasal shedding. PEDV viremia was clearly detected in the majority of both the Group A and B pigs. Only three animals (3/25; 12%) in Groups A and B did not develop a detectable viremia within the first week post-

challenge/exposure (see Table 5). No detectable viremia was present in any of the samples from the Group C pigs (see Table 5C).

Data on CT values for detection of PEDV shedding in oral fluids are shown in Figure 4. Viral nucleic acid was easily detected in oral fluids; levels were greater than nasal swabs but lower than levels detected in fecal swabs. Oral fluids pooled from the pen housing Groups A and B were PCR positive at 2 DPI and remained positive until 28 DPI. Oral fluids from Group C appeared to be positive at the time of the first successful collection point (4 DPI) and maintained positivity until 9 DPI. Subsequent oral fluids collected from Group C were PEDV negative at 14 DPI and a weak CT (>37) was observed on 21 and 28 DPI.

Environmental PEDV. Room environmental samples were collected at 14 DPI and the real-time PCR values are summarized in Figure 5. The data show that viral nucleic acid was present on the V-troughs, pens and food bins on both the Group A/B and Group C sides of the animal room. Differences between the two sides of the room were evident by the nucleic acid on the walls closest to the two pens; a CT value of 40.0 (negative) on the Group C wall and a CT value of 31.7 on the Group A/B wall.

PEDV Tissue Tropism. Tissues tested for PEDV antigen by IHC over the course of the study are summarized in Table 6. Of the tissues tested, only small intestines and mesenteric lymph nodes were positive on IHC. All other tissues were IHC negative. With regards to PEDV detection by PCR, the tissues tested and CT results are shown in Table 7. Small amounts of viral nucleic acid were detected in several tissues in addition to small intestine and lymph node, including nasal turbinates, tonsil, thymus and spleen.

Small intestinal sections and mesenteric lymph nodes from Group A pigs had the greatest amount of nucleic acid present between 4 and 7 DPI (see Table 7). Most Group A pigs (7/10;

70%) maintained positive CT values in the small intestine until the end of the study at 42 DPI; whereas, all mesenteric lymph node samples had negative CT values at this time. Interestingly, all Group B pigs had negative CT values in the small intestine at 42 DPI.

Antibody Response. Serological analysis of serum samples was performed using indirect fluorescent antibody (IFA) and serum neutralization assays. Geometric mean titers for both assays are shown in Figure 6. All pre-inoculation samples were negative for antibody and there was significant seroconversion in the Group A and B animals. Seroconversion at 14 DPI in these groups correlates well with resolution of viremia (compare Figure 6 and Table 5). There was no evidence of seroconversion in Group C, despite clear demonstration of PEDV nucleic acid in nasal and oral fluid samples. Interestingly, the Group B animals reached lower peak antibody titers and appear to have more rapid antibody decay based on the IFA antibody test (see Figure 6A). In addition, Group B animals reached peak neutralization titers 1 week after Group A at 21 DPI (see Figure 6B).

Discussion

Porcine epidemic diarrhea virus (PEDV) emerged in the U.S. in April 2013 and had spread to most pork producing states within months, resulting in significant losses due to nursing piglet mortality. PEDV pathogenesis is highly dependent on the age of the pig and in this study, we targeted 4 week-old feeder pigs to investigate transmission, antibody response, viral shedding, clinical outcome, and tissue tropism of PEDV over a 42 day period. Four week-old pigs serve as an important age group due to the high potential for viral replication and shedding combined with the low potential for mortality. As such, we were able to investigate the long term effects of PEDV infection. This study showed that PEDV nucleic acid was present in the

small intestines of 4 week-old pigs for at least 42 days post-challenge, long after clinical disease had abated; thus, this age group may serve as an important source for chronic carriage and fecal transmission.

Although PEDV is primarily transmitted through the fecal/oral route, viral transmission has also been associated with contaminated fomites such as trucks and personnel (Lowe et al., 2014), contaminated feed ingredients (Bowman et al., 2015; Dee et al., 2014; Pasick et al., 2014), and airborne virus particles (Alonso et al., 2014). Alonso et al investigated the infectious potential for PEDV collected from air samples in both an experimental setting and a field setting with mixed results (Alonso et al., 2014). In the experimental setting, PEDV was abundant in air samples collected from the room housing PEDV challenged pigs and was subsequently proven infectious in an 11 day-old pig bioassay. In the field setting, PEDV was detected in 18% of air samples and up to 10 miles away from farms with acute outbreaks; however, quantities of PEDV were significantly lower than experimental conditions and productive infections did not occur in the 11 day-old pig bioassay (Alonso et al., 2014).

Of the aforementioned modes of PEDV transmission, both contact and aerosol routes were investigated in the current study. Rapid infection was clearly demonstrated in the Group B animals, with 4/5 pigs (80%) shedding PEDV in the feces 2 days after exposure to the Group A animals (see Table 3). Transmission to Group B animals was likely due to fecal/oral direct contact with Group A animals; however, indirect contact with contaminated fomites, such as pens or food bins, cannot be eliminated. Although viral shedding patterns and clinical signs were virtually identical between Groups A and B, two important differences should be highlighted. First, most intestinal samples from Group A pigs (7/10; 70%) were PCR positive at 42 DPI whereas none of the Group B intestinal samples (0/5; 0%) were positive at this time point

($p = 0.0256$, Fisher's exact test). Second, antibody titers in the Group B pigs were typically lower and had more rapid decay when compared to Group A pigs. These differences may be due to the Group B pigs being infected with a lower PEDV dose than Group A pigs, either through fecal/oral or environmental exposure, and may be better representative of field conditions.

