Understanding the pathogenesis of porcine circovirus type 2 (PCV2)-associated diseases

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Understanding the pathogenesis of porcine circovirus type 2 (PCV2)-associated diseases

by

Tanja Ilse Opriessnig

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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ABSTRACT

Porcine circovirus type 2 (PCV2) is associated with several disease manifestations in pigs including postweaning multisystemic wasting syndrome (PMWS). The hallmark microscopic lesions of PCV2-infection are lymphoid depletion or granulomatous lymphadentitis (or both) and the presence of PCV2 antigen or nucleic acids associated with the lymphoid lesions. PCV2 alone is limited in its ability to induce the full spectrum of disease and lesions associated with PMWS in pigs.

PCV2 is widespread in the global swine population and in order to establish a PCV2 model in conventional pigs it was necessary to identify a method to derive pigs free of PCV2 or anti-PCV2 antibodies. We obtained more than 500 PCV2-free pigs from six seropositive breeding herds and demonstrated that segregated early weaning is an effective technique to derive PCV2-free pigs from positive breeding herds for research or for commercial production.

We determined that vaccination with commercially available adjuvanted bacterins enhances PCV2 replication and PCV2-associated lesions. We also found that timing of vaccination in relationship to PCV2 infection is important in the outcome of vaccine-induced enhancement of PCV2 replication and PCV2-associated lesions.

Field reports indicated that PCV2-infection may also decrease the efficacy of vaccines used at the time of PCV2 infection. When we vaccinated pigs with a modified-live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine 14 days post PCV2 inoculation we found that PCV2 infection significantly decreased the efficacy of the PRRSV vaccine as measured by gross and microscopic lesions.

It is thought that coinfections may be an important trigger for progression of PCV2 to PMWS and other PCV2-associated diseases. We experimentally confirmed that PCV2 and porcine parvovirus coinfection resulted in clinical PMWS in conventional pigs whereas singular infection does not. Coinfecting conventional pigs with PCV2 and Mycoplasma hyopneumoniae resulted in severe respiratory disease, reduced average daily gain, and severe lung and lymphoid lesions associated with PCV2-antigen in dual-infected pigs implying that M. hyopneumoniae potentiates the severity of PCV2-associated lung and lymphoid lesions and increases the incidence of PMWS.
Host and virus differences may also be important in the outcome of PCV2 infection. In our conventional pig model, we found a predisposition of Landrace pigs to PCV2-associated lesions and disease when compared to Duroc and Large White pigs. Finally, we were the first to experimentally confirm that PCV2-field isolates differ significantly in virulence in our conventional pig model.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is prepared as an alternate manuscript format. The dissertation includes a general introduction and a literature review, eight separate scientific manuscripts, and general conclusions. The appendix includes a short communication related to this project. References to literature cited in the general introduction and the general conclusions chapters are listed at the end of the dissertation. Seven manuscripts have been published in refereed scientific journals relevant to the focus of this dissertation and two manuscripts are submitted for publication. The Ph.D. candidate, Tanja Ilse Opriessnig is the primary author of the manuscripts and is the principal investigator for the experimental work described in the manuscripts.

The first manuscript describes the derivation of porcine circovirus type 2 (PCV2) free pigs from positive sow herds and has been published in the *Journal of Swine Health and Production*. The second manuscript describes the effect of vaccination with selected bacterins on PCV2-associated lesions and has been published in *Veterinary Pathology*. The third manuscript describes the effect of timing of vaccination on PCV2-associated lesions and has been published in *Veterinary Record*. The fourth manuscript describes the effect of acute PCV2-infection on the efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine and has been submitted to *Clinical Vaccine Immunology*. The fifth manuscript describes the effect of porcine parvovirus (PPV) vaccination on PCV2-associated lesions in the PPV and PCV2 coinfection model and has been published in *Veterinary Microbiology*. The sixth manuscript describes experimental reproduction of postweaning multisystemic wasting syndrome in the PCV2 and *Mycoplasma hyopneumoniae* coinfection model and has been published in *Veterinary Pathology*. The seventh manuscript describes differences in host susceptibility to PCV2-associated disease and lesions and has been published in *Veterinary Pathology*. The eighth manuscript describes differences in virulence of PCV2-field isolates recovered from pigs with and without PCV2-associated lymphoid depletion and has been submitted to the *Journal of General Virology*. 
The appendix contains a brief communication that describes cardiovascular lesions associated with natural and experimental PCV2 infection and has been published in the *Journal of Comparative Pathology*.

**Introduction**

Porcine circovirus (PCV) was first isolated from pigs suffering from postweaning multisystemic wasting syndrome (PMWS) in 1997 (Allan et al., 1998b). Subsequent characterization showed that the PMWS-associated PCV was genetically and antigenically different from the cell culture contaminating apathogenic PCV that had been identified more than 20 years earlier (Tischer et al., 1974) and the name “PCV type 2 (PCV2)” was proposed for the newly identified PMWS-associated circovirus variant (Allan et al., 1998a; Allan et al., 1998b; Ellis et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998).

PMWS was first recognized in the early 1990’s in high health commercial herds in western Canada (Harding, 1996; Harding and Clark, 1997). Shortly after the initial recognition and description of PMWS, the syndrome was almost simultaneously observed in France (LeCann et al., 1997) and in the USA (Daft et al., 1996) followed by reports from most European countries and Southeast Asia (Allan and Ellis, 2000).

In North America, PMWS usually results in low but persistent death losses and only on rare occasions severe epidemics resulting in 3- to 4-fold increases in mortality have occurred (Harding, 2004). In Europe the situation is different, and PMWS is often associated with persistent and high mortality in many areas (Done, 2001). The close association between PMWS and PCV2 has been confirmed experimentally; PMWS has been reproduced in experimental models following PCV2-infection (Bolin et al., 2001; Harms et al., 2001; Ladekjær-Mikkelsen et al., 2002) and case-control studies have proven that PMWS is strongly associated with PCV2-infection (Pogranichniy et al., 2002; Wellenberg et al., 2004). PCV2 is not only linked to PMWS but is also associated with other diseases and syndromes such as the porcine respiratory disease complex (PRDC) (Harms et al., 2002a, Kim et al., 2003b) and porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000). PCV2 is associated with sporadic outbreaks of reproductive failure mainly in gilts.
(Ladekjaer-Mikkelsen et al., 2001; O’Connor et al., 2001; West et al., 1999) and enteritis in grow/finish pigs (Kim et al., 2004a).

We have much more to learn about the epidemiology and pathogenesis of PCV2-associated diseases. Most swine herds in the global pig population are seropositive (Allan and Ellis, 2000). Its ubiquitous distribution and its presence in clinically healthy pigs makes it difficult to evaluate the true impact of PCV2 and it also makes it difficult to understand the pathogenesis of the disease. Although PCV2 has consistently been shown to be the necessary cause of PMWS, it is clear that other factors are necessary for full expression of PCV-associated disease. These factors include external factors such as coinfecting agents or immunostimulation, pig specific factors such as genetics and antibody status at the time of infection, and virus specific factors such as differences in PCV2 isolates.

The main focus area of our PCV2 research was to develop a model for PCV2-infection in conventional pigs suitable for application to the US field situation. Most available research data described experiments that were conducted in Europe where housing and vaccination practices are different from what is common practice in the US. It was also important to us to develop a model using conventional colostrum-fed pigs as opposed to gnotobiotic pigs, cesarean-derived, colostrum-deprived pigs (CDCD), or colostrum-deprived (CD) pigs. In order to develop a model for PCV2-associated diseases in conventional pigs it was first necessary to find a way to derive pigs free of PCV2 and anti-PCV2 antibodies from commercial US breeding herds. We attempted to do this by segregated early weaning (SEW).

There is evidence in the literature suggesting that immune stimulation may trigger progression of PCV2 infection to disease and lesions characteristic of PMWS. Krakowka et al. (2001) reproduced PMWS in gnotobiotic pigs stimulated with keyhole limpet hemocyanin in Freund’s incomplete adjuvant and infected with PCV2. Furthermore, commercially available vaccines have been implicated in the enhancement of PCV2 replication and PCV2-associated disease. Allan et al. (2000c) observed PMWS in 21% of PCV2-infected pigs that were vaccinated with a commercial M. hyopneumoniae and Actinobacillus pleuropneumoniae bacterin. Field trials further supported this hypothesis. Allan et al. (2002b) observed a significant increase in losses associated with PMWS in M. hyopneumoniae vaccinated pigs. A similar observation was made by Kyriakis et al. (2002) who vaccinated a
portion of the pigs in a herd experiencing a PMWS outbreak with a \textit{M. hyopneumoniae} vaccine and observed PMWS in 43\% of the vaccinated pigs compared with 11\% of the non-vaccinated pigs. The results of these European studies are not ideally suited for extrapolation to the US field situation. One of our objectives was to determine whether vaccination with bacterins commonly used in the US, when administered at a time typical of US protocol, enhances PCV2 replication and the incidence and severity of PCV2-associated microscopic lesions in conventional pigs. Vaccinations, however, play a very important role in the swine industry to minimize losses associated with pathogens such as \textit{M. hyopneumoniae}, swine influenza virus and others. Therefore, simply eliminating a successful vaccination program might be contraindicated. Therefore, another objective we addressed was to determine if there is an effect of timing of vaccination on PCV2 replication and PCV2-associated lesions. We compared commonly used two-dose regimens and one-dose regimens with the goal to understand and advise producers on the best point in time to use \textit{M. hyopneumoniae} vaccines in herds with recurrent PCV2-associated diseases.

PCV2-infection can induce varying degrees of lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. The presence of this hallmark lesion implies a temporarily impaired adaptive immune response that may compromise vaccine efficacy. Porcine reproductive and respiratory syndrome virus (PRRSV)-induced pneumonia is a major global problem. The next study was designed to investigate the effect of acute PCV2-infection on the efficacy of a modified live virus (MLV) PRRSV vaccine to protect growing pigs against PRRSV-induced respiratory disease and lesions.

