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Variation in response to both natural and experimental infection with Porcine Circovirus 2b

Theresa P. Bohnert
University of Nebraska-Lincoln, tbohnert@huskers.unl.edu

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VARIATION IN RESPONSE TO BOTH NATURAL AND EXPERIMENTAL INFECTION WITH PORCINE CIRCOVIRUS 2B

by

Theresa Peace Bohnert

A THESIS

Presented to the Faculty of
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In animals, disease progression can be influenced by genetics, environment, management practices, and the type of pathogen. Infection with porcine circovirus 2 (PCV2) causes development of diseases known as porcine circovirus 2-associated diseases (PCVAD), for which there is currently no treatment. In Trial 1, weanling unvaccinated barrows and gilts (n = 229) from two genetic lines were bled at d14, 49, and at necropsy to assess disease progression from natural infection using antibodies (ELISA) and viral copy counts (quantitative PCR). Virus was isolated from infected pigs and sequenced to determine the strain responsible for infection. At d49, 113 animals had active infection. At necropsy, clinical signs of infection were found in 16% of the pigs. In Trial 2, barrows from two genetic lines (n = 81) were infected with an inoculate known to cause PCVAD. Blood and weights were collected before infection and at d7, 14, 21, and 28 to characterize response. Pigs were classified as early responders (n = 33), late responders (n = 40), and non-responders (n = 7) based on IgM levels. Other traits were fitted to a mixed model with effects of group and line, and random effect of litter. Means of viral load throughout the 28-day period differed ($P < 0.01$) between early and late
responders and non-responders. Viremia differed among groups ($P < 0.01$) at d14, 21, and 28. Viremia of non-responders was 18% less than early and late responders at d14, and 22% less at d21. Non-responders tended to have greater growth rate than the other groups. Variation in immune response can be utilized in genomics studies to identify genetic markers for disease resistance that can be used to select for disease resistance.
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CHAPTER 2

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INTRODUCTION

Porcine circoviruses (PCV) are members of the genus *Circovirus* and the family *Circoviridae*. The most notable of the porcine circoviruses is porcine circovirus type 2, PCV2, which has been associated with several disease syndromes since its initial classification in 1996. The genome contains single-stranded, non-enveloped, circular DNA molecules, and is 1768 nucleotides in size (Gillespie et al., 2009). Two major open-reading frames have been identified; open-reading frame 1 encodes for proteins involved in viral replication, and open-reading frame 2 encodes the capsid protein surrounding the virus (Carman et al., 2008).

Porcine circovirus 2 is further classified into two categories, a and b, with a first detected in North America, and b first detected in Europe (Takahagi et al., 2009). It has been demonstrated in field studies and previous research that PCV2a is the less virulent strain of the virus. The other category, PCV2b, is most usually defined as the etiological agent of PCVAD, porcine circovirus associated diseases.

The most common characteristics in all PCV2 infections are signs of lymphoid depletion and lymphopenia in circulating blood. The virus is able to attack the immune system by affecting both macrophages and dendritic cells. Although PCV2 is observable in these cells, it has been suggested that they are not the sites of viral replication. Several studies have indicated that dendritic cells may be the source of transportation for viral cells. However, not much is known about the pathogenesis of the PCV2 virus (Opriessnig et al., 2007).

Porcine circovirus associated diseases affect several biological systems in the pig. First, the porcine respiratory disease complex (PRDC) has been linked to infection with
PCV2. Also, PCV2 can cause gastrointestinal complications in the form of PCV2-associated enteritis, reproductive issues in the form of PCV2-associated reproductive failure, and a vascular disease known as porcine dermatitis and nephropathy syndrome. The most notable disease syndrome caused by PCV2 infection is known as post-weaning multisystemic wasting syndrome (PMWS). This disease state causes a wasting condition in which pigs are unable to put on adequate weight and exhibit depletion in lymphocytes leading to an inability to fight off other infections.

The development of PCVAD is reliant on many factors. Many strains of PCV2b have been isolated and sequenced, and a difference in virulence among the strains can affect the severity of the virus and thus the severity of disease conditions. There is also a genetic effect, in which certain breeds are more susceptible to the disease than others. Studies have indicated a vast difference in the development of clinical signs in pigs of varying breeds including Large White, Pietrain, Duroc, and Landrace. Co-infections with other viruses or bacteria such as *Mycoplasma hyopneumoniae*, porcine parvovirus, porcine reproductive and respiratory disease, and swine influenza can all affect disease progression of PCV2. Host factors, such as the amount of maternal antibodies acquired from colostrum in the first hours of life, can have an effect on the development of PCV2 associated diseases.

A vaccination protocol for PCV2 exists. Four commercially available vaccines are on the market, with the first being approved for use in the United States in 2007. The vaccines vary in dosing, antigen content, and the age of pig for which they are licensed. Vaccinations have been proven successful in lowering mortality rates in both field studies and farm applications (Takahagi et al., 2009). However, every pig in the herd must be
vaccinated in order to prevent an outbreak, even though many pigs contract the virus and show no clinical signs. The use of selection to eliminate the need for vaccines against PCV2 would be ideal to save the swine industry millions in vaccination costs yearly.
1.1 LITERATURE REVIEW

Characteristics of PCV2

Porcine Circovirus (PCV) emerged in 1974, initially as a contaminant of the porcine kidney cell line PK-15 (Grau-Roma et al., 2010). This PK-15 PCV isolate did not elicit clinical disease in pigs in experimental challenges. Allan et al., 1995, demonstrated that infecting pigs with PCV that were Cesarean-delivered and colostrum-deprived resulted in replication of the virus, but no lesions or disease state was induced (Sorden, 2000).

In 1991, post-weaning multisystemic wasting syndrome, PMWS, emerged and was known to be associated with a circovirus (Allan and Ellis, 2000). Sequence analysis comparison of the two virus strains showed that the sequence from PCV and the one causing PMWS had 75-76% nucleotide sequence identity. This lead to the discovery of another type of circovirus; the non-pathogenic virus became PCV1 and the new virus became PCV2 (Opriessnig et al., 2006a). In addition to the two types, PCV2 can be broken down into two categories: PCV2a, detected in North America, and PCV2b, detected in Europe (Takahagi et al., 2009). According to Grau-Roma et al. (2010), studies show that PCV2b is more prevalent in natural infections of herds. Therefore, PCV2b may have higher virulence than PCV2a.

The porcine circoviruses are members of the genus *Circovirus* of the family *Circoviridae*. Other members of the genus *Circovirus* include beak and feather disease virus, canary circovirus, goose circovirus, and pigeon circovirus (Opriessnig et al., 2007). The *Circoviridae* family genomes contain single stranded, non-enveloped DNA molecules that are circular in shape and range in size from 1.8 to 2.3 kilobases.
(Opriessnig et al., 2007). Because the DNA molecules are non-enveloped, the virus is resistant to many disinfectants, such as those with alcohol, chlorhexidine, iodine, and phenol bases (Grau-Roma et al., 2010). The virion DNA is single-stranded and negative sense, with 1,800 bases and six open reading frames (Chae, 2005). Porcine circovirus was discovered to be the smallest virus capable of replicating independently in mammalian cells (Kim et al., 2004b).

Porcine circoviruses can be divided into type 1 or 2. Type 1 is generally regarded as non-pathogenic, while type 2 is considered pathogenic. Type 1 and 2 share about 75 percent nucleotide sequence identity (Opriessnig et al., 2006b). Because Type 1 has not been shown to induce a disease in swine, research has been more focused on Type 2 as the cause of several diseases (Finsterbusch and Mankertz, 2009).

PCV2 is an icosahedral, non-enveloped virion (Takahagi et al., 2009). Isolates of PCV2 have genomes ranging from 1767 to 1768 base pairs (Opriessnig et al., 2006b). The virion is about 17 nm in diameter (Takahagi et al., 2009). There are two major open reading frames that are associated with PCV2. The first, open reading frame 1 (ORF1), enables viral replication by encoding for replication proteins. Open reading frame 2 (ORF2) encodes the capsid protein to surround the virus (Carman et al., 2008).

Infection with PCV2 is complex in that infected pigs can remain asymptomatic. Pigs that are highly viremic with PCV2 DNA may develop diseases associated with PCV2 infection. These diseases have been classified as Porcine Circovirus 2 Associated Diseases (PCVAD), and include PMWS, PDNS, PCV2-associated reproductive failure, PCV2-associated enteritis, and porcine respiratory disease complex (PRDC).
Pathogenesis

The development and step-by-step events leading to infection and replication of PCV2 are somewhat unclear to scientists. It seems that the disease consistently causes lymphoid depletion and low levels of lymphocytes in peripheral blood. Reduction of lymphocytes in infected pigs can be attributed to several factors, including reduced production in the bone marrow, reduced proliferation in secondary lymphoid tissues, or increased loss of lymphocytes in areas such as the bone marrow, peripheral blood, or secondary lymphoid tissues (Opriessnig et al., 2007).

Dendritic cells have a distinctive ability to start an immune response and control homeostasis within lymphocytes. Because they are highly networked in many locations such as the skin, mucosal surfaces, and circulating in blood, dendritic cells are able to deal with viruses rapidly once they have been encountered. This cell type is responsible for encapsulating antigens and travelling to lymph nodes and organs to activate T cells for an increased immune response (Vincent et al., 2003). In the study carried out by Vincent and others in 2003, it was determined that both bone-marrow-derived and monocyte-derived dendritic cells are affected by PCV2 infection. In the Opriessnig et al. (2007) review, it was further described from Vincent’s study that replication in dendritic cells could not be proven. However, PCV2 was able to persist in the cells without undergoing induction of apoptosis. It was suggested that dendritic cells might be utilized as vehicles for transportation because of their migratory ability.

Porcine circovirus 2 manifests itself in both macrophages and dendritic cells, but studies using in vitro models have found that monocytes may not be the site of PCV2 replication (Opriessnig et al., 2007). The review by Opriessnig et al., 2007, describes in
detail the work of Gilpin and others, 2003, in which macrophages and monocytes were tested for their facilitation of PCV2 replication. In that study, replication was not observed, but neither was degradation in the cytoplasm of both monocytes and macrophages.

Fenaux and others in 2002 developed an infectious clone of PCV2 to help understand the role of PCV2 in systemic diseases. They infected specific pathogen-free pigs with cloned genomic PCV2 DNA by direct injection into the livers and superficial iliac lymph nodes. After 35 days post infection (d.p.i.), PCV2 antigen was detected in a variety of tissues and organs in the pigs, and lesions were found on both the lungs and lymph nodes. This study was groundbreaking because it illustrated that in vivo transfection could be used to study the relationships of PCV2 genes to assess their roles in both virus replication and pathogenesis.

Not clearly understood is the reason that some pigs will develop clinical signs of the disease upon infection, whereas other pigs will not manifest any signs. Porcine circovirus 2b has been shown to be influenced by several factors that can either enhance or prevent spread of the virus. These aspects, as explained by Opriessnig and others in the 2007 review, are: virus-dependent factors, host-dependent factors, effect of coinfections, and effect of immune modulation.

**Influential Factors - Virulence**

Virus dependent factors can have an effect on the pathogenicity and transmission of PCV2. Virulence fluctuates from strain to strain; one isolate can infect a whole herd of pigs, whereas another isolate can have no clinical manifestations. It has been reported
that most herds in the United States are infected with PCV2 (Allan and Ellis, 2000; Opriessnig et al., 2006a), but many herds do not exhibit PCV2-associated diseases, whereas other farms report high occurrence and high mortality numbers (Larochelle et al., 2003; Opriessnig et al., 2006a).

A study by Opriessnig and others (2006b) compared two isolates, PCV2-40895 and PCV2-4838, both of which were defined as PCV2a. The isolate PCV2-40895 was taken from a pig diagnosed with post-weaning multisystemic wasting syndrome, a PCV-associated disease. The pig had lymphoid depletion, histiocytic replacement of lymphoid follicles, and high PCV2 antigen counts. The second isolate, 4838, was taken from a pig from an Iowa farm that suffered from respiratory disease. *Streptococcus suis, Pasteruella multicrode, and Mycoplasma hyopneumoniae* were isolated from this pig. The pig did not show signs of PCV2-associated lymphoid lesions, but did test positive for PCV2 DNA.

Viruses from both animals were sequenced and the genomes for both were found to be 1768 base pairs. In open reading frame (ORF) 1, the two isolates had 98.9 percent sequence identity and had only 19 sequence differences. The sequence differences resulted in five nucleotide differences; two caused amino acid shifts. The ORF 2 capsid gene of the two isolates shared 98 percent nucleotide identity and 96.1 percent amino acid identity. This study demonstrated that two strains that are genetically somewhat similar could have a variety of effects on the host.

Following this study, Opriessnig and others in 2008 conducted a study in which the same strains, now referred to as ISU-40895 and ISU-4838, were used, as well as NC-16845 and Can-17639. The strain NC-16845 was isolated from a group of pigs showing a history of severe respiratory disease, specifically lymphadenitis and bronchointerstitial
pneumonia. The strain Can-17639 was taken from a group of pigs demonstrating wasting, coughing, and bronchointerstitial pneumonia. While ISU-40895 and ISU-4838 were classified as PCV2a, NC-16845 and Can-17639 were classified as PCV2b. One hundred and thirteen pigs were used for the study and were inoculated with one of the four virus isolates. Upon necropsy and sequencing, it was determined that the four genome sequences shared 95.7% identity. The two PCV2b isolates alone shared 99.9% nucleotide sequence identity. However, because none of the isolates generated clinical symptoms, and because there were no differences in macroscopic lesions between groups, the results of the study only shed light on the differences between the strains, not their effects.

Another study published by Grierson and others (2004) looked at 10 isolates of PCV2 in the Netherlands from four farms with clinical signs of PMWS and six farms without. The virus was isolated and sequenced in each case, and the sequences were aligned. It was found that the 10 strains of PCV2 had 95.6 to 100% sequence identity. However, because the strains were causing symptoms on some of the farms and no symptoms on the others, and they were genetically very similar or exactly similar, it was concluded that small differences in nucleotide sequences of PCV2 do not fully elucidate why some pigs show symptoms and others do not.