In contrast to contact transmission, productive aerosol transmission was not demonstrated in this study, despite repeated PEDV nucleic acid detection in the nares of all five of the aerosol transmission pigs. There are several reasons the PEDV transmitted to the aerosol group may not have resulted in a productive infection. First, the dose of PEDV transmitted via aerosolization may have been too low. As shown in Table 4, the CT values detected in nasal swabs of the aerosol transmission pigs were relatively low, with the greatest quantity detected at a CT of 31.45. This may have been due to the small amount of virus aerosolization or may have been due to the BSL-3 housing conditions, where air exchange occurred almost 15 times each hour. Second, the circulating PEDV detected in nasal swabs may have been inactivated by chemicals used to disinfect the room. Third, the lack of productive transmission may have been due to the number and age of the indicator animals. For example, it is possible that this level of nucleic acid may have been infectious to highly susceptible 1 day-old neonatal piglets. Future research is needed to understand the risk of airborne PEDV, the role of disinfectants in inactivating aerosolized particles, and the infectious dose necessary for productive aerosol transmission.

Four week-old feeder pigs were selected for this challenge study due to the known reduction in PEDV mortality post-weaning, enabling us to monitor viral pathogenicity over the 42 day study period. Detection of PEDV nucleic acid in fecal swabs, nasal swabs, and oral fluids was relatively consistent between 2 and 14 DPI in this age group. However, detection of PEDV viral antigen in intestinal sections was highly variable among pigs euthanized on the same day

post-challenge and inconsistencies were present within different intestinal sections collected from the same pig (see Table 2). Of the pigs that were negative on IHC for PEDV antigen between 2 and 9 DPI, all pigs (4/4; 100%) were positive for PEDV nucleic acid in fecal swabs. Specifically, one of the pigs euthanized on 7 DPI was negative on IHC but was shedding large amounts of PEDV nucleic acid in the feces collected on the same day (CT = 21.52). In addition, all pigs euthanized after 2 DPI had high levels of PEDV nucleic acid detected in small intestinal samples (Table 7). Discrepancies between IHC and PCR results were also reported in 3 week-old pigs after challenge with a different PEDV strain (Madson et al., 2014). These IHC inconsistencies warrant collection of several intestinal sections from multiple pigs to ensure initial diagnosis and complete resolution of PEDV carriage after an acute outbreak. Further, PCR may prove to be a more sensitive technique for diagnostic testing in weaned pigs.

Mild to moderate clinical disease was documented for approximately 1 week after PEDV challenge in the Group A pigs. However, PEDV shedding in fecal and nasal swabs were detected up to 28 DPI, approximately 3 weeks after clinical disease had abated. In addition, PEDV nucleic acid was detected in intestinal samples from most Group A pigs (7/10:70%) at the conclusion of the study. As 42 days was the study duration, it is unclear how long intestinal samples may have remained PCR positive after challenge. This is concerning due to the possibility of long term virus carriage and shedding in an age group where transient and mild clinical signs could be easily overlooked in a large production system. However, we do not know the infectious transmissibility of the PEDV detected in fecal swabs or intestinal samples. Moreover, each one of the seven pigs that tested positive for PEDV nucleic acid in the intestine at 42 DPI had negative fecal swabs collected on the same day, indicating the presence of intestinal PEDV with a lack of viral shedding. It is possible that chronic viral presence in the

intestine does not pose a risk for transmission. In view of these findings, Crawford et al investigated horizontal transmission of PEDV to naïve pigs in an experiment designed to mimic an endemic infection (Crawford et al., 2015). In this study, naïve pigs were introduced at weekly intervals to a group of pigs naturally exposed to PEDV. Although pigs exposed to PEDV had intermittent viral shedding through 42 DPI, naïve pigs only became infected when introduced at 7 and 14 DPI, but not at later time points (Crawford et al., 2015). These results again highlight the need for future research on understanding the minimum infectious dose for different age groups and differentiating between a positive PCR result and the presence of infectious virus.

In summary, PEDV was transmitted rapidly via natural contact but was not transmitted via the aerosol route in this study, despite recurrent detection of nucleic acid in the nasal swabs of the aerosol transmission pigs. Although the greatest amounts of virus were present in feces, PEDV was also detected in nasal swabs, oral fluids, and serum starting at 2 DPI. Clinical disease in 4 week-old pigs was transient and primarily occurred in the first week post-challenge; however, PEDV was detected in these pigs up to 6 weeks post-challenge. This study supports the notion that post-weaning pigs should be considered as possible reservoirs for PEDV transmission and may support subclinical infections long after resolution of clinical signs.

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Table 4:1 Experimental design for PEDV challenge in 4 week-old feeder pigs

Group	Treatment	*n*
A	PEDV inoculated*	23
B	None: Contact Transmission†	5
C	None: Aerosol Transmission‡	5

* Inoculation via oral and intranasal routes; 5 ml/route

† Co-mingled with Group A 6 hours post-inoculation

‡ Housed in a separate pen in the common animal room

Table 4:2 Immunohistochemistry summary of small intestinal sections collected sequentially from Group A pigs after challenge with PEDV*

DPI	Reactivity	Positive Sections (%)
0	-	0
2	-	0
4	+	100
7	+	100
7	-	0
9	-	0
9	-	0
14	+	20
14	+	20
21	+	40
21	-	0
28	+	40
28	-	0

*Pigs were randomly selected throughout the study for sequential collection. Five sections of small intestine were collected from each pig and evaluated for the presence of PEDV antigen on immunohistochemistry.

