RESEARCH REPORT
SWINE HEALTH

Title: Comparison of porcine high fever disease isolates of PRRSV to US isolates for their ability to cause secondary bacterial infection in swine - **NPB #11-119**

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Industry Summary

The appearance of highly-pathogenic porcine reproductive and respiratory syndrome virus (PRRSV) isolates in Asia necessitates investigation into the clinical repercussions of these viruses if the strains were to appear in the US. Epidemiologic data from Asian outbreaks suggest that disease severity was associated with both the PRRSV isolates from these cases and secondary bacterial infections. Previous reports have indicated that US isolates of PRRSV predispose to secondary bacterial infections as well, but outbreaks like the ones described in Asia have not been reported in the US. The objectives of this research were to compare the pathogenesis of Asian and US PRRSV isolates with regard to their ability to cause disease and predispose to secondary bacterial infections in swine. The experiment consisted of 10 groups of 9-10 pigs each. At 6 weeks of age, half the groups were inoculated with a bacterial cocktail of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis* and 1 week later 4 bacterial colonized groups and 4 non-bacterial colonized groups were inoculated with 1 of 2 Asian HP-PRRSV strains (JXwn06 or SRV07) or 1 of 2 US PRRSV strains (SDSU73 or VR2332). JXwn06 was isolated during the initial outbreaks of Porcine High Fever Disease in China in 2006, whereas SRV07 was isolated after the disease had spread to Vietnam in 2007.

The HP-PRRSV strain JXwn06 caused severe disease compared to the North American prototype strain VR2332, which caused the least disease of the isolates tested. A virus like JXwn06 could be devastating to the swine industry in the US, causing severe disease and mortality, whether entering from a foreign country or emerging from similar evolution of endemic viruses in the US. Disease caused by the Vietnamese strain SRV07 and the US strain SDSU73 fell somewhere between that of VR2332 and JXwn06. Although SRV07 is still much more potent than strains such as VR2332, these results may indicate that HP-PRRSV isolates in Asia have attenuated to some degree with time and are on par with higher pathogenic US strains such as SDSU73, which itself 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.

Results indicate that higher amounts of virus were detected in pigs infected with the JXwn06 strain of PRSSV possibly indicating broader replication and dissemination of this virus. The presence of more extensive and disseminated lesions, such as encephalitis (brain inflammation) may explain the increased severity of disease. The increased frequency and quantity of nasal virus shedding of both Asian PRRSV strains suggest they are more transmissible as well. Serum chemistries did not indicate any major organ malfunction was responsible for the severe clinical signs, although they did suggest the pigs were in a severe malnourished state. Pigs infected with JXwn06 also displayed greater measures of immune dysfunction and as a result the frequency and severity of secondary bacterial infections increased with the increasing virulence of PRRSV. Thus secondary infections do appear to play a role in the severity of disease seen with

these HP-PRRSV isolates. Furthermore, age influences mortality rates with JXwn06, as mortality in 4 week old pigs was greater than in 7 and 10 week old pigs. 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. The next step is to determine whether intervention methods such as vaccination, antibiotic treatment, or immunomodulators can diminish the devastating effects of these viruses.

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Scientific Abstract

The appearance of highly-pathogenic porcine reproductive and respiratory syndrome virus (PRRSV) isolates in Asia necessitates investigation into the clinical repercussions of these viruses if the strains were to appear in the US. Epidemiologic data from Asian outbreaks suggest that disease severity was associated with both the PRRSV isolates from these cases and secondary bacterial infections. Previous reports have indicated that US isolates of PRRSV predispose to secondary bacterial infections as well, but outbreaks like the ones described in Asia have not been reported in the US. The objectives of this research were to compare the pathogenesis of Asian and US PRRSV isolates of varying virulence with regard to their ability to cause disease and predispose to secondary bacterial infections in swine. The experiment consisted of 10 groups of 9-10 pigs each. At 6 weeks of age, half the groups were inoculated with a bacterial cocktail of *Streptococcus suis*, *Haemophilus parasuis*, and *Actinobacillus suis* and 1 week later 4 bacterial colonized groups and 4 non-bacterial colonized groups were inoculated with 1 of 2 Asian HP-PRRSV strains (JXwn06 or SRV07) or 1 of 2 US PRRSV strains (SDSU73 or VR2332). The pigs infected with JXwn06 were clinically the most severely affected (based on clinical signs, febrile response, and weight gain) while the pigs infected with SRV07 and SDSU73 were moderately affected, and pigs infected with VR2332 showed minimal clinical signs. One pig coinfected with JXwn06 and bacteria became moribund and was euthanized. An increase in the levels of proinflammatory cytokines in the sera occurred, in general, around day 6-8 post viral infection with the magnitude of increase generally correlating with the severity of clinical disease. The highest viral titers were detected in pigs challenged with JXwn06. *S. suis*, *A. suis* and/or *H. parasuis* was cultured from the lungs of 1/9 pigs from group challenged with the bacteria alone, 2/9 pigs challenged with VR2332/bacteria, 3/9 pigs challenged with SDSU73/bacteria, and from 5/9 pigs challenged with SVR07/bacteria and JXwn06/bacteria. These bacteria were not isolated from the non-challenged control pigs or pigs challenged with virus alone. Lesions consistent with bacterial pneumonia, including abscesses, were seen in the groups coinfected with PRRSV and bacteria. The levels of proinflammatory cytokines in the serum were often lower for pigs coinfected with virus and bacteria compared to pigs infected with PRRSV alone indicating an alteration in the immune response in coinfected pigs. There was a range of virulence among the PRRSV isolates and differences in their ability to predispose to secondary bacterial infection.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a widely disseminated virus of swine that was first recognized as acute outbreaks of reproductive failure in the late 1980s. PRRSV is a member of the genus *Arterivirus* in the family *Arteriviridae*, made up of linear positive-sense, single-stranded RNA. The hallmark of this virus family is its ability to undergo high frequency viral recombination, which leads to remarkable evolution and extraordinarily diverse

viruses. PRRSV is divided into Type 1 (European-like; prototype strain Lelystad) and Type 2 (North American-like; prototype strain VR-2332) which vary in nucleotide sequence by approximately 40%. Within each genotype, nucleotide variation of over 20% is seen between isolates. It is important to remember that considerable genetic, antigenic and virulence differences exist among PRRSV isolates. Depending on strain, dose and immune status, some farms may be subclinically infected with PRRSV while others experience severe reproductive and/or respiratory disease. 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. The disease is associated with newly-emergent, highly-pathogenic PRRSV strains. Bacteria and other viruses 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 as a major factor.

Following oronasal exposure, PRRSV replicates in macrophages in tonsils, upper respiratory tract, and lungs resulting in viremia by 6-12 hours post infection. Isolation of PRRSV from oropharyngeal samples for up to 157 days after experimental intranasal inoculation of conventional pigs provides evidence of persistent infection of growing pigs. 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. Infection of macrophages by PRRSV is thought to have a profound impact on the respiratory immune system of the pig. PRRSV-induced damage to pulmonary intravascular macrophages and pulmonary alveolar macrophages is believed to result in increased susceptibility to bacterial pneumonia and septicemia. Experimentally, PRRSV infected pigs showed an increased septicemia and mortality when challenged with *Streptococcus suis* and increased pulmonary infections with *Bordetella bronchiseptica*. There is also evidence of PRRSV interaction with respiratory viruses such as porcine circovirus and swine influenza virus, and alteration in the typical disease response to pathogens such as *Haemophilus parasuis*.

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. 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. *S. suis*, was often isolated from clinical cases of the PHFD outbreaks in Asia.

Epidemiologic data from Asian PHFD outbreaks suggest that disease severity was associated with both the PRRSV isolates from these cases and secondary bacterial infections. Previous reports have indicated that US isolates of PRRSV predispose to secondary bacterial infections as well, but outbreaks like the ones described in Asia have not been reported in the US. Our preliminary data indicate that the Chinese strain of PRRSV, in particular, may have a greater capacity to predispose to secondary bacterial infection, but the disease caused by the US isolate (VR2332) that was used in this study is fairly mild, so a more fair comparison would be to use a US isolate that has been shown to be more pathogenic. A controlled coinfection study with cleaner pigs will help answer the question of whether there is a difference in the virulence of these PRRSV isolates and their ability to predispose to secondary bacterial disease.

Objectives

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 US swine.

Research priorities to be addressed include (1) delineate differences between Asian and US isolates with regard to the severity of disease and alterations in the host innate immune response in pigs with no secondary infection (virus only infected groups); (2) determine if Asian and US isolates equally predispose to secondary bacterial infections (coinfected groups); (3) what is the contribution of secondary bacterial infection to the morbidity/mortality associated with PHFD (comparing single to coinfected groups); (4) what are the mechanisms by which PRRSV predisposes to secondary infection (comparing alterations in the innate immune response to viruses with varying ability to cause secondary infection).

Materials & Methods

Piglets. Ten sows seronegative for PRRSV were purchased from a high-health herd and transported to NADC about 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.

PRRSV and bacterial isolates. Four isolates of PRRSV were used, 2 U.S. isolates – VR2332, the type 2 North American prototype, 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, and 2 Asian strains - a Chinese isolate rJXwn06 that was rescued from a full-length cDNA of the JXwn06 virus and rSRV07 rescued from a full-length cDNA of a 2007 strain detected in the Socialist Republic of Vietnam (SRV07), both from cases of PHFD. Virus was propogated on MARC-145 cells (VR2332- passage 6, SDSU73- passage 3, JXwn06- passage 4, and SRV07- passage 4) and pigs were challenged with 10^4 $CCID_{50}$ per pig by the intranasal route. A sham inoculum was prepared from virus-free MARC-145 cells. A cocktail of *Streptococcus suis* (Ss), *Haemophilus parasuis* (Hp), and *Actinobacillus suis* (As) was used to colonize the pigs 1 week prior to PRRSV challenge. All three bacterial isolates were obtained from the lung lavage of pigs from a previous experiment using the JXwn06 strain of PRRSV. To prepare the inocula, Ss and As were cultured on bovine blood agar (BA) plates and Hp was cultured on Casman's agar (CAS) supplemented with 5% horse serum and 1% nicotinamide adenine dinucleotide for 24 hours. A culture suspension was prepared in phosphate buffered saline (PBS), and pigs were inoculated by the intranasal route with 10^6 CFU of each bacteria per pig.

Experimental design. Ninety-five piglets were blocked by litter and equally assigned to 1 of 10 treatment groups (see Table 1). At 6 weeks of age, pigs were inoculated with the bacterial cocktail to allow for colonization and 1 week later inoculated with the designated strain of PRRSV. Four pigs from the coinfected groups and 5 pigs from the viral only infected groups were euthanized 4 days following PRRSV challenge, the other 5 pigs from all groups were euthanized 10 days following PRRSV challenge.

