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)

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Running Title: Clinical outcome following PRRSV/PCV2 co-infection

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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 increased 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 a comprehensive DNA microarray technology, the Lawrence Livermore Microbial Detection Array, to detect over 8,000 microbes. Bacterial species, such as *Bacillus cereus*, were detected at a higher rate in the serum of worst performing pigs. 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.

Keywords: Swine; Microbiome; Porcine reproductive and respiratory syndrome virus; Porcine circovirus type 2; Clinical outcome; Virus load

1. 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 (Segales, 2012). Although PCV2 is essential for PCVAD, PCV2 infection alone rarely results in clinical signs and co-infections 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) (Opriessnig and Langohr, 2013; 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 *Arteriviridae* (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). 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 (Niederwerder et

al., 2015; Tribble et al., 2012). 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).

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 and a pan-pathogen microarray technology, the Lawrence Livermore Microbial Detection Array (LLMDA) (Jaing et al., 2015), to evaluate differences between the best and worst clinical outcomes following co-infection with PRRSV and PCV2.

2. Materials and methods

2.1. 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 single commercial source herd negative for PRRSV. The sow herd had no history of clinical disease associated with PCV2 or PRRSV. Relevant health history included a recent introduction of porcine epidemic diarrhea virus (PEDV) prior to farrowing. All piglets were confirmed to be negative for PRRS viremia using qPCR prior to challenge. 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*.

2.2. 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., 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° 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), penicillin-streptomycin (Pen Strep; 80 Units/ml and 80 µg/ml, respectively; Gibco), 3 µg/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°C in 5% CO₂, wells were examined for PRRSV induced cytopathic effects, and the 50% tissue culture infectious dose (TCID₅₀/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% CO₂, 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 TCID₅₀/ml was calculated using the method of Reed and Muench (Reed and Muench, 1938).

2.3. 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} TCID₅₀ PCV2 and 10⁵ TCID₅₀ 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.

2.4. 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. The majority of clinical signs were documented as either present or absent during daily evaluations. Five of the clinical signs, including dyspnea, lethargy, decreased body condition, diarrhea, and lameness, were scored based on severity. 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 $\geq 104^{\circ}\text{F}$. 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 $\geq 104^{\circ}\text{F}$ 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 and histopathology were performed by a board certified pathologist who

was blinded as to the source of the pigs. Tissues were collected from each pig including lung (1 section from each lobe) and tracheobronchial lymph node. Tissues were allowed to fix in 10% neutral buffered formalin for at least 7 days, routinely processed in an automated tissue processor, embedded in paraffin, and stained with hematoxylin and eosin (H&E stain). Microscopic lung lesions were estimated using a 0 to 4 scoring system as previously described (Niederwerder et al., 2015). Degree of lymphoid depletion was estimated using the following scoring system: 0, no lymphoid depletion; 1, mild or small amount of lymphoid depletion; 2, moderate or intermediate amount of lymphoid depletion; 3, severe or large extent of lymphoid depletion. 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.

2.5. Measurement of PRRSV and PCV2 viremia

Viral DNA and RNA were extracted simultaneously from 50 μ L 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 μ M. Nuclease free water was used to bring the mastermix volume to 18 μ L per reaction. The addition of 2 μ L of template nucleic acid brought the final reaction volume for each sample to 20 μ L. 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 μ L reaction volume or $\log_{10}$ PCV2 DNA starting quantity per 20 μ L reaction volume.

2.6. DNA microarray analysis

The Lawrence Livermore Microbial Detection Array (LLMDA) developed at the Lawrence Livermore National Laboratory was designed to detect all sequenced microbes. The LLMDA is not commercially available at the time of this study, but available for research collaborations. The version 5 of the LLMDA in the 12plex 135K format was used to analyze the

microbiome in pig samples. This version of the array targets all vertebrate infecting pathogens including 1856 viruses, 1398 bacteria, 125 archaea, 48 fungi, and 94 protozoa. The LLMDA oligonucleotide probes are around 60 nucleotides in length and were designed to detect all sequenced microbial families with a large number of probes per sequence (average of 30 probes) to improve sensitivity in the evaluation of microbial nucleic acids in a variety of samples. The high-density oligo LLMDA microarray and statistical analysis method have been extensively tested in numerous studies for viral and bacterial detection in pure or complex environmental and clinical samples (Gardner et al., 2010; Jaing et al., 2015; Rosenstierne et al., 2014).