Table 4:3 PEDV fecal shedding in individual animals until death or 42 DPI*

Group	Day Post-Inoculation (DPI)														
	-3	0	1	2	3	4	5	6	7	9	14	21	28	35	42
A.	40.0	40.0	40.0	36.0	37.2	21.4	21.7	25.7	25.9	30.1	23.9	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	20.8	21.7	19.2	20.0	23.8	26.3	32.0	26.5				
	40.0	40.0	40.0	34.4	20.3	19.2	20.9	21.2	22.9	28.3	28.2	27.6			
	40.0	40.0	40.0	30.9	21.2	17.5	19.7	28.0	25.3	25.4	36.1	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	34.8											
	40.0	40.0	40.0	37.3	36.6	21.9	19.9	19.6	21.5	26.9	32.8				
	40.0	40.0	40.0	38.2	36.9	25.3	22.4	22.7	22.2	32.8	26.5	40.0	40.0	40.0	40.0
	40.0	40.0													
	40.0	40.0	40.0	34.0	34.0	19.6	18.4	18.8	20.4	23.2	34.4	40.0	40.0		
	40.0	40.0	40.0	32.9	40.0	20.7									
	40.0	40.0	40.0	34.1	32.0	25.8	18.8	18.3	18.1	23.7	32.9	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	32.2	32.2	24.7	21.2	20.0	22.2						
	40.0	40.0	40.0	40.0	40.0	22.1	20.9	21.0	21.1	29.2	26.1	40.0	24.5		
	40.0	40.0	40.0	37.0	35.4	20.2	20.1	18.5	19.0	26.0	34.5	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	23.5	29.4	20.7	18.8	19.2	22.0	26.1	23.5	29.6	40.0	40.0	40.0
	40.0	40.0	40.0	34.7	34.7	25.4	19.6	17.4	16.9	22.1	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	35.4	35.5	30.0	22.3	22.9	27.4	25.2	27.7	32.5	40.0	40.0	40.0
	40.0	40.0	40.0	34.5	36.2	25.9	19.1	21.9	21.1	32.8	28.3	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	24.8	31.1	22.3	20.7	21.3	25.2	23.2					
	40.0	40.0	40.0	33.9	36.2	25.7	19.2	20.5	22.4	28.8	30.3	40.0			
	40.0	40.0	40.0	17.4	22.8	25.3	22.9	24.5	27.1	30.3					
	40.0	40.0	40.0	29.4	40.0	19.2	20.0	19.1	21.5						
	40.0	40.0	40.0	34.4	34.9	29.2	22.0	18.8	22.5	27.0	40.0	40.0	40.0	40.0	40.0
B.	40.0	40.0	40.0	35.3	36.8	23.4	19.6	20.6	24.2	31.7	36.8	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	36.5	34.2	27.1	20.8	21.0	20.0	23.9	28.4	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	39.4	36.4	28.5	19.1	21.7	22.2	24.1	40.0	37.0	40.0	40.0	40.0
	40.0	40.0	40.0	34.7	36.0	21.9	19.8	20.1	21.1	26.6	32.9	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	36.1	19.9	35.4	22.7	19.2	22.3	22.9	32.3	29.6	40.0	40.0	40.0
C.	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	39.5	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0

*Cycle Threshold (CT) values are shown for animals within the inoculated (A.), contact transmission (B.) and aerosol transmission (C.) groups. Values are color-coded based on a numerical gradient with the lowest CT values being red and the greatest CT values being green. A CT value of ≥38 was considered negative.

Table 4:4 PEDV nasal shedding in individual animals until death or 42 DPI*

Group	Day Post-Inoculation (DPI)														
	-3	0	1	2	3	4	5	6	7	9	14	21	28	35	42
A.	40.0	40.0	40.0	29.7	34.2	28.1	29.4	28.5	29.0	33.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	29.8	37.1	31.4	31.5	29.2	28.9	32.6	36.6				
	40.0	40.0	40.0	31.3	34.2	30.8	30.4	30.0	30.4	29.9	35.9	35.7			
	40.0	40.0	40.0	26.9	30.6	27.7	28.9	27.0	28.7	31.0	35.2	36.8	40.0	40.0	40.0
	40.0	40.0	40.0	32.3											
	40.0	40.0	40.0	32.2	32.0	29.1	29.6	28.1	27.4	32.1	36.6				
	40.0	40.0	40.0	32.3	33.4	32.5	29.8	29.4	31.1	32.6	40.0	40.0	40.0	40.0	40.0
	40.0	40.0													
	40.0	40.0	40.0	32.0	31.1	29.0	30.0	29.3	28.9	29.8	36.0	38.4	40.0		
	40.0	40.0	40.0	29.1	33.0	30.6									
	40.0	40.0	40.0	28.8	30.1	24.5	27.4	26.1	28.2	29.7	35.3	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	31.1	34.4	29.0	28.7	29.6	29.0						
	40.0	40.0	40.0	32.3	29.2	26.6	29.2	27.9	30.9	28.8	35.6	36.1	32.9		
	40.0	40.0	40.0	31.1	32.4	28.2	27.8	25.4	31.0	30.8	38.1	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	33.5	31.3	28.0	29.8	28.4	31.0	29.0	35.8	38.1	40.0	40.0	40.0
	40.0	40.0	40.0	29.2	33.2	28.4	28.8	30.2	21.5	29.5	37.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	29.5	32.4	28.9	27.8	29.6	30.7	30.3		40.0	40.0	40.0	40.0
	40.0	40.0	40.0	30.4	33.2	30.2	28.9	26.6	31.9	28.2	36.4	39.9	39.9	40.0	40.0
	40.0	40.0	40.0	28.8	31.2	29.6	28.7	27.9	29.3	30.0					
	40.0	40.0	40.0	29.8	31.6	30.1	29.7	30.6	29.8	32.7	35.1	40.0			
	40.0	40.0	40.0	27.6	31.3	29.4	29.5	28.2	30.8	31.2					
	40.0	40.0	40.0	30.3	32.2	29.7	29.4	31.7	30.0						
	40.0	40.0	40.0	30.7	31.1	29.1	28.8	29.6	31.0	29.5	34.8	40.0	40.0	40.0	40.0
B.	40.0	40.0	40.0	30.3	33.0	30.4	30.0	27.3	27.5	30.9	35.3	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	28.6	37.1	30.1	29.6	29.1	27.4	31.1	36.8	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	29.2	32.2	29.7	29.5	30.2	31.8	32.8	36.8	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	29.8	30.1	29.5	29.4	30.0	32.6	31.2	33.5	40.0	37.1	40.0	40.0
	40.0	40.0	40.0	33.3	31.3	22.2	29.6	27.7	28.1	29.6	38.4	37.1	40.0	40.0	40.0
C.	40.0	40.0	40.0	40.0	40.0	40.0	35.9	38.3	32.5	33.9	38.3	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	37.2	40.0	36.7	37.2	37.0	36.5	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	35.9	40.0	36.8	37.4	36.7	34.0	36.5	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	35.4	37.9	40.0	37.2	36.7	33.4	34.6	40.0	40.0	40.0	37.9	40.0
	40.0	40.0	40.0	40.0	40.0	35.6	35.7	37.0	33.1	31.5	40.0	40.0	40.0	40.0	40.0