We evaluated severity of disease 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 that were collected in buffered formalin and processed for routine histopathological examination.

We assessed viral replication dynamics 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. Nasal swabs were collected on days -7, 0, 4 and 10 relative to PRRSV challenge. Sera, nasal swabs, and lung lavage were aliquoted and stored at -80 to evaluate viral presence, burden, and shedding. Virus isolation and titration were used to assess viral presence and load.

Table 1: Experimental Outline

Group	PRRSV	Bacteria	Number
1	JXwn06	None	10
2	JXwn06	Ss, Hp, As	9
3	SRV07	None	10
4	SRV07	Ss, Hp, As	9
5	SDSU73	None	10
6	SDSU73	Ss, Hp, As	9
7	VR2332	None	10
8	VR2332	Ss, Hp, As	9
9	None	Ss, Hp, As	9
10	None	None	10

The ability to predispose to secondary bacterial infection was determined by isolation rates of bacteria from the lung and systemic dissemination. Blood samples to detect bacteremia, nasal swabs, and lung lavage were collected as described above. At necropsy swabs were also collected from the meninges, joint, peritoneum, pleura, and pericardium for bacterial isolation. Whole blood was collected into EDTA tubes and 100 µl plated onto both BA and CAS plates to evaluate bacteremia. Swab samples taken from systemic sites at necropsy were collected into 1 ml PBS and, after vortexing, 100 µl was cultured on both BA and CAS plates to

evaluate bacterial dissemination. Bacterial culture of lung lavage was also performed by plating 100 µl on both BA and CAS plates.

Alteration of the host innate immune system was evaluated by determining complete blood cell count (CBC) and serum cytokine protein levels from the blood samples taken. Lung lavage was collected for cytokine protein levels and flow cytometry to analyze cell populations. Thymus and tracheal bronchial lymph nodes were taken for flow cytometric analysis. The levels of 7 porcine cytokines (TNF-α, IL-8, IL-1β, IL-6, IL-10, IFN-α and IFN-γ) in serum and lung lavage were measured by multiplex ELISA (SearchLight, Aushon Biosystems). To evaluate cell types phenotypic analysis by flow cytometry was performed. Cells were collected and stained with antibodies to granulocytes (PG68A), lymphocytes (PG106A) and macrophages (2A10/11) to determine the percentage of each cell population.

To assess metabolic changes, serum chemistries were run on blood samples taken on days 0, 1, 2, 3, 4, 6, 8, and 10 relative to PRRSV challenge.

Results

Clinical signs: 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 VR2332. Groups infected with the other 3 PRRSV isolates all developed significant clinical disease with pigs in these groups displaying anorexia, lethargy, fever, shivering, coughing and labored breathing. The pigs infected with JXwn06 were the most severely affected while the pigs infected with SRV07 and SDSU73 were moderately affected. Vomiting and diarrhea was noted among pigs infected with JXwn06 and SRV07 and neurologic signs consisting of incoordination and lateral recumbency with paddling were observed in a pig coinfected with JXwn06 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.

Weight gains and febrile response: Non-infected control pigs and pigs infected with bacteria only did not have febrile responses (rectal temperature ≥ 40° C or 104° F). The febrile response was mild in pigs infected with VR2332, with about 60% of the pigs in these groups running low grade fevers (between 40.0° and 40.5° C = 104° to 104.9° F) for 1 or 2 days. The average temperature for these groups only reached ≥ 40° C on one day in the coinfected group (Figure 1). The average rectal temperatures for groups infected with the other 3 isolates were ≥ 40° C for multiple days during the 10 days after challenge that they were monitored (Figure 1). 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 (105° F) and often above 41.1° C (106° F). A few pigs in the groups challenged with SRV07 or JXwn06 had fevers that reached ≥ 41.7° C (107° F).

All groups infected with PRRSV had decreased weight gains as compared to the non-infected control pigs, the pigs infected with JXwn06 were the most severely affected and actually lost weight as a group during the 10 days they were monitored post challenge (Figure 2).

There did not appear to be significant differences in the febrile response or weight gains whether or not the pigs were coinfected with bacteria, with the possible exception of the febrile response in pigs infected with SDSU73 where pigs ran fevers earlier in the group infected with virus only (Figures 1 and 2).

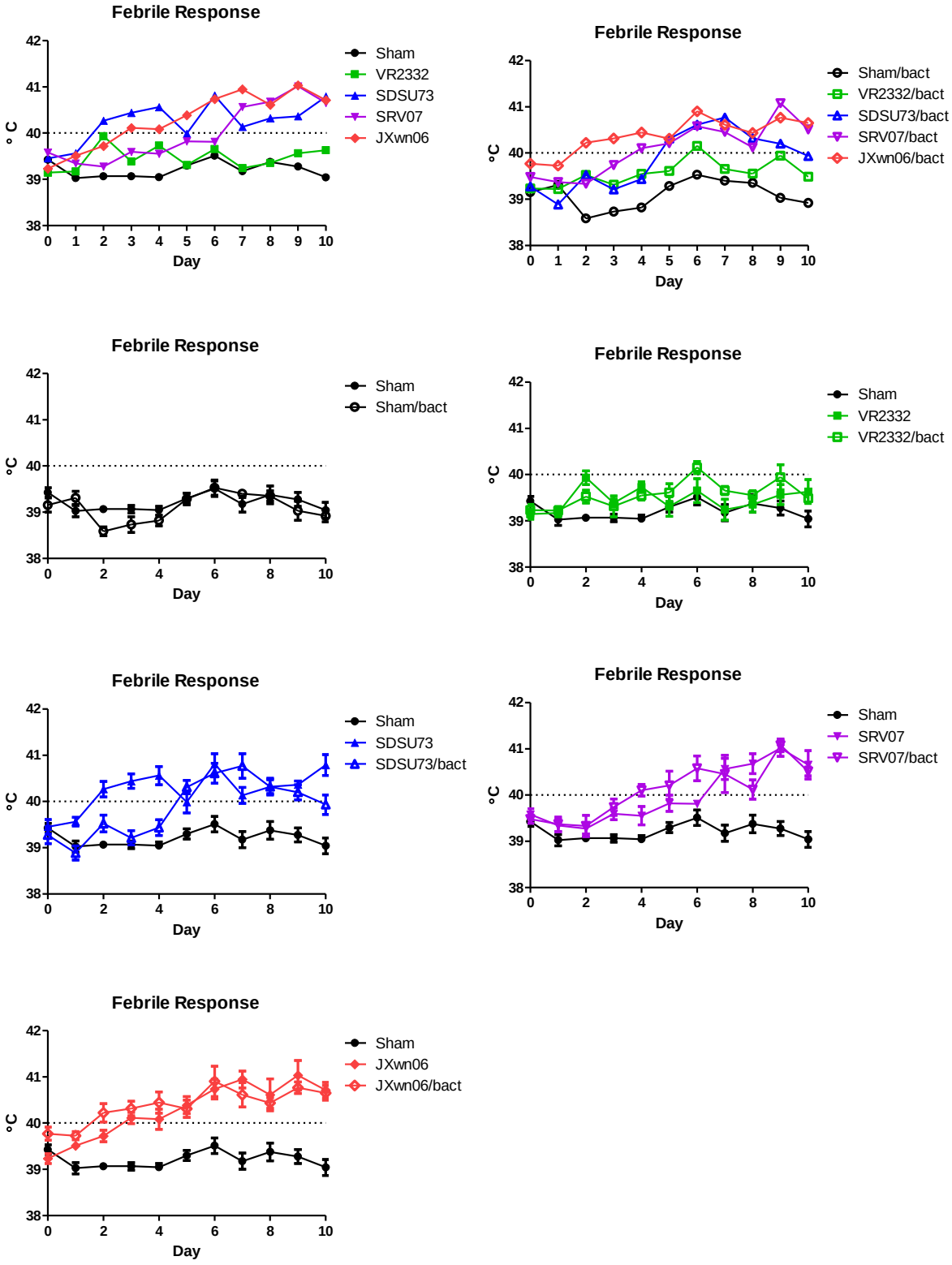


Figure 1: Average febrile response in groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

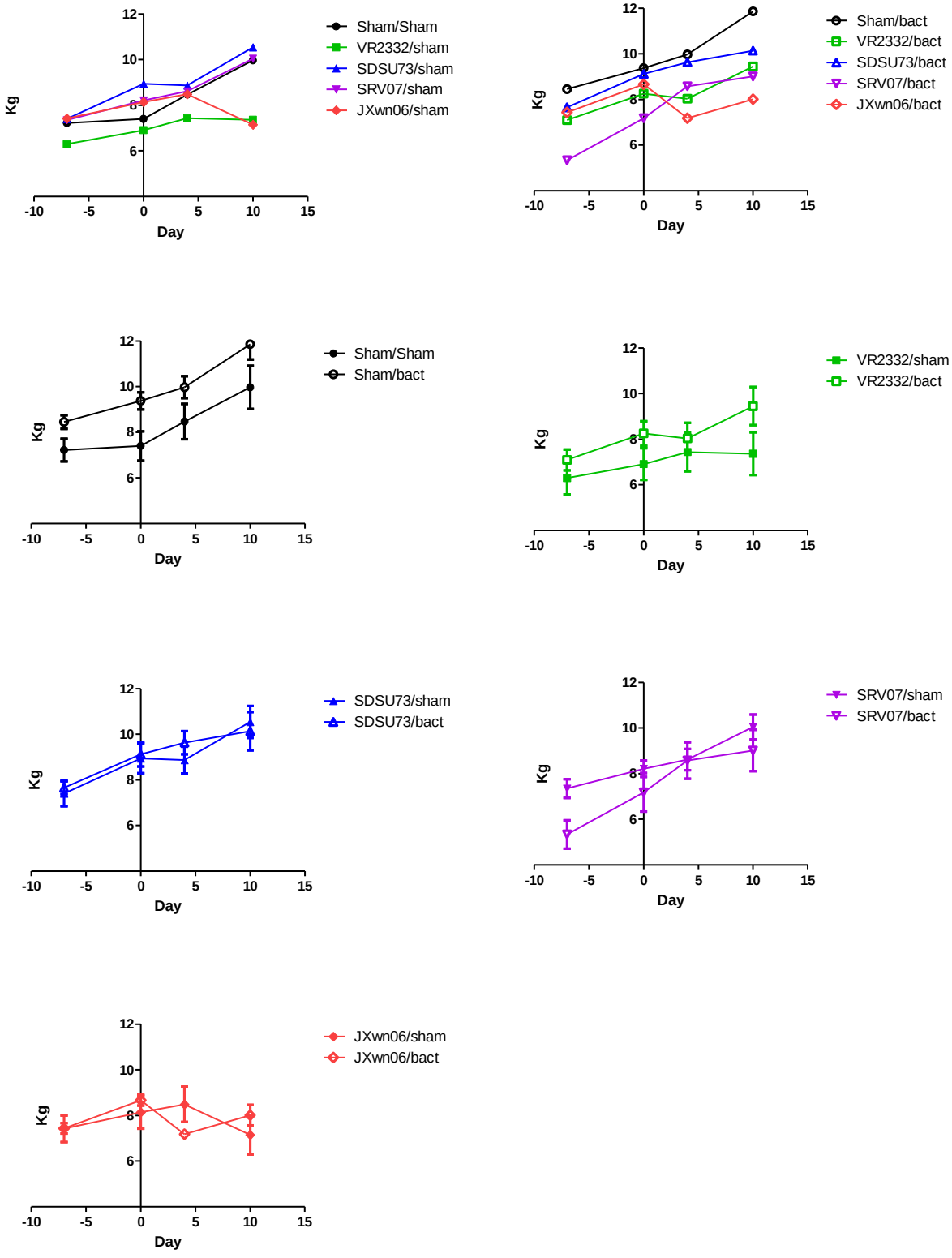


Figure 2: Average weight gains in groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