Last, de Boisseson and others in 2004 looked at 23 herds in the region of Brittany in France. The virus from each herd was isolated and sequenced. It was noted that 10 of the isolates did not cause signs of PMWS and were recorded as PMWS – (negative). Thirteen of the strains did cause PMWS and were known as PMWS + (positive) cases. It was found that there was a broader nucleotide diversity (94.6 to 99.9% sequence identity) in the PMWS – pigs as compared to a nucleotide diversity of 99.5 to 99.9% in the
PMWS + pigs. Also, two clusters were identified that the sequences of strains fit clearly into. Interestingly, Cluster I contained the strains from France, the United Kingdom, and The Netherlands exclusively, while in Cluster II fit the strains from North America, Asia, Germany, and Spain. This study concluded that different strains of PCV2 have variable effects on the development of PMWS—some infected pigs showed signs of the disease and others did not.

**Influential Factors - Breed Differences**

Host susceptibility has been shown to affect disease progression of PCV2. Previous research has demonstrated that every pig breed around the world is susceptible to infection with PCV2. However, the degree of susceptibility appears to vary across breeds.

In a study by Opriessnig and others (2006a), three breeds were tested to determine whether there is a difference in susceptibility to PCV2 infection and development of PCV2 associated diseases. The three groups of purebreds used were Duroc, Landrace, and Large White because they represent the genetic make-up of a large percentage of commercial swine herds in the United States. Pigs were inoculated with a single strain of PCV2 (ISU-40895) and then scored for clinical symptoms, tested serologically, and underwent histological examination and immunohistochemical analysis. It was found that Landrace pigs had significantly more severe lymphoid depletion in the tonsillar region as well as in the lymph node pool. Also, microscopic lesions that were characteristic of PMWS developed in 15.8% of the Landrace pigs, whereas none of the Duroc and Large White pigs developed these lesions. These three Landrace pigs also experienced moderate
to severe granulomatous enteritis, as well as moderate lymphohistiocytic inflammation in the kidney and thymus. There were no significant differences in the susceptibility to PCV2, as measured by viremia, genomic DNA copy counts, and anti-PCV2 antibody levels. Nevertheless, there was a difference in the amount of lesions consistent with PMWS that developed in the Landrace pigs versus the Duroc and Large White pigs. So while Landrace pigs are no more susceptible to contracting the virus, they may have a higher vulnerability for PCVAD.

Opriessnig and others (2009a) continued breed susceptibility research by conducting a study using 39 Landrace and 39 Pietrain pigs. The pigs were inoculated with the same strain as the one used in the previous study (ISU-40895), were clinically evaluated, slaughtered, and underwent histopathology, lymphoid lesion scoring, and immunohistochemistry. For the Pietrain pigs, 34.6% had mild depletion of lymph node follicles. However, 30.7% of Landrace pigs had moderate to severe lymphoid depletion. In addition, Landrace pigs experienced a variety of lesions that were scored as mild (n = 6), moderate (n = 5), and severe (n = 1), whereas the Pietrain pigs had only mild lesions (n = 9). The study results imply that the Landrace breed demonstrates a higher susceptibility to the development of lesions associated with PCV2 infection. It also indicates that Pietrain pigs have the propensity for becoming infected with the virus and not developing histopathological symptoms.

A study by Rose and others in 2005 used 540 pigs that were born to Pietrain or random boars and random sows known as X and Y. The study consisted of clinical evaluations and serology. It was found that the average rate of occurrence of PMWS overall was 18%. In addition, the morbidity rate in the X-derived pigs was 17.3%, while
the morbidity rate in Pietrain pigs was 16.3%. This study demonstrated that Pietrain pigs might have some genetic capability to stop the development of PCVAD, although further testing is needed to confirm this finding.

**Influential Factors - Maternally Derived Antibodies**

Sows have the ability to pass on immunity to their piglets through colostrum secreted shortly after birth. Passively acquired antibodies can bolster the immune system’s ability to ward off viruses (McKeown et al., 2005).

McKeown and others (2005) conducted a study to determine the role of maternally-derived antibodies in the prevention of PCV2 infection. The study utilized 106 piglets that were separated based on antibody titers and infected with a PCV2 inoculate. Piglets in group A were considered negative for maternal antibodies, group B had low antibodies, and groups C and D had high antibody levels. Groups A, B, and C were infected at d0 and d42. Group D was not infected with the virus until d42. The piglets in group A experienced seroconversion by 42 days post infection (d.p.i.), indicating presence of infection. The low maternal antibodies in group B had waned by 14 d.p.i., and by 42 d.p.i., four of the five piglets had PCV2 DNA. For group C, the high antibody pigs, only two of the eight piglets had PCV2 viremia by 42 d.p.i. In group D, who were inoculated later than the other groups, all five pigs became infected with PCV2 after infection at d42, when the initial maternal antibodies had waned.

At the time of second challenge for groups A, B, and C, at d 42, all but group C were negative for maternal antibodies, and only two group C pigs became viremic. The results of this study indicated that piglets exposed to PCV2 with little or no maternal
antibodies did not have protection against the virus, and therefore exhibited seroconversion and became viremic.

In a study described by Calsamiglia et al. (2007), 15 sows from seven farms were chosen and after farrowing, the piglets were followed up to seven months of age. Pathological exams were performed to check for signs of PMWS. Forty of the 63 piglets that lived to the seven-month mark and sent to necropsy (63.5%) were diagnosed as having PMWS. However, 36 out of 38 (94.7%) sows that had piglets live were tested and found PCV2 negative. Thirty-two out of the 38 (84.2%) had antibody titers in the medium to high range. In the group of sows that had piglets die before the seven-month study was finished, only 2 of 7 (28.6%) had medium to high antibody titers. It was observed that increasing maternal immunity and decreasing sow infection status could lessen the propensity for PWMS.

Opriessnig and others (2004a) designed an experiment where the objective was to deliver piglets negative for PCV2 from sows positive for PCV2. Among other characteristics, sow herd antibody profiles, the transfer of passive antibodies, and maternal antibody decay were measured. Viremic conditions were not found in the piglets, and the authors suggested that passively-acquired antibodies may be able to stop PCV2 replication until the levels of maternal antibodies decrease. At this time, piglets would be able to develop clinical symptoms of PCV2 and could experience progression to PCVAD.
**Influential Factors - Co-infection**

Porcine circovirus 2 has been found to act as a co-infecting agent in conjunction with other viruses and bacteria. According to a review published by Grau-Roma et al. in 2010, there are at least eight viruses, three mycoplasmas, and three bacterial agents that have been found as co-infectors in PMWS cases. These viruses include PRRSV, PPV, porcine epidemic diarrhea virus, Aujeszky’s disease virus, Hepatitis E virus, torque teno virus (TTV), porcine teschovirus, and swine influenza virus (SIV). The Mycoplasma most associated with PCV2 is *Mycoplasma hyopneumoniae*, although two others, *M. hyorhinis* and *M. suis* have been noted as PCV2 co-infectors. Several bacteria, including *Salmonella cholera suis*, *Escherichia coli*, and *Haemophilus parasuis*, have been found in conjunction with PCV2.

In a field study completed by Pallares et al. in 2001 at the Iowa State Veterinary Diagnostic Laboratory, the experimenters reviewed 4,688 tissue submissions for presence of PCV2 and PMWS. Thirty-seven percent (1,751) of the tissues tested positive for PCV2 using immunohistochemistry. Conversely, 484 (10.3%) of the 4,688 samples tested positive for PMWS. Most (80%) of the cases used in the study came from Iowa, followed by North Carolina, Oklahoma, and Minnesota. Infection with both PCV2 and PRRSV was the most common combination, with 164 cases; following that was PCV2 with *M. hyopneumoniae* (92), PCV2 with bacterial septicemia (68), PCV2 with bacterial pneumonia (37), and PCV2 with swine influenza. In addition, 77 pigs experienced infections of PCV2, PRRSV, and *M. hyopneumoniae*. Only nine of 4,688 cases showed singular infection of only PCV2. Of the 384 cases demonstrating PMWS, 369 ages were provided. It was found that PMWS occurred between 8 and 18 weeks in 294 of the 369
pigs. PMWS was found most in pigs 10 weeks of age. This age of presentation was normally distributed and corresponds with the findings of other reports (Pallares, 2001).

A study by Krakowka and others (2000) looked at the effects of a dual infection of PCV2 and PPV in gnotobiotic pigs. Twenty-six piglets delivered by C-section were divided into infection groups, PCV1 alone (n = 3), PCV2 alone (n = 3), PPV alone (n = 3), PCV1 and PPV (n = 4), PCV2 and PPV (n = 4), and PCV1 and PCV2 (n = 4). Three pigs were kept as controls. Those pigs receiving only one infectious agent remained normal, with only a small enlargement of the lymph nodes. The four piglets receiving PCV1 and 2 together also remained clinically normal. Pigs inoculated with the combination dose of PCV2 and PPV developed acute gross lesions characteristic of PMWS. All four pigs developed a jaundice condition as well as subcutaneous edema. This caused the need for euthanasia on 30 d.p.i.. Three of the four piglets in this group experienced a decrease in thymic size, and three had moderate histological lesions. This was vastly different from piglets receiving a single infectious dose. The piglets with PPV alone never became viremic. Only half of the PCV1 pigs became viremic. This study confirmed that PCV2 is the viral pathogen, because PCV1 was unable to manifest clinical symptoms. Also, it was determined that in gnotobiotic piglets, the presence of PCV2 was consistent in all PMWS cases, but not enough alone to cause clinical symptoms.

Numerous studies have been conducted to understand the importance of PRRSV infection in the development of PCVAD. A brief report by Allan and others (2000) explained attempts to infect colostrum-deprived piglets with both PCV2 and PRRSV. Four pigs were inoculated with PRRSV alone, three with PCV2 alone, and five with an equal mixture of PCV2 and PRRSV. In the pigs infected with one virus, no clinical
disease or gross lesions developed. One pig with dual infection suffered from enlarged lymph nodes, and thymic atrophy was observed in another dual infection pig. Also, as is common with PMWS, lymphoid, liver, renal, and vascular lesion were seen in four of the five PRRSV/PCV2 pigs. Because disease developed in only those pigs dually-infected, it was suggested that infection with PRRSV could enhance the replication of PCV2 and thus lead to clinical symptoms of PCVAD.

Another study consisting of PRRSV and PCV2 was conducted by Harms and others in 2001. They utilized 59 piglets from 10 sows and placed them into four treatment groups: controls (n = 10), PCV2 (n = 19), PRRSV (n = 13), and PCV2/PRRSV (n = 17). No respiratory symptoms were noticed in the control pigs. The PCV2 pigs had mild clinical respiratory disease. One pig from the PCV2 group died of severe exudative epidermitis and three died from hemorrhagic gastric ulcers before the study was completed. In addition, two pigs from the PCV2 group were euthanized early due to severe depression, lethargy, and jaundice.

The PRRSV pigs developed acute respiratory problems after the seventh day post infection that were significantly worse than the PCV2 and control pigs; however, no pigs died or were euthanized from this group. The PCV2/PRRSV pigs had similar respiratory problems to the PRRSV pigs. Between 10 and 20 d.p.i., 10 of 11 pigs contracted acute dyspnea and depression. Four of these pigs died early, and six were euthanized for the sake of animal welfare. The remaining PCV2/PRRSV pig died at 20 d.p.i.. The PCV2/PRRSV pigs had more severe and recurrent hepatic lesions than PCV2 pigs, as well as more severe lymphoid depletion. In addition, the dually infected pigs all
experienced necrotizing hepatitis and interstitial pneumonia. It was concluded that PRRSV could enhance the progression of PCV2 in the pig model.

A study by Opriessnig and others (2004b) incorporated 67 crossbred piglets into four groups. Seventeen pigs received no inoculations, whereas 17 received *Mycoplasma hyopneumoniae* alone, 16 received PCV2 alone, and 17 received an infectious dose containing both *M. hyopneumoniae* and PCV2. Single *M. hyopneumoniae* infections exhibited mild to moderate respiratory disease whereas in dual *M. hyopneumoniae*/PCV2 infections it was mild to severe. The severity of *M. hyopneumoniae*-associated lesions were similar between single and dual infections. Pigs in the PCV2 alone group and the dual infection group experienced a two to three times normal size enlargement of lymph nodes. Pigs in the *M. hyopneumoniae* group did not illustrate this condition. All treatment pigs developed interstitial pneumonia, and four of the 17 dually infected pigs showed signs and lesions pertaining to PMWS. None of the PCV2 alone group exhibited signs of PMWS. The authors concluded that *M. hyopneumoniae* could strengthen the effects of PCV2 and lead to more severe clinical disease.

Last, Pogranichniy et al. (2002) conducted a field-based case control study in which pigs from four to 16 weeks of age showing signs of PMWS were observed. Thirty-one infected pigs and 56 controls were utilized and tested for PCV2, PRRSV, PPV, porcine enterovirus, SIV, porcine respiratory coronavirus (PRCV), transmissible gastroenteritis virus (TGEV), porcine endogenous retrovirus (PERV), porcine lymphotropic herpesvirus (PLHV), and bovine viral diarrhea virus (BVDV). All animals tested positive for PERV, which is known as omnipresent in swine. Porcine circovirus 2 was present in 29 of 31 (94%) PMWS pigs, as well as in 35 of 56 (63%) controls. In
addition, PRRSV was detected in 42% of the PMWS animals and 20% of the controls. After statistical analysis, it was determined that the odds ratio for the association between PCV2 and PMWS was 9.3, meaning that a pig with PCV2 is 9.3 times more likely to develop PMWS as compared to pigs not viremic for PCV2. The analysis also indicated that an animal is 31.2 times more likely to develop PMWS if infected by both PCV2 and PRRSV.

All of these studies currently lead to no concrete conclusions about whether a single pathogen perpetuates the infectious nature of PCV2. According to a review by Opriessnig and others from 2007, it appears that several infectious agents of varying pathogenicity play a role in the development and progression of PCVAD.

Transmission

Porcine circovirus 2 can be transmitted both horizontally and vertically. The high occurrence of PCV2 viral DNA in herds across the world reaffirm the fact that PCV2 is effective in terms of transmission (Kristensen et al., 2009). Horizontal transmission occurs when infected and non-infected pigs are mixed together (Grau-Roma et al., 2010). One study indicated that pigs kept in the same pen with direct contact were able to transmit the disease to other pigs more efficiently than pigs in separate pens (Andraud et al., 2008). The most common route of infection appears to be intra-nasally, although PCV2 can be shed in tonsillar, bronchial, and ocular secretions. Feces, saliva, milk, and semen have also been shown to contain PCV2 DNA and therefore could be modes of transmission for PCV2 (Krakowka et al., 2000).
In a study performed by Yang and others (2003), fecal and intestinal samples were obtained from 97 piglets, 63 with signs of enteric disease and 34 piglets that had no signs of gastrointestinal problems. Of the 63 piglets with enteric disease, 18 tested positive via PCR for porcine circovirus. Because PCV2 DNA was present in 14 intestinal samples and four fecal samples, it becomes evident that PCV2 can be shed in feces and therefore can be transmitted in fecal matter.

