Comparison of Asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to United States isolates for their ability to cause disease and secondary bacterial infection in swine

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Abstract

Epidemiologic data from Asian outbreaks of highly-pathogenic (HP) porcine reproductive and respiratory syndrome virus (PRRSV) suggest that disease severity was associated with both the virulence of the PRRSV isolates and secondary bacterial infections. Previous reports have indicated that U.S. isolates of PRRSV predispose to secondary bacterial infections as well, but the severity of disease that occurred in Asia in pigs infected with these HP-PRRSV strains has not been reported in the U.S. The objectives of this research were to compare the pathogenesis of Asian and U.S. PRRSV isolates with regard to their ability to cause disease and predispose to secondary bacterial infections in swine. To address these objectives groups of pigs were infected with 1 of 2 Asian HP-PRRSV strains (rJXwn06 or rSRV07) or 1 of 2 U.S. PRRSV strains (SDSU73 or VR-2332) alone or in combination with *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis*. Pigs infected with rJXwn06 exhibited the most severe clinical disease while the pigs infected with rSRV07 and SDSU73 exhibited moderate clinical disease, and pigs infected with VR-2332 exhibited minimal clinical signs. The frequency

of secondary bacterial pneumonia was associated with the clinical severity induced by the PRRSV strains evaluated. The levels of proinflammatory cytokines in the serum were often lower for pigs coinfecting with virus and bacteria compared to pigs infected with PRRSV alone indicating an alteration in the immune response in coinfecting pigs. Combined our results demonstrate that severity of disease appears to be dependent on virulence of the PRRSV strain, and development of secondary bacterial infection.

Keywords: porcine reproductive and respiratory syndrome virus, PRRSV, highly pathogenic PRRSV, pathogenesis, virulence, secondary bacterial infection, *Haemophilus parasuis*, *Actinobacillus suis*, *Streptococcus suis*, porcine respiratory disease complex, PRDC

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widely disseminated virus of swine that is a member of the genus *Arterivirus* in the family *Arteriviridae*, made up of linear positive-sense, single-stranded RNA. The virus is divided into 2 major genotypes, Type 1 (European) and Type 2 (North American), that differ by as much as 40% at the nucleotide level, while strains within each genotype can differ by more than 20% at the nucleotide level (Nelsen et al., 1999; Faaberg et al., 2006). The hallmark of this virus family is its ability to undergo frequent recombination and mutation leading to extraordinarily diverse viruses with regard to sequence and virulence. In 2006 large outbreaks of a high morbidity/high mortality disease that became known as “porcine high fever disease” (PHFD) began to occur across China, and in 2007 Vietnam began to experience swine disease outbreaks causing similar clinical signs (Li et al., 2007; Metwally et al., 2010; Tian et al., 2007). The disease was associated with newly-emergent, highly-pathogenic (HP) Type 2 PRRSV strains that eventually spread to many Asian countries.

Following oronasal exposure, PRRSV replicates in macrophages in tonsils, upper respiratory tract, and lungs resulting in viremia by 6-12 hours post infection. PRRSV infection results in destruction and decreased function of pulmonary alveolar macrophages and pulmonary intravascular macrophages, changes in T-cell subpopulations, and possibly in decreased function of antigen presenting cells such as dendritic cells and macrophages (Thanawongnuwech et al., 1998a and 1998b; Rodriguez-Gomez, et al., 2013). PRRSV-induced damage to pulmonary intravascular macrophages and pulmonary alveolar macrophages is believed to result in increased susceptibility to bacterial pneumonia and septicemia (Thanawongnuwech et al., 1998a, 1998b, and 2000). Experimentally, PRRSV infected pigs have increased septicemia and mortality when challenged with *Streptococcus suis* and increased pulmonary infections when challenged with *Bordetella bronchiseptica* (Brockmeier et al., 2000, Galina et al., 1994, Thanawongnuwech et al., 2000).

S. suis, *H. parasuis*, and *Actinobacillus suis* are important pathogens of swine that cause sporadic “stress-associated” disease among young pigs in conventional herds and high mortality outbreaks in naïve pigs of any age in high health status herds (MacInnes and Desrosiers, 1999). These bacteria are early colonizers of the respiratory tract and can cause disease ranging from pneumonia to acute septicemic disease, including polyserositis, arthritis, and meningitis. They are often isolated from the upper respiratory tract of healthy pigs and vaccination, serodiagnostic testing, and even serotyping are complicated by the presence of multiple serotypes, cross-reactive antigens, and the absence of clear markers for virulence. All three of these bacteria contribute to the porcine respiratory disease complex (PRDC) often in association with respiratory viruses such as PRRSV.

Bacteria, including *S. suis*, were often isolated from clinical cases of the PHFD outbreaks

in Asia and it was speculated that the cause of the swine deaths in PHFD was a multifactorial syndrome with PRRSV serving as the major contributing factor (Li et al., 2007; Metwally et al., 2010; Xu et al., 2010). Previous experiments conducted in conventional weaned pigs with the Chinese HP-PRRSV isolate rJXwn06 resulted in severe disease with a 70-100% occurrence of secondary bacterial pneumonia (Guo et al., 2013a and 2013b; Lager et al., 2014). These data indicate that the Chinese strain of PRRSV, in particular, may have a greater capacity to predispose to secondary bacterial infection. To gain a broader and more relevant assessment of PRRSV virulence and ability to predispose to secondary bacterial infections, a comparison of U.S. and Asian PRRSV isolates with expected virulence differences were examined in a coinfection study performed in pigs with a controlled pathogenic bacterial burden under low stress conditions. The objective of this study was to determine if North American and Asian PRRSV isolates are equally pathogenic with regard to their ability to cause disease alone and predispose to secondary bacterial infection in U.S. swine. Research priorities were to delineate differences between Asian and U.S. isolates with regard to the severity of disease and alterations in the host innate immune response in pigs, to determine if Asian and U.S. isolates equally predispose to secondary bacterial infections, to determine the contribution of secondary bacterial infection to the morbidity/mortality associated with PHFD, and gain insight to the mechanisms by which PRRSV predisposes to secondary infection.