Virus replication and shedding: 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 3). Peak serum viral titers were not significantly different among the groups infected with VR2332, SDSU73, or

SRV07 during the time frame measured. Viremia was detected slightly later in pigs infected with SRV07 and peak titers for these occurred on day 10 post challenge, the last day of the study, so it is possible that titers may have peaked higher later. There were not significant differences in the serum viral titers whether or not the pigs were coinfected with bacteria.

Although mean viral titers in the lung lavage (BALF) were highest for the groups infected with JXwn06 the differences, in general, were not as extreme as those seen in the serum (Figure 4). At 4 days post challenge with PRRSV, titers for the groups infected with VR2332, SDSU73, and JXwn06 were not significantly different from each other, but all were significantly greater than the groups infected with SRV07. At 10 days post challenge with PRRSV, the titer for groups infected with JXwn06 was significantly greater than the titers for groups infected with VR2332 or SRV07, but not SDSU73, and the difference in titers for the groups infected with VR2332, SDSU73, or SRV07 were not significantly different from each other. There were not significant differences in the BALF viral titers whether or not the pigs were coinfected with bacteria.

At 4 days post challenge with PRRSV, nasal shedding of virus was significantly higher in the JXwn06 infected groups compared to the groups infected with the other strains of PRRSV. At 10 days post challenge with PRRSV, nasal virus shedding in both the SRV07 and JXwn06 groups was significantly higher than in the SDSU73 and VR2332 infected groups. In addition, 5% of the pigs infected with VR2332, 32% of the pigs infected with SDSU73, 68% of the pigs infected with SRV07, and 95% of the pigs infected with JXwn06 had nasal swabs that were positive by virus isolation for PRRSV on either day 4 or 10 post challenge. There were not significant differences in the nasal viral titers whether or not the pigs were coinfected with bacteria.

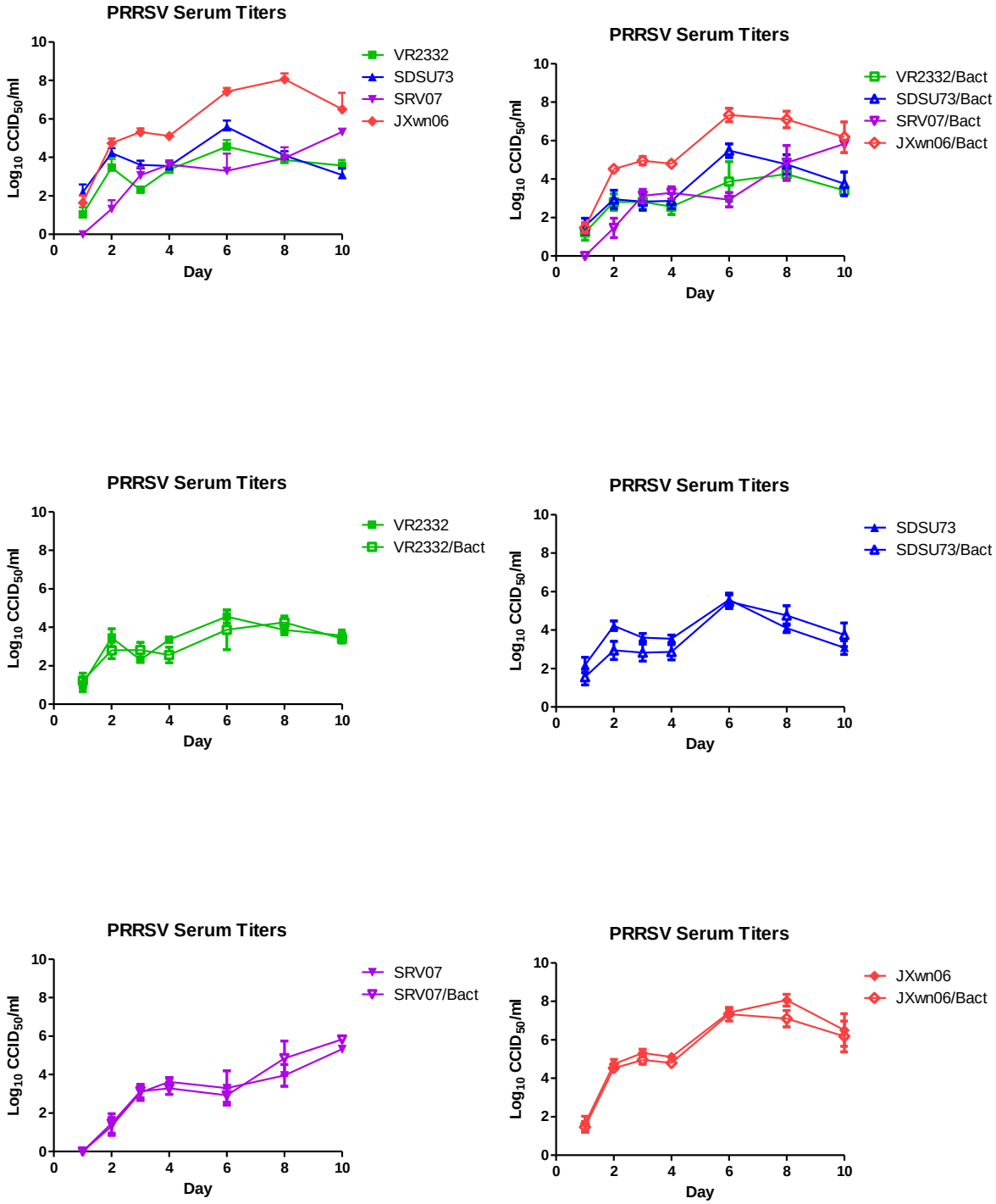


Figure 3: Average virus titer in the serum of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

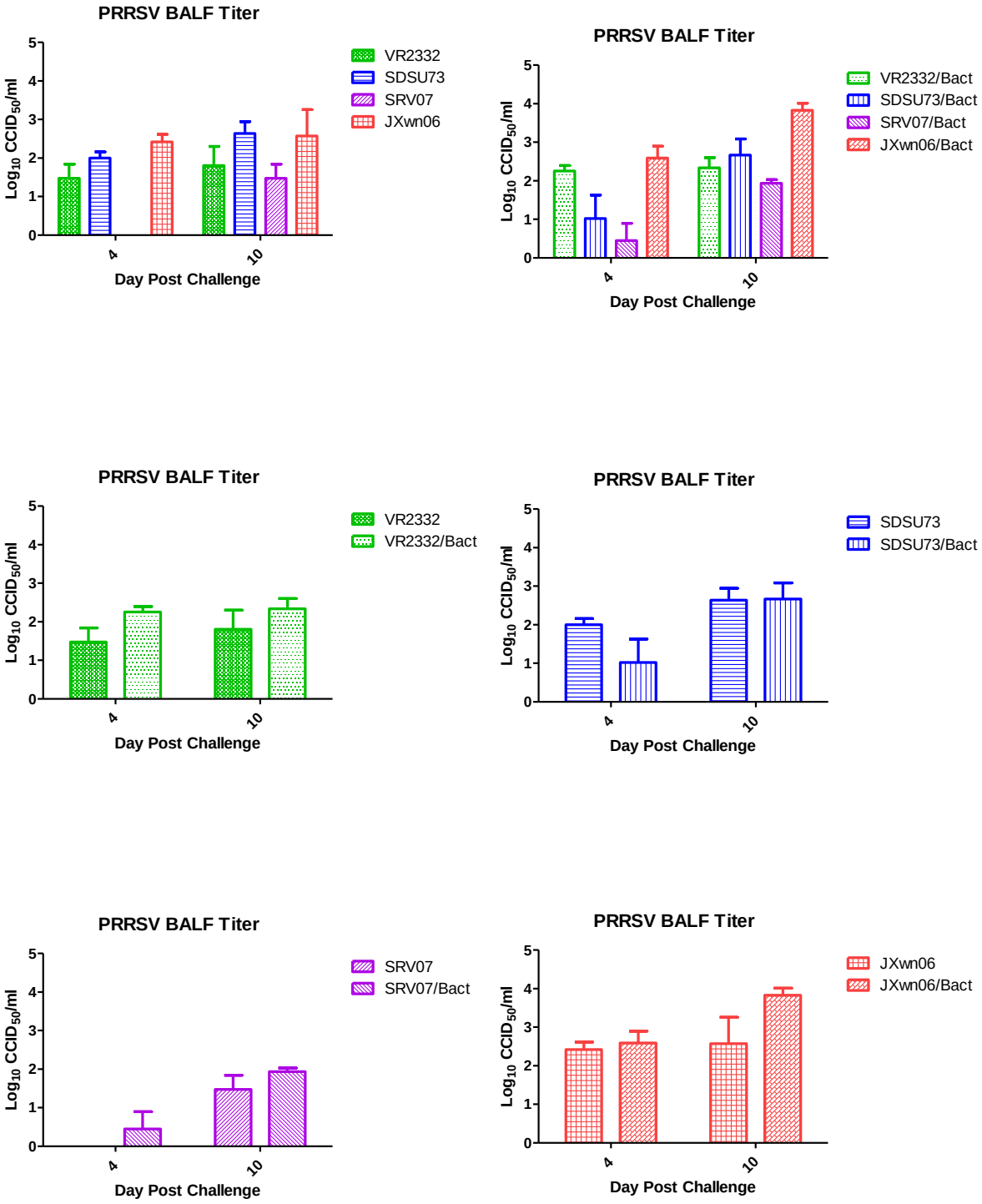


Figure 4: Average virus titer in the lung lavage (BALF) of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