The possible existence of a respiratory route of infection was demonstrated by Magar and others in 2000, when they found that the virus is able to infect the nasopharynx and tonsils. From there the virus was able to spread throughout the body by way of blood or lymph (as reported in a review by Dupont et al., 2009).

Indirect infection, when pigs are kept in separate pens but can still spread the disease, occurs as well. Kristensen and others (2009) conducted a study with 12 herds of pigs where eight herds had signs of PMWS and four did not. In some cases, the pigs were mixed inside the pens; in other cases, the non-PMWS pigs were placed in pens next to PMWS-pigs; additionally, some affected pigs were kept in pens across the aisle from pigs not affected by PMWS. When the pigs were mixed within the pens, 10 of 18 with direct contact began to illicit signs of PMWS, while three of 18 in neighboring pens and one of 18 across the aisle also showed signs. This study concluded that direct and indirect contact may induce clinical signs of PMWS.

Vertical transmission, from dam or sire to piglet, has been proven by several studies. Trans-placental infection can occur after intra-nasal infection of pregnant sows before farrowing (Ha et al., 2008). PCV2 was found in both pigs born alive and pigs that
were aborted. Numerous studies have been conducted to find whether semen or colostrum containing PCV2 DNA can be infectious to piglets.

Madson and others in 2009 implemented a study to attempt to induce infection in PCV2-free sows by artificial insemination with PCV2-laced semen. Nine specific pathogen-free sows underwent estrus synchronization and insemination using a dose of semen mixed with three mL of either PCV2a or PCV2b. In the three sows inseminated with semen and PCV2a, no piglets were born live, stillborn, or mummified. The sows failed to maintain any pregnancy to gestation. In the three sows inseminated with semen and PCV2b, 35 piglets were delivered; however, only eight were born alive, two were stillborn, and 25 were mummified. Of the pigs coming from these dams, only the two stillborns acquired lesions, and these were classified as due to chronic myocardial dysfunction and cardiac dilatation. The authors concluded that intrauterine infection of sows could induce viremia in both the sow and fetuses (Madson et al., 2009).

Larochelle et al. in 2000 and Schmoll et al. in 2008 indicated that PCV2 can be shed in semen. In addition, a study performed by Patterson and others in 2010 sought to demonstrate and understand shedding and infection dynamics of PCV2. They used 26 pigs and inoculated them with the virus in a variety of methods: pooled oral samples using intraperitoneal (IP) administration, pooled fecal samples using IP, pooled nasal samples using IP, pooled oral samples administered orally, pooled fecal samples administered orally, pooled nasal samples administered intranasally (IN), and intramuscular (IM) injections to administer the virus. All three sources of IP administration resulted in PCV2 viremic conditions and seroconversion to anti-PCV2 antibodies. Nasal samples administered IN also resulted in PCV2 infection. Fecal
samples administered orally resulted in infection in two of four pigs. One of the three pigs with the IM injection became viremic. However, the oral samples administered orally did not result in viremia for PCV2. The conclusions from the study were that PCV2 is shed in large amounts nasally, orally, and in fecal secretions in an experimental infection.

In a study performed by Opriessnig and others, the authors wanted to determine if PCV2 was transmissible when fed to other pigs via meat and bone meal. Because PCV2 has been found in lymphoid tissue and bone marrow (Bolin et al., 2001), as well as in heart tissue (Kennedy et al., 2000), it is important to know the relationship between oral consumption and PCV2 infection. As found by Allan and others in 1994, PCV1 is stable at a pH of 3, and because meats in slaughter houses and packing plants do not typically reach a pH that low, it is likely that meat from a PCV2-positive animal could still contain an infectious form of the virus through storage and possible use afterward (Opriessnig et al., 2009b).

The authors collected tissue samples including skeletal muscle, spleen, tonsil, lymph nodes, and bone marrow from three pigs previously infected with PCV2. Then, they brought in 15 two-week old, colostrum-deprived crossbred piglets and separated them into treatments based on the tissues they would consume: skeletal muscle orally, bone marrow orally, lymphoid tissues orally, or PCV2 inoculate via a stomach tube. Necropsy on the piglets was performed, and the virus from the pigs was isolated and sequenced. It was found that consumption of PCV2 positive lymph nodes, bone marrow, and skeletal muscle caused viremic conditions after only seven days. All of the pigs in the study became PCV2 positive, and microscopic lesions due to lymphocytic depletion and
histiocytic replacement of lymph follicles were found in nine of the 15 pigs. It was determined that infection can be transmitted through uncooked skeletal muscle, bone marrow, and lymphoid tissue by way of oral consumption by PCV2-naïve pigs.

**Diseases Caused by PCV2**

*Post-weaning Multisystemic Wasting Syndrome*

Post-weaning multisystemic wasting syndrome, known as PMWS, occurs throughout the world (Chae, 2005). This disease was first discovered in 1991 in Canada, and affects primarily piglets from five to twelve weeks old (Gagnon et al., 2007). Symptoms of the disease include wasting and the inability to gain weight, enlargement of the inguinal lymph nodes, jaundice, and lymphocytic depletion (Gagnon et al., 2008). In addition, pigs with PMWS experience a decline in immunological function due to a marked reduction in circulating lymphocytes (Takahagi et al., 2009). The disease manifests itself in 10 to 30% of infected pigs (Darwich et al., 2004). This indicates that many pigs have developed a susceptibility to the syndrome, despite infection with PCV2. In herds that undergo an acute outbreak, mortality due to PMWS can reach 10%; in herds that are endemically affected, mortality totals are lower (Allan and Ellis, 2000).

Post-weaning multisystemic wasting syndrome is characterized by lesions that involve clusters of globular intracytoplasmic inclusion bodies (Sorden, 2000). Lymphoid lesions can be found in almost all tissues of an infected animal, including the tonsils, Peyer’s patches, spleen, thymus, and most commonly, lymph nodes (Darwich et al., 2004). Existence of these lesions is indicative of PMWS; however, these lesions are not
always present in an animal suffering from the disease. Therefore, there are several other methods to precisely detect PCV2 infection that can lead to PMWS.

Post-weaning multisystemic wasting syndrome can manifest itself in a variety of symptoms. When an infected animal is sent for necropsy, often it is found that the lungs are unable to collapse and are tan in color. The kidneys may have white streaks or spots. Pigs with PMWS may also experience enlargement of several lymph nodes, including mesenteric, mediastinal, and superficial inguinal (Gillespie et al., 2009).

In addition to the presence of lesions and other clinical signs, PMWS can alter cytokine expression in infected pigs. The disease causes an over-expression of interleukin 10 (IL-10) in the thymus, which can lead to thymic depletion as well as atrophy in the thymus. Also, PMWS increases the expression of interferon-gamma (IFN-γ) in the tonsils (Darwich et al., 2003). In the study Darwich and others published, they used 10 eight-week old piglets that were showing signs of wasting and respiratory disorder. Three pigs had such severe thymic atrophy that their thymus could not be found at necropsy. In the other seven pigs, enlargement of the lymph nodes, interstitial pneumonia, and lymphoplasmacytic hepatitis were observed. Histological lesions had mild to severe lymphocyte depletion. In addition, a marked increase in IL-10 was observed. Interleukin-10 is responsible for having an anti-inflammatory effect in the body, and also regulates natural killer and B-cell growth; thus, an increase in IL-10 indicates the presence of an active immune stressor. Another observation from the study was a decrease in IL-2 and IL-12 in the spleen, likely due to extensive T-cell damage.

Development of PMWS depends on numerous factors. Sows vaccinated against PCV2 can protect piglets via passive immunity. Experimental infections by Tomas et al.
in 2008 indicated that piglets that did not receive colostrum from sows had a higher incidence of PMWS. A study by Calsamiglia et al. in 2007 also demonstrated that piglets born to sows with low maternal antibodies had higher mortality rates than piglets born to sows with higher antibody titers. In addition, it was found that sows with high PCV2 viremia had higher piglet mortality rates (Grau-Roma et al., 2010). Also, management flaws such as drafts, overcrowding, mixing ages of pigs, and deficient air quality can worsen PMWS conditions (Harding and Clark, 1997). Post-weaning multisystemic wasting syndrome is known as multifactorial; PCV2 is needed for development of the disease, but infection with PCV2 is not necessarily enough to create a clinical condition (Calsamiglia et al., 2007).

In addition to sow immune status, another factor affecting PMWS development is virulence of the infecting strain. Numerous studies have confirmed that PCV2b is the strain most common in natural infections with PCV2 (Grau-Roma et al., 2010). Infection with PCV2a has been demonstrated to be associated with non-PMWS-affected farms (Carman et al., 2008; Grau-Roma et al., 2008). Therefore, the virulence of PCV2a is considered to be lower than the virulence of PCV2b.

In 2000, Sorden determined a definition of PMWS in which all factors must be present for accurate diagnosis. These factors included: presence of clinical symptoms such as weight loss, respiratory disease, and wasting; also, the presence of PCV2-associated lesions, which includes lymphoid depletion or histiocytic replacement of follicles in lymphoid tissues; and, the presence of PCV2 antigen or nucleic acid as determined by immunohistochemistry (Opriessnig et al., 2007).
In order for a complete and accurate diagnosis of PMWS, formalin-fixed tissue samples should be submitted for testing. Portions of the lung work best for diagnosis; other suitable tissues include lymphoid tissues (tonsil, thymus, spleen), pancreas, liver, and kidney. Fresh samples may be used to rule out the presence of other pathogens, including fresh lung, tonsil, and spleen (Sorden, 2000). In their paper, Opriessnig and others (2004b) explained a scoring system of seven lymphoid tissues to determine the extent of PCV2 infection. These tissues included: superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric lymph nodes; spleen; and tonsil. The scoring system ranged from zero to three for the categories of lymphoid depletion, granulomatous inflammation score, and PCV2-immunohistochemical score. This system enabled a subjective but consistent evaluation of tissues affected by infection.

There seem to be no herds safe from a PMWS outbreak. The disease can affect farrow-to-finish, farrow-to-feeder, off-site nursery, and grower pig operations. It has been found in small operations with as little as 50 sows and large operations with as many as 1200 sows (Harding and Clark, 1997).

Because many PCV2 cases experienced co-infection, it was postulated that various pathogens may use the same mechanism to evade the immune system. Another idea is that infection with PCV2 may make pigs more susceptible to the acquisition of other pathogens. Infection with PCV2 and other viruses simultaneously, such as porcine parvovirus or PRRSV, may perpetuate the effects of the virus (Krakowka et al., 2000).
Porcine circovirus 2-associated enteritis is not as well reported but can have effects on many systems in the body. Pigs with the disease experience diarrhea and inflammation of the colon (Kim et al., 2004a). The diarrhea starts out yellowish in color and progresses to black. Piglets with PCV2-associated enteritis go through periods of decreased weight gains. The disease manifests itself in pigs between 40 and 70d old (Chae, 2005).

Enteritis caused by PCV2 infection is another PCV associated disease. In a scenario described by Kim et al. (2004a), 150 Landrace x Large White sows experienced an outbreak of PCV2 induced enteritis, where 30% of the piglets developed diarrhea symptoms within a 3-week period. The diarrhea was non-responsive to antibiotic treatment. Two pigs were selected from the herd and sent to a diagnostic laboratory for necropsy. Two more pigs were selected from two other herds and submitted. The pigs were euthanized and samples of lung, heart, inguinal lymph node, tonsil, thymus, spleen, intestines, liver, kidney, and pancreas were collected, formalin-fixed and paraffin-embedded. Both small and large intestines were observed for the presence of several viral pathogens including PCV2, PPV, PRRSV, and classical swine fever virus (CSFV).

The pigs consistently demonstrated granulomatous inflammation and lymphoid depletion in the Peyer’s patches in both the large and small intestines. Two of the pigs suffered from multifocal necrosis and ulceration with fibrin in both the cecum and colon. PCV2 was isolated from the lymph nodes of two of the pigs as well. A hybridization signal for PCV2 was found in Peyer’s patches from the six pigs. The scientists found,
using in situ hybridization, that PCV2 replicated inside the macrophages of the Peyer’s patches.

Diagnosis criteria of PCV2-associated enteritis include three factors. First, the pigs must be suffering from clinical signs of diarrhea; next, the Peyer’s patches must contain those lesions consistent with PCV2 infection, but the lesions must NOT be present in the lymph nodes; last, lesions in the Peyer’s patches must contain traces of PCV2 (Chae, 2005). Diagnosis of the disease can create difficulty because the symptoms are similar both clinically and histopathologically to PMWS. The general rule is that if lesions are observed in the lymph node, the pig suffers from PMWS; lesions in the Peyer’s patches are due to PCV2-associated enteritis (Kim et al., 2004a).

*Porcine Respiratory Disease Complex*

Porcine respiratory disease complex (PRDC) clinically affects pigs around 16 to 22 weeks of age. Symptoms of the disease include retarded growth, lower feed efficiencies, anorexia, cough, and shortness of breath (Kim et al., 2003). An increase in mortality due to either single or multiple bacterial infections characterizes the disease complex (Chae, 2005). Pneumonia caused by PRDC can be due to both bacterial and viral pathogens. Co-infection with PCV2 and other viruses such as PRRSV, swine influenza virus, or *Mycoplasma hyopneumoniae* can intensify the clinical symptoms of the disease. For instance, co-infection with PCV2 and porcine parvovirus can produce severe lesions (Kim et al., 2003). In addition, experimental evidence suggests that a synergy between PCV2 and PRRSV exists (Chae, 2005).
In a study done by Kim and others in 2003, the results illustrated that PCV2 is common in pigs that suffer from PRDC. Eighty-five pigs out of 105 showed positive cells for PCV2, and 43 of the 85 pigs illustrated co-infection by PCV2, PRRSV, and porcine parvovirus (PPV) together. PCV2 was tested by in situ hybridization and consistently showed up in the lung lesions, which may mean that PCV2 plays an integral role in the development of PRDC (Kim et al., 2003).