2. Materials and methods

2.1. Piglets. Ten sows seronegative for PRRSV were purchased from a high-health herd and transported to the National Animal Disease Center (NADC) approximately 2 weeks prior to farrowing. Piglets were weaned at 3-4 days-of-age and moved into isolation rooms and fed a

milk replacer that was gradually replaced by dry feed over the next 10 days. These measures were done to minimize transmission and colonization of the piglets with common bacterial pathogens.

2.2. PRRSV and bacterial isolates. Four isolates of PRRSV were used, 2 U.S. isolates – VR-2332, the Type 2 North American prototype (Collins et al., 1992), and SDSU73, an isolate from 1996 from a herd with a high prevalence of abortions, increased sow mortality, and severe illness among nursery age pigs described as atypical PRRS at the time (Mengeling et al., 1998; Brockmeier et al., 2012), and 2 Asian strains - a Chinese isolate rJXwn06 that was rescued from a full-length cDNA of the JXwn06 virus (Zhou et al., 2009) and rSRV07 rescued from a full-length cDNA of a 2007 strain detected in the Socialist Republic of Vietnam (SRV07) (Metwally et al., 2010, Guo et al., 2013b), both from cases of PHFD. Viruses were propagated on MARC-145 cells (VR-2332- passage 6, SDSU73- passage 3, rJXwn06- passage 4, and rSRV07- passage 4) and pigs were challenged with 10^4 50% cell culture infectious dose (CCID₅₀) in 2ml (1 ml/nare) per pig by the intranasal route. A sham inoculum was prepared from virus-free MARC-145 cells and given in a similar volume.

A cocktail of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis* was used to colonize some of the pigs 1 week prior to PRRSV challenge. All three bacterial isolates were obtained from the lung lavage of pigs from previous experiments using the rJXwn06 strain of PRRSV (Guo et al., 2013a and 2013b). To prepare the inocula, *S. suis* and *A. suis* were cultured on sheep's blood agar (BA) plates and *H. parasuis* was cultured on Casman's agar (CAS) supplemented with 5% horse serum and 1% nicotinamide adenine dinucleotide (NAD) for 24 hours. A culture suspension was prepared in phosphate buffered saline (PBS), and pigs were inoculated by the intranasal route (3 ml total volume, 1.5 ml/nare) with 10^6 colony forming units

(CFU) of each bacterium per pig.

2.3. Experimental design. Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the NADC Institutional Animal Care and Use Committee. Ninety-five piglets were blocked by litter and equally assigned to 1 of 10 treatment groups (see Table 1). At 6 weeks-of-age, half of the groups were inoculated intranasally with the bacterial cocktail to allow for colonization. One week later one bacterial inoculated group and one non-bacterial inoculated group was challenged intranasally with one of the designated strain of PRRSV. Four pigs from the bacterial inoculated groups and 5 pigs from the viral only infected groups and non-infected control group were euthanized 4 days following PRRSV challenge; the other 5 pigs from all groups were euthanized 10 days following PRRSV challenge.

Severity of disease was evaluated by 1) comparing mortality rates; 2) recording and comparing febrile response by taking rectal temperatures daily ; 3) comparing the reduction in weight gains by recording body weights on days -7, 0, 4 and 10 relative to PRRSV challenge; 4) comparing the occurrence and frequency of clinical signs by recording daily the number of pigs exhibiting general signs (lethargy/depression, anorexia), respiratory signs (increased respiration rate, thumping, coughing, sneezing, or cyanosis), signs of bacterial dissemination (lameness, and neurologic signs); 5) comparing the distribution, character, and severity of gross and microscopic lesions seen at necropsy including: recording percent of lung affected as well as character of lesion (mottled, consolidated, hemorrhage, pleuritis, etc.); recording other gross lesions; examining and recording microscopic lesions seen in samples of brain, tonsil, thymus, lymph node, lung, heart, spleen, liver, salivary gland, trachea, pancreas, gastrointestinal tract (stomach, small intestine , large intestine), bone marrow, and kidney.

Viral replication dynamics were determined by measuring viremia, nasal shedding, and viral titers in lung lavage. Blood samples were collected on days 0, 1, 2, 3, 4, 6, 8, and 10 relative to PRRSV challenge and lung lavage was collected at necropsy on days 4 and 10 post PRRSV challenge by instilling 50 ml of PBS into the lung through the distal trachea followed by aspirating fluid for collection. Nasal swabs were collected on days -7, 0, 4 and 10 relative to PRRSV challenge. The ability to predispose to secondary bacterial infection was determined by bacterial isolation from the lung and systemic samples. Bacterial isolation was performed on blood samples, nasal swabs, and lung lavage. At necropsy swabs were also collected from the meninges, joint, peritoneum, pleura, and pericardium for bacterial isolation. Alteration of the host innate immune system was evaluated by determining lymphocyte numbers in the peripheral blood and cytokine protein levels in the serum and lung lavage. To assess metabolic changes, serum chemistries were evaluated on blood samples taken on days 0, 2, 4, 6, 8, and 10 relative to PRRSV challenge.