Coinfecting pathogens have been shown to trigger PCV2-infection to progress to full blown PMWS. Ellis et al. (1999) reproduced most of the lesions typical of PMWS in gnotobiotic pigs inoculated with filtered cell culture material and filtered lymphoid tissues from pigs with naturally acquired PMWS. Both PCV2 and porcine parvovirus (PPV) were detected in the experimentally infected pigs. It was determined, that the same PPV that is typically associated with reproductive disease is able to enhance PCV2-replication and PCV-associated disease in growing pigs (Allan et al., 1999a; Kennedy et al., 2000). PPV is not a pathogen typically associated with grow-finisher operations but practitioners were increasingly interested in eliminating or minimizing the potential effect of PPV on promoting
PCV2-infection. Another objective of our work was to determine if co-infection of conventional SPF pigs with PCV2 and PPV induces an increase in the incidence of PMWS compared to singular PCV2 infection, and to determine if vaccination against PPV protects pigs against PMWS associated with PCV2/PPV co-infection.

Recent diagnostic investigations of field cases have indicated that *M. hyopneumoniae* vaccination in herds affected by PMWS may actually increase the incidence of PMWS. This information has led some practitioners to discontinue the use of *M. hyopneumoniae* vaccination programs in affected herds. However, *M. hyopneumoniae* vaccines are well established in the field and have been shown to be economically beneficial in reducing losses associated with respiratory disease. Thus, the fifth animal trial was conducted to investigate the interactions between *M. hyopneumoniae* and PCV2 and to evaluate the effect of *M. hyopneumoniae*/PCV2 co-infection.

Essentially all farms are infected with PCV2; however, relatively few experience PCV2-associated disease. Differences in susceptibility among pigs might be one possible explanation for the variability in disease manifestation seen with PCV2. We designed and conducted an experiment to determine whether host genetic differences in the susceptibility to PCV2-associated disease exist among selected breeds of pigs. It is also not clear if PCV2 isolates vary in virulence. If so, this may explain much of the difference in severity of PCV2-associated diseases from farm-to-farm. Distinct, purified PCV2 isolates had not yet been compared in a controlled study in SPF pigs. Thus, the last objective of the work included in this thesis was to compare the pathogenicity of PCV2 isolates obtained from field cases with or without PCV2-associated lymphoid lesions and disease.
CHAPTER 2. LITERATURE REVIEW

Historical Background

Porcine circovirus (PCV) was first recognized as a contaminant of the continuous pig kidney cell line PK-15 (ATCC-CCL31) in 1974 in Germany and described as picornavirus-like virus (Tischer et al., 1974). PCV is a very small, nonenveloped, single stranded DNA virus with a circular genome (Tischer et al., 1982). Under experimental conditions, the PCV-PK-15-isolate did not produce disease in pigs (Allan et al., 1995; Tischer et al., 1986). In the late 1990’s PCV was associated with a newly emerged disease syndrome in pigs “postweaning multisystemic wasting syndrome” (PMWS) (Allan et al., 1998b). Sequence analysis of the PMWS-associated PCV revealed differences compared to the earlier described PCV (Allan et al., 1998a; Allan et al., 1999b; Ellis et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). In order to distinguish the pathogenic PMWS-associated PCV from the non-pathogenic PCV, the first was designated porcine circovirus type 2 (PCV2) and the later porcine circovirus type 1 (PCV1).

The presence of PCV2 can be dated back to 1985 in Canada (Magar et al, 2000b), Belgium, and Spain (Rodríguez-Arrioja, 2003b), to 1973 in Ireland (Walker et al., 2000), 1970 in the UK (Grierson et al., 2004a), and 1969 in Belgium (Sanchez et al., 2000). Magar et al. (2000) tested 177 serum samples from 1985, 145 serum samples from 1989, and 147 serum samples from 1997. All sera were obtained from Canadian slaughterhouses and tested by immunofluorescence assay (IFA) for antibodies to PCV1 and PCV2. In 1985, 8% of the sera were positive for PCV1 and 13.6% were positive for PCV2. In 1989, 41.4% of the sera were positive for PCV1 and 72.4% were positive for PCV2. In 1997, 38.1% of the sera were positive for PCV1 and 66.7% were positive for PCV2 (Magar et al., 2000b). Walker et al. (2000) detected PCV2-antibodies in the majority of pig serum samples collected in Northern Ireland from 1973 until 1999. The percentage of sera containing PCV2-antibodies showed an increased incidence in the samples collected in 1988 (100%; 80/80) and 1999 (92.1%; 129/140) when compared to 1973 (69.1%; 56/80), 1981 (61.3%; 49/80), and 1984 (55%; 44/80). Sporadic cases of PMWS were identified in archived tissues before the emergence of PMWS in the 1990’s. Grierson et al. (2004a) reviewed formalin-fixed tissues from 68
porcine cases that had been submitted to a laboratory in England between 1970 and 1997 by PCR, open reading frame (ORF) 2-sequencing, and immunohistochemistry (IHC) for PCV2 specific nucleic acids and antigen. The authors found PCV2-specific nucleic acids in 41% (9/22) of submission from the 1990’s, in 31% (4/13) of submission from the 1980’s, and in 32% (8/25) of submissions from the 1970’s. Sequence analysis of 5 archived tissues revealed a high identity to PCV2 sequences obtained from a 2000 porcine dermatitis and nephropathy syndrome (PDNS) case implying that a similar PCV2 isolate has been present in the UK pig population for more than 30 years (Grierson et al., 2004a). Rodríguez-Arrioja (2003b) investigated archived tissues from 189 pigs for the presence of PCV2-DNA by in-situ-hybridization (ISH) and archived sera from 388 pigs for the presence of PCV2-specific antibodies by indirect immunoperoxidase monolayer assay (IPMA) collected from 1985 to 1997 in Spain and found 41.3% (78/189) tissues ISH positive and 72.7% (282/388) sera IPMA positive. This is indicative of enzootic infection in Spain since 1985 (Rodríguez-Arrioja, 2003b). Sanchez et al. (2001) tested 50 serum samples from 1969, 50 serum samples from 1975, and 50 serum samples from 2000 obtained in slaughter houses in Belgium. All serum samples were positive for PCV2-specific antibodies by IPMA (Sanchez et al., 2001).

**Taxonomy**

Both, PCV1 and PCV2 are members of the *Circoviridae* family (Todd et al., 2005). The *Circoviridae* family is divided into the genera *Circovirus* (*Circo* indicates that the virus has a circular conformation) and *Gyrovirus* (*Gyro* is a derivation from the Greek work “gyrus” meaning “ring” or “circuit”). The genus *Circovirus* contains the following species: beak and feather disease virus (BFDV), canary circovirus (CaCV), goose circovirus (GoCV), pigeon circovirus (PiCV), PCV1, and PCV2, and the tentative species in this genus are duck circovirus (DuCV), finch circovirus (FiCV), and gull circovirus (GuCV). The genus *Gyrovirus* contains only chicken anemia virus (CAV) (Todd et al., 2005).

Viruses that belong to the *Circoviridae* family have characteristic virions that exhibit icosahedral symmetry and do not possess an envelope. The genomes are covalently closed, circular, ssDNAs, which range in size from 1.8 to 2.3 kb. The genome organization of CAV is negative sense, whereas those of the other circoviruses are ambisense (Todd et al., 2005).
CAV, PCV2, and BFDV were found to have an icosahedral T=1 structure containing 60 capsid protein molecules arranged in 12 pentamer clustered units (Crowther et al., 2003). Circoviruses are host-specific or exhibit a narrow host range and the majority of those reported to date infect avian species (Todd et al., 2005). Subclinical infections are common; however, circovirus infections are associated with clinical disease in some cases such as with infectious chicken anemia, psittacine beak and feather disease, circovirus disease of pigeons, and PMWS in pigs. Circovirus infections in all species cause varying degrees of lymphoid depletion and are thought to be immunosuppressive (Todd et al., 2005).

Phylogenetic analysis of PCV1, avian circovirus, plant geminiviruses, and nanoviruses classified PCV1 as most closely related to BFDV and were intermediate between the two plant viral groups (Niagro et al., 1998). Furthermore, it has been proposed that a predecessor to PCV1 and BFDV may have originated from a plant nanovirus that infected a vertebrate host and recombined with a vertebrate-infesting RNA virus, most likely a calicivirus (Gibbs and Weiller, 1999).

**Biological and Physical Properties**

Allan et al. (1994) demonstrated that PCV1 was stable at pH 3, was stable at 56°C and at 70°C for 15 minutes, and was resistant to inactivation after exposure to chloroform. The buoyant density of PCV1 in CsCl has been reported to be 1.37 g per cm³ by Tischer et al. (1974), and it has been reported to be 1.36-1.37 g per ml CsCl by Allan et al. (1994). The sedimentation coefficient (S) was determined to be 57S when compared with the sedimentation coefficient of a bovine enterovirus (Allan et al., 1994).

PCV2 is readily isolated from tissue samples that have been stored at -70°C (Ellis et al., 1998). PCV2 was shown to be resistant to some disinfectants (Nolvasan®, DC&R®, Weladol®, or ethanol) but virus titers were significantly reduced by sodium hydroxide, Virkon® S, and others (Royer et al., 2001).

**Replication and Binding Strategy**

All viruses of the *Circoviridae* family are thought to have a similar replication strategy. A circular, double stranded replicative form DNA intermediate is produced using
host cell DNA polymerases during S phase of cell division. The replicative form serves as template for generation of viral ssDNA using the rolling circle replication mechanism (Todd et al., 2005). Viral DNA intermediates are generated in nuclei and require host cell enzymes for completion of the replication cycle (Tischer et al., 1987). Virions are assembled in both nuclei and cytoplasm and released from infected cells in the absence of viral cytopathic effects.

Misinzo et al. (2005) studied the binding characteristics of PCV2 in the porcine monocytic line 3D4/31 and found that PCV2 enters 3D4/31 cells predominantly via clathrin-mediated endocytosis and requires an acidic environment for infection.

Meerts et al. (2005a) found that PCV2 capsid protein was expressed between 6 to 12 hours post inoculation and nuclear relocation occurred around 12 to 24 hours post inoculation in PK-15 cells. In porcine alveolar macrophages or fetal cardiomyocytes, nuclear localized antigens appeared approximately at 48 hours post inoculation and in fewer cells compared to PK-15 cells.

When seven different PCV2 strains were compared in PK-15 cells, it was found that abortion-associated strains had different replication kinetics compared with PMWS or PDNS-associated PCV2 isolates. A higher number of infected cells were observed at 24 hours post inoculation and the percentage of infected cells with nuclear localized antigens was lower compared to that of the other strains. The same authors found also differences in susceptibility to PCV2 infection between porcine alveolar macrophages from different pigs (Meerts et al., 2005a).