*Cycle Threshold (CT) values are shown for animals within the inoculated (A.), contact transmission (B.) and aerosol transmission (C.) groups. Values are color-coded based on a numerical gradient with the lowest CT values being red and the greatest CT values being green. A CT value of ≥38 was considered negative.

Table 4:5 PEDV viremia in individual animals until death or 42 DPI*

Group	Day Post-Inoculation (DPI)									
	1	2	3	4	5	6	7	9	14	42
A.	40.0	40.0	40.0	40.0	36.2	35.8	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	
	40.0	40.0	30.0	31.0	30.5	29.8	32.5	37.4	40.0	
	40.0	33.3	37.0	35.6	35.5	34.6	40.0	40.0	40.0	40.0
	40.0	40.0								
	40.0	40.0	40.0	36.9	37.3	37.5	37.4	40.0	40.0	
	40.0	40.0	33.7	34.5	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	32.5	32.9	32.3	40.0	40.0	
	40.0	40.0	36.9	36.7						
	40.0	40.0	40.0	33.6	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	30.8	37.5	37.3	36.3	40.0			
	40.0	40.0	40.0	37.4	37.3	40.0	40.0	40.0	40.0	
	40.0	40.0	34.6	31.6	29.9	29.5	30.0	40.0	40.0	40.0
	40.0	40.0	40.0	36.8	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	35.4	36.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	35.9	40.0	36.9	40.0	40.0	40.0
	40.0	40.0	40.0	36.0	33.7	38.3	40.0	40.0	40.0	40.0
	40.0	33.7	30.4	36.3	40.0	40.0	40.0	40.0		
	40.0	40.0	40.0	36.9	40.0	40.0	40.0	40.0	40.0	
	40.0	32.6	34.2	31.7	32.2	34.2	36.9	40.0		
	40.0	40.0	38.1	40.0	40.0	40.0	40.0			
	40.0	40.0	40.0	36.0	33.4	34.2	35.3	37.4	40.0	40.0
B.	40.0	40.0	35.4	31.5	32.4	33.1	33.2	39.3	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	32.4	34.2	34.6	37.3	37.2	40.0	40.0	40.0
	40.0	40.0	40.0	36.9	36.2	36.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	36.6	36.3	36.6	37.3	40.0	40.0
C.	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0

*Cycle Threshold (CT) values are shown for animals within the inoculated (A.), contact transmission (B.) and aerosol transmission (C.) groups. Values are color-coded based on a numerical gradient with the lowest CT values being red and the greatest CT values being green. A CT value of ≥38 was considered negative.

Table 4:6 Summary of tissues stained by the immunohistochemical method for PEDV*

	Day Post-Inoculation												
Tissue	0	2	4	7	7	9	9	14	14	21	21	28	28
Stomach	KI	KI	KI	KI	KI	KI	K	KI	KI	KI	K	KI	K
Small intestines	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI
Large intestines	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI
Pancreas	KI	K	KI	KI	KI	KI	K	K	KI	KI	KI	K	KI
Turbinates	K	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI
Trachea	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI
Lungs	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI
Lymph nodes	KI	K	KI	KI	KI	K	KI	KI	KI	K	K	KI	K
Tonsil	K	NP	K	K	K	K	K	K	K	K	K	K	K
Tongue	-	KI	-	KI	-	-	-	KI	-	K	-	K	-
Esophagus	K	KI	-	K	KI	KI	KI	KI	KI	K	K	KI	K
Thymus	K	K	K	K	K	K	KI	K	K	K	-	K	-
Thyroids	KI	K	K	KI	-	K	K	-	KI	-	-	-	K
Adrenals	KI	K	-	K	-	K	K	-	-	-	-	K	-
Bone marrow	-	-	-	-	-	-	KI	KI	KI	-	-	-	-
Liver	KI	-	-	KI	KI	KI	-	-	-	-	-	-	-
Kidney	KI	-	-	K	-	K	-	-	-	-	-	-	-
Spleen	K	K	-	K	-	K	-	-	-	-	-	-	-
Heart	KI	-	K	KI	KI	KI	KK	KI	KI	K	-	K	-