2.4. Viremia and viral burden. Sera, nasal swabs, and lung lavage were aliquoted and stored at -80 °C to evaluate viral presence, burden, and shedding. Virus isolation and titration was used to assess viral presence and load in the serum, nasal swabs, and lung lavage samples. Virus isolation was performed by adding 50 µl of serum or lung lavage to a monolayer of MARC-145 cells in 1 well of a 24 well plate. Each well was examined for cytopathic effect (CPE) and assessed as positive or negative daily for one week of culture. Titration was completed by preparing 10-fold dilutions of each positive sample and adding 50 µl of each dilution to 4 wells of a monolayer of MARC-145 cells in a 96 well plate. The Reed and Muench method was used to calculate the 50% end point titer (Reed and Muench, 1938). Results were converted to log₁₀ values for graphing and statistical analysis. To quantitatively assess viral load

in tissues, an RT-qPCR assay was performed to measure target genomic RNA levels for each PRRSV isolate in thymus, tracheobronchial lymph node, lung, tonsil, liver, kidney, and spleen samples as previously described (Spear and Faaberg, 2015).

2.5. Bacterial isolation. One hundred μ l of whole blood collected into EDTA tubes and lung lavage were plated onto BA and CAS plates to evaluate bacterial presence in the blood and lung. Swab samples taken from nasal and systemic sites at necropsy were collected into 1 ml PBS and, after vortexing, 100 μ l was cultured on both BA and CAS plates to evaluate bacterial colonization and dissemination. Bacteria that were isolated were identified based on colony morphology and confirmed by species specific PCR for *A. suis*, *H. parasuis*, and *S. suis*, or by 16S rRNA PCR and sequencing for unknown species as described previously (Guo et al., 2013a).

2.6. Multiplex cytokine assay. The levels of 9 porcine cytokines (pro-inflammatory cytokines TNF- α , IL-8, IL-1 β , IL-6, anti-inflammatory cytokine IL-10, antiviral cytokine IFN- α , and adaptive T-cell cytokines IL-2, IL-4, and IFN- γ) in serum and cell-free lung lavage were measured by SearchLight multiplex ELISA performed according to the manufacturer's recommendations (Aushon Biosystems, Billerica, MA). The average of duplicate samples for each sample was used for statistical analysis.

2.7. Gross and microscopic lesions. During macroscopic examination, the percentage of lung with viral interstitial pneumonia, characterized by multifocal to diffuse, tan to red mottled areas with irregular borders, was estimated and a score of 0-4 was given as follows: 0 = no interstitial lung lesions noted, 1 = 1-25% of the lung affected, 2 = 26-50% of the lung affected, 3 = 51-75% of the lung affected and 4 = 76-100% of the lung affected. In addition, an estimate of the percentage of lung with bacterial bronchopneumonia, characterized by plum colored

hepatization or abscess formation with clearly demarcated borders, was assigned on the basis of the percentage of each lung lobe affected and the percentage of total lung volume represented by each lobe. Percentage of total lung volume of each lobe was estimated as 10% for each cranial lobe, 10% for each middle lobe, 27.5% for each caudal lobe, and 5% for the intermediate lobe.

Samples of brain (medulla, cerebellum, thalamus, cerebrum), tonsil (palatine, pharyngeal), thymus, lymph nodes (tracheobronchial, mandibular, small and large intestinal mesenteric), lung, heart, spleen, liver, salivary gland, trachea, pancreas, gastrointestinal tract (stomach, small intestine, large intestine), bone marrow, and kidney were collected in buffered formalin and processed for routine histopathological examination.

2.8. Serum chemistries. Serum chemistries were run on a Vet Scan analyzer (Abaxis) using the comprehensive diagnostic profile rotor which provides complete chemistry and electrolyte analysis including alkaline phosphatase (ALP), alanine transaminase (ALT), amylase, total protein, albumin, globulin, total bilirubin, blood urea nitrogen (BUN), creatinine, glucose, calcium, phosphorus, sodium, and potassium.

2.9. Statistical analysis. Data were compared using a two-way analysis of variance with multiple comparisons at a significance level of $P < 0.05$ using GraphPad Prism software.

3. Results

3.1. Severity of clinical disease depends on the strain of PRRSV. No clinical signs were noted for non-infected control pigs or pigs infected with bacteria only, and mild clinical signs were noted for both groups infected with PRRSV VR-2332. Groups infected with the other 3 PRRSV isolates, both alone and coinfecting with bacteria, all developed significant clinical disease with pigs in these groups displaying anorexia, lethargy, fever, shivering, coughing and

labored breathing. The pigs infected with rJXwn06 were the most severely affected while the pigs infected with rSRV07 and SDSU73 were moderately affected. Vomiting and diarrhea was noted among pigs infected with rJXwn06 and rSRV07 and neurologic signs consisting of incoordination and lateral recumbency with paddling were observed in a pig coinfecting with rJXwn06 and bacteria. The pig displaying neurologic signs had to be euthanized on day 9 after viral challenge, but this was the only pig to succumb to disease during the experiment.