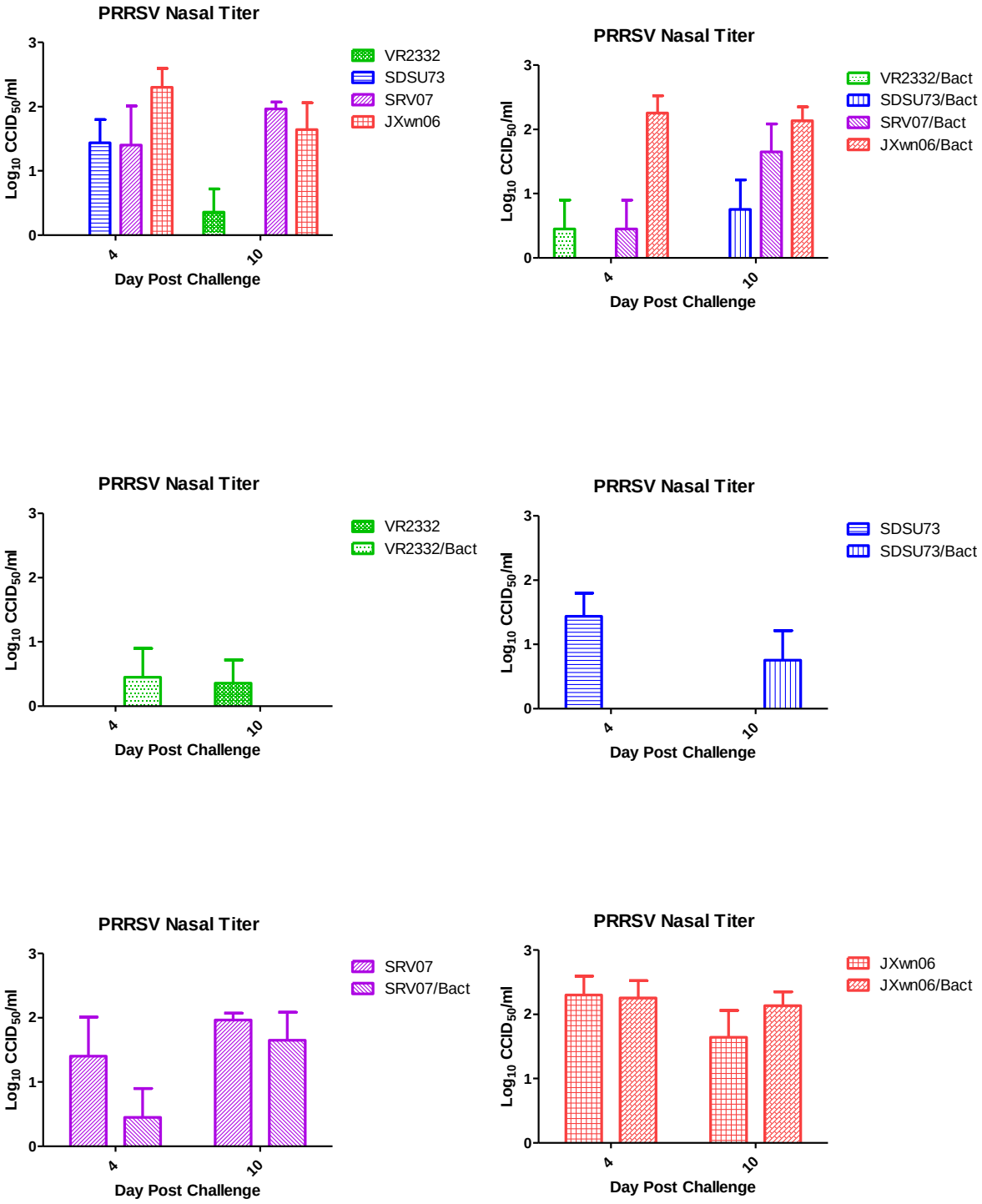


Figure 5: Average virus titer in the nasal swabs of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

Gross lung lesions: Gross lung lesions consistent with PRRSV infection were seen in all groups infected with the virus (Figure 8). At day 4 post challenge with PRRSV, the viral associated lesions were mild and there were no differences in severity among the groups (Figure 6). At day 10 post challenge with PRRSV, gross viral lung lesions were most severe for the groups infected with JXwn06 and SDSU73 and affected significantly greater lung area, on average, than lung lesions in the VR2332 and SRV07 groups. There were not significant differences in gross viral lung lesion scores whether or not the pigs were coinfected bacteria.

Lesions consistent with bacterial pneumonia, including plum-colored, well-demarcated consolidation and abscess formation, were seen in all the groups infected with bacteria (Figure 8). 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 coinfected with JXwn06 and bacteria had significantly greater lung involvement than all the other groups (Figure 7).

Bacterial isolation: *A. suis*, *S. suis*, and/or *H. parasuis* was cultured from 1/9 pigs from the group challenged with the bacteria alone, from 2/9 pigs challenged with VR2332/bacteria, from 3/9 challenged with SDSU73/bacteria, and from 5/9 pigs challenged with SVR07/bacteria and JXwn06/bacteria (Table 2). These bacteria were not isolated from 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 group coinfected with JXwn06 and bacteria, this was not the pig that had neurologic signs and had to be euthanized.

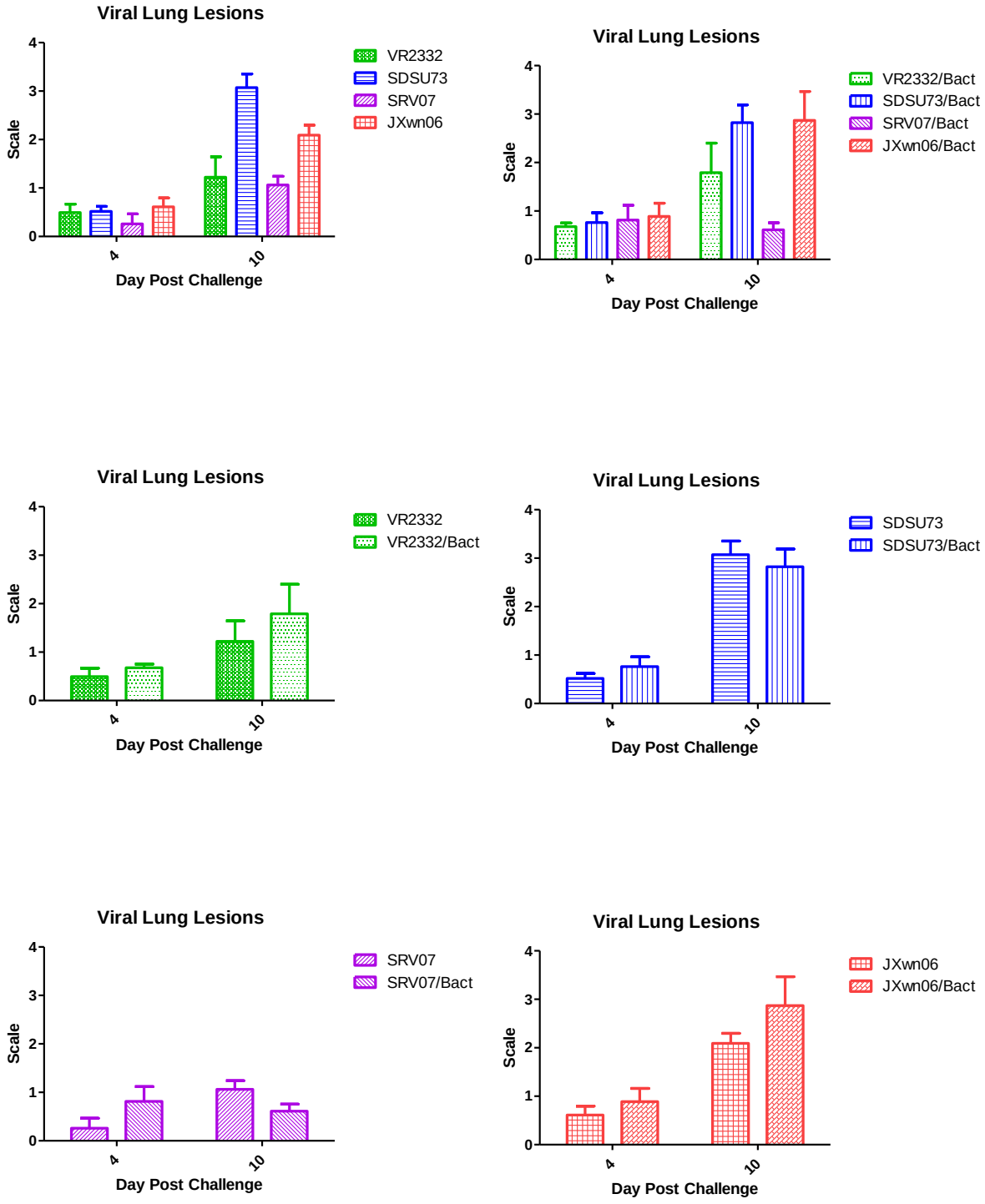


Figure 6: Average gross viral lung lesion scores of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 with or without coinfection with *H. parasuis*, *S. suis*, and *A. suis*.

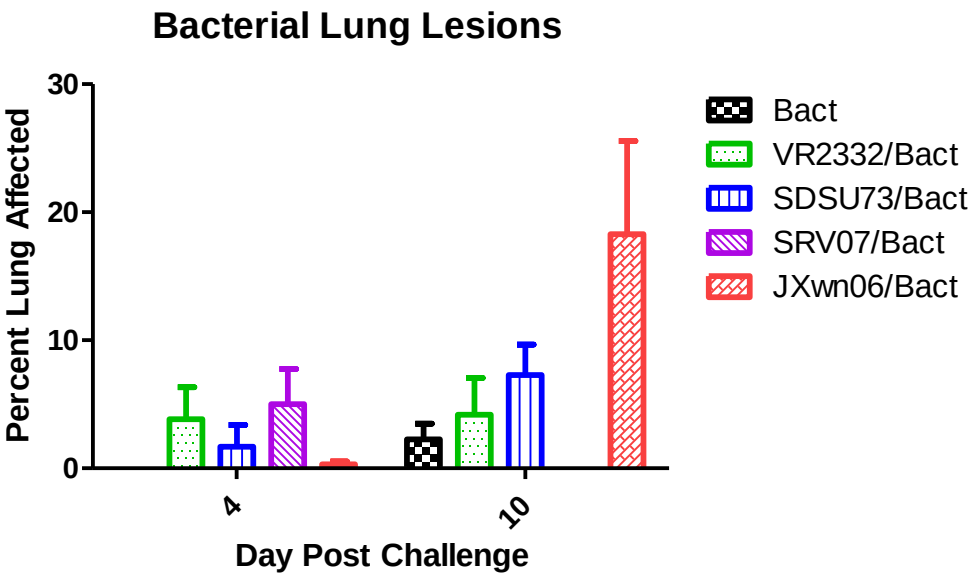


Figure 7: Average percentage of lung affected by bacterial lung lesion of groups of pigs coinfected with PRRSV VR2332, SDSU73, SRV07, or JXwn06 and *H. parasuis*, *S. suis*, and *A. suis*.