*Only small intestine and mesenteric lymph nodes were IHC positive

K = IHC stained at Kansas State Veterinary Diagnostic Laboratory

KI = IHC stained at Kansas State and Iowa State Veterinary Diagnostic Laboratories

- = Tissue not stained

NP = Tissue not processed

Table 4:7 Real-time PCR detection of PEDV in tissues collected sequentially throughout the study or at 42 DPI*

Group	DPI	Nasal Turbinate	Tonsil	Trachea	Lung	Lymph Node	Thymus	Spleen	Small Intestine
A.	0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	39.9
	2	36.2	38.4	40.0	40.0	40.0	36.3	40.0	36.8
	4	40.0	35.3	40.0	39.1	27.7	40.0	40.0	13.6
	7	39.7	36.6	40.0	40.0	23.3	39.9	36.7	14.5
		38.7	35.5	40.0	40.0	26.7	34.5	40.0	25.0
	9	40.0	35.2	40.0	40.0	31.7	40.0	29.2	26.3
		40.0	40.0	40.0	40.0	33.5	38.3	33.5	27.8
	14	40.0	38.9	40.0	40.0	30.7	40.0	38.2	22.0
		40.0	40.0	40.0	40.0	31.9	40.0	35.5	29.7
	21	38.6	38.6	40.0	40.0	32.9	40.0	35.1	19.9
		40.0	40.0	40.0	40.0	35.3	40.0	37.3	28.2
	28	40.0	40.0	40.0	40.0	30.1	40.0	38.3	27.5
		40.0	36.5	40.0	40.0	34.7	35.6	40.0	18.9
A.	42					40.0			32.6
						40.0			40.0
						40.0			40.0
						40.0			32.1
						40.0			32.0
						40.0			35.8
						38.8			38.4
						40.0			32.4
						40.0			34.3
						39.0			35.3
B.	42					40.0			40.0
						40.0			40.0
						40.0			40.0
						40.0			40.0
						40.0			40.0
C.	42					40.0			40.0
						40.0			40.0
						40.0			40.0
						40.0			40.0
						40.0			40.0

*Cycle Threshold (CT) values are shown for inoculated (A.), contact transmission (B.) and aerosol transmission (C.) groups sequentially collected during the study or at 42 DPI. Values are color-coded based on a numerical gradient with the lowest CT values being red and the greatest CT values being green. A CT value of ≥38 was considered negative.

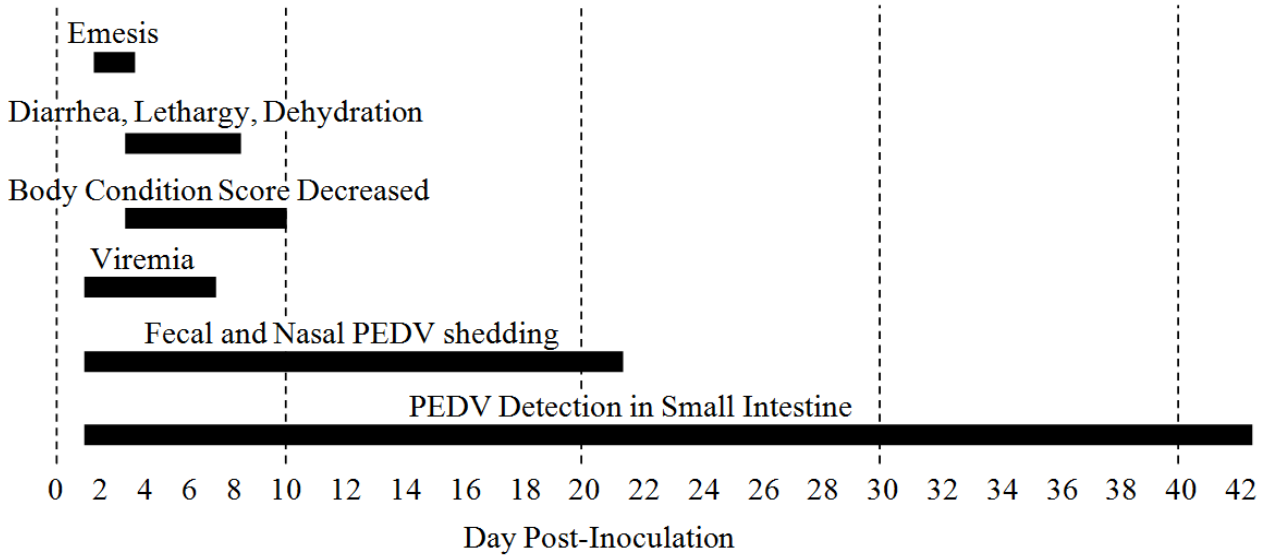

Figure 4:1 Time course of clinical disease and PEDV detection in Group A pigs after PEDV inoculation.

Clinical disease was assessed as described in the materials and methods. Clinical signs consistent with PEDV were not present after 10 DPI.