Porcine respiratory disease complex generally elicits similar symptoms to PMWS. However, PRDC usually occurs in older pigs (16 to 22 weeks) as compared to PMWS (8 to 16 weeks of age). PRDC caused by PCV2 also has several specific histological signs, including widespread granulomatous inflammation, multinucleated cells, and intracytoplasmic basophilic viral inclusion bodies (Kim et al., 2003). And, PCV2 consistently causes lesions that demonstrate bronchointerstitial pneumonia with peribronchial and peribronchiolar fibrosis (Chae, 2005).

Specific criteria have been established in order to diagnose the presence of PCV2 as the cause of the disease complex. First, there must be the presence of respiratory symptoms that do not respond to antibiotic treatments. Next, the pig must have presence of pulmonary lesions; also, PCV2 must be present in the lesions. Last, the pig must not have the characteristic lesions associated with PMWS. If a pig meets all four criteria, PCV2 infection status is the confirmed cause of PRDC (Chae, 2005).

**PCV2-associated Reproductive Failure**

The first report of reproductive failure associated with PCV2 viral DNA surfaced in Canada in 1999 (Gillespie et al., 2009). Clinical signs from commercial farms showing
infection of PCV2 include higher incidence of abortions, stillbirths, and delivery of fetal mummies. Several studies conducted in the early 2000s and reported in a review paper by Chae in 2005 indicate that infection of the dam with PCV2 can lead to stillborn near-term fetuses and premature piglets.

Sanchez and others in 2001 determined that PCV2-associated reproduction problems can occur throughout all phases of gestation. In addition to infection of the dam, it has been demonstrated that PCV2 can be shed in semen of an infected boar. In a study by Larochelle and others, four boars were intra-nasally infected with two concentrations of PCV2 isolate LHVA-V53. The boars were housed singularly and semen was collected 10 times after infection with the virus. In addition, serum samples were taken eleven times to test for the presence of PCV2. It was found that the boars all recurrently shed PCV2 DNA in their semen. However, it was not determined if the PCV2 DNA was infectious, and the authors suggested that follow-up studies should be conducted to find out if PCV2 shed in the semen could induce infection in sows that were either naturally mated to infected boars, or sows that were artificially inseminated with infectious semen (Larochelle et al., 2000).

A study conducted by Kim and others (2004b) used 350 aborted and stillborn fetuses from 321 Korean farms to test for the incidence of PCV2, porcine parvovirus, and porcine respiratory and reproductive syndrome. Forty-three percent of the piglets that underwent reproductive complications tested positive for at least one of the three viruses. A total of 35 of the 350 cases tested positive for PCV2 alone. This study promoted the idea that PCV2-associated lung lesions are not always present in aborted fetuses. Of 12 fetuses that were aborted in late gestation, only four exhibited lesions. Two of six
stillborn piglets showed lesions as well. The lesions that were present were found in varying locations, and were moderately severe. The authors suggested that PCV2 might be able to induce reproductive failure in all stages of gestation - early, middle, and late.

O’Connor et al. (2001) described an outbreak at a 3,000-sow farm where acute reproductive losses were experienced. The rate of mummified fetuses rose to almost 15% during an 8-week period. In addition, the rate of stillborns and pre-weaning mortality hit highs of 8% and 11%, respectively, for that same 8-week period. Piglets from about 40 litters were sent to necropsy labs, where myocarditis and myocardial necrosis were found. Immunohistochemical staining exposed high amounts of PCV2 antigen in myocardial lesions in six of the piglets sent to necropsy. Samples from two stillborn piglets and two that died soon after birth were submitted and tested for presence of PCV2. The virus was found in the hearts of two piglets and in the pooled lung and splenic tissues of the other two. This study demonstrated that PCV2 could undergo vertical transmission and infects piglets in utero.

Park and others in 2004 described a study where six pregnant sows were infected with a strain of PCV2 isolated from an aborted fetus. Two sows remained uninfected as controls. The six infected sows delivered a total of 65 stillborn and 10 liveborn piglets, while the control pigs delivered a total of 18 liveborn piglets and no stillborns. Mild pneumonia was discovered in 15 of the stillborn, and five of the liveborn pigs that came from infected sows. The authors proposed that PCV2 may be able to infect sows and then cross the placenta to infect the piglets. Also, it was demonstrated that PCV2 could replicate in fetuses after intra-fetal infection.
Porcine dermatitis and nephropathy syndrome (PDNS) was first discovered in 1993 in the United Kingdom (Gillespie et al., 2009). The disease syndrome typically affects nursery and pigs in the grow-finish stage. However, PDNS has been described in finishing pigs and replacement gilts as well (Chae, 2005). Clinical signs of PDNS include fever, lethargic behavior, and occasionally loss of appetite (Harding, 2004). In addition to these signs, there are often physical manifestations of the disease. Red-purple skin lesions may arise in several locations, first starting in the hindquarters, limbs, and abdomen (Harding, 2004). These lesions usually progress to scabs containing black centers and are located on the rear legs (Gillespie et al., 2009). In addition to visual clinical signs, pigs experiencing PDNS often have enlarged, pale kidneys with hemorrhages (Harding, 2004). Other gross lesions include edema, enlarged lymph nodes, and gastric ulcerations (Drol et al., 1999). Pigs showing signs of anorexia, weight loss, and skin lesions combined usually die quickly; mortality rates in pigs showing a multitude of symptoms can reach 20% (Chae, 2005).

Porcine dermatitis and nephropathy syndrome is a vascular disease, with the primary lesion being a systemic necrotizing vasculitis. Lesions associated with PDNS mirror effects of immune complex-mediated disorders that have the ability to commence an acute inflammation (Drol et al., 1999).

The association between PDNS and PCV2 was recognized in 2000 (Opriessnig et al., 2007). Although PDNS has commonly been associated with PCV2, it remains unclear whether PCV2 is the only virus capable of causing the syndrome. In a study described by a review by Gillespie et al. in 2009, Krakowka and others in 2008 found that PDNS was
isolated from pigs infected with PRRSV and TTV, but these pigs were PCV2-free.

Another study by Wellenberg et al. (2004) looked at three pigs from each of 10 Dutch herds showing clinical signs of PDNS. The pigs demonstrated fever, lethargy, and were affected by skin lesions on several locations of the body. Upon necropsy, the pigs exhibited enlarged grey-brown kidneys with white spots on the renal cortex, as well as hemorrhagic lymph nodes. It was found that 100% of the pigs contained PCV2 DNA. In addition, 33% of the pigs tested positive for PPV and 83% of the pigs tested positive for PRRSV. Therefore, although it is known that PDNS is immune-mediated, there has been no conclusive evidence that it is related only to PCV2, and that PCV2 is the main cause of PDNS.

A study by Sipos and others (2005) observed the changes in cytokine levels in five pigs known to have PDNS, five pigs known to have PMWS, and five controls. In the PDNS pigs, amounts of the cytokines interleukin 1α (IL-1α), interleukin 6 (IL-6), and interleukin 10 (IL-10) were significantly greater than in the controls. This was likely due to an inflammatory response against the pathogen. In addition, interferon gamma (IFN-γ) levels were markedly greater (approximately two-fold) than in controls. The authors concluded that this may imply that cytotoxic T cells were important at this stage of infection. Lastly, levels of interleukin 2 (IL-2) and interleukin 4 (IL-4) both decreased. Since both are found in leukocytes, this could be due to a decrease in the amount of overall leukocytes.
**Diagnosis**

The presence of clinical signs has thus far been the most straightforward way to diagnose PCV2. Characteristics of PWMS, PDNS, and PRDC are generally recognizable on farms and in herds. Other associated diseases can be determined by various lab procedures. If PCV2 antigen is contained in more than one lymphoid tissue, or a lymphoid tissue and an organ system such as the kidney or lung, it is generally regarded that PCV2 is present in the body in an infectious capacity. Chronic severe PCV2 is demonstrated by severe lesions even in the presence of only limited PCV2 antigen content (Gillespie et al., 2009).

Immunohistochemistry (IHC) and in situ hybridization (ISH) are methods that have been established to recognize PCV2. Both tests use tissues from the animal that must first be paraffin-embedded and formalin-fixed. The IHC method utilizes both polyclonal antisera and monoclonal antibodies to detect presence of PCV2, and is a rapid process with quicker results than ISH. However, a study conducted by McNeilly et al. in 1999 and discussed in a review by Sorden, 2000, suggested that ISH has the ability to detect more positive cells than immunohistochemistry.

Both virus isolation and polymerase chain reaction (PCR) can be utilized to identify the presence of PCV2. Virus isolation is not as sensitive as immunohistochemistry or in situ hybridization, but is useful for the production of vaccines for further research. Using PCR, one can successfully detect between PCV1 and PCV2. However, it is a method that can easily incur contamination and show false positives. Sorden (2000) points out that PCR may be too sensitive a test to determine an accurate PMWS diagnosis. And because most pigs experience PCV2 infection at some point in
their lives, and PCR can pick up as little as one genomic copy, sensitivity is further an issue (Opriessnig et al., 2007).

Serology can be used to detect exposure to the virus using a test that can be performed on large numbers of pigs at once. Modes of serologic testing include indirect fluorescent antibody assays (IFA), immunoperoxidase monolayer assay (IPMA), enzyme-linked immunosorbent assay (ELISA), and a serum-virus neutralization assay. An IFA is subjective and is not automated. An IPMA is also subjective in terms of endpoints, and is not automated as well. The ELISA is sensitive and can aid in determination of when the infection occurs by comparing immunoglobulin G and immunoglobulin M levels. Last, the serum-virus neutralization assay requires fluorescent antibody or immunoperoxidase staining to conclude if viral replication is present (Opriessnig et al., 2007).

Serologic tests are generally used for diagnosis of herds, not individual pigs. Part of the reason for this is that pigs may be seropositive and not show any clinical symptoms of PCVAD. This should be taken into consideration when serologic tests are performed (Gillespie et al., 2009).

Grau-Roma and others, in a 2010 review, mentioned that quantitative real-time PCR (qPCR) is another method that can be used for determination of infection. Threshold levels can be set for blood samples or swabs to test for PCV2; these thresholds will differ from lab to lab and therefore consistency can be a problem. Using live animals limits the capacity of qPCR, however; there have been issues with specificity and sensitivity, and whether qPCR can replace histopathological examinations remains to be seen.
Prevention and Control

No treatment practices have been developed to combat PCV2 infection thus far. Because there is a wide variety of symptoms and range of reactions to infection, it had been a challenge to formulate a catchall procedure. Prognosis upon exposure to PCV2 relies on many factors, including immune status, age at infection, and specific syndrome that is manifested.

Prevention practices are also variable and inconsistent. Even the farm with the strictest vaccination and sanitation protocols can experience an outbreak. Prevention of co-infections by vaccinating for other bacteria and viruses can help avert the spread of PCV2, but this becomes impractical when so many co-infectious agents exist.

Disinfection to thwart PCV2 outbreaks is critical in large farms and research settings. In their 2007 review, Opriessnig and others discussed several methods of reducing viral replication. These methods, tested in laboratory settings, include: sodium hydroxide, Virkon S (Antec International, UK), Roccal D Plus (Parmacia and Upjohn, US), Clorox Bleach (Clorox Company, US), and Tex-Trol (Bio-Tek Industries Inc, US). Although not tested in commercial settings, these disinfectants have been successful in inhibition of the spread of PCV2, when used according to manufacturer’s directions.

Deterrence of PCV2 infection involves many basic strategies that farms may already have in place. Adequate housing management is a vital factor in the prevention of PCV2. Reduction of stress on the animals, proper hygiene of employees, and the use of an all in/all out strategy can reduce the risk of PCV2 contraction (Gillespie et al., 2009). In 2000, Madec and others devised a strategy known as ‘Madec’s 20-point plan’ to combat the spread of PCV2. Their plan included procedures such as limited animal
contact, proper disinfection, and the isolation or euthanasia of disease pigs in the herd.

Harding in 2004 also suggested that maintenance of ideal pen stocking density and segregation of pigs by age could decrease the spread of PCV2.

In addition, a list of factors that could decrease the risk of PMWS was discussed in Grau-Roma et al. review (2010). These factors include: separate flush pits for separate rooms of pigs, shower facilities for employees, sorting pigs by sex at the nursery stage, vaccination of sows against atrophic rhinitis, use of oxytocin during farrowing, and use of spray-dried plasma in nursery rations. Also outlined was a list of practices that might increase the incidence of PMWS, including: high levels of cross-fostering, large range in weight and age entering the nursery, and vaccination of gilts against PRRSV.

**Vaccinations**

Several vaccines have been developed to stop the progression of PCV2 associated diseases. The first vaccine, Circovac® by Merial, was approved for use in 2007. Since then, the FDA has approved four more vaccines for use in the United States.

Circovac® is a killed-PCV2 virus vaccine for sows that uses mineral oil as the adjuvant. The drug requires 2 mL intramuscular injections about three to four weeks apart at least two weeks before mating. A booster vaccine once every gestation period, at least two to four weeks before farrowing is scheduled, is also required. The drug is available for purchase in both Canada and Europe (Iowa State website).

Circumvent™ PCV by Intervet is an inactivated Baculovirus that uses two mL intramuscular injections three weeks apart. The vaccine is designed for healthy pigs three weeks or older and is sold in the United States and in Canada (Iowa State website).
Another vaccine by Intervet, Porcilis® PCV, is a PCV2 capsid that is designed for piglets as well, and is delivered with a Diluvac Tocopherol adjuvant. It also requires two injections (Burch, 2008).

Suvaxyn PCV2® One Dose was developed for healthy pigs four weeks or older. The vaccine is available in the United States, and is an inactivated PCV1-2 chimera. The vaccine is marketed by Fort Dodge® Animal Health (Iowa State website). It is developed with SL-CD aqueous as an adjuvant (Burch, 2008).

Last, Ingelvac® CircoFLEX™ is PCV2 expressed in inactivated Baculovirus and is delivered in a single dose of one mL intramuscularly. It is designed for piglets three weeks of age and older, and is marketed in United States by Boehringer Ingelheim (Iowa State website).