It has been demonstrated that treatment of PK-15 cell cultures with IFN-γ causes a 20 times higher production of PCV2 progeny (Meerts et al., 2005b). The enhancing effect of IFN-γ on PCV2 infection was found to be due to increased internalization of PCV2 virion-like particles. Expression of PCV2 proteins in infected cells were not altered by IFN-γ treatment (Meerts et al., 2005b).

During productive infection of PK-15 cells, nine RNAs, capsid RNA, five Rep-associated RNAs (Rep, Rep’, Rep3a, Rep3b, and Rep3c), and three NS-associated RNAs (NS515, NS672, and NS0) are synthesized by PCV2 (Cheung, 2003c), and it was demonstrated that Rep and Rep’ are essential for PCV2 replication (Cheung, 2003b). PCV1
and PCV2 were found to differ from each other in expression levels of NS- and Rep3c-associated RNAs (Cheung, 2003a).

**Genome Organization, Genetic Characterization, and Proteins**

The non-pathogenic PCV1 and the pathogenic PCV2 share less than 80% nucleotide sequence identity (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998). The lengths of the genomic DNAs of the PCV1 isolates range from 1,758 to 1,760 bp (Fenaux et al., 2000). Sequence analyses of the complete genomes of PCV-2 isolates showed that the complete genome of each PCV2 isolate is 1,767 to 1,768 bp in length (Fenaux et al., 2000; Mankertz et al., 2000). Analysis and comparison of PCV2 isolates demonstrated that PCV2 isolates share 94.6-99.0% nucleotide sequence identities (de Boissèson et al., 2004; Fenaux et al., 2000). The two main viral genes are ORF1 and ORF2 which are oriented in opposite directions and which represent 93% of the PCV2 genome. ORF1 is associated with replication, it is highly conserved among isolates, and it encodes a protein of 35.7 kDa (Mankertz et al., 1998). The ORF2 gene encodes for the 27.8 kDa capsid protein (Nawagitgul et al., 2000a, 2002) and shares about 91-100% nucleotide sequence identity and about 90-100% amino acid sequence identity among PCV2 isolates (Fenaux et al. 2000; de Boisseson et al., 2004). In addition to ORF1 and ORF2, it is predicted that there are another four potential ORFs encoding proteins larger than 5kDa (Meehan et al., 1998). Recently, it has been shown that ORF3 protein is not essential for PCV2 replication *in vitro* but is involved in PCV2 induced apoptosis by activating caspase-8 and caspase-3 pathways (Liu et al., 2005).

**Host Range**

**Pigs**

PCV2 was first detected and described in pigs where it is widely distributed. PCV2-specific antibodies have been demonstrated in domestic pigs (Allan and Ellis, 2000) in most countries and most continents. PCV2 was associated with PMWS in 6-week-old wild boar piglets raised under free-range conditions (Ellis et al., 2003). Similarly, PMWS was observed in a 10-month-old male European wild boar in Germany (Schulze et al., 2004), and in a 6-month-old Spanish wild boar (Vicente et al., 2004).
Ruminants

Tischer et al. (1995a) tested 90 bovine serum samples and found a weak reactivity to PCV1 antigen (35% reacted positive) as determined by IFA. Nayar et al. (1999) found PCV2-nucleic acids in cattle with respiratory disease and in aborted bovine fetuses. The authors investigated 100 cases of bovine respiratory disease and detected PCV2 nucleic acids in six of 100 lung tissue samples. In addition, PCV2-antigen was detected by IHC in one of these cases. The virus was tentatively named bovine circovirus (BCV) and it was found to be more than 99% homologous to PCV2 (Nayar et al. 1999). No other reports have confirmed these findings.

In contrast, sera of 200 sheep, 350 cattle, and 150 goats were found to be negative for PCV1-specific antibodies as determined by an IFA (Allan et al. 1994). In addition, Allan et al. (2000e) tested 185 bovine serum samples from 40 herds from Northern Ireland, 120 bovine serum samples from France, and 120 sheep serum samples from 14 flocks in Northern Ireland by IFA and enzyme-linked immunosorbent assay (ELISA) for PCV2-specific antibodies. All sera were found negative for antibodies to PCV2 (Allan et al., 2000g). Ellis et al. (2001) tested 100 serum samples from beef and dairy cattle with various clinical conditions collected from 42 Canadian farms and 100 colostrum samples collected randomly from dairy cows from 53 Canadian farms for PCV1 and PCV2 antibodies by ELISA and found no evidence of PCV2- or PCV1-specific antibodies. Rodríguez-Arrioja et al. (2003a) tested tissues from 26 cattle, 24 sheep, and 8 goats for PCV2-nucleic acids by ISH and serum from 20 cattle and 20 goats for PCV2-antibodies by IPMA. In total, 58 samples were tested by ISH and 40 samples were tested by IPMA and all were found negative for PCV2-nucleic acids and PCV2-specific antibodies. Six, one-day-old lambs free of PCV2-antibodies were inoculated oronasally with a PCV2 stock that had been previously shown to be pathogenic in the pig model (Allan et al., 2000e). Two lambs were necropsied at 13, 26, and 36 days post inoculation (DPI). There were no microscopic lesions observed in the animals, PCV2 antigen was not detected by IFA, and the lambs did not seroconvert to PCV2 (Allan et al., 2000e). Ellis et al. (2001) inoculated one 1-day-old calf and six 6-month-old beef calves with PCV2. The 1-day-old calf was necropsied at 14 DPI and no gross or microscopic lesions were detected and IHC for PCV2 was negative on all tissues examined. Blood was collected from
the six beef calves and tested for PCV2-and PCV1-specific antibodies on the day of inoculation and again 6 weeks later. No antibodies to PCV2 or PCV1 were detected before inoculation or 6 weeks later (Ellis et al., 2001).

**Mice**

Reactivity to PCV1 antigen by IFA was found in serum from mice (Tischer et al., 1995a). Seventy-three mice were tested and found to be negative for PCV2-specific antibodies by IPMA (Rodríguez-Arrioja et al., 2003a) and 50 mice were found to be negative for PCV1-specific antibodies as determined by IFA (Allan et al. 1994).

Several studies have investigated the susceptibility of mice for PCV2-infection. One study using BALB/c mice found detectable PCV2 antigen in the nuclei and cytoplasm of histiocytes and apoptotic cells in germinal centers of lymph follicles as well as in hepatocytes in the liver (Kiupel et al., 2001). This group also showed mild lymphoid depletion and histiocytic infiltration of lymphoid follicles. The mice in this study were infected intraperitoneally as well as intranasally with a PCV2-isolate that had been passaged four times in cell culture and originated from a case with naturally occurring PMWS. Four mice were necropsied at 7, 14, 28, and 42 DPI, respectively. Microscopic lesions were first detected at 7 DPI, were most prominent at 14 and 21 DPI in bronchial and mesenteric lymph nodes and spleen and Peyer’s patches, and were less severe at 42 DPI (Kiupel et al., 2001). PCV2 replicated in the mice and upregulated caspase 1, 2, 3, 6, 7, 8, 11, and 12, the transcripts of apoptosis inhibitors bcl-2, bcl-w, bcl-X, and apoptosis promoters bax, bak and bad, and was associated with apoptosis in spleens, Peyer’s patches and lymph nodes of infected BALB/c mice (Kiupel et al., 2005).

Another study infected ICR-CD1 mice with the 4th cell culture passage of a PCV2-isolate from another case of naturally occurring PMWS (Quintana et al., 2002). Three intraperitoneally PCV2-infected mice were necropsied at 3, 7, 10, 14, and 20 DPI, respectively. Despite the detection of PCV2-DNA in sera, there was no evidence of PCV2 antigen or nucleic acids in microscopic sections; however, this group apparently did not examine the relevant lymphoid tissues (lymph nodes, spleen) that are the major sites of PCV2-associated lesions in pigs (Quintana et al., 2002).
Three different mouse lines (BALB/c, C57BL/6, and C3H/HeN) were used to investigate possible differences in host susceptibility to PCV2 (Opriessnig et al., 2004b). C57BL/6 and C3H/HeN were selected based on markedly different responses to certain pathogens compared to BALB/c mice. Forty-eight mice of each line were purchased at 4 weeks of age. Thirty-six mice in each line were inoculated with $10^4$TCID$_{50}$ PCV2-isolate ISU-40895. The inoculation was done twice intranasally and intramuscularly at 5 and 6 weeks of age. At the time of inoculation, half of the mice in each line received additional immunostimulation in the form of subcutaneous injections with keyhole limpet hemocyanin in incomplete Freund’s adjuvant. Necropsies were performed at 12, 17, 22, 27, 32, and 37 DPI. Mice within all breeds were found to be positive for PCV2-nucleic acids based on PCR performed on tissue homogenates. There was a trend towards more PCR positive mice in the BALB/c group. PCV2-associated microscopic lesions were not observed and efforts to detect PCV2 antigen by IHC and PCV2 nucleic acids by ISH were unsuccessful (Opriessnig et al., 2004b).

**Rabbits**

Quintana et al. (2002) inoculated 16 female New Zealand rabbits intranasally with each PCV2 and PCV1. Necropsies were performed at 3, 7, 10, 14, and 20 DPI. None of the rabbits seroconverted to PCV1 or PCV2, PCV2 nucleic acids as determined by ISH were not detected in any of the rabbits, and there were no PCV2-associated microscopic lesions (Quintana et al., 2002). Fifteen rabbits were found to be negative for PCV2-specific antibodies by IPMA (Rodriguez-Arrioja et al., 2003a) and 60 rabbits were negative for PCV1-specific antibodies by IFA (Allan et al. 1994). Rabbits are commonly used to produce polyclonal anti-PCV2-antibodies for usage in immunohistochemistry assays (Sorden et al., 1999).

**Humans**

Tischer et al. (1995a) found weak reactivity to PCV1 antigen in serum samples from healthy humans (116 tested, 20.07% positive), blood donors (175 tested, 8.6% positive), and hospitalized adult patients with fevers (188 tested, 23.9% positive) by IFA. In contrast, sera
of 200 humans were found to be negative for PCV1-specific antibodies by IFA (Allan et al. 1994) and 120 randomly selected samples from healthy humans in Northern Ireland were found to be negative by IFA and ELISA for PCV2-specific antibodies (Allan et al., 2000e). Ellis et al. (2000b) tested serum from 54 swine veterinarians and 6 academic veterinarians and laboratory workers (involved in PCV2 research for several years) with a competitive PCV2 ELISA, a whole cell PCV2 ELISA, and a whole cell PCV1 ELISA. All samples were found to be negative for PCV2 and PCV1 specific antibodies (Ellis et al., 2000b).