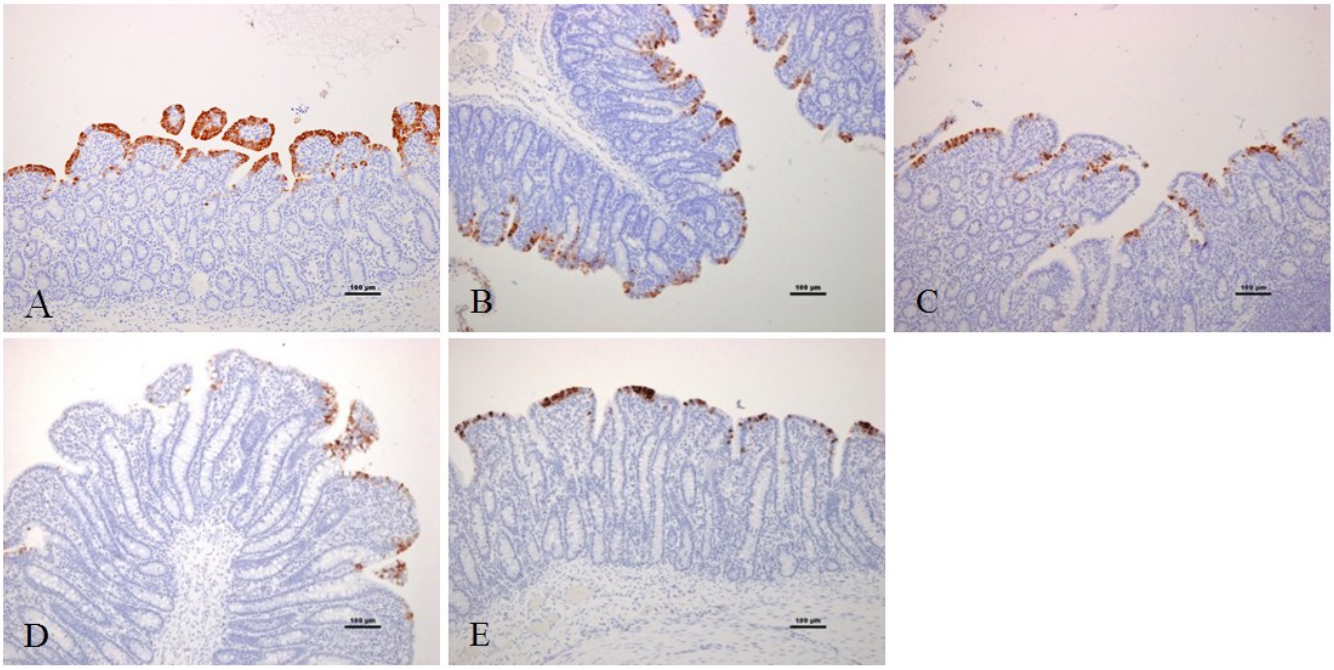

Figure 4:2 PEDV immunohistochemistry of jejunal sections from Group A pigs sequentially collected after PEDV challenge.

Images are shown from tissues collected: A. 4 DPI; B. 7 DPI; C. 14 DPI; D. 21 DPI; and E. 28 DPI.

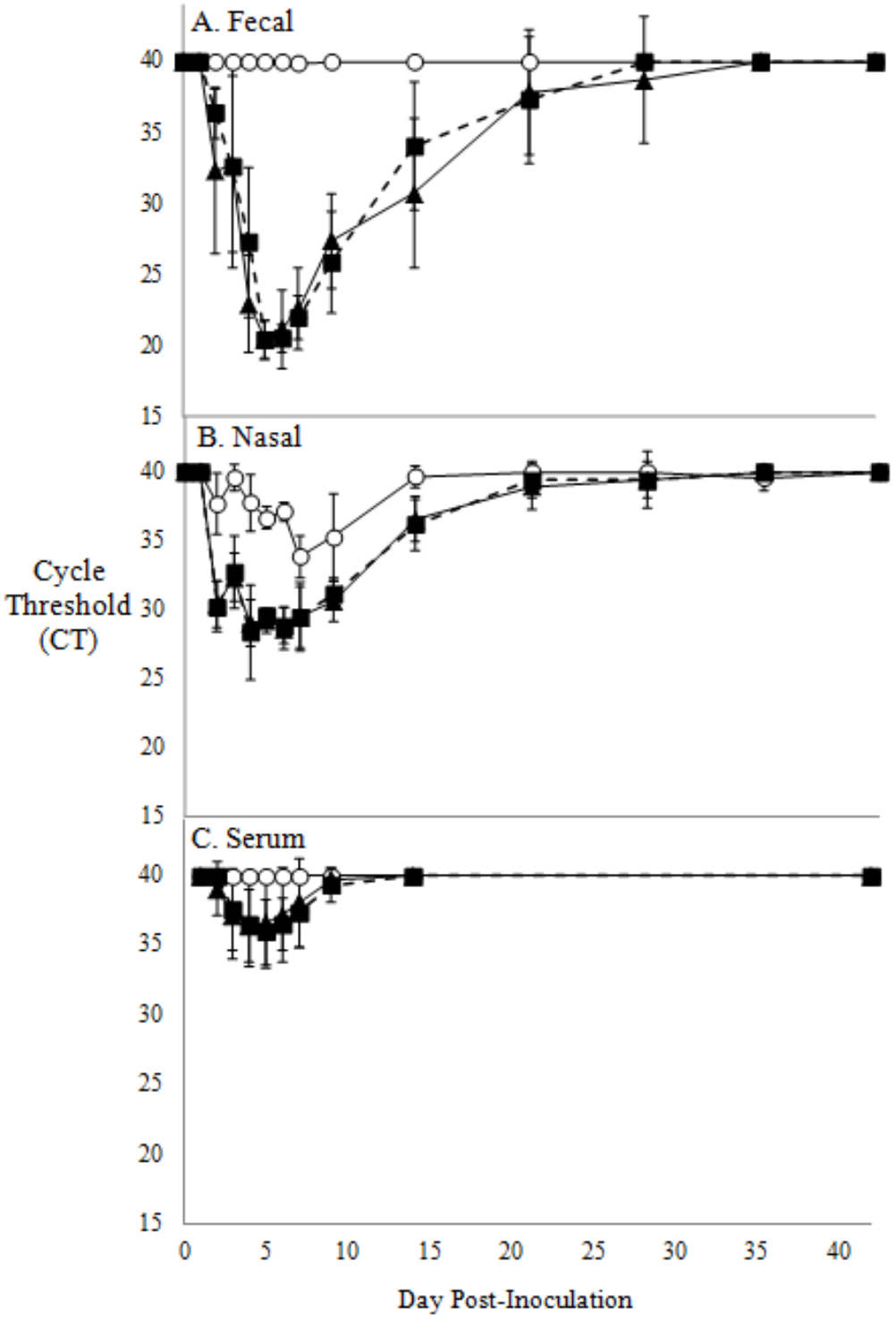

Figure 4:3 PCR detection of PEDV in fecal swabs (A), nasal swabs (B) and serum (C) after PEDV inoculation.