Numerous studies have been carried out to determine the efficacy of these vaccines since their approval in the past years. One such study was performed by Lyoo and others (2010) and tested the use of Circumvent™ PCV, Ingelvac® CircoFLEX™, and Suvaxyn PCV2®. Four pigs from 20 litters in a natural farm setting were selected and assigned one of the above doses, with the fourth pig remaining as a control. All pigs were vaccinated using the protocols as established by their respective companies. It was determined that the three vaccine groups differed in average daily gain (ADG), in which the pigs receiving the Circumvent™ PCV and Ingelvac® CircoFLEX™ vaccines had higher ADG than control pigs ($P < 0.05$). In terms of viremia, only 16.9% of the pigs vaccinated with Circumvent™ PCV tested positive for PCV2 DNA; 18.1% of the Ingelvac® CircoFLEX™ pigs tested positive; and 15.2% of the Suvaxyn PCV2® pigs tested positive. Although they had PCV2 DNA, they demonstrated no clinical symptoms.
Another study by Takahagi et al. (2009) tested two vaccines, Porcilis® PCV and Ingelvac® CircoFLEX™, according to their manufacturer regulations. A third group was not vaccinated and used as controls. A total of 997 pigs were used. It was found that at 24 weeks, the mortality rate of the Porcilis® PCV-vaccinated pigs was 11%; for Ingelvac® CircoFLEX™, the mortality rate was 13%; and for the controls, the mortality rate was 32%. Mortality rates were drastically reduced after vaccination and therefore use of a vaccination against PCV2 is a viable option to reduce the incidence of PCV2 associated diseases.

Although vaccines for PCV2 are relatively new to producers and scientists, they seem to be capable of decreasing infection and mortality rates and also increasing ADG. Further testing is needed to completely understand the mechanisms and full effects of the vaccines.

**Genetic Parameters for Selection**

Although there is no infallible method for treatment or prevention of PCV2 infection, it may be possible to select for animals with an increased resistance to PCV2. In a dissertation by Bates in 2008, a selection experiment was carried out to estimate genetic and environmental effects of PCVAD. The extensive study involved 3,271 pigs from two genetic lines. The lines had been selected over many generations for increased reproduction rates and growth. All pigs were transported to one of four locations, which included confinement buildings and outdoor lots. The pigs were scored for symptoms of PCVAD, including muscle wasting, slowed growth respiratory issues, and diarrhea. These scorings were performed approximately every seven to 10 days. Pigs receiving a
score of 0 displayed no symptoms and were considered normal. Pigs receiving a one 
score had minor symptoms and were thought of as potential PCVAD pigs. Pigs with a 
two score had obvious signs of PCV2 infection. Pigs receiving a two were sent to 
necropsy, and immunohistochemistry and real-time PCR was performed. All pigs tested 
negative for PRRSV, but co-infection with *M. hyopneumoniae* was found in 19 pigs. 
Eighteen pigs experienced a co-infection with *Streptococcus suis*, and four also had *Lawsonia intracellularis*.

Once the data were analyzed, several significant correlations and heritabilities 
were estimated. The PCVAD scores were compared with weights at birth weight, 
weaning weight, d65 weight, and d180 weight. It was found that birth weight and 
PCVAD score had a moderate positive correlation (r = 0.34, *P* < 0.10). Also, d65 weight 
had a strong negative correlation with PCVAD score (r = -0.53, *P* < 0.01). It was found 
that IgG levels at d90 were strongly negatively correlated with PCVAD score 
(r = -0.67, *P* < 0.001). In terms of direct heritability, PCVAD score had a low direct 
heritability (0.16 +/- 0.04). Birth weight had a moderate heritability (0.27 +/- 0.07). The 
heritability of weaning weight was 0.16 +/- 0.06, and that of both d65 weight and d180 
weight was 0.23 +/- 0.06.

There were also differences in weights at all time points. At birth, pigs with a 
PCVAD score of 0 differed from those with a score of 1 by 0.10 kg. At weaning, the 
difference was 0.51; at d65, the difference was 3.0; at d180, the difference was 20.9 
(all *P* < 0.001). This provided significant evidence that infection with PCV2 diminishes 
weight gain, and the problem is exponentially worse the longer the pig is alive.
The author suggested that a selection index could be utilized to lower the occurrence of PCV2 infection. An index using estimated breeding values (EBV) for traits such as PCVAD score and weight could accomplish this. If a pig having low weight at d180 and had a positive PCVAD score, this pig could be excluded from the breeding herd.

Bates discussed that selection could help in populations where PCV2 infection was consistently high. Although vaccinations appear to help control the spread of the virus, they can be costly to producers. Also, every pig must be vaccinated in order to gain protection against the virus. Using selection to control disease spread is an alternative that would not raise costs significantly to the producer. Selection against PCVAD is reliant on exposure to the virus. A pig must receive maternal antibodies, and it turn have them wane, to come into contact with the virus. Therefore, selection for PCVAD resistance is dependent on exposure to PCV2.

**Summary**

Porcine Circovirus 2 is a complex disease with no current treatment regime. While the complete pathology of the virus is not understood, steps can be taken to prevent outbreaks of the diseases that the virus causes. Vaccines are available but at a high cost to producers, so it is becoming increasingly evident that an alternative is needed. Genetic selection for disease resistance may become the best option for decreasing the prevalence of PCVAD. Because a definite difference in susceptibility of certain breeds exists, selection of candidates for reproduction can be used to decrease the incidence of PCV2. Because of the high economic impact of viral pathogens in swine
herds, genetic resistance is becoming more important as a mechanism to stop the spread of infectious diseases such as PCV2.
LITERATURE CITED


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CHAPTER 2: Variation in Response to Both Natural and Experimental Infection
with Porcine Circovirus 2b

2.1 INTRODUCTION

Porcine circovirus 2 (PCV2) has caused economical problems for producers and other members of the swine industry since its discovery in 1996. Infection with PCV2 can cause a host of other diseases known as PCV2 associated diseases (PCVAD). Diseases associated with PCV2 infection elicit a variety of symptoms. The largest losses from PCV2 infection have occurred following diagnosis of post-weaning multisystemic wasting syndrome, PMWS, which is characterized by an inability to gain weight creating a wasting condition, as well as jaundice and lymphocytic depletion (Gagnon et al., 2008). Another diseases caused by PCV2 infection include PCV2-associated enteritis, which causes diarrhea and inflammation of the colon (Kim et al., 2004). Porcine Respiratory Disease Complex elicits retarded growth, lower feed efficiencies, and coughing, and is known to be at least partially associated with PCV2 (Kim et al., 2003). PCV2-associated reproductive failure, causing abortion and stillborns; and porcine dermatitis and nephropathy syndrome (PDNS), which elicits fever, lethargy, and skin lesions (Harding, 2004). All of these symptoms cause unfit pigs that do not grow enough to reach their genetic potential.

Because there is no treatment for PCV2 infection, it is important to find alternative methods to stop the virus. Management practices and preventative measures have been introduced and in some cases implicated, but have not been successful in
complete eradication of the virus. Vaccines have been proven effective in several studies (Lyoo et al., 2010; Takahagi et al., 2009). Although they can efficiently prevent the spread of PCV2, vaccines also raise costs for producers and therefore put a strain on the industry.

The studies reported here investigated individual responses to both natural and experimental infections with PCV2 in pigs of two crossbred lines. The data were used to compare the two genetic lines and understand susceptibility differences between them. The objectives were to identify pigs with varying patterns of immune response and to determine if there were differences in other measurable traits, including growth, antibody level, and viremia. Another goal was to ascertain what components determine why some pigs show clinical signs and others do not. These studies represent preliminary efforts to establish a large database of tissues, blood samples, and phenotypes from infected animals in order to understand the factors that cause variation in immune responses to PCV2.
2.2 MATERIALS AND METHODS

2.2.1 TRIAL 1

*Animals:* All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. A composite Large White x Landrace cross selected for 29 generations for increased litter size and lean growth rate (Line 2, \( n = 166 \) representing 41 litters) and a control line with a similar genetic background (Line 16, \( n = 63 \), 16 litters) were used. Pigs were sired by a total of 25 boars. Pigs were farrowed at the University Swine Farm located in Mead, Nebraska, where routine testing for major pathogens occurs. All pigs were known to be free of porcine reproductive and respiratory syndrome virus (PRRSV). Following weaning at 21 days of age, 229 randomly-selected pigs of both sexes were transferred to a finishing barn at the Haskell Agriculture Laboratory in Concord, Nebraska. Pigs were placed randomly into 16 pens within one room. Pigs selected for the study were not vaccinated for PCV2; however, their dams were. All pigs were allowed ad libitum access to a standard, balanced diet supplied by a commercial feed mill.

*Serology:* Blood samples were collected on d14, d49, and at necropsy. The blood was collected in 7.5 mL BD Vacutainer tubes (Reference #367987, Franklin Lakes, New Jersey, USA). After collection, the samples were refrigerated until they could be centrifuged at 3500 rpm for 15 minutes at 4°C. The serum was poured off into 1.5 mL tubes for storage and the red blood cells remaining in the tube were discarded. Serum
samples were tested for the presence of PCV2–IgG and IgM using ELISA (Ingezim, Madrid, Spain). Samples were considered positive for IgG if the calculated sample-to-positive ratio was greater than 0.4. Samples were considered positive for IgM if the calculated sample-to-positive ratio was greater than 0.3.

The initial objective of the trial was to uniformly infect the pigs on the same day once maternal antibodies had waned. After infection, the plan was to monitor responses in viremia, immunoglobulin levels, and weight gain. However, it was found after the second bleeding that the pigs had already been exposed to the virus. The first sign of this was some of pigs showing clinical signs of PCV2 infection, including wasting and rough hair coats. This was determined by the IgM and IgG levels in the serum, as well as viral copy counts, collected at d 49. Because natural infection had occurred, the decision was made to not infect the pigs and to perform necropsy on all pigs as soon as possible.

Clinical Evaluation and Necropsy: Pigs were weighed at move-in (d0), d14, and d49. Necropsy was performed on four days because pigs were transported to the UNL Animal Science facility where necropsies were performed. Therefore, necropsy was performed with 63 pigs on d62, 59 pigs on d63, 54 pigs on d66, and 41 pigs on d69 of the experiment. Samples of spleen, lung, and mesenteric and bronchial lymph nodes were collected at necropsy. Half of each sample was snap-frozen in liquid nitrogen for future gene expression analyses and the other half preserved in formalin for histological examination.
**PCV2 Quantification:** Viral DNA was extracted from blood samples using the QIAamp DNA Minikit (Qiagen, Valencia, California, USA). DNA extracts were used for quantitative real time PCR quantification (qRT-PCR) and sequencing of PCV2 genomic DNA. Two pairs of primers were used for amplification of the PCV2b genome. The PCR was performed using GoTaq Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA) and standard protocols. Products from the PCR were purified using ExoSAP-IT (USB Corporation, Santa Clara, California, USA) and sequenced from both directions using dye terminators on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). A list of primers and probes used for both qRT-PCR and sequencing of the PCV2 genomic DNA is included in Table 1. BioEdit software (Ibis Therapeutics, Carlsbad, California, USA) was used to assemble and analyze the sequences.

**Genotyping:** A total of 110 pigs were genotyped using the PorcineSNP60 BeadChip by Illumina Inc. (San Diego, California, USA). These pigs were selected using SAS PROC PRINCOMP, principal component analysis, a multivariate method to look at relationships among many variables. The principal component analysis indexes animals based on the score given by the analysis, and the top 55 and bottom 55 were selected for genotyping. The PorcineSNP60 chip has both allelic-specific primer extension and single-base extension. The assay is based on direct hybridization of the whole genome-amplified DNA to a bead array of 50-mer specific probes. Once attached to the probe, the beads are pooled, arrayed randomly, and assembled into the wells of the slide. The hybridization of probe to DNA is followed by an enzymatic-based extension assay using DNA
polymerase and labeled nucleotides. The labels are stained and each single nucleotide polymorphism (SNP) is then scored.

**Sequence Analysis and Alignment:** The viral DNA was isolated from ten individuals showing signs of disease and sequenced. Sequencing was performed from both directions using dye terminators on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). This sequence, which matched in each individual, was aligned and entered into BLAST (Basic Local Alignment Search Tool) to find the PCV2b strain with the highest sequence identity.

**Statistical Analysis:** Correlations among variables (IgG, IgM, weight changes, and viremia) were estimated using PROC CORR of SAS software (SAS Institute Inc., Cary, North Carolina, USA). No fixed effects were included in the model.

The genotypes of each SNP were analyzed using GenCall Data Analysis Software (Illumina, San Diego, California, USA). Each SNP genotype was given a GenCall Score to sort out failed genotypes, DNA, or loci. This GenCall Score is based on the reliability of the SNP assay, DNA, or ability of a genotype call to cluster in genotype groups. The data was analyzed using JMP software (SAS, Cary, North Carolina, USA) and sorted by SNP position. This information was used to make overlay plots for both minor allelic frequency and Hardy-Weinberg Chi Square tests for both Trial 1 and Trial 2.

Gene associations were made using ASREML software (VCN International, Hemel Hempstead, United Kingdom). The software is a statistical package to fit linear mixed models using restricted maximum likelihood to fit large data sets. Each SNP from
BeadArray was assessed for association with PCVAD phenotypes using the ASREML package, with pen as a random effect, genotype as a fixed effect, and based on an extensive pedigree over six generations. Additive and dominance effects as well as an F value for each SNP were estimated. A false discovery rate was estimated for additive and dominance effects for every marker. Following analysis in ASREML, the data was entered into JMP for the creation of Manhattan plots to illustrate the genome-wide association studies.

2.2.2 TRIAL 2

**Animals:** All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. The experiment included 91 crossbred barrows from 24 litters. Pigs were either a two-way cross of Large White and Landrace (W x R, n = 72) or three-way crosses produced by mating Duroc sires with Large White x Landrace sows [D (W x R), n = 19]. Pigs were farrowed at the University of Nebraska Swine Farm in Mead, Nebraska, where routine testing for major pathogens occurs. All pigs were known to be free of porcine reproductive and respiratory syndrome virus (PRRSV). Pigs selected for the study were not vaccinated for PCV2, although their dams were.

At approximately 35 days of age, the pigs were moved to the Animal Science research facility for the infection experiment. The barrows were housed in one room of the building and randomly assigned into one of 18 identical pens. The pens contained a
combination of slatted and solid surface flooring. The pens allowed each pig approximately 0.65 m² of floor space, which exceeds standard space requirements for pigs of that age. All pigs were allowed ad libitum access to a standard, balanced diet manufactured at the ARDC feed mill.

