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Increased microbiome diversity at the time of infection is associated with improved growth rates of pigs after co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most important pathogens affecting the swine industry worldwide. Co-infections are common on a global scale, resulting in pork production losses through reducing weight gain and causing respiratory disease in growing pigs. Our initial work demonstrated that the fecal microbiome was associated with clinical outcome of pigs 70 days post-infection (dpi) with PRRSV and PCV2. However, it remained uncertain if microbiome characteristics could predispose response to viral infection. The purpose of this study was to determine if microbiome characteristics present at the time of virus exposure were associated with outcome after co-infection. Using the Lawrence Livermore Microbial Detection Array, we profiled the microbiome in feces prior to infection from pigs identified retrospectively as having high or low growth rates after co-infection. High growth rate pigs had less severe interstitial pneumonia, reduced virus replication, and a significant increase in average daily weight gain throughout the study. At the level of the fecal microbiome, high growth rate pigs had increased microbial diversity on both a family and species level. Shifts in the microbiome composition of high growth rate pigs included reduced *Methanobacteriaceae* species, increased *Ruminococcaceae* species, and increased *Streptococcaceae* species when compared to low growth rate pigs. The results indicate that both microbiome diversity and composition at the time of virus exposure may play a role in the subsequent response of pigs to PRRSV/PCV2 co-infection.

1. Introduction

Pork is the most widely consumed protein around the world and global pork production is forecast to reach a record of 111 million tons in 2017 (USDA, 2016). Worldwide, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most significant pathogens affecting the swine industry, costing billions in lost production over the last 3 decades. PRRSV is a single-stranded RNA virus in the family *Arteriviridae* (Benfield et al., 1992) which causes reductions in weight gain and respiratory disease in growing pigs. It is currently considered the most costly disease of swine production worldwide (Chand et al., 2012). PCV2 is a single-stranded DNA virus in the family *Circoviridae* and is associated with a group of disease syndromes termed porcine circovirus associated disease (PCVAD), which includes muscle wasting, respiratory disease, and enteric disease (Segales, 2012). Both viruses result in systemic infections, cause primary lung pathology, and modulate the immune response. For example, PRRSV suppresses innate immunity through antagonizing type I interferon production (Chen et al., 2010; Han and Yoo, 2014) and PCV2 depletes lymphocytes in lymphoid tissues (Opriessnig and Langohr, 2013). Co-infection with the two viruses enhance disease when compared to single infections alone and can result in a wide range of overt clinical signs; however, overall morbidity is typically less than 30% (Niederwerder et al., 2015), leaving the majority of pigs to support

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subclinical infections.

Playing an essential role in both nutrient availability and immunity (Honda and Littman, 2016; Turnbaugh et al., 2006), the microbiome, or collection of microorganisms within the gastrointestinal tract, has been associated with outcome during systemic viral infections in both humans and mouse models. Microbiome associations have been found with viruses primarily affecting the respiratory tract, such as respiratory syncytial virus and influenza virus (Fujimura et al., 2014; Ichinohe et al., 2011), as well as viruses considered immunomodulatory, such as human immunodeficiency virus (Mudd and Brenchley, 2016). Disease progression, airway inflammation, immune response, and morbidity can all have associations with microbiome composition and diversity during these viral infections.

The role of the microbiome and its impact on the response to systemic infections in swine is an emerging area of study and the focus of our work. To investigate the porcine microbiome, we utilized the Lawrence Livermore Microbial Detection Array (LLMDA), which was developed to detect all known microbes for which whole genome sequences are available. Current as of June 2013, this technology allows for the detection of 8101 microbes. The LLMDA has been used in the detection of known and unknown microbes in various porcine samples, including feces, serum, lung, oral fluids, lymph node suspension and tonsil (Jaing et al., 2015; Niederwerder et al., 2016).

In a recent study, we investigated the associations between the microbiome and clinical outcome in pigs following PRRSV/PCV2 co-infection (Niederwerder et al., 2016). Best and worst clinical outcome pigs were selected based on the presence and severity of clinical disease as well as weight gain after co-infection. The fecal microbiomes of best clinical outcome pigs were characterized by increased diversity and increased prevalence of *Escherichia coli* 70 days after co-infection. While this initial study confirmed a potential role for the microbiome in outcome following PRRSV/PCV2 co-infection, important gaps in our knowledge remained, such as microbiome characteristics that predispose outcome and the role of the microbiome in subclinical infections.