Table 2: Bacterial isolation from the lung.

Group	Total	Ss	Hp	As
BacT	1/9	0	0	1
VR2332/BacT	2/9	0	0	2
SDSU73/BacT	3/9	1	2	1
SRV07/BacT	5/9	1	3	2
JXwn06/BacT	5/9	1	5	0

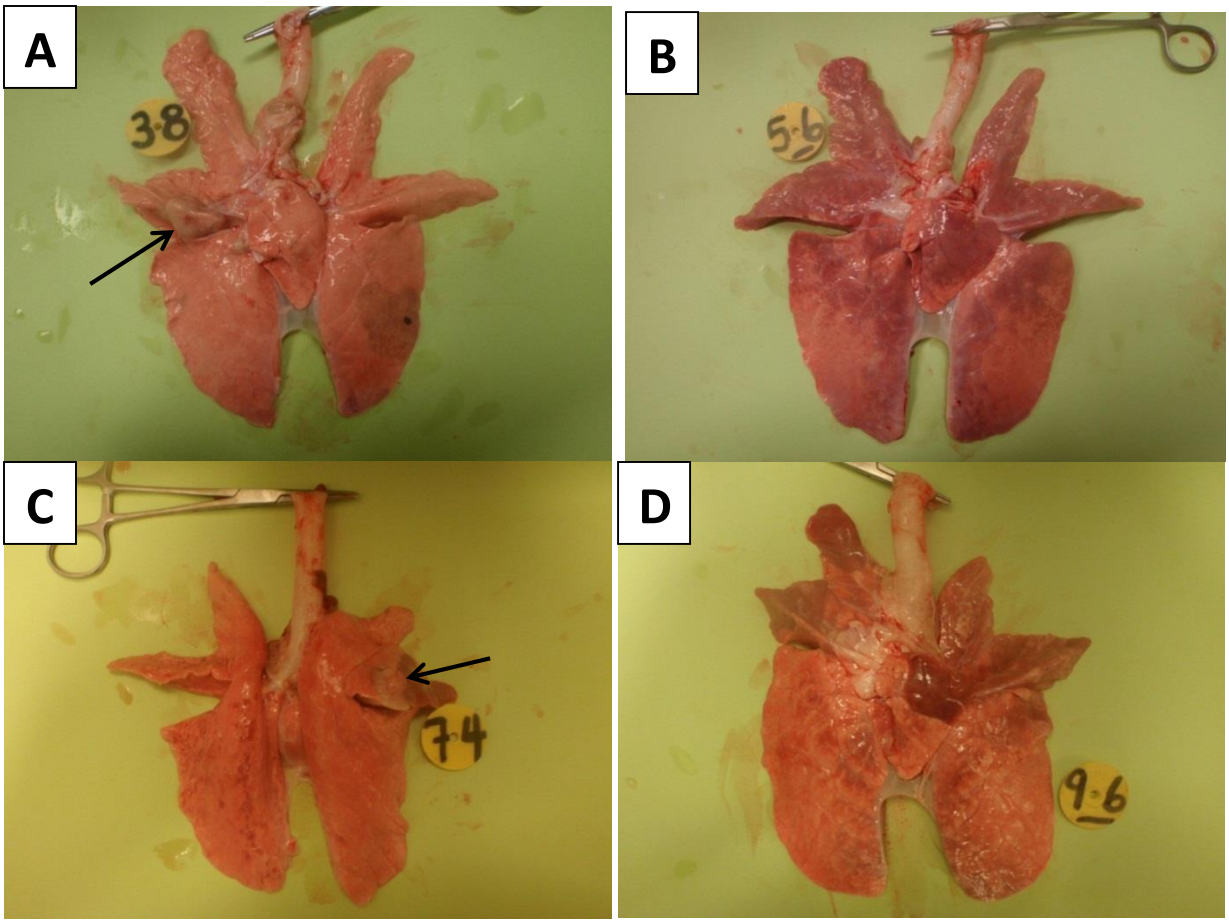


Figure 8: Gross lung lesions.
A. Lung from a pig infected with VR2332/BacT - d10 – showing mild viral lesions and an abscess affecting the right middle lung lobe (arrow). *A. suis* was isolated from this lung.
B. Lung from a pig infected with SDSU73/BacT - d10 - showing severe viral lesions but no bacterial lesions. No bacteria were isolated from this lung.
C. Lung from a pig infected with SRV07/BacT – d4 - showing mild viral lesions and consolidation with an abscess affecting the right middle lung lobe (arrow). *A. suis* and *S. suis* were isolated from this lung.
D. Lung from a pig infected with JXwn07/BacT - d10 - showing severe viral lesions and consolidation indicating bacterial pneumonia affecting the cranial, middle, and accessory lung lobes. *H. parasuis* was isolated from this lung

Serum chemistries: Serum chemistry panels were run on samples taken from the groups infected with virus alone. Total protein levels for most groups were fairly stable, except for the JXwn06 infected group, which saw a rise in total protein levels between 8 and 10 days post challenge. When breaking total protein levels down to albumin and globulin levels however, results showed that the level of albumin in the three PRRSV groups infected with SDSU73, SRV07, and JXwn06 tended to decrease over time while globulin levels tended to rise (Figure 9). The protein levels in VR2332 infected pigs were similar to those in sham infected control pigs. Serum enzyme levels such as alkaline phosphatase (ALP) and aspartate aminotransferase (AST), which tend to be released with tissue injury, did not rise, but in fact decreased over time in groups infected with SDSU73, SRV07, and JXwn06 (Figure 10). Young animals tend to have higher baseline levels of ALP due to the active remodeling of bone in animals that are still growing. The enzyme levels in pigs infected with VR2332 tended to be very similar to control animals. Calcium and phosphorus levels also tended to decrease over time in groups infected with SDSU73, SRV07, and JXwn06, but not in pigs infected with VR2332 (Figure 11). However, sodium and potassium levels were maintained during the 10 days monitored after infection in all groups (data not shown). Serum blood urea nitrogen (BUN) and creatinine, indicators of kidney function, did not change appreciably during the 10 days they were monitored after challenge (data not shown). Total bilirubin and glucose levels

likewise were not altered during the time monitored (data not shown). The serum chemistries did not indicate any major organ malfunction, but generally indicated pigs to be in an anorectic or malnourished state. Since these pigs were probably somewhat dehydrated as well the changes may have been more severe than indicated. The one exception was the increase in globulin levels, which could reflect dehydration or true increase or a combination of both. Increases in globulin could be a result of inflammatory processes or, as has been reported from an increase in immunoglobulin production.

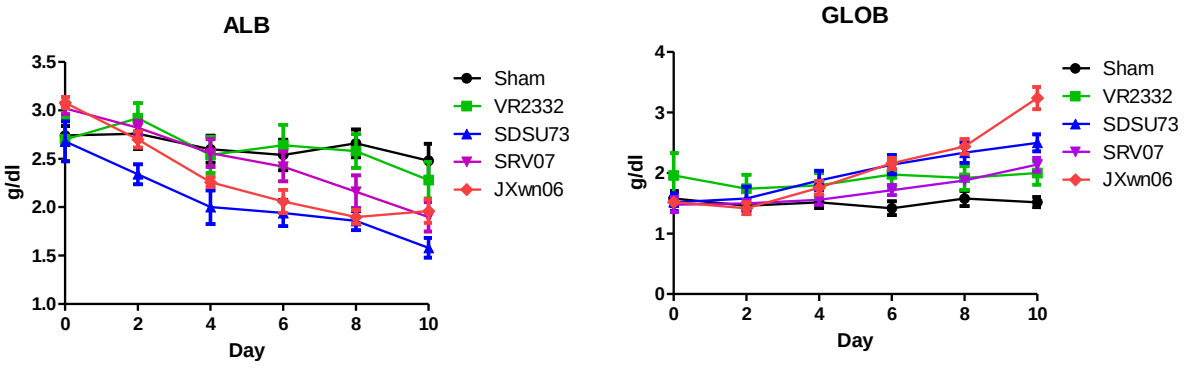


Figure 9: Average serum albumin (ALB) and globulin (GLOB) levels of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.

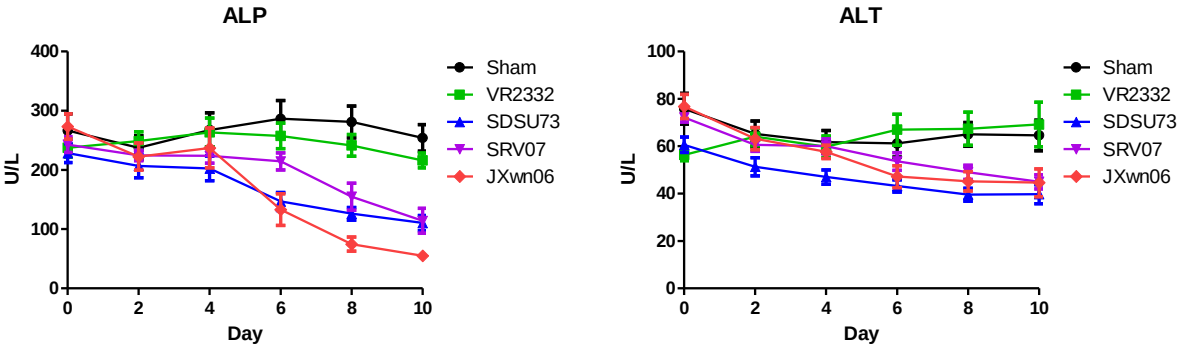


Figure 10: Average alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.