Non-infected control pigs and pigs infected with bacteria only did not have febrile responses (rectal temperature $\geq 40^{\circ}\text{C}$). The febrile response was mild in both groups of pigs infected with VR-2332, with about 60% of the pigs in these groups running low grade fevers (between 40.0 and 40.5°C) for 1 or 2 days. Mean rectal temperature for these groups only reached $\geq 40^{\circ}\text{C}$ on one day in the coinfecting group (Figure 1A). Mean rectal temperatures for groups infected with the other 3 isolates were $\geq 40^{\circ}\text{C}$ for multiple days during the 10 days after challenge that they were monitored (Figure 1A). There were minor differences among these groups but in general all pigs surviving to 10 days ran fevers lasting 4 to 9 days that reached above 40.5°C and often above 41°C . A few pigs in the groups challenged with rSRV07 or rJXwn06 had fevers that reached $\geq 41.5^{\circ}\text{C}$.

All groups infected with PRRSV had decreased average daily weight gains post infection compared to the non-infected control pigs. Average daily gains of 0.05 and 0.12 kg/day were recorded for the groups infected with VR-2332 and VR-2332/bacteria, respectively; 0.16 and 0.10 kg/day for the groups infected with SDSU73 and SDSU73/bacteria, respectively; 0.18 kg/day for both groups infected with rSRV07; and -0.10 and -0.07 kg/day for pigs infected with rJXwn06 and rJXwn06/bacteria, respectively. Non-infected control pigs and pigs colonized with the bacteria alone had average daily gains of 0.26 and 0.25 kg/day during this same time period.

There were no significant differences in the febrile response or weight gains whether or not the pigs were coinfecting with bacteria.

The main changes seen in serum chemistry profiles were a decrease in calcium, phosphorus, albumin and enzyme (alkaline phosphatase and aspartate aminotransferase) levels over time in virus infected pigs suggesting the pigs were in an anorectic and malnourished state (data not shown). There was also an increase in globulin levels over time which could be a result of inflammatory processes or from an increase in immunoglobulin production (data not shown). The magnitude of the changes tended to follow overall virulence with the greatest differences seen in pigs infected with rJXwn06 and very little difference seen in pigs infected with VR2332 compared to sham infected controls. No changes in sodium, potassium, blood urea nitrogen, creatinine, total bilirubin, or glucose levels were seen in any of the groups (data not shown).

3.2. Viremia and viral shedding was highest for pigs infected with JXwn06 and is not affected by bacterial coinfection. The serum virus titers were significantly higher in pigs challenged with rJXwn06, with 2 log higher peak average titers for these groups compared to groups infected with the other 3 strains of PRRSV (Figure 1B). Peak serum viral titers were not significantly different among the groups infected with VR-2332, SDSU73, or rSRV07 during the time frame measured. Viremia was detected and peaked later in pigs infected with rSRV07. No significant differences were found in the serum viral titers whether or not the pigs were coinfecting with bacteria.

Although mean viral titers in the lung lavage (BALF) tended to be highest for the groups infected with rJXwn06, the differences, in general, were not as extreme as those measured in the serum (Figure 1C). At 4 days-post-challenge (dpc) with PRRSV, titers for the groups infected with VR-2332, SDSU73, or rJXwn06 were not significantly different from each other, but all

(except the SDSU73/bacteria group) were significantly greater than the groups infected with rSRV07. At 10 dpc with PRRSV, the titer for groups infected with rJXwn06 was significantly greater than the titers for groups infected with VR-2332 or rSRV07, but not SDSU73, and the difference in titers for the groups infected with VR-2332, SDSU73, or rSRV07 were not significantly different from each other. There were no significant differences in the BALF viral titers whether or not the pigs were coinfecting with bacteria.

At 4 dpc with PRRSV, nasal shedding of virus was significantly higher in the rJXwn06 infected groups compared to the other PRRSV groups (Figure 1D). At 10 dpc with PRRSV, nasal virus shedding in the rSRV07 and rJXwn06 groups was significantly higher than the SDSU73 and VR-2332 challenged groups. In addition, 5% of the pigs infected with VR-2332, 32% of the pigs infected with SDSU73, 68% of the pigs infected with rSRV07, and 95% of the pigs infected with rJXwn06 had nasal swabs that were positive by virus isolation for PRRSV on either day 4 or 10 post challenge. There were no significant differences in the nasal viral titers whether or not the pigs were coinfecting with bacteria.

To quantitatively measure viral load, a RT-qPCR assay was performed to evaluate viral genomic RNA levels in serum and tissue samples of thymus, tracheobronchial lymph node, lung, tonsil, liver, kidney, and spleen from the viral only infected groups on the days of necropsy (Figure S2). Viral copy number detected in the serum and lung tissue was consistent with results obtained with virus titration of the serum and BALF. For the other tissues tested, viral copy numbers were not drastically different among groups and there was no trend for higher viral load any particular group.

3.3. PRRSV infection causes interstitial pneumonia and predisposes to secondary bacterial bronchopneumonia in a strain dependent manner. Gross lung lesions consistent with PRRSV infection were seen in all groups infected with the virus. At day 4 post challenge with

PRRSV, the viral associated lesions were mild and there were no differences in severity among the groups (Figure 2A). At day 10 post challenge with PRRSV, gross viral lung lesions were most severe for the groups infected with rJXwn06 and SDSU73 and affected significantly greater lung area, on average, than lung lesions in the VR-2332 and rSRV07 groups (Figure 2A). There were no significant differences in gross viral lung lesion scores whether or not the pigs were coinfecting with bacteria.