In the current study, we investigated the early microbiome properties that predispose high and low growth rates after co-infection in subclinically affected pigs. Our results demonstrate that both microbiome diversity and composition prior to infection play a role in weight gain following PRRSV/PCV2 co-infection.

2. Materials and methods

2.1. Animals and housing

All use and experimentation incorporating animals and viruses were done 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. This 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; a subset of pigs from this project were included in the current study. Three week old barrows (n = 50; average age 23.5 ± 2.6 days) were obtained at weaning from a high health commercial herd negative for PRRSV. While the 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 without regards to maternal antibody. All pigs were housed in a single environmentally controlled room at the Kansas State University Large Animal Research Center under BSL-2 conditions. The piglets were randomly distributed and housed in groups of 8–10 pigs per 13.4 m^2 pen. All pigs were given a period of approximately 4 weeks to acclimate to their new environment prior to co-infection. Pigs were given access to food and water *ad libitum*.

2.2. Viruses

The PRRSV and PCV2b viral isolates used to prepare the inoculum for this study were originally derived from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described (Trible et al., 2012). PRRSV (isolate KS62; GenBank accession no. KM035803) was isolated by propagation on MARC-145 cells. Since wild-type PCV2b (GenBank accession no. JQ692110) does not propagate to high levels in cell culture, we took advantage of the heat stability of the virus to prepare a lymph node suspension enriched for PCV2 as previously described (Niederwerder et al., 2015, 2016). The isolated PRRSV was recombined with the heat-treated PCV2 homogenate to co-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. Analysis of the inoculum yielded negative results for most heat-stable agents, but was positive for two viruses ubiquitous to swine, including Torque teno sus virus (T-TSuV) and porcine endogenous retrovirus (PERVs) (Jaing et al., 2015).

PCV2b was titrated on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of PCV2 challenge stock were plated in quadruplicate onto rapidly dividing ST cells in a 96-well tissue culture plate (BD Falcon). Dilutions were prepared in Eagle's minimal essential medium (EMEM; Sigma-Aldrich) supplemented with 7% fetal bovine serum (FBS; Sigma-Aldrich) and 50 μg/mL of gentamicin (Lonza). Following a 3-day incubation at 37° C in 5% CO_2, cells were fixed and permeabilized with 80% acetone. Cells were 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 50% tissue culture infectious dose ($TCID_{50}$/mL) was calculated using the method of Reed and Muench (Reed and Muench, 1938).

MARC-145 cells were used for the titration of PRRSV. Briefly, virus was serially diluted 1:10 in minimal essential medium (MEM; Corning) supplemented with 7% FBS (Sigma-Aldrich), penicillin-streptomycin (Pen Strep; 80 Units/mL and 80 μg/mL, respectively; Gibco), 3 μg/mL amphotericin B (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% CO_2, cells were examined for PRRSV-induced cytopathic effects. The $TCID_{50}$/mL was calculated using the method of Reed and Muench (Reed and Muench, 1938).

2.3. Experimental design and sample collection

At approximately 8 weeks of age (average age 54.5 ± 2.6 days), all 50 pigs were infected with PRRSV and PCV2b. The viruses were recombined to yield a 2-mL dose consisting of $10^{3.6}$ $TCID_{50}$ PCV2b and 10^5 $TCID_{50}$ PRRSV in MEM. The 2-mL dose was split, with 1 mL being delivered intranasally and 1 mL being delivered intramuscularly. Body weights of individual pigs were collected upon arrival and on 0, 7, 14, 21, 28, 35, and 42 days post-infection (dpi). Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi. Fecal samples were collected from all 50 pigs during the week prior to co-infection. At 35 dpi, 20 pigs were selected to represent high growth rate pigs (n = 10) and low growth rate pigs (n = 10). To select these two groups, the average daily gain (ADG) was calculated between 0 and 35 dpi as the change in weight over the change in time and reported in kg. Pigs in the high growth rate group had the highest ADG and pigs in the low growth rate group had the lowest ADG. The two groups were balanced according to initial weight on 0 dpi. Any pig that had overt clinical disease requiring veterinary medical treatment (as described below) was excluded from selection. At 42 dpi, all 20 pigs were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals and complete necropsies were performed.