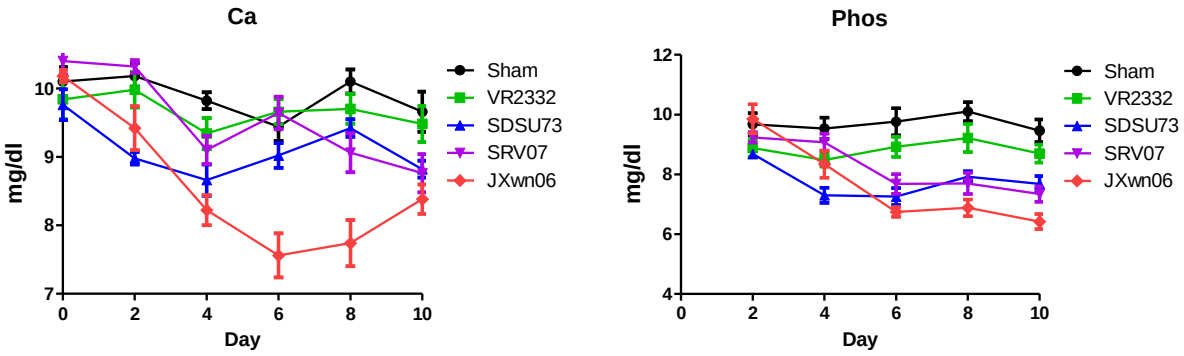


Figure 11: Average calcium (Ca) and phosphorus (Phos) levels of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.

Circulating lymphocytes: There was a sharp decline in circulating lymphocytes between 1 and 2 days post challenge for pigs challenged with VR2332, SDSU73, and JXwn06 and between days 2 to 4 for pigs infected with SRV07 (Figure 12). This decline was reflected in most subpopulation of lymphocytes (Figure 13). There were no differences detected in serum cortisol levels among the PRRSV infected groups compared to sham challenged controls (data not shown).

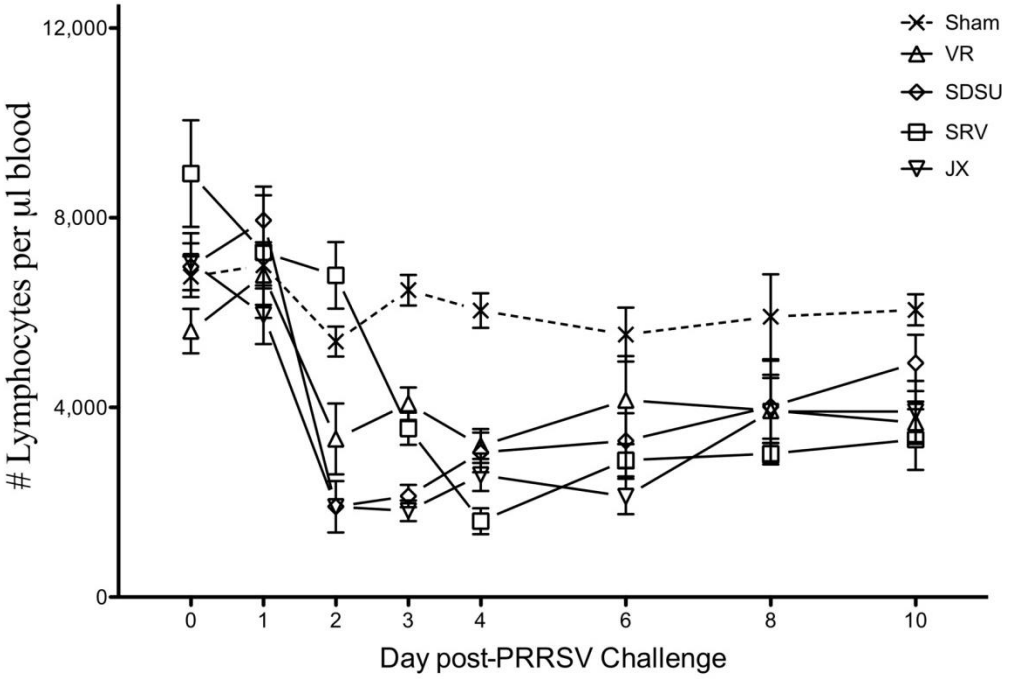


Figure 12: Average circulating lymphocyte numbers in the blood of groups of pigs infected with PRRSV VR2332 (VR), SDSU73 (SDSU), SRV07 (SRV), JXwn06 (JX), or sham inoculated controls.

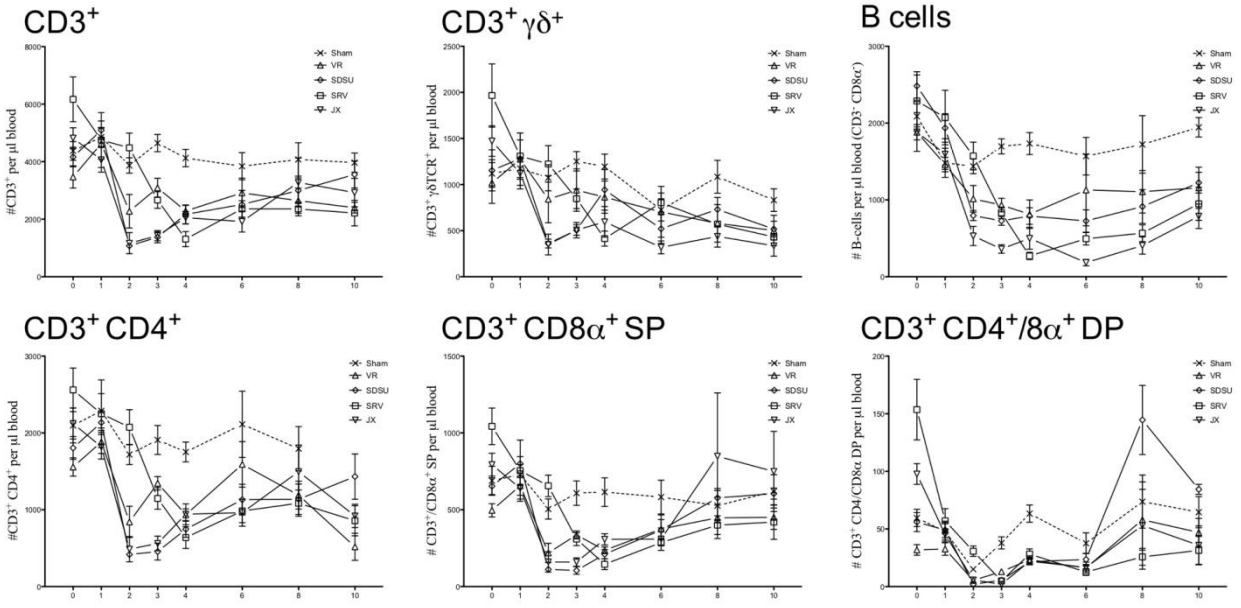


Figure 13: Average circulating lymphocyte subpopulation numbers in the blood of groups of pigs infected with PRRSV VR2332 (VR), SDSU73 (SDSU), SRV07 (SRV), JXwn06 (JX), or sham inoculated controls.

Thymic atrophy: There was thymic atrophy in all groups infected with PRRSV, but it was most pronounced for pigs infected with JXwn06 (Figure 15). Flow cytometry performed on cells collected from the thymus revealed and increased percentage of dead cells in all PRRSV infected groups at both days 4 and 10 post challenge with PRRSV (Figure 14). The greatest percentage of dead cells were in the thymi from pigs infected with JXwn06 at both days 4 and 10 post challenge and pigs infected with SDSU73 at day 10 post challenge.

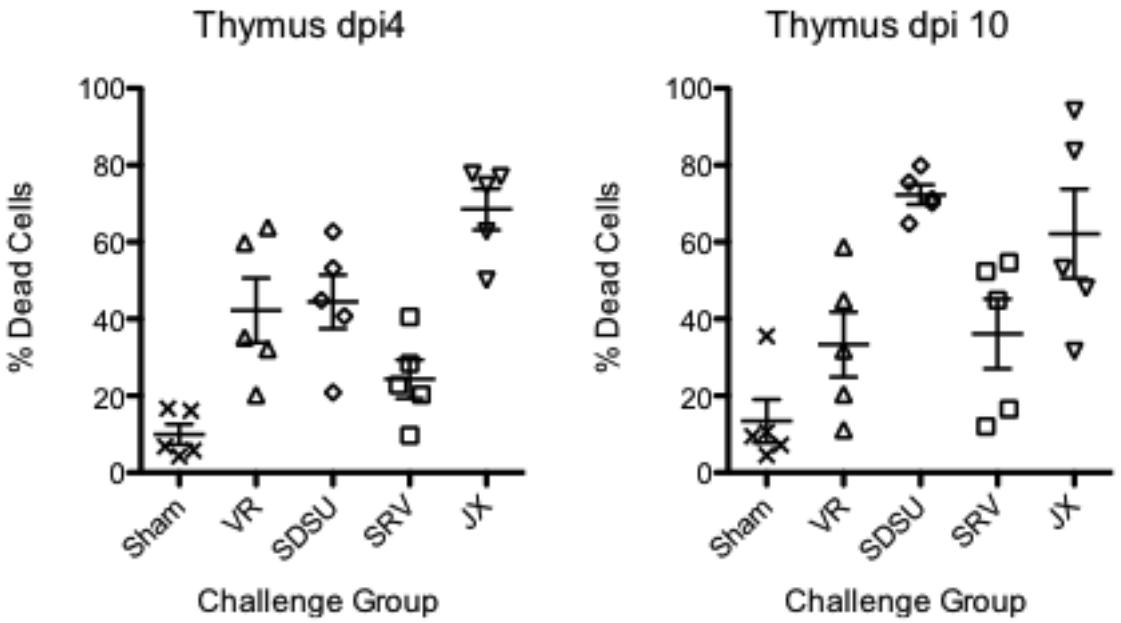


Figure 14: Percentage of dead cells in the thymus as determined by flow cytometry in pigs infected with PRRSV VR2332 (VR), SDSU73 (SDSU), SRV07 (SRV), JXwn06 (JX), or sham inoculated controls at day 4 and day 10 post challenge with PRRSV.

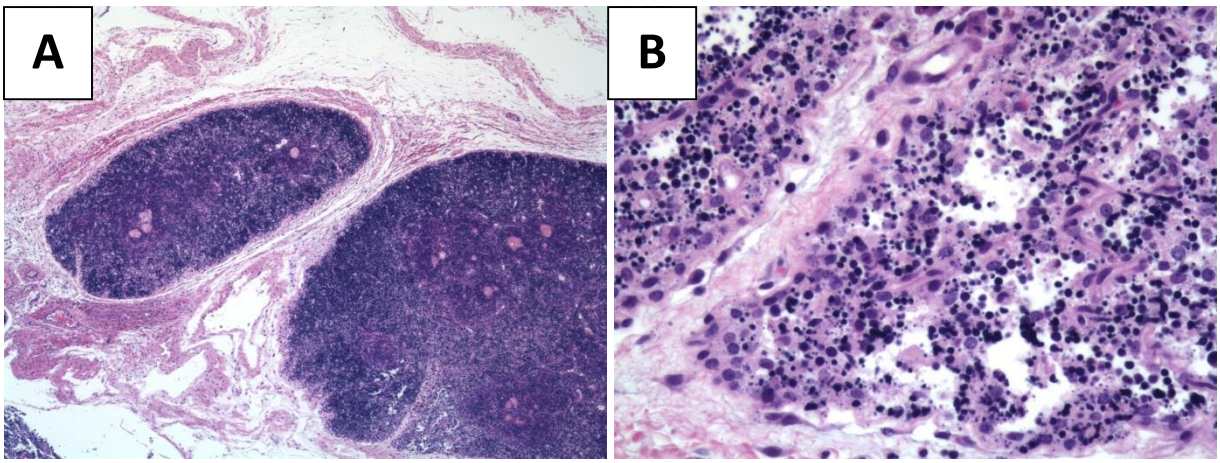


Figure 15: Photomicrographs of a thymus from a pig infected with the JXwn06 strain of PRRSV 10 days post challenge. Note lack of clear delineation of cortex and medulla (A) and myriad apoptotic bodies (nuclear remnants) from dead cells (B).