**Poultry, Horses, Guinea pigs, Cats, and Dogs**

Two-hundred turkey, 200 chickens, and 200 ducks were found to be negative for PCV1-specific antibodies as determined by an IFA (Allan et al. 1994). Rodríguez-Arrioja et al. (2003a) tested 20 poultry by ISH. All were found negative for PCV2-specific nucleic acids. Ellis et al. (2001) tested sera from 100 Canadian horses for PCV-antibodies by ELISA and found no evidence of PCV2- or PCV1-specific antibodies. Seven horses were negative by ISH for PCV2-specific antibodies and 21 horses were negative for PCV2-specific antibodies by IPMA (Rodríguez-Arrioja et al., 2003a). Three Guinea pigs and 21 dogs were negative for PCV2-nucleic acids by ISH, and 13 dogs and 15 cats were negative for PCV2-antibodies by IPMA (Rodríguez-Arrioja et al., 2003a).

**Growth in cell culture**

Allan et al. (1994) found PCV1 growth confined to porcine derived cell cultures and Vero cultures. Primary bovine and ovine cell cultures were also found to be susceptible; however, passage of cell lysates resulted in reduction or elimination of cytoplasmic staining. There was no indication of growth in avian-derived cell cultures (Allan et al., 1994). Hattermann et al. (2004) infected 9 different human cell lines with PCV1 and PCV2 to investigate whether PCV2 can infect and replicate in human epithelial cells and lymphocytes. The results indicated that although PCV gene expression and replication took place in human cells, the infection was non-productive, since the infection was not passed when virus-free cells were inoculated with supernatant of PCV-infected human cells. The authors suggested protein targeting to be disturbed in human cells (Hattermann et al. 2004).
Epidemiology

Prevalence of anti-PCV2-antibodies

The mean PCV2-antibody half-life in weanling pigs is estimated to be 19.0 days and the window for PCV2-passive antibody decay within a population is relatively wide (Opriessnig et al., 2004d). Serological studies have demonstrated that passively-acquired PCV2 antibodies decay during the lactation and nursery periods to negative or near negative levels at the end of the nursery period (7 weeks of age) followed by active seroconversion of the majority of the population starting around 12 weeks of age (Roriguez-Arrioja et al., 2002). Allan et al. (1994) showed a similar trend for PCV1. Larochelle et al. (2003) did a comparative serologic and virologic study in 5 PMWS-affected herds and 2 herds without PMWS in Quebec. Sixty blood samples were collected in each herd in 4-week-intervals from 3-to 23-week-old pigs and it was found that all herds had similar PCV2 profiles: low PCV2-antibody levels were present at 3 weeks of age and reached very low-to-negative levels by 11 weeks of age. PCV2-infection as determined by PCR took place from 11 to 15 weeks of age at which time PCV2 seroconversion occurred. All pigs were seropositive at 23 week of age.

PCV2 passively acquired antibodies present at 10 to 12 days of age were found to decay below ELISA cutoff level by approximately 4.9 ± 1.2 weeks of age in piglets with low levels of antibodies at weaning, by approximately 8.1 ± 1.9 weeks of age in piglets with moderate levels of antibodies at weaning, and by approximately 11.1 ± 2.5 weeks of age in piglets with high levels of antibodies at weaning (Opriessnig et al., 2004d).

Serological surveys found that PCV-antibodies are present globally in almost all swine herds tested and in up to 100% of individual pigs within herds. There is a high prevalence of PCV infection in the global swine populations of Canada, France, Germany, New Zealand, Northern Ireland, United Kingdom, and the United States (Allan et al., 1994; Dulac and Afshar, 1989; Edwards and Sands, 1994; Hines and Lukert, 1995; Horner, 1991; Magar et al., 2000b; Tischer et al., 1982, 1986, 1995a; 1995b; Walker et al., 2000). Magar et al. (2000b) found that PCV2 appeared to be the main PCV2 type circulating in Canadian pig herds and that serological evaluation using PCV1 underestimated the seroprevalence of PCV2. Most U.S. breeding herds and the majority of the sows within those herds were found to be seropositive for PCV2 (Opriessnig et al., 2004d). Sibila et al. (2004) determined the
presence of PCV2-antibodies by ELISA in 5 farms without a history of PMWS and in 4 farms with PMWS. Serum antibodies were detected in a higher percentage of pigs from PMWS farms but overall a high prevalence of PCV2 infection was found (Sibila et al., 2004).

**Persistence of PCV2**

There is evidence from experimental inoculations that persistent infections may be a feature of PCV2 (Bolin et al., 2001). Viral DNA was detected in some cesarian derived, colostrum deprived (CDCD) PCV2-inoculated pigs up to 125 days post infection. To confirm presence of infectious virus, viral isolation was done on homogenates of tissues that were PCR positive. Virus was isolated from all tissues in which viral DNA was detected (Bolin et al., 2001). Proof of persistent infection in the field is contradictory. PCV2 viremia was detected in the same animals for at least 8 weeks by PCR confirming persistence of PCV2 in pigs after natural exposure (Larochelle et al., 2003). Rodríguez-Arrioja et al. (2002) found a long duration of PCV2 viremia (up to 28 week of age) in a high percentage of naturally-PCV2-infected pigs on a PMWS-affected farm in Spain. PCV2 nucleic acids were detected in sera from 52.6% of 386 healthy slaughter-age pigs (Liu et al., 2002). In another survey however, researchers were unable to demonstrate microscopic lymphoid lesions or PCV2 nucleic acids at slaughter suggesting that pigs typically clear the virus (Quintana et al., 2001).

**Transmission of PCV2**

Transmission of PCV2 is thought to occur through direct contact via oronasal, fecal, and urinary routes (Bolin et al., 2001; Magar et al., 2000a). Shibata et al. (2003) investigated PCV2 shedding in experimentally infected CDCD pigs and in pigs with naturally acquired PMWS by PCR. Sixteen pigs were inoculated with PCV2, and oropharyngeal and nasal swabs, feces, whole blood, and serum became PCV2-DNA positive immediately after inoculation and all samples with the exception of oropharyngeal swabs (1/2 positive) remained positive up to 70 DPI. In field samples collected from 313 pigs, PCV2 was detected in 95 (30.4%) of the whole blood samples, 60 (19.2%) of the nasal swabs, and 64 (20.4%) of the feces implying that whole blood and serum are the best samples for detection
of PCV2 by PCR (Shibata et al., 2003). Segalés et al. (2005a) quantified PCV2 DNA in tonsillar, nasal, tracheo-bronchial, urinary and fecal swabs of pigs with and without PMWS. The authors were able to detect PCV2 DNA in a high percentage of the samples and concluded that PCV2 is most likely excreted through respiratory secretions, oral secretions, urine, and feces of both PMWS-affected and clinically healthy pigs, with higher viral loads in the PMWS-affected pigs. Yang et al. (2003) detected PCV2 nucleic acids in feces in pigs with (14/54 intestines, and 4/9 feces) and without (3/14 intestines and 16/20 feces) enteric disease by PCR suggesting fecal-oral transmission of PCV2 in feces.

Direct contact with pigs inoculated with virus 42 days previously resulted in transmission of virus to 3 of 3 control CDCD pigs (Bolin et al., 2001). Vertical transmission has been demonstrated to occur in individual sows in the field (Ladekjær-Mikkelsen et al., 2001; O’Conner et al., 2001) and experimentally (Johnson et al., 2002). There are reports of vertical intrauterine transfer of PCV2 resulting in viremic or persistently infected piglets at birth (West et al., 1999). Vertical PCV2 infection may not always cause fetal death, and virus, antibody, or both were detected in clinically normal fetuses (Sanches 2003).

Larochelle et al. (2000) infected four 7-month-old boars intranasally with PCV2. Serum samples were collected at 4, 7, 11, 13, 18, 21, 25, 28, 35, 55, and 90 DPI and were tested for the presence of PCV2 DNA by nested PCR. PCV2 DNA was detected as early as 4 DPI in 3 of the 4 boars and serum samples were positive up to 35 DPI but negative by 90 DPI. Semen was collected at 5, 8, 11, 13, 18, 21, 25, 28, 33, and 47 DPI and analyzed by nested PCR. PCV2 was detected as soon as 5 DPI in semen of two of the boars and intermittently through 47 DPI in all 4 boars. The semen of 2/4 infected boars was positive for PCV2 DNA at 47 DPI (Larochelle et al., 2000). Kim et al. (2003c) tested semen from ninety-eight 1-year-old boars from 49 herds in Korea and found 13 of 98 semen samples to be positive for PCV2 by first round (conventional) PCR, 26 of 98 semen samples were positive by semi nested PCR, and 11 of 98 semen samples were positive by virus isolation. The same study also investigated prevalence of PCV2 in seminal fluid, non-sperm cells, and sperm heads, and detected the greatest amount of PCV2 DNA in the seminal fluid and nonsperm fraction (Kim et al., 2003c). The frequency of PCV2 DNA in semen from naturally infected boars was found to be low and sporadic (McIntosh et al., 2005). The authors concluded that
boars seropositive for PCV2 may have persistent shedding of the virus in semen. PCV2 DNA in semen didn’t appear to affect the percent of morphologically-normal or live sperm cells in PCV2-shedding boars and boars older than 17.5 month of age did not appear to shed PCV2 DNA in semen (McIntosh et al., 2005).

PCV1 and PCV2 DNA were detected in porcine-derived commercial pepsin (Fenaux et al., 2004c). It was found that the PCV-contaminated pepsin lacks infectivity in \textit{vitro} and also in \textit{vivo} as determined by culture in PK-15 cells and experimental inoculation of pigs (Fenaux et al., 2004c).

**Derivation of PCV2-free pigs**

It has been demonstrated that PCV2-free pigs can be achieved by derivation of pigs through cesarean-section and colostrum deprivation (Tucker et al., 2003). Segregated early weaning (SEW) techniques are also effective for derivation of PCV2-free pigs (Opriessnig et al., 2004d).

**Pathogenesis**

The pathogenesis of PCV2 infection and the major cell types that support PCV2 replication are poorly understood. It has been suggested that PCV2 initially replicates in the tonsil. Allan et al. (2000b) reported that in porcine parvovirus (PPV) and PCV2 coinfected pigs, PPV antigen predominates in the tissues of the pigs killed between 3 and 14 DPI with a maximum observed between 6 and 14 DPI. PCV2 antigen was first observed in minimal amounts in mesenteric lymph nodes at 10 DPI with increasing density and distribution of PCV2 antigen at 14, 17, 21, and 26 DPI (Allan et al., 2000b).