**Experimental Infection:** The objective was to infect pigs with PCV2b virus after protection from maternal antibodies had waned. Another factor in the timing of the infection was to infect as soon as possible because of the threat of a natural infection. Blood was drawn from pigs at the University Swine Farm in Mead, Nebraska, before the pigs were transported to the Animal Science facility. The blood was analyzed for immunoglobulin G (IgG), which is an indicator of maternal antibodies, using an ELISA (Ingezim, Madrid, Spain). The sample-to-positive ratio of IgG in all individuals selected for the experimental infection was less than 0.3 at the time of inoculation. This level is specified by Ingezim as negative for antibodies. In addition, immunoglobulin M (IgM) was tested as an indicator of self-antibodies produced in response to infection. Sample-to-positive ratio was less than 0.4, the cut-off recommended by Ingezim, showing that natural infection had not occurred.

The virus inoculum contained the titer of $10^{4.0}$ 50% tissue culture infection dose (TCID₅₀)/mL in minimum essential media with 50 µg/mL gentamicin and 5% fetal calf serum. The original experimental protocol was to infect each pig with 2 mL of inoculum intramuscularly (IM) and 3 mL intranasally (IN). However, approximately 20 minutes after inoculation of 20 pigs, anaphylactic shock occurred in 10 pigs and eight of them died. Twelve pigs inoculated with 2 mL IM and 3 mL IN survived. The dose
administered to the remaining pigs was therefore altered. Most of the pigs (n = 58) received 1 mL IM and 3 mL IN. The rest of the pigs received a variation of this dose: 1 mL IM with 0.5 mL IN (n = 4) or 2 mL IM with 0.5 mL IN (n = 8). The pigs used for these treatments were chosen at random. Ten pigs were selected as negative controls, assigned to separate pens, and not inoculated.

**PCV2b Isolate:** Isolate UNL_VBMS was taken from a pig showing symptoms of PCV2b infection. Viral DNA was isolated using QIAamp DNA Minikit (Qiagen, Valencia, California, USA). Two pairs of primers were used to amplify the entire PCV2b genome. Amplification of DNA was performed using GoTaq Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA), and PCR products were purified using ExoSAP-IT (USB Corporation, Santa Clara, California, USA). Viral DNA was sequenced from both directions using dye terminators and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). Sequence analysis using BLAST revealed that the strain used for inoculation was most similar to strain FMV-05-6507. This strain was identified first in Quebec, Canada in 2005 and is known to induce clinical signs of PMWS and cause an increase in mortality rate.

**Serology:** Blood samples were collected before inoculation and at 7, 14, 21, and 28 d.p.i. (days post infection). The blood was collected into sterile 7.5 mL tubes (BD Reference #6530) and then separated into two tubes: half the blood went into the BD Vacutainer tubes used in trial 1, and the other half went in 7.5 mL Tempus Blood RNA tubes. These tubes contain an RNA stabilization solution that must be shaken vigorously once in
contact with blood, and are manufactured by Applied Biosystems (Carlsbad, California, USA). The blood in the BD tubes was refrigerated until it could be centrifuged at 3500 rpm for 15 minutes at 4°C to obtain serum. The Tempus tubes were frozen at -20°C for storage. Levels of PCV2-specific antibodies, IgG and IgM, were measured from serum using ELISA. Samples were considered positive if the calculated sample-to-positive ratio was greater than 0.3 for IgG and 0.4 for IgM. Antibody data were normalized based on positive control values obtained on each plate.

Clinical Evaluation and Necropsy: Pigs were observed daily for clinical signs of infection, and were weighed at 0, 7, 14, 21, and 28 d.p.i. Necropsy was performed at 28 d.p.i. Lung, spleen, and mesenteric and bronchial lymph nodes were collected for histological examinations and gene expression analysis.

PCV2 Quantification: Viral DNA was extracted from serum collected at 7, 14, 21, and 28 d.p.i. using QIAamp DNA Minikit. Estimates of viral copy number were obtained by quantitative real-time PCR using TaqMan Master Mix and ABI7900 (Applied Biosystems, Carlsbad, California, USA). A list of primers and probes used for both qRT-PCR and sequencing of the PCV2 genomic DNA is included in Table 1. The area under the curve (AUC) was calculated to estimate total viral load throughout the 28-day experimental infection.

Genotyping: All infected pigs were genotyped using the PorcineSNP60 BeadChip by Illumia Inc. (San Diego, California, USA). This chip is described in Section 2.2.1.
**Sequence Analysis and Alignment:** The PCV2 strain used in experimental infection was isolated from a pig with PCVAD symptoms, cultured and sequenced. Viral DNA from four pigs of each IgM group (early, late, and no responders) was sent for sequencing to confirm that they were infected with the same strain. Sequences were aligned using Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Once aligned, the sequences were entered into BLAST (Basic Local Alignment Search Tool) to find the sequence with the highest sequence identity.

**Statistical Analysis:** Least Squares Means (LSM) were obtained using mixed-model procedures including the immune response pattern with crossbred lines as fixed effects and pen and litter as random effects. The variables included in the model were IgG, IgM, viremia, and ADG at all time points. Analysis of the IgM antibody profiles during the challenge revealed that infected individuals could be characterized by three patterns of immune response and could be separated in three groups. Most pigs clearly fitted into one of these groups, but in some cases pigs were placed in a group based on a subjective judgment. Correlations among traits were calculated from variances and covariances adjusted for line effects.

The genotypes of each SNP were analyzed using GenCall Data Analysis Software as described in Section 2.2.1. This information was used to make overlay plots for both minor allelic frequency and Hardy-Weinberg Chi-Square tests using the data from both trials.

Gene associations were made using ASREML software (VCN International, Hemel Hempstead, United Kingdom) as described in Section 2.2.1. The model included
pen, litter, and genetic line as random effects, and genotype as a fixed effect. Following analysis in ASREML, the data were entered into JMP for the creation of Manhattan plots to illustrate the genome-wide association studies.
2.3 RESULTS AND DISCUSSION

2.3.1 TRIAL 1

Serology: All pigs used for the challenge came from dams that were vaccinated against PCV2. The pigs received maternal antibodies, specifically IgG, through colostrum from the dam during nursing following birth. Results from ELISA indicated that on d14, which was the first bleeding time point, 23 of 217 pigs (10.6%) were positive for IgG. Because the pigs were not bled before moving into the facility to check for maternal antibodies, it was deduced that the differences among pigs was due to the rate of maternal antibody decay varying from one individual to the next. However, IgG levels were much higher at d49 with 148 of 217 pigs (68.2%) being positive, indicating that infection had occurred between these time points and the immune system was mounting a secondary response to the virus. At necropsy, 198 of 217 pigs (91.2%) had a high enough sample-to-positive ratio to be considered positive for IgG.

In terms of IgM, a very low percentage of pigs were positive at d14, only two of 217 (0.009%). Because IgM is a measure of individual primary response to an immune stressor, it was determined that these pigs were experiencing PCV2 infection. The two infected pigs were housed in pens two and six. Because so many pigs were serologically positive for IgM at the next bleeding on d49 (77.4%), it seemed that these two pigs could have spread the virus throughout their pens and consequently to other pens. This is possible because the pens that housed the pigs had metal bars with approximately six inches of distance in between. This was enough room for the pigs to touch snouts
between the bars. Because nose-to-nose contact is a common mode of transmission, it is logical to assume that the virus started in two pens and was spread throughout the barn in a 35-day period. The number of positive pigs was somewhat lower at necropsy (72.4%). The modest decrease could be due to the fact that some of the pigs had already mounted an immune response against the virus. Once anti-PCV2 IgM had peaked after infection, it tended to decline.

This decline in IgM values has been demonstrated in previous studies done by Opriessnig et al. (2006). PCV2-specific IgM antibodies in the pigs infected with the strain PCV2-4838 peaked at d21 and began to decline by d28 post-infection. The levels of IgM in the pigs infected with the strain PCV2-40895 in their study were even higher, but followed the same declining trend. At necropsy, IgM response had started to drop for some of the pigs, but remained high for others. Also, new infections could have occurred between d49 and necropsy, so pigs that had had lower IgM values could have started to see an increase.

**PCV2 DNA in Serum:** Real-time quantitative PCR establishment of viral copy counts indicated that at d14 all pigs were negative for PCV2 viremia. At d49, the second bleeding, least squares means of all pigs was 3.75 viral DNA copies per mL serum (log 10) \( (P < 0.0001) \) for test of mean greater than 0. At the last bleeding at necropsy, least squares means for all pigs was 3.58 viral DNA copies per mL serum (log 10) \( (P < 0.0001) \). There were no differences between lines for viremia at any time point (Figure 1). For area under the curve for viremia, the least squares mean was 127.9 viral
DNA copies per mL serum (log 10) \((P < 0.0001)\). This statistic helps to explain the entire viral load that each animal experienced throughout the trial.

These results indicated that there was a steady increase in viremia throughout the infection period. This also showed that the pigs were unable to mount an adequate defense against the virus. As more pigs came into contact with the virus, it became increasingly harder to avoid becoming ill. It seemed that the viral copy counts peaked somewhere between d49 and necropsy. This sheds a little light on the timing in which the pigs first contracted the virus. This information can be combined with the fact that IgM levels spiked from d14, when only two pigs were positive, to d49, when 168 pigs were positive. Likely, around d49 the highest number of pigs were infected, and the highest viral copy counts were present in their DNA. Unfortunately, without more frequent testing, it is impossible to know the exact point of infection for each individual.

**Clinical Evaluation:** At necropsy, the following clinical signs were observed: evidence of mycoplasma \((n = 14)\), enlarged mesenteric lymph nodes \((n = 10)\), interstitial pneumonia \((n = 3)\), enlarged bronchial lymph nodes \((n = 2)\), edema \((n = 2)\), abscesses of the spleen and liver \((n = 2)\), peritonitis \((n = 1)\), and polyserositis \((n = 1)\). In addition, 12 pigs died before the conclusion of the trial, either in the pens or during the transportation process to the kill facility. Necropsy could not be performed on these animals.

**Genetic Characterization of the PCV2b Isolate:** Virus was isolated from ten individuals showing signs of disease and sequenced. It was established that the source of infection was a PCV2b strain, and was named UNL_ANSCI. It was found to have three
nucleotides that differed from the strain being developed for inoculation of the second round of pigs, Trial 2.

**Genotyping:** The PorcineSNP60 BeadChip was used to genotype 110 animals from Trial 1, and provides sufficient density in order to identify either markers or groups of markers that are in association with deviations of physiological traits. There were 62,163 SNPs, and the low GenCall Score Cutoff was 0.15. The GenCall Score Cutoff is calculated for each genotype and ranges from zero to one. Because the scores decline when the sample is further from the center of the cluster when the data point is associated, genotypes with a GenCall score of less than 0.15 are considered not “quality” and are not given a genotype. The average call rate of the 110 animals was 99.53%, and the average number of calls per animal was 59,613. The average number of no calls, in which the genotype could not be called with accuracy, was 281. The average heterozygosity was 24.9% for the 110 pigs genotyped.

Figure 2 illustrates overlay plots of minor allelic frequency versus SNP position at each chromosome for all pigs genotyped in both trials. For example, on SSC 11, between 32 and 41 Mb, the minor allelic frequency of a large number of SNPs is below 0.2. A similar approach was used to identify a chromosomal region where clusters of SNPs are in Hardy Weinberg genetic equilibrium (Figure 3). An example of this is on SSC1, at approximately 14 Mb, where there is a cluster of SNPs. Another cluster on SSC1 is between 27 and 28 Mb. SSC9 shows a cluster occurring between 60 and 61 Mb. These plots can be used to discover errors in genotyping methods as well. On SSC1, a SNP at
17 Mb appears to be informative because of its spread, but is likely due to genotyping error since it is one data point and not a cluster.

Both approaches can be used to uncover regions of the genome where selection is affecting the frequency of beneficial alleles by driving them either up or down. Genetic dissection of this region could uncover functional polymorphisms for traits of economic importance.

**Gene Associations:** The software package ASREML was used to determine genome-wide associations between the SNP genotypes for the 110 pigs genotyped and PCV2-specific traits- IgM, IgG, viremia, and ADG.

**Correlations:** There was a moderate positive correlation between the changes in weight from d1 to d14 with IgG at d49 (0.24, \( P < 0.05 \)). Another positive correlation existed between change in weight from d1 to d14 and IgG at necropsy (0.16, \( P < 0.05 \)). Correlations are given in Table 2. Because these correlations were weak, they cannot be used to draw exact conclusions about the variations in immune reaction that occurred during the trial.

**Implications:** The pigs in this natural infection experienced a variable response to infection with PCV2. Evidence of infection was found in 77.4% of pigs at d49 in terms of IgM response. Also the least square mean of viremia at this time point was 3.75 viral DNA copies per mL serum (log 10) at this time point. However, only 16.1% of the pigs had developed clinical symptoms of PCVAD. This is indicative that the patterns of
immune response differed among the pigs. Some of the pigs that were infected showed no clinical signs, and continued to grow at a normal rate throughout the experiment. These pigs were ideal because they could overcome the effects of the virus and still eventually reach a suitable market weight. Only a few of the pigs were very negatively affected by the virus and suffered from respiratory complications and stunted growth. It is important to be able to select against the pigs that showed clinical signs in order to have pigs remain healthy and grow to their potential.

Although it was difficult to draw definitive conclusions because of a variance in both timing of infection and amount of virus each pig was exposed to, it is important to understand that the immune response mounted for each pig differed. This preliminary study adequately set up the next trial to understand individual responses to infection with PCV2 in two different lines.

2.3.2 TRIAL 2

**Serology:** All pigs used for the challenge came from dams that were vaccinated against PCV2. The pigs received maternal antibodies, specifically IgG, through colostrum from the dam at birth. Antibody decay varies in piglets but on average will wane by 5 to 21 weeks. Of the initial 120 candidates for infection, 75% had antibody levels low enough to begin the infection process. This was determined by ELISA and a sample-to-positive ratio of less than 0.3 for IgG. Overall, 82% of the Large White x Landrace (W x R) pigs had IgG levels below the threshold (n = 82). Comparatively, 58% of the Duroc x Large
White x Landrace [D (W x R)] had levels low enough to infect (n = 31). The levels of IgG in the two crossbred groups did not differ ($P > 0.1$) before infection. Throughout the trial, there was no difference in IgM (Figure 4) or IgG (Figure 5) levels between the two genetic lines, W x R and [D (W x R)].