Cytokine expression: An increase in the levels of the proinflammatory cytokines IL-1β, TNFα, and IL-8 in the sera occurred after day 6 post viral challenge, and IFNα peaked between days 2-4 (Figure 16). The magnitude of increase was greatest for the group infected with JXwn06 and least or non-existent for the group infected with VR2332. Similarly,

the regulatory cytokines IFNγ, IL-2, and IL-10 in the sera were elevated after day 6 post challenge and the magnitude of the increase was greatest for pigs infected with JXwn06 (Figure 17). However, the levels of proinflammatory cytokines in the serum were often lower for pigs coinfected with virus and bacteria compared to pigs only infected with PRRSV (Figure 18).

In general, levels of both proinflammatory and adaptive cytokines in the lung lavage (BALF) were highest, on average, for pigs infected with JXwn06 and lowest for pigs infected with VR2332 (Figure 19). IL-1β and TNFα were both elevated on day 10 post challenge with JXwn06 and IL-1β was also elevated in pigs infected with SDSU73 on day 10. IL-6 and IL-8 were elevated at both days 4 and 10 post challenge with JXwn06. IL-6 and IL-8 were somewhat elevated in pigs infected with SRV07 on day 4 post challenge, and IL-8 was somewhat elevated on days 4 and 10 post challenge in pigs infected with SDSU73. Similar to serum values, IFNα levels peaked at day 4 post challenge and were highest for groups infected with JXwn06 and SDSU73. Levels of IL-10 were mainly elevated in pigs infected with JXwn06 at day 10 post infection, and IFNγ was elevated in both SDSU73 and JXwn06 groups at day 10 post infection. IL-2 levels were elevated in JXwn06 infected pigs at both days 4 and 10 post infection.

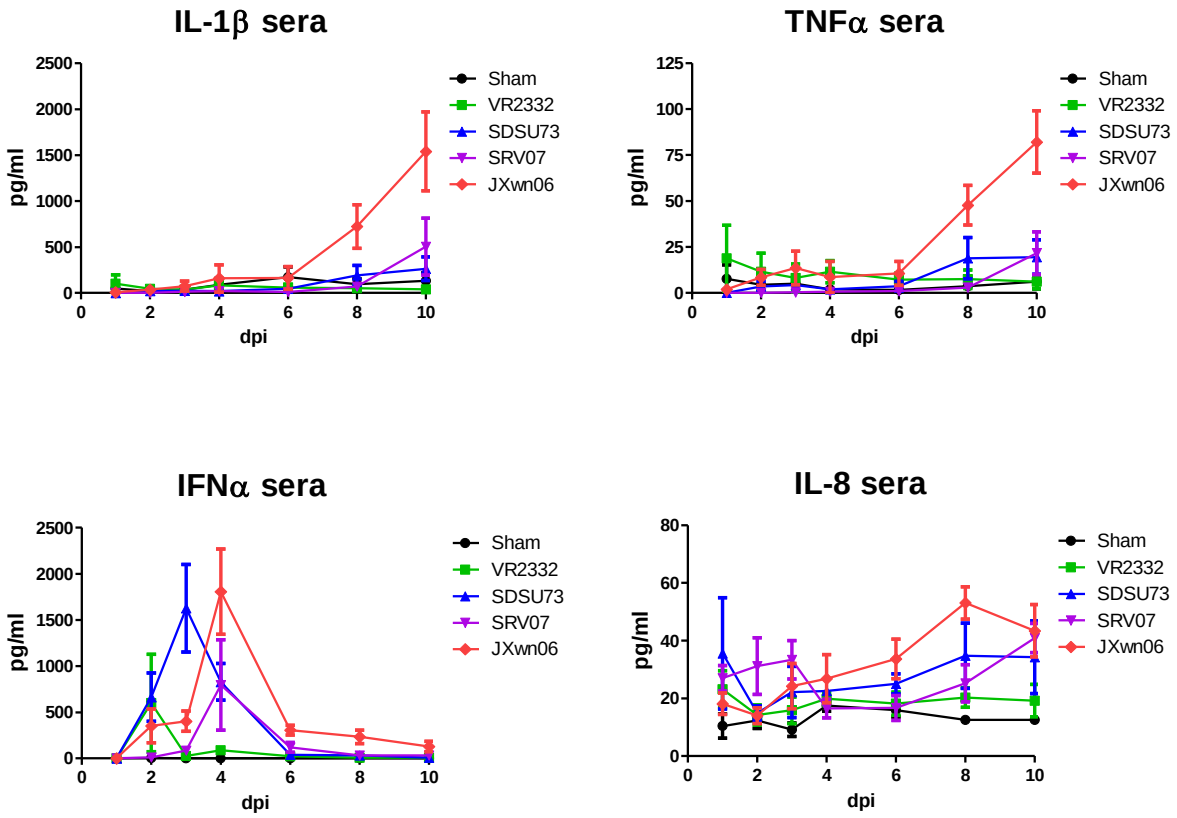


Figure 16: Average cytokine levels of IL-1β, TNFα, IFNα, and IL-8 in the serum of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.

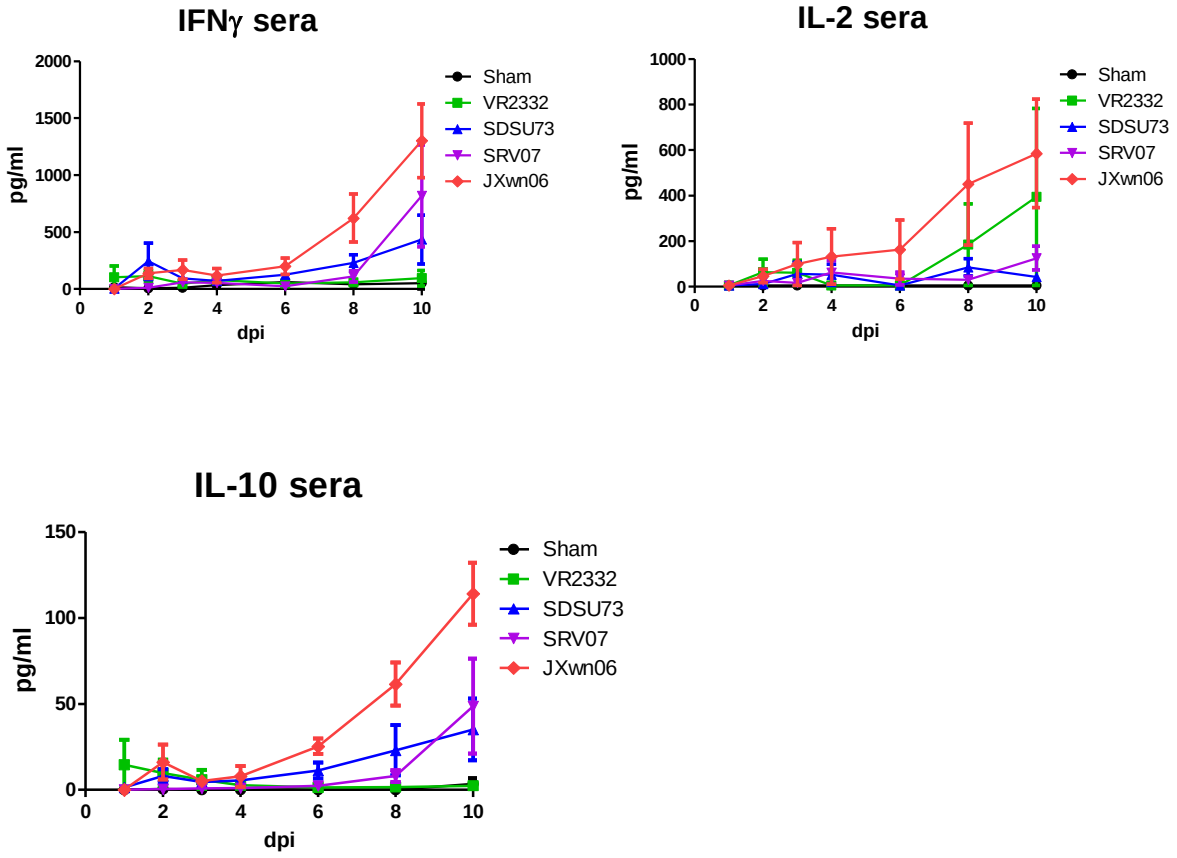
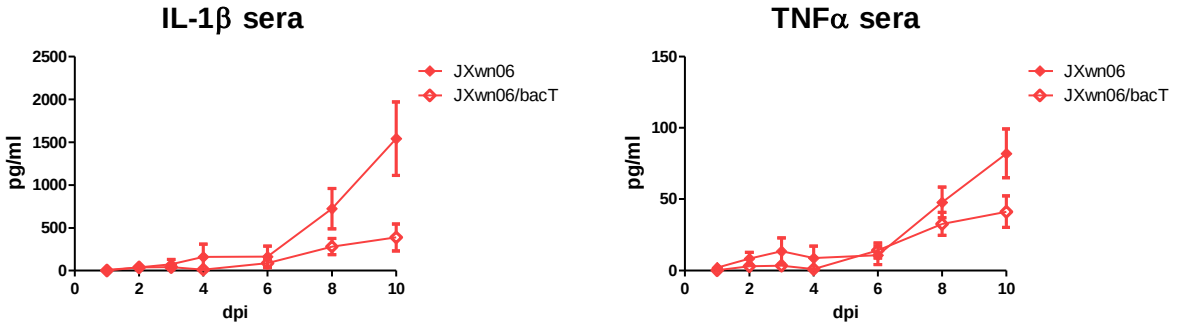


Figure 17: Average cytokine levels of IFNγ, IL-2, and IL-10 in the serum of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.