Large numbers of PCV2 antigen or nucleic acids are often detected in the cytoplasm of macrophages and dendritic cells by IHC or ISH (Allan and Ellis, 2000; Sorden, 2000). A recent study found that antigen presenting cells in general, and not only macrophages stained positive by IHC for PCV2 antigen (Chianini et al., 2003). In contrast, PCV2 antigen in lymphocytes was only sporadically detected. In thymus, PCV2 was only detected in few histiocytes in the medulla suggesting that thymocytes and T cells might be more resistant to PCV2 infection (Chianini et al., 2003). To a lesser extent, PCV2 antigen is also
found in epithelial cells in lungs and kidneys, in smooth muscle cells, and in endothelial cells in several tissues in pigs experimentally infected with PCV2 (Kennedy et al., 2000) as well as in pigs with naturally occurring PCV2-associated PMWS (McNeilly et al., 1999; Rosell et al., 1999). Kennedy et al (2000) demonstrated PCV2-antigen in infiltrating macrophages in the tunica albuginea, in interstitial macrophages and in germinal epithelial cells in the testes, and in infiltrating macrophages in the epididymides of boars 24 to 29 days after they had been coinfected with PCV2 and PPV at 3 days of age. PCV2 was also found in the parenchyma of the secondary sex glands in a naturally infected boar (Opriessnig et al., 2006d). PCV2 targets mainly cardiomyocytes, hepatocytes, and macrophages during fetal life, and mainly monocytes in early post-natal life (Sanchez et al., 2003).

**PCV2 and macrophages**

Viruses that replicate in the monocyte/macrophage lineage, PPV (Allan et al., 1999a; Opriessnig et al., 2004a) and porcine reproductive and respiratory virus (PRRSV) (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002), have been shown to increase the replication of PCV2 in coinfected pigs and increase the incidence of PMWS. Despite the presence of PCV2 in macrophages and dentritic cells, recent *in vitro* studies suggest that monocytic cells may not represent the primary target for PCV2 replication (Gilpin et al., 2003). Monocytes and macrophages were tested for the ability to support PCV2-replication *in vitro*. PCV2-replication in these cell types was not observed; however, PCV2 was not degraded and was stored in the cytoplasm of the cells (Gilpin et al., 2003). Similarly, no evidence of *in vitro* virus replication in dendritic cells was found by Vincent et al. (2002); however, PCV2 did persist in dendritic cells without loss of infectivity or the induction of cell death. It has been speculated that because of their migratory capacity, dendritic cells can provide a potent vehicle for transport of the virus throughout the host without the need for replication (Vincent et al., 2003).

**PCV2 and lymphoid depletion**

Lymphoid depletion and lymphopenia in peripheral blood is a consistent feature in pigs that develop clinical PMWS. The reduction of lymphocytes might be due to reduced
production in the bone marrow, reduced proliferation in secondary lymphoid tissues, or due to increased loss of lymphocytes in the bone marrow or peripheral blood or secondary lymphoid tissues via necrosis or apoptosis. The lymph node reaction can be graded as initial, intermediate, and end stage (Sarli et al., 2001). Studies on pigs with naturally acquired PMWS revealed that associated with absence of follicles and depletion of lymphocytes there was a reduction in number of interfollicular dendritic cells and interdigitating cells as well as a reduction or absence of B cells and CD4+ T lymphocytes (Sarli et al., 2001). By IHC characterization of PCV2-associated lesions in naturally PMWS-affected pigs, Chianini et al. (2003) found increased numbers of macrophages and partial loss and redistribution of antigen presenting cells throughout lymphoid tissues when compared to healthy control pigs. In particular, a reduction or loss of CD79α and 3C3/9-positive cells and diminution in T-cell areas of CD3-positive lymphocytes was observed. Decrease of proliferation of both, lymphoid and medulla-like tissues in the initial stage of PMWS, but not in the intermediate or final stage has been reported. It was also demonstrated, that there is increase of subcapsular and peritubular lysozyme-positive macrophages, and partial loss and redistribution of BL2H5-positive antigen presenting cells throughout lymphoid tissues (Chianini et al., 2003).

Shibahara et al. (2000) found a loss of lymphocytes in B-cell areas and attributed this to increased PCV2-induced apoptosis since PCV2-antigen was found in nuclei of macrophages and apoptotic lymphocytes. In contrast, Mandrioli et al. (2004) investigated inguinal lymph nodes of 10 pigs with naturally acquired PMWS and found that decreased cell proliferation and not increased apoptosis seemed to be the most important variable leading to lymphoid depletion in PMWS. Krakowka et al. (2004) investigated tissues from experimentally PCV2 infected pigs for TUNEL staining and found positive signals only within the cytoplasm of virus-positive phagocytic mononuclear cells. It was concluded that apoptosis is not the primary mechanism of lymphoid depletion and hepatocyte loss in PMWS (Krakowka et al., 2004).

**PCV2-induced alterations in peripheral blood cells**

Segalés et al. (2000) studied hematological parameters of 50 pigs with naturally
acquired PMWS, 8 experimentally-PCV2-infected pigs, and 11 PCV2-free control pigs. PMWS-affected pigs had significantly lower red blood cells counts and hematocrit values which was associated with the presence of gastric ulcerations rather than with PCV2-infection. The mean lymphocyte percentages were lower in PMWS-affected pigs; however, this was not statistically significant (Segalés et al., 2000). Segalés et al. (2001a) analyzed changes in peripheral blood leukocyte populations in pigs with PMWS by comparing data obtained from 13 PMWS-affected pigs with 11 clinically healthy pigs. It was found that pigs suffering from PMWS had lower proportions of CD4+ and IgM+ cells in blood than clinically healthy pigs (Segalés et al., 2001). Darwich et al. (2002) used twenty-four 8- to 12-week-old PMWS affected pigs and 17 healthy control pigs from conventional farms in Spain to determine changes in peripheral blood monocytes. The authors found that PMWS-affected pigs had significantly ($P < 0.05$) decreased numbers of CD8+ and double-positive cells compared to control pigs. The amount of PCV2 in lymphoid tissues was correlated to the degree of cell depletion and to the relative decrease in IgM+ and CD8+ cells in peripheral blood (Darwich et al., 2002). Nielsen et al. (2003) found primarily lymphopenia in experimentally PCV2-inoculated pigs. All T lymphocyte subpopulations were susceptible to PCV2 induced lymphopenia with the conclusion that memory/activated Th-lymphocytes might be affected more than other T cell subpopulations. Only pigs that subsequently developed PMWS had lymphopenia (Nielsen et al., 2003). Bassaganya-Riera et al. (2003) demonstrated significant B-cell depletion in PCV2-infected pigs and found that the most depleted B-cell subset (IgM+) expressed an immature phenotype characterized by expression of a myeloid marker (SWC3). Segalés et al. (2004) determined the serum concentrations of acute phase proteins, haptoglobin, and pig-major acute phase protein, in pigs with and without PMWS and found that mean haptoglobin and pig-major acute phase protein levels were significantly increased in PMWS-affected pigs. In a second study, pigs were divided into 2 groups based on PCV2-PCR status done on serum samples and no significant differences between groups were detected. The authors concluded that acute phase proteins levels are significantly increased in pigs that develop PMWS but not in pigs with subclinical PCV2 infection (Segalés et al., 2004).
**PCV2, cytokines, and chemokines**

The majority of studies investigating cytokine profiles in PCV2-infected pigs that have been published to date where done on pigs with naturally-acquired PMWS. Sipos et al. (2004) found an increase of IL-1α and IL-10 mRNA and down-regulation of IL-2 and IL-2Rα mRNA associated with PMWS by comparing 10 pigs with naturally acquired PMWS with 10 clinically healthy pigs. Pigs with naturally acquired PMWS pigs had an altered cytokine mRNA expression pattern with overexpression of IL-10 mRNA in thymus and IFN-γ mRNA in tonsil (Darwich et al., 2003b). Mononuclear cells from pigs with naturally acquired PMWS did not produce normal levels of IFN-γ, IL-1β, IL-2, IL-4 or IL-8 when they were challenged with a T-cell mitogen such as phytohemaglutinin or superantigens but they did produce some IFN-γ or IL-10 when challenged with recall PCV2 antigens (Darwich et al., 2003a). Sipos et al. (2005) compared 5 pigs affected by PDNS, 5 pigs with naturally acquired PMWS, and 5 healthy control pigs and found IL-1α, IL-6, and IFN-γ mRNA expressions elevated as well as an increased capacity of peripheral blood mononuclear cells to produce IL-2, IL-4, IL-6, IL-12, TNF-α, and IFN-γ in PDNS pigs compared to PMWS pigs. In PMWS pigs, IL-10 mRNA was significantly decreased compared to control pigs (Sipos et al., 2005).