After infection, pigs were classified into one of three groups according to IgM response. This is demonstrated in Figure 6. Those pigs that had the highest change in IgM levels before d14 were classified as early responders (n = 33), and were placed in Group 1. Individuals that had the highest change in IgM levels after d14 fit into Group 2 (n = 40) and were called late responders. Group 3 consisted of pigs that did not respond to infection (n = 7); that is, they did not have a noticeable change in IgM levels.

IgM levels during the experiment indicated that an infection had occurred and that the barrows were building a primary response. At d7, there was no difference in IgM levels between the three groups and the controls. At d14, Group 1 had the highest IgM level with a mean of 1.38, whereas the mean for Groups 2 and 3 were 1.04 and 0.89, respectively. At d21, Group 2 had the highest IgM levels at 2.02, whereas Group 3 remained the lowest and Group 1 was intermediate. At necropsy (d28), Group 2 remained highest and Group 3 lowest. This indicates that in Group 3, with baseline IgM levels of about 0.89 throughout the challenge, no quantifiable change in IgM levels occurred, and therefore those pigs did not undergo a primary immune response (Figure 7).

There was no differences among the three groups in IgG levels, at d0 and 7. At d14, Group 1 and Group 2 differed in IgG levels ($P < 0.05$). At d21, Group 1 differed from Group 3 ($P < 0.05$), and there was a difference between Group 2 and 3 ($P < 0.05$) as
well. At necropsy, Group 1 differed from Group 3 \((P < 0.001)\) and Group 2 differed from Group 3 \((P < 0.0001)\) (Table 3).

Differences in both the magnitude and time of IgM response were observed during the experimental challenge with PCV2b. The three groups of IgM response experienced variance in response patterns (Figure 6). The chief difference between Group 1 and Group 2 was in the time of response. This was demonstrated by the peaks of IgM before d14 in Group 1 and between d14 and d21 for Group 2. Because all of the pigs were infected at the same time, and because there was no difference in IgG levels at d0 when infection occurred, it is unlikely that they had sufficient maternal antibodies to attack the virus.

Group 3 differed from the other two groups in the magnitude of response. Levels of IgG were higher in Group 1 than Group 3 at d21 and necropsy \((P < 0.05\) and \(P < 0.001\), respectively). Also, Group 2 had higher levels of IgG at d21 \((P < 0.05)\) and necropsy \((P < 0.0001)\) (Table 3). Therefore, Group 3 was utilizing some other mechanism to prevent infection from spreading throughout the body. There seemed to be an inhibition of viral replication that allowed these pigs to remain healthy and avoid subclinical signs of the virus.

**PCV2 DNA in Serum:** Quantitative PCR results for viral copy count indicated that all barrows were PCV2 DNA negative at the day of infection. At all other time points, all of the pigs that received the inoculation tested positive for infection with PCV2. Differences in viral copy numbers between the two crossbred lines existed at d7 \((P < 0.006)\) and d14 \((P < 0.01)\) as illustrated in Figure 8. However, DNA copy number followed the same
trend throughout the challenge for both lines: there was a steady increase in copy number through d21, then a sharp decline thereafter.

Pigs in Group 3, those who had no noticeable change in IgM values, maintained lower viral copy numbers than pigs in both Groups 1 and 2. At d7, Group 1 had the highest response at 4.56 viral DNA copies per mL serum (log 10), whereas the response in Group 3 was 3.85. At d14, Group 2 had the highest response with a mean of 5.24, whereas the mean for Group 3 was only 4.27. At d21, the mean for Group 2 continued to be highest, and Group 3 was lowest. At d28, the day necropsy was performed, Group 2 remained highest in viral copy count, while Group 3 remained lowest. Viremia area under the curve (AUC), a measure of overall viral load throughout the challenge, revealed Group 3 had the lowest AUC and thus the least viral load and Group 2 the highest. Viremia in the control pigs remained baseline during the challenge and had no peaks or spikes (Table 3).

The dose of inoculate administered to the pigs did not influence viral copy count, with the exception of viremia results on d7 (P < 0.05). However, only four pigs showed significant differences from the others, and they received the lowest dose of all groups of infected pigs.

**Clinical Evaluation:** Only one death occurred during the 28-day experimental challenge. This individual died during the fourth week of the challenge and displayed signs of a wasting condition, as well as lethargy and a rough hair coat. According to quantitative real-time PCR, this individual had the highest viral load three weeks after the challenge, and was categorized in Group 1, the early response group.
The only difference in growth occurred between Group 1 and 2 in terms of average daily gain (ADG) during the entire 28-day period. Group 1 had an ADG for the whole challenge of 0.42 kg, whereas pigs in Group 2 had an ADG of 0.36 kg for the challenge \( P < 0.05 \). Pigs in Group 3, those with no detectable immune response, had the greatest ADG and a trend toward having the best growth rate \( P = 0.19 \).

**Genetic Characterization of the PCV2b Isolate:** The strain isolated, sequenced, and aligned from 12 infected individuals belongs to a PCV2b cluster and has the highest similarity with strain FMV-05-6507. This strain has been shown to induce clinical signs of PMWS and cause an increase in mortality rate. Upon alignment of the sequence used for infection and the 12 sequences recovered from infected pigs, it was determined that the strain used for infection was identical to the strain found in the 12 pigs whose DNA was sequenced.

**Genotyping:** All 81 individuals from Trial 2 were genotyped using the PorcineSNP60 chip. The number of SNPs was 62,163 and the Low GenCall Score Cutoff was 0.15. The average call rate was 99.65%, and the average number of calls for the 81 pigs was 59,687. The average heterozygosity of the 81 pigs was 33.8%. Figure 2 illustrates overlay plots of minor allelic frequency versus SNP position at each chromosome for all pigs genotyped in both trials. A similar approach was used to identify a chromosomal region where clusters of SNPs are in Hardy Weinberg genetic equilibrium (Figure 3). These figures are explained in the Section 2.3.1.
**Gene Associations:** The software package ASREML was used to determine genome-wide associations between the SNP genotypes and PCV2-specific traits- IgM, IgG, viremia, and ADG. The association between SNP genotypes and IgM at d21 is illustrated in Figure 9.

On chromosome 1, two SNPs were discovered. The first gene connected with a SNP was syntaxin 11, also known as STX11. This gene encodes a member of the protein group of syntaxins that works in intracellular movement. Syntaxin 11 is primarily expressed in phagocytes and antigen presenting cells. Its role in vesicle trafficking may play a role in the granule secretory pathway. This gene is known to cause familial hemophagocytic lymphohistiocytosis in humans (Danielian et al., 2009).

Another SNP found on chromosome 1 was GNAQ, also known as guanine nucleotide binding protein. This protein encodes the alpha subunit of heterotrimeric G proteins. The role of these proteins is to attach the seven-transmembrane domain receptors to allow for intracellular signaling (Van Raamsdonk et al., 2010). Mutations in the protein cause a mutation known as dominant dark skin that is characterized by increased dermal melanin (Emley et al., 2011).

A SNP on chromosome 6 associated with IgM was close to the interleukin 34 (IL-34) gene. Interleukin 34 is found in many tissues of the human, including lung, liver, kidney, and in abundance in the spleen. This member of the cytokine family plays a role in regulation of myeloid cell growth and differentiation. It has also been shown to encourage the formation of a macrophage progenitor in human bone marrow cultures (Lin et. al, 2008).
In addition, there were two possible SNPs associated with IgM on chromosome 14. The first, positioned near the G protein-coupled receptor kinase 5 gene (GRK5), is in the family of serine/threonine kinases that assist phosphorylation of seven-transmembrane-spanning G protein-coupled receptors. The variety of receptors that they have effects on include adrenergic, muscarinic, and chemokine receptors. The fifth kinase in the family specifically phosphorylates a transcriptional factor and tumor suppressant, p53, which is a regulator of both apoptosis and proliferation. In mice, deletion of GRK5 results in hypothermia, hypoactivity, tremors, and increased salivation (Chen et al., 2010).

Last, also on chromosome 14 was a SNP located near the TIAL1 locus (TIA1 cytotoxic granule-associated RNA binding protein-like 1). This protein is involved in nuclear and cytoplasmic RNA metabolism, and is important in splicing regulation in mammals. It also has been found to be a translational regulator to assist cellular and virus-induced apoptosis, specifically by adapting the cellular response to deal with metabolic stress and inflammation (Bossowski et al., 2010).

**Correlations:** Moderate negative correlations existed between average daily gain (ADG) and viremia area under the curve (AUC) for several time points. These values are located in Table 4. Viremia AUC from d0 to d7 was correlated with ADG for the whole challenge (-0.22, \( P < 0.05 \)). Viremia AUC from d0 to d14 had a correlation of -0.24 with ADG for the whole challenge (\( P < 0.05 \)). The correlation between AUC from d0 to d21 and ADG from d0 to d28 was -0.27 (\( P < 0.05 \)). The strongest correlation was between AUC for the whole trial and ADG for the whole trial at -0.29 (\( P < 0.01 \)). This correlation indicated that the higher the viral load of the pig, the less it would be able to put on
weight and grow to its genetic potential. Figure 10 illustrates the correlation between the Z score values for AUC PCV2 copy number and ADG, with each dot representing a pig. Pigs in the lower right corner demonstrated the most desirable phenotype, with the highest grow rates and the lowest viremia.

These correlations help to understand the effects of disease progression of PCV2 on pigs in the grow-finish stage. Pigs with high viremic conditions struggle to catch up with their littermates and siblings because with viral replication comes certain wasting conditions. Though it has not been demonstrated that feed intake suffers during a period of experimental infection such as this, it has been shown in numerous studies that growth has been stunted in pigs infected with PCV2.

A study done by Blanchard et al. (2003) compared vaccinated pigs to unvaccinated pigs in terms of PMWS disease progression. They challenged 28 pigs of various immune statuses, with one group being unvaccinated. Ten to 14 days after infection, all of the challenge-control group (pigs that were not vaccinated and were challenged with the virus) developed both pyrexia and growth retardation. On the other hand, pigs inoculated against the virus had significantly higher growth at both the second and third week post infection.

Albina et al. (2001) found similar results when they studied the effects of direct transmission on piglets. Two of the piglets with signs of PMWS before the experiment showed signs of emaciation, weakness, and had a high rate of weight loss. These two piglets had to be euthanized before the trial ended. The rest of the piglets underwent one of their nine trials and were either inoculated with PCV2 or kept as controls. It was
shown that all infected pigs developed both pyrexia and growth retardation after about 13 days of infectious status.

**Implications:** Differences in the magnitude and time of IgM response was observed during experimental challenge with PCV2b. The difference between Group 1 and Group 2 was in the time of response, while the difference between Groups 1 and 2 and Group 3 was in the magnitude of response. Group 3 had the lowest viral copy count and the highest growth, most likely due to a mechanism that inhibited viral replication. The pigs in Group 3 demonstrated the most desirable phenotype, one in which the pigs’ immune system never mounted a response to the disease while also having the propensity toward having the best growth rates. Following studies will be aimed at understanding and characterizing the mechanism in which these pigs deal with a viral infection such as PCV2. Once research has been completed to explain the response to viruses, selection against those pigs susceptible for PCVAD will lead to healthier, faster growing pigs.
2.4 CONCLUSION

The results of this preliminary research provide evidence of a significant variation in immune response from one individual to another. The natural infection illustrated that some infected pigs showed clinical signs to the disease while other infected pigs did not elicit symptoms. It also helped explain transmission patterns and virus progression. The experimental challenge revealed three groups of pigs that differed greatly in both antibody response and viral copy count numbers. It also demonstrated that some pigs utilized a mechanism of inhibition of viral replication that is not clearly understood.

With a virus like PCV2, several challenges exist. First, the virus is present everywhere in the world, and is very hard to disinfect against. Almost every herd in the United States has been exposed to the virus. This makes the development of pigs that are specific pathogen free for PCV2 extremely difficult. Sows must to be vaccinated against the virus to protect against stillborns, abortions, and fetal mummies. This creates logistical problems because piglets must be virtually free of maternal antibodies to accurately simulate a disease challenge. Therefore, a waiting period must be undergone that differs for every pig. Knowing and synchronizing when each pig is ready for infection is a practical factor to consider. In the case of a natural infection, it becomes difficult to understand infection dynamics when there is such a variation in infection. When the exact timing of the occurrence of infection cannot be determined, the variation in response times and magnitudes is due to numerous uncontrollable factors. In addition to the standard environmental and litter effects, pigs infected at different times with different amounts of virus cannot be uniformly studied.
Another challenge is that pigs must be closely monitored to catch clinical symptoms and subclinical infections. This means frequent bleedings to test for anti-PCV2 antibodies and viremic conditions. Regular bleedings, more frequent than once a week, can put a strain on the pigs, as well as require an immense amount of labor and time. Sensitive tests such as ELISA work well for diagnosis, but make the question of exact timing of infection difficult unless the pig is bled daily, a practice that can put the animal in danger.

In Trial 2, the experimental infection, there were clear differences in the groups of pigs that had an early or late response versus the group showing no response. A lack of immune response has several explanations. One such elucidation is that the pigs were infected with different strains of the virus. Since pigs are so susceptible to pathogens in new environments, this argument is relevant. However, upon isolation and sequencing of the virus from four pigs in each IgM group (1, 2, and 3), the virus infecting the animals was found to be identical. This indicates that the lack of response to infection of the Group 3 pigs cannot be attributed to a difference in virulence.

Another possible suggestion is that the pigs had variable levels of maternal antibodies (IgG) circulating in their system from colostrum at the time of birth. However, the IgG ELISA confirmed that pigs in each group had no significant differences in IgG levels at d0 before infection ($P > 0.05$). All three groups of pigs, as well as the controls, were negative for maternal antibodies, thus the reason they were selected for the experiment. A difference in maternal antibodies cannot explain the variation in IgM response of the three groups.
A genetic effect could certainly exist. Because the groups of pigs constituted two genetic lines, the incidence of viral infection could be due to a susceptibility factor. However, analysis of the two lines indicated that there was no significant difference in viremia, IgG, IgM, or weight gain between the Large White x Landrace pigs and the Duroc x (Large White x Landrace) pigs. Although a genetic component to disease resistance has been demonstrated in several studies, the variation in response in this study cannot be solely attributed to genetics.