Gross lesions consistent with bacterial pneumonia, including plum-colored, well-demarcated consolidation and abscess formation, were seen in all the groups infected with bacteria. The only significant difference in the percent lung affected by lesions consistent with bacterial pneumonia among the groups was at 10 days post challenge with PRRSV where the group coinfecting with rJXwn06 and bacteria had significantly greater lung involvement than all the other groups (Figure 2B).

Microscopic lung lesions seen on day 4 in groups infected with PRRSV alone consisted of mild interstitial pneumonia consisting of alveolar septal thickening due to infiltrates of lymphocytes and macrophages, type II pneumocyte hyperplasia, and alveolar ectasia. In addition to these lesions, the lungs of some of the pigs in groups infected with both PRRSV and bacteria had evidence of bronchopneumonia with alveoli and larger airways containing neutrophils and/or alveolar necrosis with neutrophilic infiltrates and bacterial colonies often present (abscess formation) consistent with bacterial pneumonia and these lesions were most pronounced in pigs from which bacteria were isolated from the lung lavage. There were no microscopic lesions seen in the lungs of pigs infected with bacteria alone at this time point.

Microscopic lung lesions were more extensive and severe by day 10 post infection with PRRSV. There was moderate to severe interstitial pneumonia with septal infiltrates of

lymphocytes and macrophages, type II hyperplasia, and alveolar ectasia in all the groups infected with virus only and alveoli containing fibrin and necrotic debris in the groups infected with rJXwn06 or SDSU73 alone (Figure 3A-D). The severity of the microscopic lung lesions for these groups generally followed the order of rJXwn06 $\geq$ SDSU73 > VR-2332 $\geq$ rSRV07. Similar to day 4 post PRRSV infection, there were pigs in all coinfecting groups that, in addition to the preceding lesions, had purulent bronchopneumonia and/or pulmonary abscess formation consistent with bacterial pneumonia and again these lesions were most pronounced in pigs from which bacteria were isolated from the lung lavage (Figure 3E-H). One of the five pigs in the group infected with bacteria alone also had lesions of purulent bronchopneumonia and pulmonary abscesses.

No A. suis, S. suis, and/or H. parasuis was isolated from the nasal swabs taken from the pigs prior to bacterial challenge (day -7). All three bacterial species were isolated from nasal swabs taken from pigs challenged with bacteria on days 0, 4, or 10 of the experiment indicating pigs had been colonized with the bacteria, while nasal swabs taken on these days from the pigs not challenged with bacteria remained negative for these three species. *A. suis, S. suis, and/or H. parasuis* were cultured from the lung of 1/9 pigs from the group challenged with the bacteria alone, from 2/9 pigs challenged with VR-2332/bacteria, from 3/9 challenged with SDSU73/bacteria, and from 5/9 pigs challenged with rSVR07/bacteria and rJXwn06/bacteria (Table 2). *A. suis, S. suis, and/or H. parasuis* were not isolated from the lung of any of the non-challenged control pigs or any of the pigs challenged with virus alone. *H. parasuis* and *S. suis* were also isolated from the brain of 1 pig from the group coinfecting with rJXwn06 and bacteria. In addition to these 3 bacterial species, a few other bacterial species were isolated from the lungs of pigs. Specifically, *Actinomyces hyovaginalis* was isolated in combination with *A. suis* in 5 of

the 6 pigs from which *A. suis* was isolated. *Escherichia coli* was isolated from 3 coinfecting pigs, one that was infected with rSRV07 and two that were infected with SDSU73, all three isolations were in combination with either *A. suis*, *H. parasuis*, or *S. suis*. *Trueperella pyogenes* was isolated from the lung of one rSRV07 coinfecting pig in combination with *A. suis* and *S. suis*. Very few bacterial species were isolated from the BALF of pigs infected with virus alone; *E. coli* was isolated from the lung of one pig challenged with rJXwn06, *T. pyogenes* was isolated from one pig challenged with rSRV07, and a *Lactobacillus spp.* was isolated from one pig infected with VR-2332.

3.4. PRRSV induces increased levels of proinflammatory and adaptive cytokines in a strain dependent manner that is mitigated by bacterial coinfection. An increase in the quantity of both proinflammatory and adaptive cytokines was detected in the serum of PRRSV infected pigs the magnitude of which, for the most part, followed the overall virulence, with highest levels detected in pigs infected with rJXwn06 and lowest levels in pigs infected with VR-2332 (Figure 4). The rise in many of the cytokines occurred on or after day 6 post viral challenge, however IFN α peaked between days 2-4 and there were biphasic peaks with a lower magnitude early peak around days 2-3 for some of the cytokines, such as IFN γ , IL-10 and IL-4. Interestingly, serum cytokine levels were often lower for pigs coinfecting with virus and bacteria compared to pigs infected with PRRSV alone which was particularly pronounced for pigs infected with rJXwn06. There was a significant decrease in peak levels of IFN α , IL-1 β , and TNF α in pigs coinfecting with rJXwn06 and bacteria compared to rJXwn06 alone and that trend appeared in all groups for IFN α (Figure 4).