Kim and Chae (2004c) observed maximum expression of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) at 17 and 21 DPI in lymphoid tissues of experimentally PCV2-infected pigs by ISH and semiquantitative reverse transcriptase PCR. MCP-1 was correlated with PCV2 as determined by ISH done on serial sections of lymph nodes from PMWS-affected pigs, whereas IL-8 was not detected in the lesions (Kim and Chae, 2003c). Injection of granulocyte-macrophage colony stimulating factor (GM-CSF) encoding DNA had no significant effect on PCV2 replication in 5-week-old specific pathogen free (SPF) pigs injected with a tandem-cloned PCV2 DNA when compared to non-GM-CSF-injected pigs (Loizel et al., 2005). Further *in vitro* studies found that in the presence of intracytoplasmic PCV2, the function of natural interferon producing cells (conventional blood dendritic cells and plasmacytoid dendritic cells) is disrupted which would impair the maturation of associated myeloid dendritic cells favoring the establishment of concurrent infections (Vincent et al., 2005).
**PCV2-associated Diseases**

**Postweaning multisystemic wasting syndrome (PMWS)**

a. **Definition**

PMWS is considered a multifactorial disease in pigs, in which a necessary factor is the presence of PCV2. Since PCV2 is ubiquitous in the pig population and infection doesn’t necessarily equal disease, a definition for PMWS was proposed (Sorden, 2000). Based on this definition, a diagnosis of PMWS requires (1) the presence of clinical signs such as wasting, weight loss, and respiratory disease, (2) the presence of the hallmark PCV2-associated microscopic lesions (lymphoid depletion or histiocytic replacement of follicles in lymphoid tissues or both), and (3) PCV-antigen or nucleic acids associated with the microscopic lesions as determined by IHC or ISH (Sorden, 2000).

b. **Historical background**

PCV2-associated PMWS was first identified in Canadian high health herds in 1991 (Harding, 1996; Harding and Clark, 1997). Affected herds were free of major enteric and respiratory pathogens including *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), *Actinobacillus pleuropneumoniae* (APP), salmonellosis, swine dysentery, transmissible gastroenteritis virus (TGEV), pseudorabies virus, and PRRSV. The herd sizes varied from small (50 sows) to large (1,200 sows) and genetics also varied widely. In 1996, interstitial pneumonia and lymphadenopathy was observed in a 6-week-old pig in California (Daft et al., 1996). Also in 1996, workers in France observed a piglet wasting disease (LeCann et al., 1997). Subsequently, PCV2 was isolated from material of affected French and Californian pigs (Allan et al., 1998b).

c. **Clinical disease, macroscopic and microscopic lesions**

PMWS is a disease complex characterized by wasting and pneumonia in pigs in the late nursery and early grower stages typically affecting pigs between 5 and 20 weeks of age (Allan and Ellis, 2000; Harding, 2004). Harding (2004) emphasizes six fundamental clinical signs of PMWS: wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice. While all the fundamental clinical signs may not be noted in a single pig, affected farms will present with the majority of them over a period of time. Other clinical signs include coughing fever, gastric ulceration, meningitis, and sudden death (Harding et al., 2004).
Macroscopic lesions associated with PCV2 infection include generalized lymphadenopathy, non-collapsed, rubbery lungs with mottling and increased firmness, enlarged spleen, and enlarged kidneys (Clark, 1997).

Characteristic microscopic lesions associated with PCV2-infection and PMWS include lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. The lymphocellular depletion affects both, lymphoid follicles and parafollicular zones (Rosell et al., 1999). Mild-to-severe granulomatous inflammation in lymphoid and other tissues also is commonly observed (Allan and Ellis, 2000; Sorden, 2000). Syncytial cells can be seen frequently, especially in lymph nodes, Peyer’s patches, and lamina propria of the intestinal villi (Rosell et al., 1999). Macrophages in affected lymphoid tissues may contain sharply demarcated, spherical, basophilic cytoplasmic inclusion bodies (Clark, 1997; Rosell et al., 1999). The inclusions are either large and single or smaller and multiple with groups of up to 12 inclusions (Rosell et al., 1999).

d. Prevalence

PMWS has been reported from almost all swine producing countries. Morbidity due to PMWS reportedly varies from as little as 4% to as high as 30% and mortality in affected pigs is typically 70 to 80% (Allan and Ellis, 2000, Harding and Clark, 1997; Madec et al., 2000; Rodríguez-Arrioja et al., 2002). Rodríguez-Arrioja et al. (2002) collected blood samples from 3-, 7-, 12-, and 28-week-old pigs from a PMWS-affected herd. Clinical PMWS started when the pigs were 8 weeks old and had a prevalence of 30% in 8- to 10-week-old pigs.

There are two forms of PMWS, an endemic and an epidemic form. The endemic form is seen in North America, where PMWS usually results in low grade but persistent death losses. Rarely, 3- to 4-fold increase in postweaning mortality can be observed. The epidemic form, which is characterized by persistent mortality, appears to be restricted to Europe and based on recent evidence, eastern Canada (Delay et al., 2005) and North Carolina. Pork producers in Eastern Canada and North Carolina have experienced a devastating disease in growing pigs manifest as wasting and pneumonia and resulting in persistent mortality of 20-40% of the pigs between 10 weeks and market age (25 weeks). The recent severe outbreaks most resemble porcine circovirus type 2 (PCV2) associated postweaning multisystemic
wasting syndrome (PMWS); however, the severity and persistence of the disease and apparent ability of the disease to spread quickly throughout the area is different than expected with classic PCV2-associated PMWS.

e. Experimental reproduction

Experimental PCV2-infection models indicate that PCV2 is an opportunist, depending on immunostimulation (Allan et al., 2000c and 2001; Krakowka et al., 2001) or coinfecting agents like PPV (Allan et al., 1999a and 2000b, Ellis et al., 1999; Hasslung et al., 2005; Kennedy et al., 2000; Kim and Chae, 2004a; Krakowka et al., 2000; Opriessnig et al., 2004a; Ostanello et al., 2005), PRRSV (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002), or M. hyopneumoniae (Opriessnig et al., 2004c) for PCV2-infection to progress to clinical PMWS. While most research groups have not been able to reproduce clinical disease in pigs inoculated singularly with PCV2, some groups have succeeded in this regard.

Experimental infection of gnotobiotic pigs. Krakowka et al. (2000) inoculated 1-day-old gnotobiotic pigs intranasally with PCV2 (n = 3), PCV2 and PCV1 (n = 4), and PCV2 and PPV (n = 4), and found that all PCV2/PPV developed clinical PMWS whereas the singular infected pigs and PCV1/PCV2-inoculated pigs did not. Microscopically, hepatic necrosis with severe granulomatous hepatitis and prominent bile retention was observed (Krakowka et al 2000).

Experimental infection of colostrum deprived cesarian derived (CDCD): Bolin et al. (2001) inoculated twenty-three 20- to 25-day-old CDCD pigs intranasally and intramuscularly with PCV2 and induced clinical PMWS in the majority of the pigs. Harms et al. (2001) inoculated 36 CDCD pigs with PCV2 (17 of the 36 pig were concurrently infected with PRRSV) and induced severe clinical disease consistent with PMWS in singular and coinfectected pigs. Pogranichnyy et al. (2000) inoculated five 8-week-old CDCD pigs intranasally and intramuscularly with PCV2, necropsied all pigs at 35 DPI, and observed mild microscopic lesions associated with PCV2.

Experimental infection of colostrum deprived (CD) pigs: Allan et al. (1999a) inoculated eleven 1- to 2-day-old CD pigs with PCV2 (5 of 11 pigs were coinfectected with PPV, and 2 of 11 pigs were coinfectected with a formalin-treated PCV2/PPV mixture) and 3 of the 11 pigs died from clinical PMWS (one singular and two coinfectected pigs). Allan et al.
(2000a) inoculated eight 1- to 2-day-old CD pigs intranasally with PCV2 (5 of 8 pigs were coinfected with PRRSV) and found enhancement of PCV2 but no clinical signs or gross lesions in dual-infected pigs. Allan et al. (2003) inoculated thirteen 4-day-old CD pigs intranasally with PCV2 (9 of 13 pigs were coinfected with PPV), and reproduced clinical PMWS in 1 of 4 singular PCV2-inoculated and 9 of 9 PCV2/PPV coinfected pigs.

**Experimental infection of conventional pigs.** Albina et al. (2001) inoculated four 6- to 7-week old specific pathogen free piglets intratracheally with a lung tissue homogenate obtained from two French naturally PMWS-affected pigs. Thereafter, 7 passages of 6 to 8 pigs per group were subsequently intratracheally and intramuscularly infected with lymph node homogenates from the previous passage. PMWS was reproduced in 4 of 55 (7.2%) of the pigs (Albina et al., 2001). Clinical PMWS was recently induced in 3 of 5 non-immunostimulated and in 1 of 5 with keyhole limpet hemocyanin in Freund’s incomplete adjuvant (KLH/ICFA) stimulated 3-week-old conventional colostrum-fed pigs singularly-infected with PCV2 (Ladekjær-Mikkelsen et al., 2002). Balasch et al. (1999) infected eight 8-week-old conventional pigs intranasally with tissue homogenates from naturally PMWS-affected pigs and reproduced PMWS-like lesions in the pigs. Allan et al (2000) inoculated 28 conventional pigs at 13, 31, and 47 days of age oronasally with PCV2. Half of the pigs were vaccinated with a *M. hyopneumoniae* vaccine at 13 and at 31 days of age and with an APP vaccine at 47 days of age. Three of fourteen vaccinated pigs developed clinical PMWS.

**f. PCV2 infectious genomic DNA**

Fenaux et al. (2002, 2003) investigated the infectivity of an US PCV2 molecular DNA clone when infected directly into the liver or inguinal lymph nodes. Mild PCV2-associated lesions were observed in 4-week-old conventional pigs confirming the role of PCV2 in PMWS. Similar results (mild microscopic lesions and no clinical disease) were observed in another study using a European clone (Roca et al. 2004). Grasland et al. (2005) reported of the reproduction of PMWS in immunostimulated (injections of KLH/ICFA and thioglycollate medium) conventional pigs inoculated with a European PCV2 DNA clone.

**g. Coinfections present in cases of naturally occurring PMWS**

Ellis et al. (2000a) demonstrated PPV and PCV2 coinfection in 12/69 cases of naturally acquired PMWS. A retrospective analysis of 484 PMWS cases in the Midwest
United States revealed that PCV2 alone was found in only 9 of 484 (1.9%) cases investigated (Pallarés et al., 2002). PRRSV was detected in 251 of 484 (51.9%) cases, *M. hyopneumoniae* was found in combination with PCV2 in 172 of 484 (35.5%) cases, and SIV was detected in 26 of 484 (5.4%) cases (Pallarés et al., 2002). Aujeszky’s disease virus infection concurrent with PMWS was demonstrated in pigs associated with multifocal necrotizing tonsillitis and lymphadenitis (Rodríguez-Arrioja et al., 1999). Pogranichniy et al. (2002) did a case control study on pigs with a clinical history of wasting and microscopic lesions characteristic of PMWS (*n* = 31) and on control pigs without clinical signs or microscopic lesions typical of PMWS (*n* = 56). Among all viruses tested, PCV2, PRRSV, PPV, porcine enterovirus types 1-3, swine influenza virus (SIV), porcine respiratory coronavirus, TGEV, porcine endogenous retrovirus, porcine lymphotropic herpesvirus type 1, and bovine virus diarrhea virus, PCV2 had the strongest association with PMWS. The risk for PMWS was much higher if the pig was PCV2/PRRSV coinfected (Pogranichniy et al., 2002). Wellenberg et al. (2004a) did a case-control study to investigate the presence of coinfections in the Netherlands. They compared 60 pigs affected with PMWS from 20 different farms and 180 pigs without clinical signs of PMWS. Concurrent PRRSV-infection was found in 83% of the PMWS-affected pigs and in 35% of the pigs from PMWS-free herds (Wellenberg et al., 2004a).