There is significant evidence that selection for disease resistance is not only possible but also may answer many questions. However, without a panel of reliable markers, selection criteria are lacking. Genome-wide association studies to better understand the genes responsible for resistance to diseases may be key to understating a complex disease like PCV2. More research is needed to establish an extensive database of not only phenotypes but also genotypes, SNPs, and candidate genes to better understand progression and prevention of the virus. In order to achieve this, many more experimental infections must be conducted to better understand the mechanisms, timing, and variance in response to PCV2 infection.
2.5 LITERATURE CITED


Table 1. Primers and probe sequences used for qRT-PCV and cDNA sequencing of PCV2b - Trial 1 and 2.

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>5’ – 3’</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2_q_F1</td>
<td>TGGCCCCGCAGTATTCTGATT</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>PCV2_q_R1</td>
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<td>qRT-PCR</td>
</tr>
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<td>PCV2_F1</td>
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</tr>
<tr>
<td>PCV2_R1</td>
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<td>DNA Sequencing</td>
</tr>
<tr>
<td>PCV2_F2</td>
<td>GACAGCAAAAGATAACAAACTCCAC</td>
<td>DNA Sequencing</td>
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<td>PCV2_R2</td>
<td>CCATGAGGAGCAGGAAGG</td>
<td>DNA Sequencing</td>
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Table 2. Correlations between IgM, IgG, and change in weight at all timepoints - Trial 1.

<table>
<thead>
<tr>
<th></th>
<th>Change in Wt, d1 to d14</th>
<th>Change in Wt, d14 to d49</th>
<th>Wt Change Total</th>
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</thead>
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<td>IgM d14</td>
<td>-0.02</td>
<td>0.10</td>
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<td>IgG d14</td>
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<td>0.13</td>
<td>0.07</td>
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<td>IgM d49</td>
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<td>0.03</td>
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<tr>
<td>IgG d49</td>
<td>0.24&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.12</td>
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<td>IgM Necropsy</td>
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<tr>
<td>IgG Necropsy</td>
<td>0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.10</td>
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<sup>a</sup>Indicates $P < 0.05$
Table 3. Least Squares Means of the parameters profiled during challenge in the groups of individuals that showed different patterns in immune response to infection - Trial 2.

<table>
<thead>
<tr>
<th>Trait</th>
<th>1 n = 33</th>
<th>2 n = 40</th>
<th>3 n = 7</th>
<th>Control n = 9</th>
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<tbody>
<tr>
<td>ADG, 0 to 7 d.p.i.</td>
<td>0.21</td>
<td>0.15</td>
<td>0.18</td>
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<td>ADG, 7 to 14</td>
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<td>0.32</td>
<td>0.37</td>
<td>0.37</td>
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<td>ADG, 14 to 21</td>
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<td>0.43</td>
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<tr>
<td>ADG, 21 to 28</td>
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<td>0.53</td>
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<tr>
<td>ADG, 0 to 28</td>
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<td>0.36(^b)</td>
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<tr>
<td>IgG 0</td>
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<td>0.68</td>
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<tr>
<td>IgG 7</td>
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<td>0.78(^c)</td>
<td>0.75(^c)</td>
<td>0.94(^d)</td>
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<tr>
<td>IgG 14</td>
<td>0.68(^a)</td>
<td>0.61(^b)</td>
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<td>0.69</td>
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<tr>
<td>IgG 21</td>
<td>1.71(^c,a)</td>
<td>1.83(^e,a)</td>
<td>0.91(^b)</td>
<td>0.70(^f,d)</td>
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<tr>
<td>IgG 28</td>
<td>1.89(^g,e)</td>
<td>2.08(^g,i)</td>
<td>0.74(^f,j)</td>
<td>0.56(^h)</td>
</tr>
<tr>
<td>Viremia 7</td>
<td>4.56(^c)</td>
<td>4.37(^c)</td>
<td>3.85</td>
<td>3.22(^f,d)</td>
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<tr>
<td>Viremia 14</td>
<td>5.16(^g,a)</td>
<td>5.24(^g,a)</td>
<td>4.27(^g,b)</td>
<td>2.13(^h)</td>
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<tr>
<td>Viremia 21</td>
<td>5.8(^g,e)</td>
<td>6.17(^g,e)</td>
<td>4.67(^f,d)</td>
<td>3.79(^h)</td>
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<tr>
<td>Viremia 28</td>
<td>4.66(^g)</td>
<td>4.7(^g,a)</td>
<td>3.96(^c,b)</td>
<td>2.56(^h,d)</td>
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<tr>
<td>Viremia AUC</td>
<td>124.11(^g,d)</td>
<td>127.53(^g,d)</td>
<td>104.06(^c)</td>
<td>72.991(^h,d)</td>
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</table>

\(^{1}\)The infected individuals were separated in groups based on their response to infection as 1) early 2) late and 3) no response to infection.

Significant differences between groups are represented with different superscripts in the same row as follows: \(^{a,b}\) \(P < 0.05\), \(^{c,d}\) \(P < 0.01\), \(^{e,f}\) \(P < 0.001\) and \(^{g,h}\) \(P < 0.0001\) or \(^{i,j}\) \(P < 0.0001\).

\(^{2}\)Units for ADG are kilograms of gain per day; units for IgG are sample-to-positive ratio; units for viremia are viral DNA copies per ml serum (log 10).
Table 4. Correlations between weight gain and viral load - Trial 2.

<table>
<thead>
<tr>
<th></th>
<th>ADG 0 to 7</th>
<th>ADG 0 to 14</th>
<th>ADG 0 to 21</th>
<th>ADG 0 to 28</th>
<th>AUC 0 to 7</th>
<th>AUC 0 to 14</th>
<th>AUC 0 to 21</th>
<th>AUC 0 to 28</th>
</tr>
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<tbody>
<tr>
<td>ADG 0 to 7</td>
<td>1.00</td>
<td>0.83</td>
<td>0.74</td>
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<tr>
<td>ADG 0 to 21</td>
<td>0.74</td>
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<td>0.92</td>
<td>-0.17</td>
<td>-0.17</td>
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<tr>
<td>ADG 0 to 28</td>
<td>0.66</td>
<td>0.82</td>
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<td>-0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.27&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>AUC 0 to 7</td>
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<td>-0.22</td>
<td>1.00</td>
<td>0.96</td>
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<td>AUC 0 to 14</td>
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<td>AUC 0 to 21</td>
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<td>-0.29</td>
<td>0.78</td>
<td>0.90</td>
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<sup>a</sup> Indicates $P < 0.05$ and <sup>b</sup> indicates $P < 0.01$
FIGURES

Figure 1. Log 10 of viral copy number for two crossbred lines - Trial 1.
Figure 2. Overlay plots of Minor Allelic Frequency vs. SNP Position for each chromosome - Trial 1 and 2.
Figure 3. Overlay plots of Hardy Weinberg Chi-Square Values vs. SNP Position for each chromosome - Trial 1 and 2.
Figure 4. IgM sample to positive ratios (S/P) during the experimental challenge of two crossbred lines - Trial 2. Large White x Landrace pigs are denoted W x R, while Duroc (Large White x Landrace) pigs are denoted D (W x R).
Figure 5. IgG sample to positive ratios (S/P) during the experimental challenge of two crossbred lines - Trial 2. Large White x Landrace pigs are denoted W x R, while Duroc (Large White x Landrace) pigs are denoted D (W x R).
Figure 6. Individual patterns of IgM response to an experimental PCV2 infection - Trial 2. 

Infected individuals display different patterns of response to infection as: 1) early response - solid black; 2) late response - solid grey; 3) no response - dashed black.

\[\text{IgM (normalized based on PCV2 positive control)}\]

Days post inoculation
Figure 7. Least Square Means of IgM sample to positive ratios (S/P) profile during PCV2b experimental infection based on grouping by IgM response - Trial 2.
Figure 8. Log 10 of viral copy number for the two crossbred lines - Trial 2. Large White x Landrace pigs are denoted W x R, while Duroc (Large White x Landrace) pigs are denoted D (W x R).
Figure 9. Genome wide associations between SNP genotypes and PCV2-specific IgM at d21 - Trial 2¹.

¹The X-axis represents the location of the SNPs from SSC1 to 18, SSCX and a group of SNPs (represented in grey) without a precise location. Y-axis represents the F value. Each dot represents the F value corresponding to the additive effect of a SNP.
Figure 10. Individual correlations between ADG and viremia AUC based on grouping by IgM response - Trial 2. Axes represent Z score values for ADG on the x axis and AUC PCV2 copy number on the y axis.

Overall correlation: $r = -0.29$ (p < 0.01)
APPENDIX

Appendix Ia. Genome wide associations between SNP genotypes and traits measured in Trial 1 - additive effects.

**ADG d0 to d14 (t > 21.6 corresponds to a FDR < 0.11)**

![Graph showing ADG d0 to d14](image1)

**ADG d14 to d49 (t > 19.0 corresponds to a FDR < 0.1)**

![Graph showing ADG d14 to d49](image2)

**ADG d0 to d49 (t > 9.3 corresponds to a FDR < 0.3)**

![Graph showing ADG d0 to d49](image3)
IgG d14 (t > 8.6 corresponds to a FDR < 0.4)

IgG d49 (t > 17 corresponds to a FDR < 0.78)

IgG Necropsy (t > 9.5 corresponds to a FDR < 0.4)
IgM d14 (t > 11.2 corresponds to a FDR < 0.2)

IgM d49 (t > 13.1 corresponds to a FDR < 0.4)

IgM Necropsy (t > 8.9 corresponds to a FDR < 0.7)
Viremia d49 (t > 8.7 corresponds to a FDR < 0.6)

Viremia Necropsy (t > 9.2 corresponds to a FDR < 0.9)

Viremia AUC (t > 5.9 corresponds to a FDR < 0.81)
Appendix Ib. Genome wide associations between SNP genotypes and traits measured in Trial 1 - dominance effects.

**ADG d0 to d14 (t > 5.37 corresponds to a FDR < 0.9)**

**ADG d14 to d49 (t > 13.7 corresponds to a FDR < 0.2)**

**ADG d0 to d49 (t > 13.6 corresponds to a FDR < 0.3)**
IgG d14 (t > 6.0 corresponds to a FDR < 0.7)

IgG d49 (t > 13.2 corresponds to a FDR < 0.71)

IgG Necropsy (t > 9.0 corresponds to a FDR < 0.6)
IgM d14 (t > 17.4 corresponds to a FDR < 0.1)

IgM d49 (t > 6.3 corresponds to a FDR < 0.8)

IgM Necropsy (t > 7.6 corresponds to a FDR < 0.5)
Viremia d49 (t > 9.9 corresponds to a FDR < 0.3)

Viremia Necropsy (t > 13.9 corresponds to a FDR < 0.6)

Viremia AUC (t > 6.0 corresponds to a FDR < 0.9)
Appendix IIa. Genome wide associations between SNP genotypes and traits measured in Trial 2 - additive effects.

**ADG d0 to d7 (t > 10.0 corresponds to a FDR < 0.2)**

**ADG d7 to d14 (t > 20.3 corresponds to a FDR < 0.45)**

**ADG d14 to d21 (t > 26 corresponds to a FDR < 0.085)**
ADG d21 to Necropsy (t > 11.5 corresponds to a FDR < 0.1)

IgG d0 (t > 10.8 corresponds to a FDR < 0.3)

IgG d7 (t > 5.9 corresponds to a FDR < 0.4)
IgG d14 (t > 6.8 corresponds to a FDR < 0.6)

IgG d21 (t > 7.2 corresponds to a FDR < 0.6)

IgG Necropsy (t > 7.1 corresponds to a FDR < 0.5)
IgM d0 (t > 9.3 corresponds to a FDR < 0.9)

IgM d7 (t > 11.0 corresponds to a FDR < 0.4)

IgM d14 (t > 15.2 corresponds to a FDR < 0.1)
IgM d21 (t > 6.2 corresponds to a FDR < 0.5)

IgM Necropsy (t > 9.8 corresponds to a FDR < 0.2)

Viremia d7 (t > 11.2 corresponds to a FDR < 0.7)
Viremia d14 (t > 5.8 corresponds to a FDR < 0.4)

Viremia d21 (t > 8.8 corresponds to a FDR < 0.3)

Viremia Necropsy (t > 8.5 corresponds to a FDR < 0.5)
Viremia AUC (t > 25.6 corresponds to a FDR < 0.1)
Appendix IIb. Genome wide associations between SNP genotypes and traits measured in
Trial 2- dominance effects.

**ADG d0 to d7 (t > 5.5 corresponds to a FDR < 0.7)**

![Graph showing ADG d0 to d7 (t > 5.5 corresponds to a FDR < 0.7)](image)

**ADG d7 to d14 (t > 12.0 corresponds to a FDR < 0.999)**

![Graph showing ADG d7 to d14 (t > 12.0 corresponds to a FDR < 0.999)](image)

**ADG d14 to d21 (t > 9.4 corresponds to a FDR < 0.6)**

![Graph showing ADG d14 to d21 (t > 9.4 corresponds to a FDR < 0.6)](image)
ADG d21 to Necropsy (t > 5.0 corresponds to a FDR < 0.5)

IgG d0 (t > 11.2 corresponds to a FDR < 0.3)

IgG d7 (t > 7.6 corresponds to a FDR < 0.3)
IgG d14 (t > 5.6 corresponds to a FDR < 0.7)

IgG d21 (t > 7.2 corresponds to a FDR < 0.7)

IgG Necropsy (t > 7.0 corresponds to a FDR < 0.5)
IgM d0 (t > 11.4 corresponds to a FDR < 0.7)

IgM d7 (t > 11.8 corresponds to a FDR < 0.3)

IgM d14 (t > 13.3 corresponds to a FDR < 0.3)
IgM d21 (t > 5.9 corresponds to a FDR < 0.8)

IgM Necropsy (t > 7.0 corresponds to a FDR < 0.4)

Viremia d7 (t > 11.5 corresponds to a FDR < 0.9)
Viremia d14 (t > 6.8 corresponds to a FDR < 0.6)

Viremia d21 (t > 10.2 corresponds to a FDR < 0.2)

Viremia Necropsy (t > 8.1 corresponds to a FDR < 0.9)
Viremia AUC ($t > 10.5$ corresponds to a FDR < 0.4)