In general, levels of both proinflammatory and adaptive cytokines in the lung lavage (BALF) of pigs infected with PRRSV were highest, on average, for pigs infected with rJXwn06

and lowest for pigs infected with VR-2332 (Figure 5). IL-1 β , TNF α , IL-6, IL-10, IFN γ , and IL-2 were all significantly elevated in the BALF of pigs infected with rJXwn06 compared to sham inoculated animals, and IL-1 β , IL-8, IFN α , and IFN γ were all significantly elevated in the BALF of pigs infected with SDSU73 compared to sham inoculated animals. Although cytokine levels in the BALF of pigs infected with VR2332 or rSRV07 were often higher than those of sham inoculated animals, at no time were the differences statistically significant. There was a trend for cytokine levels in the BALF to be higher in pigs coinfecting with bacteria and virus in the groups infected with VR2332 and SDSU73, although none of these differences were significant (data not shown). There was no such trend for the pigs coinfecting with bacteria and virus in the groups infected with rSRV07 or rJXwn06. Cytokine levels in the BALF of coinfecting pigs in these two groups were either similar to virus only infected groups, or for the cytokines IL-1 β and TNF α were significantly lower in coinfecting pigs infected with rJXwn06 (data not shown).

3.5. The JXwn06 strain of PRRSV induces extensive microscopic lesions including thymic atrophy, encephalitis and evidence of widespread apoptosis in the tissues. Thymic atrophy was observed in all groups infected with PRRSV, but it was most pronounced for pigs infected with rJXwn06. By day 4 post infection with PRRSV, there were already microscopic thymic lesions in both groups infected with rJXwn06 in about half the pigs, mainly consisting of multiple single cell apoptosis. By day 10 post infection with PRRSV, the thymus of all pigs infected with rJXwn06 was hypocellular with lack of clear delineation of cortex and medulla and myriad apoptotic bodies (Figure 3I-L). The thymi of pigs in other PRRSV infected groups had some of the same changes but the lesions were typically not as extensive or severe and not affecting as many animals. The severity of microscopic thymic lesions for the different groups in general

followed the order rJXwn06 > rSRV07 > SDSU73 > VR-2332. There were no thymic lesions seen in noninfected control pigs or pigs infected with bacteria alone.

In addition to the thymic atrophy, there were extensive microscopic lesions in the brain in pigs infected with rJXwn06 on day 10 post infection. The majority of these pigs had lesions of encephalitis and meningitis with perivascular inflammatory cell infiltrates and single cell to extensive apoptosis throughout all sections of brain taken (medulla, cerebellum, thalamus, and cerebrum) (Figure 3O). To a lesser extent similar brain lesions were also seen in SDSU73 and rSRV07 infected pigs but the lesions had a more limited distribution, were less severe, and fewer pigs were affected (Figure 3M and N). No lesions were seen in the brain sections from noninfected pigs, pigs infected with bacteria alone, or pigs infected with VR-2332. The other notable microscopic findings in pigs infected with rJXwn06 beyond what has already been mentioned was the presence of renal hemorrhage and the presence of apoptotic bodies in nearly all tissues examined, a feature not seen in the other groups.

4. Discussion

The current study, as well as others, indicates that the HP-PRRSV strains out of Asia are highly virulent; however, the inclusion of the higher virulent U.S. strain SDSU73 demonstrates that the magnitude of difference in virulence among some of the Asian HP-PRRSV strains and U.S. strains are not as great as when compared to VR-2332. Although rSRV07 is still relatively virulent compared to strains such as VR-2332, these results may indicate that HP-PRRSV isolates in Asia have attenuated with time and are on par with higher pathogenic U.S. strains such as SDSU73, which itself is a strain that was isolated in association with outbreaks of higher morbidity/mortality known at the time as “atypical” or “acute severe” PRRSV in the US in the

late 1990s (Mengeling et al., 1998). This cycle of emergence of PRRSV strains with increased virulence has occurred on several occasions including in Eastern Europe with Type 1 lineages and again recently with Type 2 strains in the U.S. suggesting these viruses continue to evolve and the need for a better understanding of the pathogenesis of this virus (Karniychuk et al., 2010; Wang et al., 2015).

Mortality and the incidence of secondary bacterial infections in pigs infected with rJXwn06 reported here was less than what was seen in previous experiments (Guo et al. 2013a and 2013b; Lager et al., 2014; Schlink et al., 2016). One of the main differences was the use of early weaned pigs that were farrowed at NADC and kept in isolation rooms in the current study compared to the use of weaned pigs from commercial herds in the previous studies. Pathogenic bacterial burden, stress of weaning and transport, and mixing of pigs probably contributed to the development of secondary bacterial infections in previous experiments. Coinfection with PRRSV and *A. suis*, *S. suis* and *H. parasuis* did increase the frequency of bacterial isolation and severity of gross and microscopic lesions associated with these secondary bacterial infections in a strain dependent manner. Thus secondary infections do appear to play a role in the severity of disease seen with these HP-PRRSV isolates.

Age appears to be another factor that influenced mortality rates with rJXwn06. In previous experiments infecting 4 week old pigs there was 100% mortality beginning as early as 4 days post challenge, whereas in 10 week old pigs there was mortality of 33-45% which occurred between 7 to 14 days (Guo et al. 2013a and 2013b; Lager et al., 2014; Schlink et al., 2016). Pigs in the current experiment were 7 weeks old when challenged with PRRSV, and this probably played a role in the reduced mortality as well. In addition, the experiment was terminated at day 10, thus extrapolating from prior experiments more deaths may have occurred had the

experiment continued longer. Based on experimental results to date, severity of disease and mortality rates appear to be dependent on virulence of the PRRSV strain, rate of secondary infection, and age of the pig.