**PCV2-associated pneumonia**

Recent field investigations suggest that PCV2 may be playing an important role in some cases of porcine respiratory disease complex (PRDC) (Harms et al., 2002a; Kim et al., 2003b). PRDC is a condition observed mainly in 8- to 26-week-old pigs and associated with multiple respiratory pathogens including PRRSV, SIV, and *M. hyopneumoniae*. PRDC is characterized by decreased rate of growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea. There is diagnostic overlap between PMWS and PCV2-associated pneumonia. The presence of prolonged and unusually severe clinical respiratory disease, granulomatous bronchointerstitial pneumonia with bronchiolitis and bronchiolar fibrosis, and abundant PCV2 antigen associated with the lesions is suggestive of PCV2-associated pneumonia and PRDC. There is experimental evidence of unique lung lesions associated with PCV2 antigen in the literature. Interstitial pneumonia with bronchiolitis was reported in the first cases of PMWS.
Magar et al. (2000a) reproduced bronchointerstitial pneumonia in addition to the hallmark lymphoid lesions of PMWS in conventional pigs inoculated with PCV2. Bolin et al. (2001) induced mild respiratory disease and multifocal interstitial pneumonia in CDCD pigs inoculated with PCV2. Rovira et al. (2002) induced mild interstitial pneumonia and lymphoplasmacytic rhinitis in conventional pigs inoculated with PCV2. Mild granulomatous bronchointerstitial pneumonia was reproduced in conventional pigs using an infectious DNA clone of PCV2 (Fenaux et al., 2002).

Coinfections with PCV2 and other pathogens in lung lesions have been reported. *Pneumocystis carinii* was reported to occur in approximately 0.5% of cases with PMWS in western Canada (Clark, 1997). Pulmonary aspergillosis was diagnosed in a pig affected by PMWS (Segalés et al., 2003). Harms et al. (2002a) demonstrated a high prevalence of coinfection with PRRSV in cases of PRDC in the Midwestern United States. Coinfection with SIV and *M. hyopneumoniae* was also reported in this study (Harms et al., 2002a).

Proliferative and necrotizing pneumonia (PNP) which is characterized microscopically by lymphohistiocytic interstitial pneumonia with marked proliferation of type 2 pneumocytes, multifocal coagulates of necrotic cells in the alveoli, hyaline membranes, intra-alveolar lymphoplasmacytic infiltrates, and bronchiolar necrosis has been implicated to be the result of coinfection with PRRSV and PCV2 (Larochelle et al., 1999). Drolet et al. (2003) did a retrospective study on 60 cases of PNP diagnosed between 1988 and 2001 and found PRRSV antigen in 55/60 PNP cases, SIV antigen in 1/60 PNP cases, and PCV2 in 25/55 PNP cases. The authors concluded that coinfection with PCV2 is not a determining factor in PNP and that PRRSV is consistently and predominately associated with PNP (Drolet et al., 2003).

**PCV2-associated lymphoid depletion**

PCV2-associated lymphoid depletion is a hallmark lesion of PMWS but can also be observed in subclinically infected pigs (Quintana et al., 2001). Sanchez et al. (2004) investigated the development of PCV2-associated lymphoid lesions in pigs that had been inoculated *in utero* at 92 to 104 gestational days (*n* = 12) or at 1 day of age (*n* = 14) and found that high PCV2 titers in lymphoid organs may lead to the development of severe microscopic lymphoid lesions without causing clinical manifest disease. The infection
appears to occur mainly in infiltrating monocytic cells and to a lesser extend in cells bearing lymphocyte markers (Sanchez et al., 2004).

Kim and Chae (2005) described seven field cases of PCV2-associated necrotizing lymphadenitis. The main lesion was follicular necrosis in the center of prominent lymphoid follicles. PCV2 was detected in all lymph nodes with necrotic foci but was absent in other lymph nodes. No other infectious cause could be found and granulomatous inflammation and intracytoplasmic inclusion bodies were not observed in these cases (Kim and Chae, 2005). In three pigs experimentally infected with PCV2, viral antigen was also associated with obliterated blood vessels in areas of granulomatous and necrotizing lymphadenitis (Opriessnig et al., 2006c).

**PCV2-associated abortions and reproductive failure**

There have been several reports (O’Conner et al., 2001; Ladekjær-Mikkelsen et al. 2000) of PCV2-associated reproductive failure since the original report of West et al. (1999) in western Canada in 1999. Consistent signs on affected farms include increased numbers of abortions, stillbirths, fetal mummification, and increased pre-weaning mortalities. Affected herds are typically gilt-start-ups or new populations. A nonsuppurative-to-necrotizing or fibrosing myocarditis associated with abundant PCV2 antigen are the major lesions in stillborn and neonatal pigs from field cases (Mikami et al., 2005). PCV2-infection has been confirmed in PMWS outbreaks in CDF piglets (Harms et al., 1999; Jolie et al., 2000) suggesting that a vertical transmission is possible. Experimental intrauterine infection of fetuses with PCV2 resulted in virus replication in the fetuses and supports the heart as primary site of PCV2 replication in fetuses (Sanchez et al. 2001). Johnson et al. (2002) inoculated 37 fetuses from 3 pregnant sows at 86, 92, and 93 days of gestation intramuscularly and observed 24 normal pigs and 13 mummified, stillborn, or weak-born pigs at farrowing confirming that PCV2 can infect late-term fetuses and cause reproductive abnormalities.

A retrospective study failed to detect PV2 antigen and nucleic acids in cases of reproductive failure prior to 1999 in areas of endemic PCV2 infection (Bogdan et al. 2001). The authors concluded that reproductive failure may be a new clinical manifestation of PCV2 infection, and that vertical transmission may not have been the primary mechanism of initial
dissemination of the virus in the pig population. Farnham et al. (2003) tested 171 sera from stillborn fetuses from 3 different farms for PCV2-specific antibodies by IPMA and found 28 of 171 positive. Thirteen of the 28 sera were also positive for PCV2 nucleic acids as determined by PCR, and 9 of 13 PCR PCV2 positive sera were positive for PCV2 by virus isolation. Kim et al. (2004b) tested 350 aborted fetuses and stillborn pigs from 321 Korean farms submitted between 2000 and 2002 for PCV2, PPV, and PRRSV by PCR and virus isolation. PCV2 was found by PCR in 46 of 350 (13.1%) fetuses and by virus isolation in 25 of 350 (7.1%) fetuses and it was associated with all stages of gestation.

Mauch et al. (2004) observed abortions and death loss in dams after 94 gilts in mid-pregnancy were transported to four PCV2 positive farms. Pensaert et al. (2004) inoculated four 8-month-old nonpregnant gilts with a PCV2 strain isolated from a dead fetus in Canada. The gilts seroconverted to PCV2 by 21 DPI. Viral DNA was found in plasma between 14 and 49 DPI and in peripheral blood monocytes between 7 and 63 DPI. The 21 DPI PCV2 was determined to be infectious by pig inoculation. Park et al. (2005) inoculated 6 pregnant sows 3 weeks before expected farrowing date intranasally with a PCV2 recovered from an aborted fetus. Four of the 6 sows aborted between 7 and 21 DPI and 2 sows farrowed 4-5 days prematurely. The six sows delivered a total of 65 stillborn and 10 live-born piglets. PCV2 DNA was detected by ISH in lymph node, spleen, thymus, lung, tonsil, and liver from stillborn and live-born piglets.

**PCV2-associated myocarditis and vasculitis in growing pigs**

Abundant intracytoplasmic PCV2 antigen was associated with myocardioyte swelling or necrosis, or myocardial fibrosis (or both) in three naturally-infected pigs aged 4 to 7 weeks from three different farms (Opiessnig et al., 2006c). Marco (2002) reported of cardiomegaly, especially on the right side of the heart with pericardial effusion in Spanish pigs naturally affected by PMWS. PCV2-associated myocarditis was found to be the primary cause of congestive heart failure in these cases (Marco, 2002).

Severe diffuse segmental to circumferential lymphohistiocytic and plasmacytic periarteritis and endarteritis in several organs was observed in a pig naturally infected with PCV2 (Opiessnig et al., 2006c). PCV2 antigen was demonstrated in endothelial cells and
inflammatory cells in the arterial walls.

**Porcine dermatitis and nephropathy syndrome (PDNS)**

PDNS is characterized clinically by acute onset of skin lesions (raised purple skin lesions progressing to multifocal raised red scabs with black centers most prominent on the rear legs), fever, and lethargy and is almost always fatal. Macroscopically, there are enlarged tan waxy kidneys with white foci and streaks. Microscopically, there is systemic vasculitis with dermal and epidermal necrosis and necrotizing and fibrinous glomerulonephritis. The microscopic hallmark lesions of PDNS, generalized vasculitis and glomerulonephritis, are suggestive of a type III hypersensitivity reaction which is characterized by deposition of antigen-antibody aggregates or immune complexes at certain tissue sites. Many pathogens including viruses (PRRSV; Choi and Chae, 2001; Thibault et al., 1998) and bacteria such as *Pasteurella multocida*, *Streptococcus suis* type 1 and 2, *E. coli*, *Proteus sp.*, *Haemophilus parasuis*, APP, *Bordetella bronchiseptica*, *Arcanobacterium pyogenes*, *Staphylococcus aureus*, or *Salmonella sp.* (Thomson et al., 2002) have been implicated in the etiology of PDNS. Lainson et al. (2002) further differentiated *Pasteurella multocida* isolates from PDNS cases and from cases without PDNS by pulsed-field gel electrophoresis and found a single *Pasteurella multocida* variant associated with PDNS. PDNS was first associated with PCV2 in 2000 (Rosell et al., 2000). Allan et al. (2000d) investigated PDNS cases submitted in Northern Ireland in 1990 when PRRSV had not been introduced into the area and found granulomatous lymphadenitis associated with PCV2 antigen in affected pigs.