Each 1 mL sample for extraction consisted of 250 μ L sample and 750 μ L 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 μ L 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 μ g of glycogen was added to the aqueous phase along with 500 μ L 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 μ L 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 μ L (approximately 7.0-12.0 μ g) of extracted RNA was used as input to generate double stranded cDNA following the standard Roche NimbleGen protocols using the Invitrogen Double-Stranded cDNA Synthesis Kit. 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, 5 μ L 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). Microarray probes with fluorescent signals that were equal to or greater than the 99% threshold of random control signals were included for CLiMax analysis. A minimum of 8 probes or at least 20% of overall probes designed against a target sequence was used as the threshold for positive detection of a target sequence.

2.7. Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.00 software. Average daily gain, viremia, and area under the curve were analyzed and compared between groups using the unpaired t-test. Weekly weights were compared between groups using repeated measures analysis. Microbial families detected in feces as well as lung and lymphoid lesion scores were compared between groups using the Mann-Whitney U test. Correlation analyses were performed using Spearman rank correlation coefficients. 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.

3. Results

3.1. 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 conclusion of the 70-day study period, necropsies were performed and tissues were collected from all 20 pigs. All 10 of the worst outcome pigs had lesions consistent with PRRS, PCVAD, or virus associated immunosuppression, such as chronic polyserositis. Representative pictures of gross and microscopic lesions associated with the worst performing group are presented in Figure 2. 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. As summarized in Table 3, microscopic lesions were assessed in all 20 pigs through the evaluation of lung and lymph node tissues. The severity of interstitial pneumonia was significantly greater in worst performing pigs ($p = 0.027$, Mann-Whitney U test). A similar trend occurred for worst outcome pigs in the severity of lymphoid depletion; however, this difference was not significantly different ($p = 0.166$, Mann-Whitney U test). Overall, lesions in best performing pigs were considered mild and did not fall into the moderate or severe scoring categories (Table 3).

Mean weights for the two groups are shown in Figure 3. 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 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 mean weights of the two groups diverged quickly after challenge and were significantly different starting at 1 week post-infection. 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 4 and 5 for the worst outcome group, which correlated with peak PCVAD (see Figure 1C).

3.2. PRRSV and PCV2 viremia

The results for PRRSV and PCV2 viremia are shown in Figure 4. 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 4). 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 4B. 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.

3.3. DNA microarray results for serum

DNA microarray results for microbial species detected in serum, feces and lung are presented in Figures 5 through 8. The results for each bacterium or virus were ranked according to the highest log likelihood score and the first species in each microbial family with the greatest percentage of target-specific probes is shown.

A total of 12 bacterial species were detected in serum by the microarray. Those bacteria that were only found at a low prevalence (10% or less of pigs) included *Campylobacter lari*, *Staphylococcus haemolyticus*, *Frankia sp.*, *Enterobacter sp.*, *Enterobacter asburiae*, *Heliobacterium modesticaldum*, and *Cronobacter turicensis*. The remaining bacteria are listed in Figure 5. 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 log₁₀ copies/PCR reaction for the best clinical outcome and worst clinical outcome groups, respectively ($p = 0.14$, unpaired t-test). 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 log₁₀ 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 log₁₀ 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.

3.4. DNA microarray results for lung

Viral and bacterial species detected by microarray in lung samples are shown in Figure 6. 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.

3.5. 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 of pigs) 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 7. 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 5); 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 7. 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 under the defined threshold. The results are presented in Figure 8. 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).

4. 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% (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 several weeks after virus challenge. As such, it is interesting to note that there were significant differences between the mean weights of the two groups as early as one week after challenge. 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 is noteworthy 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 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. Because all pigs were co-infected, we were unable to compare results with non-challenged controls; thus, differences in microbial presence due to exposure to PRRSV and PCV2 are unknown. Overall, the DNA microarray detected the microbial signatures of 18 species in serum, 6 species in lung and 37 species in feces.