One of the biggest differences among the viruses is in the viremic load that results from infection with rJXwn06, which was about 2 log-folds higher than the other viruses. This may reflect broader tropism and/or cellular production of virus. Previously it was demonstrated that the number of virus positive cells in the lungs of rJXwn06 infected pigs was no greater and was limited to alveolar and interstitial macrophages (Guo et al., 2013a). The difference in viral load in the lung and other tissues tested in the current experiment was not great. However, some evidence indicates that PRRSV strains with higher virulence may infect a broader population of cells of the monocyte-macrophage lineage (Morgan et al., 2014; Frydas et al., 2015) and there was greater distribution of lesions in tissues of pigs infected with rJXwn06. The rJXwn06 virus has been shown to replicate to higher titers in MARC cells as well (Guo et al., 2013b). Thus the cumulative effect of increased viral production and an overall greater distribution of macrophage type cells infected throughout the animal may combine to explain the higher titers of virus found in the serum. One of the sites in particular of increased replication of PRRSV strains with increased virulence may be in the upper respiratory tract of pigs (Frydas et al., 2013; Frydas and Nauwynck, 2016). There was an increased frequency and quantity of nasal virus shedding in pigs infected with the Asian HP-PRRSV isolates in the present study and that may indicate that these strains have an increased transmissibility as well.

The replication dynamics with the rSRV07 virus were slightly different from the other viruses in that there was a delay in viral detection and the magnitude of viremia was still rising by the end of the experiment, which may explain some of the differences seen when comparing

this group to the others especially at early time points. The impression from this and other experiments with the rSRV07 virus, which have all terminated by 10-14 days post challenge, is that the clinical disease may not have peaked (Guo et al., 2013b; Lager et al., 2014). The replication dynamics of this virus in MARC cell culture are not significantly different from that of rJXwn06 or VR-2332 other than peak titers (Guo et al., 2013b). However since infectious virus was derived from an infectious clone generated from viral RNA it is possible that an introduction of a genetic change that resulted in altered *in vivo* growth characteristics occurred and that over time the virus was adapting in the host. Future swine infection studies using the pig-adapted virus must be performed in order to determine it has enhanced viral replication *in vivo*.

Bacterial infection did not appear to alter the dynamics of the viral infection; however, cytokine levels in the serum were typically lower in pigs coinfecting with PRRSV and bacteria. Contrary to the results reported here, previous studies have shown an increase in the production of proinflammatory cytokines in pigs coinfecting with PRRSV and bacteria (Thanawongnuwech et al., 2004; Van Gucht et al., 2004). Several explanations may elucidate the apparent contrasting results between these reports and the present study. The experimental designs were different with regard to PRRSV strains, bacterial species, and timing of infection. In the previous studies bacterial (or LPS) exposure was either simultaneous or subsequent to PRRSV infection, which could lead to different dynamic with regards to cell signaling and cytokine production. The previous studies only looked at cytokine levels in the BALF, not serum, which is where we saw a general decrease in cytokine levels in coinfecting pigs. In fact, there was a trend toward an increased production of cytokines in the BALF of coinfecting animals given VR2332 or SDSU73,

while coinfecting pigs given rSRV07 and rJXwn06 had similar or decreased levels of cytokines in the BALF, thus this may reflect a unique response to the HP-PRRSV strains

The mechanism by which pre-exposure to the bacteria altered the magnitude of the cytokine response, or whether one species or some combination was responsible for this effect is not clear at this time. However, all three of these bacteria can reside as commensal members of the respiratory microbiota and the microbiota in turn has been shown to influence the immune response to viral infections both positively and negatively (Karst, 2016). In one example bacteria of the intestinal microbiota were shown to suppress type I interferon production in response to viral infection (Baldridge et al., 2015). Exposure to *Streptococcus pneumoniae* has been shown to suppress inflammatory responses associated with allergic airway disease as well (Preston et al., 2011).

In addition to lung lesions, the severity of lesions seen in the thymus and a wider distribution of lesions with evidence of cell death (apoptotic bodies or nuclear remnants) was associated with increased virulence of the PRRSV strains. Studies have shown an induction of apoptosis and necrosis in PRRSV infected cells (Miller and Fox, 2004; Sur et al., 1997; Gomez-Laguna et al, 2013; Costers et al., 2008; Kim et al. 2002). Although the viral load in these tissues does not appear to be greater for the HP-PRRSV strains cell death has also been reported in non-infected bystander cells and increased virulence could be due to an increase in this bystander effect leading to greater cell death (Sirinarumitr et al., 1998; Sur et al., 1998; Labarque et al., 2003). An increase in cell death has been associated with the production of apoptogenic cytokines such as TNF α IL-1, and IL-10 which were elevated to a greater extent in the HP-PRRSV infected pigs (Labarque et al., 2003; Choi and Chae, 2002). Thymic atrophy, especially with HP-PRRSV strains, has been reported previously and may also be associated with increased

bystander cell death (Wang et al., 2015). The degree of immune cell death may be a cause of immunosuppression and increased susceptibility to secondary bacterial infections that appears to rise with enhanced virulence. The increased severity of brain lesions (encephalitis and meningitis) associated with the higher pathogenic PRRSV strains could also explain the worsening of clinical signs including fever, lethargy, vomiting and increased mortality as well.