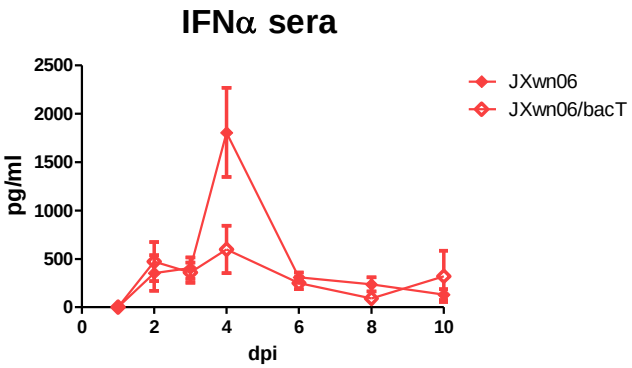


Figure 18: Average cytokine levels of IL-1β, TNFα, and IFNα in the serum of groups of pigs infected with either JXwn06 alone or coinfected with JXwn06 and *H. parasuis*, *S. suis*, and *A. suis* (JXwn06/bacT).

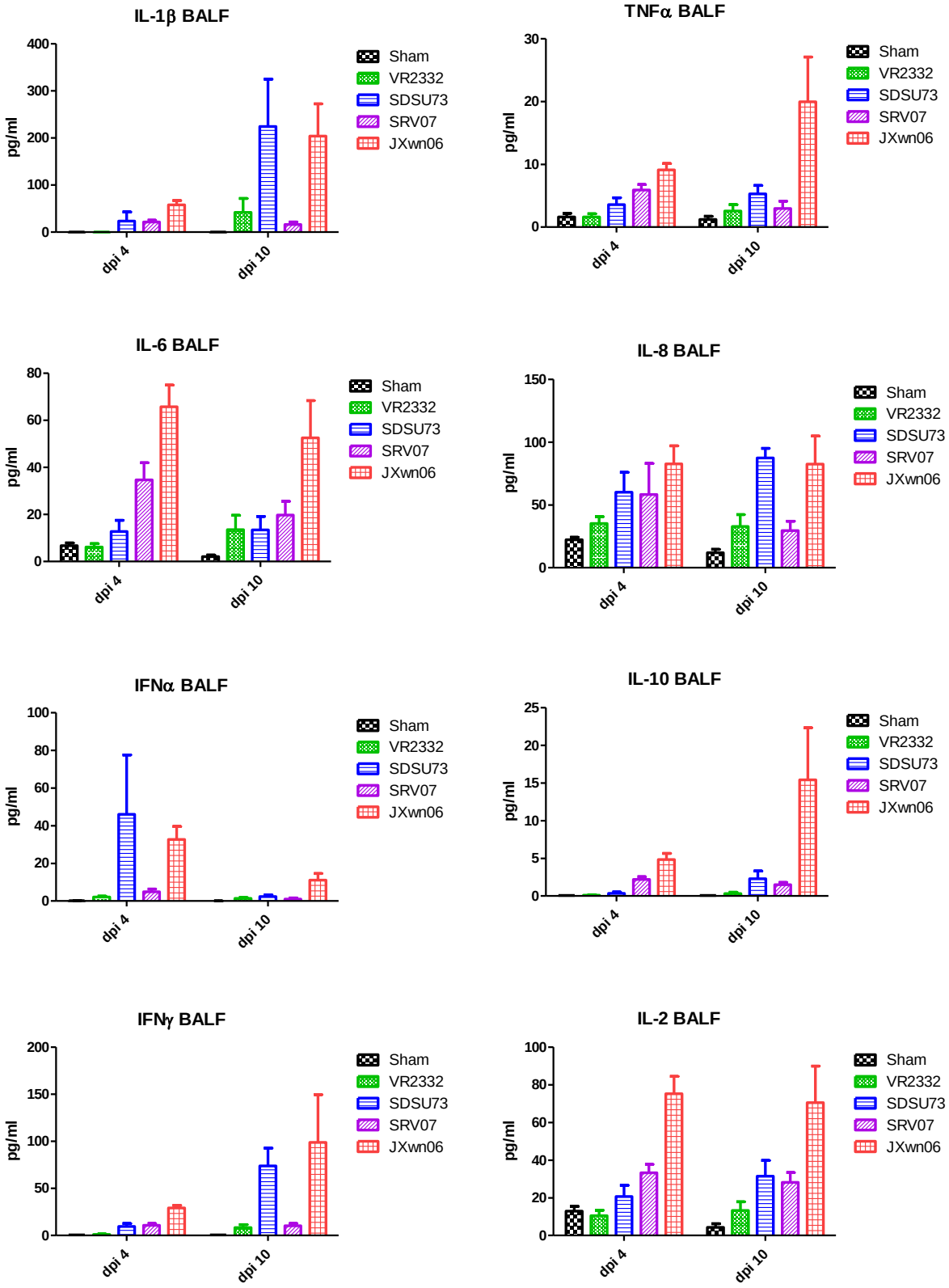


Figure 19: Average cytokine levels in the lung lavage (BALF) of groups of pigs infected with PRRSV VR2332, SDSU73, SRV07, JXwn06, or sham inoculated controls.

Histology: Encephalitis characterized by perivascular infiltration with mononuclear cells, mostly lymphocytes and some macrophages, was seen in pigs infected with both SRV07 and JXwn06 in groups infected with virus alone and those coinfected with bacteria at 10 days post challenge (Figure 20).

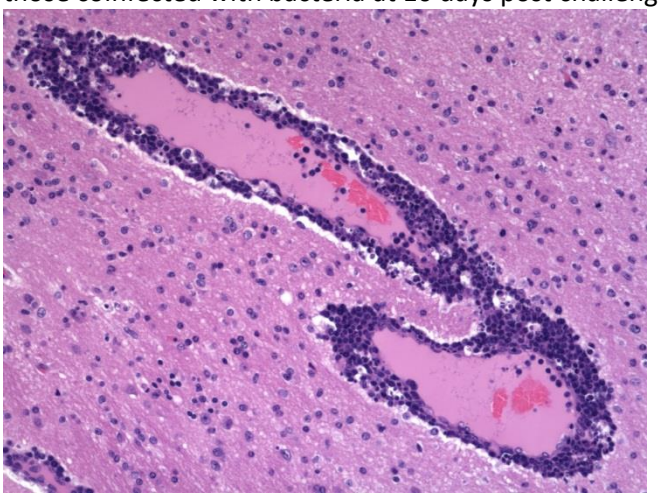


Figure 20: Photomicrocraphs of a thalamus of the brain from a pig infected with the JXwn06 strain of PRRSV 10 days post challenge showing mononuclear perivascular infiltration.

Discussion:

In 2006 outbreaks of a high morbidity/high mortality disease that became known as "porcine high fever disease" began to occur across China, and in 2007, Vietnam began to experience swine disease outbreaks causing similar clinical signs. The disease was associated with newly-emergent, highly-pathogenic PRRSV strains that evolved from endemic type 2 PRRSV and contained a common set of discontinuous deletions in nsp2 that have been used as a molecular marker for these isolates but do not appear to be solely responsible for the phenotype. Bacteria and other viruses 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 HP-PRRSV as a major factor.

The current study, as well as others, indicates that the HP-PRRSV strain JXwn06 is highly virulent especially compared to the North American prototype strain VR2332, which was the least virulent of the isolates tested. The HP-PRRSV strain SRV07 and the US strain SDSU73 fell somewhere between the virulence of VR2332 and JXwn06. Although SRV07 is still relatively virulent compared to strains such as VR2332, these results may indicate that HP-PRRSV isolates in Asia have attenuated with time to some degree and are on par with higher pathogenic US strains such as SDSU73, which itself 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.

The ultimate question is by what means are strains such as JXwn06 more virulent? One of the biggest differences among the viruses is in the degree of viremia that results from infection with JXwn06, which was about 2 log-folds lower than the other viruses. This may reflect either greater tropism or cellular production of virus. Increased tropism may indicate greater number of macrophages infected, either by more widely distributed infection (macrophages in more organs) or greater percentage of macrophages that are susceptible, or both, or cells beyond the macrophage lineage becoming permissive. Further examination of tissues for viral load and viral staining may help delineate these possibilities. Interestingly the difference in viral load in the lung lavage was not as dramatic as was seen in the serum. This may argue for a broader distribution of virus. The wider distribution of inflammatory infiltrates and lesions with associated evidence of cell death (apoptotic bodies or nuclear remnants) in pigs infected with JXwn06 also points toward this possibility. Serum chemistries did not indicate any major organ malfunction although they did suggest the pigs were in an anorectic or malnourished state. Pigs infected with JXwn06 did have increased systemic production of both innate and adaptive cytokines which may contribute to clinical signs such as a febrile response, cachexia and promote inflammation as well as immune dysregulation. Additionally, the extensive thymic atrophy seen in these pigs may be further evidence of a greater degree of immunosuppression. The presence of lesions such as encephalitis may have contributed to the severity of clinical disease as well.

There were a few similarities that the two Asian isolates shared that were not common to the US isolates tested; the occurrence of gastrointestinal signs such as vomiting and diarrhea, increased frequency and quantity of nasal virus

shedding, and lesions of encephalitis. These results may indicate that these Asian HP-PRRSV strains are affecting a greater number organ systems have an increased transmissibility. The SRV07 strain was clearly less virulent than JXwn06 which and one clear difference was in the replication dynamics of the virus. There was a delay in detection of virus and in general a decrease in viral titers seen with this virus. However, as seen in this study the magnitude of viremia was still rising by the end of the experiment, and the impression from this and other experiments with this virus, which have all terminated by 10-14 days post challenge, is that the clinical disease may not have peaked. Thus further studies observing a longer post challenge time period may be prudent to fully understand the clinical effects of this virus.

Mortality rates in pigs infected with JXwn06 in this experiment were less than we saw in previous experiments. In the current study, we did not see the same degree of secondary bacterial infection in pigs infected with virus alone as in previous studies. In the earlier studies bacterial isolation rates were 70-100% from the lungs of the JXwn06 infected pigs (compared to 0-20% of the pigs infected with VR2332) and there were increased numbers of bacterial isolations from systemic sites as well. One of the main differences was the use of early weaned pigs that were farrowed at NADC and kept in isolation rooms 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 predisposed pigs to the development of secondary bacterial infection. We did see an increase in secondary infections in the coinfected groups and the frequency and severity of secondary bacterial infections increased with the increasing virulence of PRRSV. Thus secondary infections do appear to play a role in the severity of disease seen with these HP-PRRSV isolates. In general, bacterial infection did not appear to alter the dynamics of the viral infection, but cytokine levels were typically lower in pigs coinfected with PRRSV and bacteria. Furthermore, age probably influences mortality rates with JXwn06. In two 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. 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 based on past 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.