In serum, the array detected *Bacillus cereus* in a significantly greater proportion of worst outcome pigs compared to best outcome pigs. *B. cereus* can be used as a probiotic in feed (phylum Firmicutes) and this finding may represent 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). 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 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 from swine did not affect outcome after challenge with *Mycoplasma hyopneumoniae*, another common respiratory pathogen (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 Figures 1 and 6). 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). 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 hyopneumoniae*. 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).

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. Although the worst outcome pigs had not received antibiotics for an average of 25 days prior to sample collection, reduced family diversity in the feces from this group 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 and the number of microbial families detected in feces for each of the worst outcome pigs; no significant association was detected ($p = 0.81$, Spearman rank correlation; data not shown). Although there

does not appear to be a significant correlation between antibiotic doses received and fecal microbial diversity, the overall effect of antibiotic administration cannot be eliminated. However, it could 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 that 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), increased body weight and fat deposition in rats (Karlsson et al., 2011), and prevention of cachexia in mice after infection with respiratory and gastrointestinal pathogens (Schieber et al., 2015). 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. 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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7. Figure captions

Figure 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. Gross and microscopic lesions in worst performing pigs 70 days after co-infection with PRRSV and PCV2. A) Gross lung showing moderate interstitial pneumonia. B) Thoracic cavity showing diffuse fibrous adhesions between the pleura, lungs, and pericardium. C) H&E-stained lung showing severe diffuse edema and interstitial fibrinosuppurative pneumonia with increased numbers of alveolar macrophages (10X). D) H&E-stained lymph node showing lymphoid depletion with severely decreased numbers of lymphoid follicles (2X).

Figure 3. Mean weights over time for pigs with the best and worst clinical outcomes. Data is shown as mean weight $\pm$ one standard deviation with regression lines. Asterisks identify statistically significant differences between groups ($p < 0.005$, unpaired t-test using repeated measures analysis).

Figure 4. PRRSV and PCV2 viremia in pigs with best and worst clinical outcomes. The figure shows mean PCR values $\pm$ 1 standard deviation after challenge with PRRSV and PCV2. Asterisks identify statistically significant differences between groups ($p < 0.05$, unpaired t-test).

Figure 5. 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 6. 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 7. 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 8. 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).

Table 1

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

Table 2

Table 2. Average daily gain between 0-70 dpi			
Worst Performing		Best Performing	
Pig	ADG (kg)	Pig	ADG (kg)
12	0.149	30	0.779
50	0.280	55	0.797
3	0.492	43	0.805
47	0.493	98	0.808
61	0.495	29	0.827
16	0.506	15	0.831
1	0.537	62	0.848
28	0.542	6	0.883
24	0.555	63	0.889
88	0.698	54	0.903
*Mean	0.475	*Mean	0.837
SD	0.153	SD	0.042
*Significant difference between means, $p < 0.0001$, unpaired t-test			

Table 3. Microscopic lung and lymph node lesions in pigs with the best and worst clinical outcome on 70 dpi with PRRSV and PCV2

Score	No. (%) of pigs	
	Worst Performing	Best Performing
Microscopic lung lesion score ^a		
0	1 (10)	3 (30)
1	3 (30)	6 (60)
2	2 (20)	1 (10)
3	1 (10)	0 (0)
4	3 (30)	0 (0)
Lymphoid depletion score ^b		
0	2 (20)	3 (30)
1	4 (40)	7 (70)
2	3 (30)	0 (0)
3	1 (10)	0 (0)

^a Determined by evaluation of lung sections stained with hematoxylin and eosin. Scores were assigned as follows: 0, no significant lesions; 1, mild multifocal interstitial pneumonia with <50% lung lobe involvement; 2, mild to moderate multifocal interstitial pneumonia with 50 to 75% lung lobe involvement; 3, moderate to severe multifocal interstitial pneumonia with 50 to 75% lung lobe involvement; 4, severe diffuse interstitial pneumonia with >75% lung lobe involvement. Best clinical outcome pigs had significantly lower scores compared to worst clinical outcome pigs ($p = 0.027$, Mann-Whitney U test).

^b Determined by evaluation of lymph node sections stained with hematoxylin and eosin. Score were assigned as follows: 0, no lymphoid depletion; 1, mild or small amount of lymphoid depletion; 2, moderate or intermediate amount of lymphoid depletion; 3, severe or large extent of lymphoid depletion. Scores were not significantly different between the two groups ($p = 0.166$, Mann-Whitney U test).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8