PRRSV virulence is a multifactorial and complicated issue and has yet to be definitively associated with specific genetic changes in the virus or different pathogenic mechanisms induced in the pig. The virus is clearly capable of altering the innate and adaptive immune responses in its favor leading to success in maintaining infection in the host and increasing the potential for secondary infection. However management factors such as managing bacterial burden, age at the time of exposure, and minimizing stress can play an important role in the outcome of infection. Continued studies to clarify the pathogenesis and virulence of this very adaptive virus are needed to improve the potential for successful control of disease.

Acknowledgements/Conflict of interest statement

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Figure 1: Febrile response (A) and virus titers in the serum (B), BALF (C), and nasal swabs (D), of groups of pigs infected with PRRSV VR-2332, SDSU73, rSRV07, rJXwn06, or sham inoculated with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis* (Bact). Data are expressed as the mean $\pm$ SEM. Rectal temperatures and serum titers are based on 9 or 10 pigs through day 4 and 5 pigs on days 5-10. BALF and nasal swab titers are based on 4 or 5 pigs on day 4 and 5 pigs on day 10. Statistically significant differences in virus titer were detected in the serum of rJXwn06 challenged pigs compared to the other groups, mean viral titers for BALF and nasal swabs with different letter superscripts are statistically different $p \leq 0.05$.

Figure 2: Gross viral lung lesion scores (A) and percentage of lung affected by bacterial lung lesion (B) of groups of pigs infected with PRRSV VR-2332, SDSU73, rSRV07, or rJXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis* (Bact). Data are expressed as the mean $\pm$ SEM based on 4 or 5 pigs on day 4 and 5 pigs on day 10, and different letter superscripts are statistically different $p \leq 0.05$.

Figure 3: Photomicrographs of lungs from a pigs infected with VR-2332 (A), SDSU73 (B), rSRV07 (C), or rJXwn06 (D) strains of PRRSV 10 days post challenge. Note interstitial pneumonia in all the groups and alveoli containing neutrophils, fibrin, and necrotic debris in the groups infected with rJXwn06 or SDSU73. Photomicrographs of lungs from a pigs coinfectd with VR-2332 (E), SDSU73 (F), rSRV07 (G), or rJXwn06 (H) strains of PRRSV and *H. parasuis*, *S. suis*, and *A. suis* showing abscess formation and purulent bronchopneumonia. Photomicrographs of thymi from pigs infected with VR-2332 (I), SDSU73 (J), rSRV07 (K), or rJXwn06 (L) strains of PRRSV 10 days post challenge. Note loss of cortex with increasing

virulence of the virus and myriad apoptotic bodies (nuclear remnants) from dead cells of pig infected with the rJXwn06 strain of PRRSV (insert L). Photomicrographs of cerebra from a pigs infected with SDSU73 (M), rSRV07 (N), or rJXwn06 (O) strains of PRRSV 10 days post challenge. Note encephalitis with perivascular infiltrates of mononuclear cells in all and apoptotic cell bodies especially in B and C. Magnifications 40X for A, C, I, K; 100X for D, E, G, J, L; 200X for B, F, H, N, O; and 400X for M.

Figure 4: Mean proinflammatory and adaptive cytokine levels in the serum of groups of pigs infected with PRRSV VR-2332, SDSU73, rSRV07, rJXwn06, or sham inoculated with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis* (Bact).. Data are expressed as the mean $\pm$ SEM of the 5 pigs in each group necropsied at day 10. * indicates statistically significant differences in peak levels($p \leq 0.05$) as follows: rJXwn06 statistically different from all other groups for IL-1 β , TNF α , and IL-10; rJXwn06 statistically different from sham and VR-2332 groups for IFN γ and IL-4.

Figure 5: Mean cytokine levels in the lung lavage (BALF) of groups of pigs infected with PRRSV VR-2332, SDSU73, rSRV07, rJXwn06, or sham inoculated controls. Data are expressed as the mean $\pm$ SEM of 5 pigs on days 4 and 10 and different letter superscripts denote statistical differences $p \leq 0.05$.

Table 1. Experimental groups.

Group	PRRSV	Bacteria	Number
1	rJXwn06	None	10
2	rJXwn06	Ss, Hp, As	9
3	rSRV07	None	10
4	rSRV07	Ss, Hp, As	9
5	SDSU73	None	10
6	SDSU73	Ss, Hp, As	9
7	VR-2332	None	10
8	VR-2332	Ss, Hp, As	9
9	None	Ss, Hp, As	9
10	None	None	10

Ss = *Streptococcus suis*; Hp = *Haemophilus parasuis*; As = *Actinobacillus suis*.

Table 2: Bacterial isolation from the BALF.

Group	Number of pigs bacteria isolated from/ Number pigs in group	Ss	Hp	As
Bact	1/9	0	0	1
VR-2332/Bact	2/9	0	0	2
SDSU73/Bact	3/9	1	2	1
rSRV07/Bact	5/9	1	3	2
rJXwn06/Bact	5/9	1	5	0

Ss = *Streptococcus suis*; Hp = *Haemophilus parasuis*; As = *Actinobacillus suis*.