Data is represented by real-time PCR group mean cycle thresholds (CT) ± 1 standard deviation. Inoculated (▲), contact transmission (■) and aerosol transmission (○) groups are shown. A CT value of ≥38 was considered negative.

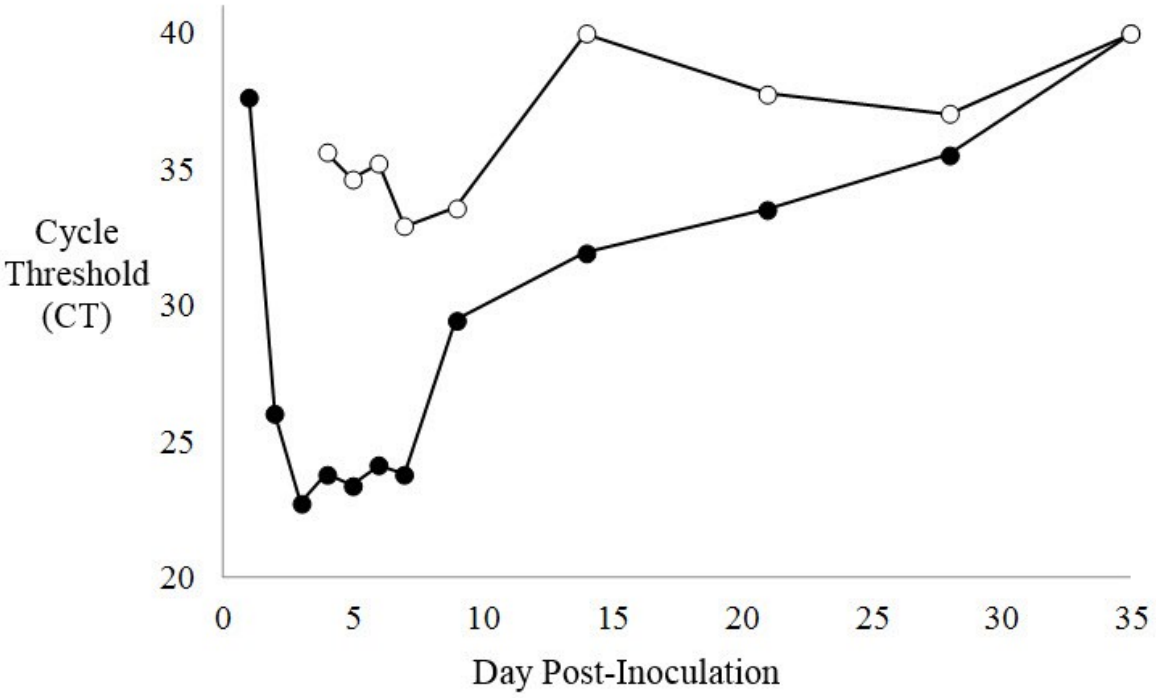

Figure 4:4 PCR detection of PEDV in pooled oral fluid samples after PEDV challenge.

Data is represented by real-time PCR cycle thresholds (CT). Pen samples from Groups A and B (●) and Group C (○) are shown. A CT value of ≥38 was considered negative.

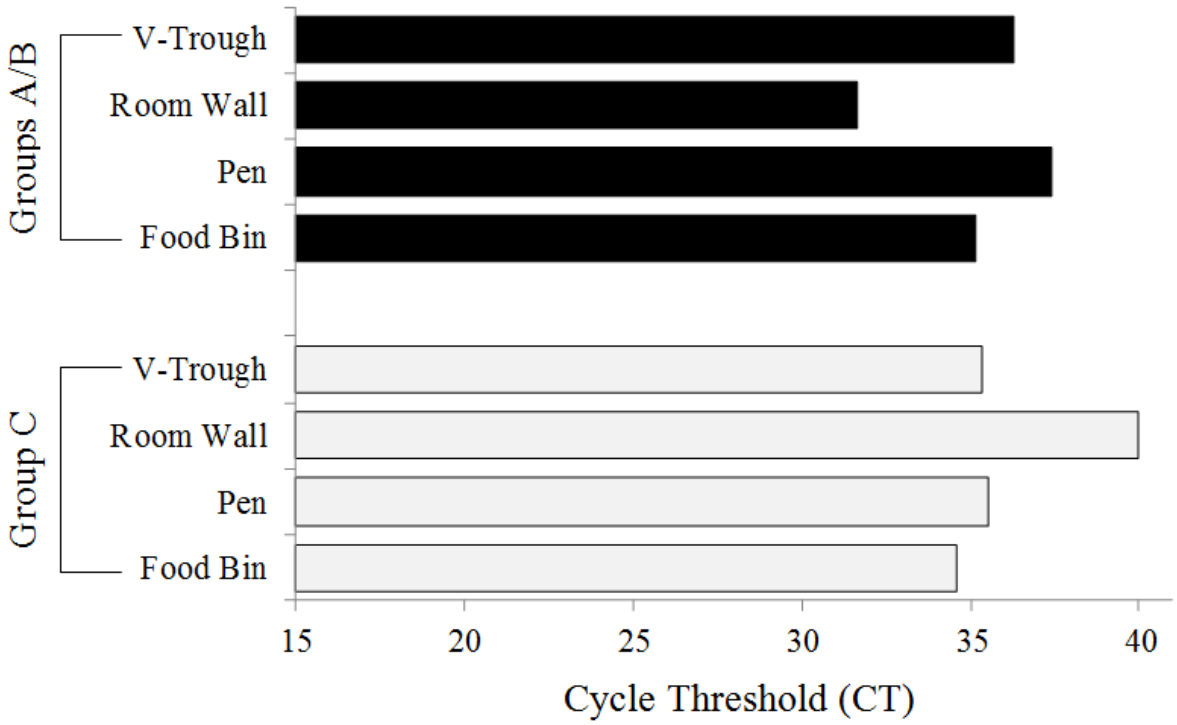

Figure 4:5 PEDV detection in environmental samples collected on 14 DPI.

Swabs were collected from both the Groups A and B side and the Group C side of the common animal room. Data is represented by real-time PCR cycle thresholds (CT). A CT value of ≥ 38 was considered negative.

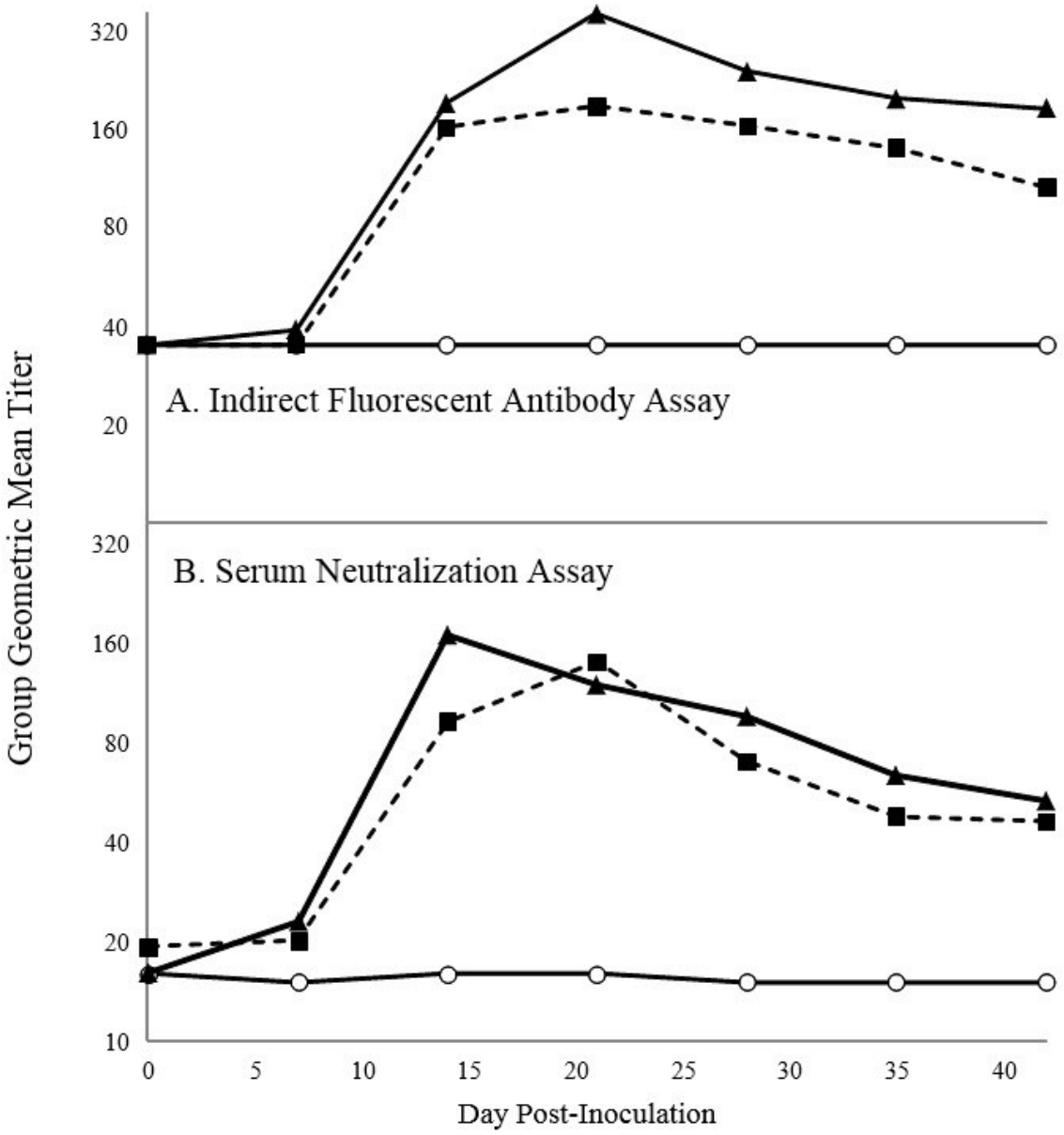

Figure 4:6 Antibody response after PEDV challenge in all 3 experimental groups.

Data is represented by the group geometric mean indirect fluorescent antibody titer (A) and serum neutralization titer (B) during each week post-inoculation. Inoculated (▲), contact transmission (■) and aerosol transmission (○) groups are shown. A. Antibody titers < 40 adjusted to 35 and B. Antibody titers of < 20 adjusted to 15 for improved visibility.