There are two forms of PDNS described: the sporadic form and the epizootic form. With the sporadic form the mortality is rarely above 0.5%. The epizootic form was first observed in 1999 in England, when there was a sudden, marked increase of PDNS cases (Thomson et al., 2000, 2002). The within-herd mortality was reported to range from 0.25-20% and similar observations were made in the Netherlands (Elbers et al., 2000; Wellenberg et al., 2004b). Investigations into this “outbreak” found that there was a clear temporal association of PMWS and PDNS; PDNS cases usually followed PMWS cases on the same farms. Studies have determined that the mean age of pigs affected by PMWS ranges from 6 to 14 weeks whereas the mean age of pigs affected by PDNS ranges from 12 to 16 weeks.
(Gresham et al., 2000). Similar observations were made in Korea and these authors speculated that the presence of both PMWS and PDNS in the same herd but in different age groups was probably due to different strains of PCV2 or varying susceptibility of the pigs (Choi et al., 2002a). A recent case-control study investigating PDNS in the Netherlands found that there was a significant association of high antibody titers to PCV2 and the development of PDNS (Wellenberg et al., 2004b). The authors were not able to show PCV2-antigen by IHC in all of the PDNS cases but they were able to confirm the presence of PCV2 by PCR in all cases of PDNS. Importantly, the authors were able to show that porcine parvovirus or PRRSV nucleic acids were not present in many of the PDNS cases as determined by PCR (Wellenberg et al., 2004b). A study comparing PCV2 serum load in PMWS and PDNS cases found that PDNS cases had significantly lower numbers of PCV2 in serum compared to healthy, subclinical PCV2-infected pigs (Olvera et al., 2004). This study further confirms that PDNS pigs are infected with PCV2. Mauch et al. (2004) observed abortions and death loss in dams after 94 gilts in mid-pregnancy and seronegative for PCV2-antibodies were transported to four PCV2 positive farms. Within 2 months of entry, 34 of the 94 gilts showed typical PDNS like lesions, 8 of 34 gilts aborted, and 4 of 34 gilts died. The gilts had necrotizing vasculitis in skin, renal cortex, lymph nodes, spleen ileum, and in the uterus wall (Mauch et al. 2004).

PCV2-associated enteritis

Cases of PCV2-associated enteritis are relatively uncommon. Most of the PCV2-associated enteritis cases are from grow-finish pigs. PCV2-associated enteritis cases often clinically and grossly resemble subacute or chronic ileitis. The intestinal mucosa is grossly thickened and mesenteric lymph nodes are enlarged. Microscopic examination confirms the presence of granulomatous enteritis which can be associated with abundant PCV2-antigen by IHC (Halbur and Opriessnig, 2004). Kim et al. (2004a) diagnosed PCV2-associated enteritis in six weanling pigs. PMWS or PDNS were not seen in the herd of origin and the six pigs had no lymphoid depletion or histiocytic replacement of follicles in lymphoid tissues. The authors proposed that diagnosis PCV2-associated enteritis occur only (1) if diarrhea is present, (2) if characteristic lesions are present in Peyer’s patches but not in other lymph
nodes, and (3) if PCV2-antigen or nucleic acids are present within the lesions (Kim et al., 2004a).

There are many reports of concurrent infections with PCV2-associated enteritis. *Cryptosporidium parvum* and PCV2 were demonstrated in a 3-month-old Iberian pig (Núñez et al., 2003). Since *Cryptosporidium parvum* is a rare primary intestinal pathogen in post-weaning and growing pigs, the authors speculated that concurrent PCV2 infection caused immunosuppression (Núñez et al., 2003). Granulomatous enteritis was observed in Iberian pigs naturally infected with PCV2 and *Lawsonia intracellularis* (Segalés et al., 2001b). Intestinal chlamydial infection concurrent with PMWS was observed in a 12-week-old Spanish pig (Carrasco et al., 2000). Finally, Jung et al. (2006) identified PCV2 in pig naturally infected with porcine epidemic diarrhea virus (PEDV). The authors found 32.7% (35/107) small intestinal samples from 87 pig herds diagnosed with PEDV-infection positive for PCV2 nucleic acids by PCR and 29.9% (32/107) PCV2 positive by ISH. Since most of the pigs in this study were less than 3 days old, it was concluded that prenatal *in utero* PCV2 infection may be common (Jung et al., 2006).

**PCV2-associated hepatitis**

Hepatic lesions have been described as lymphohistiocytic inflammatory infiltration in portal zones, single cell necrosis of hepatocytes, swelling and vacuolation of hepatocyte cytoplasm and karyomegaly (Clark, 1997). PCV2 associated hepatitis has been reported in experimentally infected pigs coinfected with PCV2 and PPV (Allan et al., 1999a; Kennedy et al., 2000). Harms et al. (2001) observed severe necrotizing hepatitis lesions in CDCD pigs inoculated with PCV2 or concurrently inoculated with PCV2 and PRRSV, whereas hepatitis was not observed in pigs singularly infected with PRRSV. The authors concluded that hepatitis observed in field cases of atypical PRRSV likely results from concurrent PRRSV/PCV2 infection (Harms et al., 2001). Moderate-to-severe necrotizing and granulomatous hepatitis was observed in PCV2 and PCV2/PPV coinfected gnotobiotic piglets (Krakowka et al., 2000). Acute hepatitis with centrilobular necrosis of hepatocytes was observed in a 24-day-old CD pig experimentally infected with a tissue homogenate from a pig with naturally-acquired PMWS (Hirai et al., 2003). Ladekjær-Mikkelsen (2002)
observed moderate to severe hepatic atrophy associated with nonsuppurative cholangiohepatitis in experimentally-PCV2-infected pigs. In field cases, hepatic disease is a common clinical and pathological feature in pigs with PMWS and has been associated with concurrent PPV infection (Ellis et al., 2000a). Coinfection with Aujezsky’s disease virus and PCV2 has been reported in pigs with severe PMWS and hepatic lesions (Rodríguez-Arrioja et al., 1999).

**PCV2-associated CNS disease**

Congenital tremors in pigs are associated with demyelination of brain and spinal cord. The most common form in North America is transmissible and classified as type A2. This form is characterized by clonic contractions of skeletal muscles of varying severity that decrease with time and usually resolves by 4 weeks of age. Hines and Lukert (1994) were the first to report on a potential association between PCV and congenital tremors. Four PCV seronegative sows were experimentally infected with PCV obtained from a piglet with congenital tremor. The sows didn’t develop any clinical signs, but all of the piglets had congenital tremors of varying degrees. Stevenson et al. (2001) demonstrated PCV2-antigen (IFA) and nucleic acids (ISH and PCR) intralesional in affected neonatal pigs from four different farms in the Midwestern US.

In contrast to this, Kennedy et al. (2003) investigated central nervous system and nonneural tissue of 40 pigs affected by congenital tremor from Spain, the United Kingdom, Ireland, and Sweden by IHC, ISH, and PCR (Spanish cases only). PCV1 or PCV2 nucleic acids or antigen were not found in any of the pigs (Kennedy et al., 2003).

**PCV2-associated exudative epidermitis**

Wattrang et al. (2002) described an outbreak of exudative epidermitis in a Swedish SPF herd associated with PCV2. At the time of the outbreak, seroconversion to PCV2 was observed. *Staphylococcus hyicus* was isolated from skin swabs from affected pigs and PCV2 nucleic acids were demonstrated by PCR in lymphoid tissues from affected pigs (Wattrang et al., 2002). Harms et al. (2001) observed severe, diffuse exudative dermatitis in 42% (8 of 19 pigs) of CDCD pigs experimentally infected with PCV2 and the presence of *Staphylococcus*
hyicus was confirmed by bacterial culture. The dermatitis lesions appeared at 3 to 7 DPI and progressed to a severe and diffuse form between 10 to 21 DPI. In addition, mild exudative dermatitis was observed in 6/18 PCV2 infected pigs and in 2/10 control pigs. Exudative dermatitis was not observed in PRRSV and PRRSV/PCV2 coinfected pigs (Harms et al., 2001). Kim and Chae (2004b) investigated 142 tissues from pigs diagnosed with exudative epidermitis on the basis of bacterial isolation and microscopic lesions. The cases had been submitted between 1997 and 2002. Sections of skin and lymph node were tested by ISH for the presence of PPV and PCV2. PCV2 was found in 12 of 142 (8.5%) cases, PPV was found in 16 of 142 (11.3%) cases, and both, PCV2 and PPV were found in 60 of 142 (42.3%) cases. Distinct positive labeling was found throughout the inflammatory area in the dermoepidermal junction and superficial dermis. PCV2 and PPV DNA were detected occasionally in macrophages in lymphoid tissues. The authors concluded that PCV2 and PPV are highly prevalent in pigs with exudative epidermitis (Kim and Chae, 2004b).

Factors that play a Role in PCV2-associated Diseases

Coinfections
a. Porcine parvovirus (PPV)

Ellis et al. (1999) reproduced most of the lesions typical of PMWS in gnotobiotic pigs inoculated with filtered cell culture material and filtered lymphoid tissues from pigs with naturally acquired PMWS. Both PCV2 and PPV and antibodies to these viruses were detected in the experimentally-inoculated pigs. Since then, several groups have demonstrated that CD pigs dually-inoculated with PCV2 and PPV develop more severe disease and lesions than pigs with singular PCV2 infection (Allan et al., 1999a; Kennedy et al., 2000). Krakowka et al. (2000) further confirmed the synergistic effect of PCV2 and PPV in gnotobiotic pigs by reproducing clinical disease and lesions typical of PMWS in coinfected pigs but not in pigs infected with PCV2 or PPV alone. Kim et al. (2003a) coinfected twenty-four 28-day-old CD pigs with a Korean PCV2 isolate and PPV and observed clinical PMWS in all pigs. Opriessnig et al. (2004a) were able to demonstrate the development of PMWS in conventional SEW pigs coinfected with PCV2 and PPV. The mortality in PCV2/PPV-infected pigs was 6.7%, which is similar to PMWS-associated mortality in affected herds in
the US. Hasslung et al. (2005) reproduced PMWS in Swedish and Danish CD pigs concurrently infected with PCV2 and PPV at 3 days of age. Ostanello et al. (2005) coinfected eight 3-week-old conventional pigs confirmed to have passively acquired antibodies to PPV and PCV2 oronasally and intramuscularly with PPV and PCV2. Half of the pigs (4/8) were vaccininated with a commercial APP vaccine at 3 DPI. None or the pigs developed clinical disease and all pigs were necropsied at 42 DPI. It was found that passive immunity against PCV2 can prevent PMWS but it can not prevent subclinical PCV2 infection (Ostanello et al., 2005).

b. Porcine reproductive and respiratory syndrome virus (PRRSV)

Allan et al. (2000a) inoculated 1- to 2-day-old CD pigs with PCV2 and PRRSV and observed upregulation of PCV2 replication in coinfected pigs. However, the replication and distribution of PRRSV in concurrently infected pigs was not enhanced compared to that observed in single PRRSV infected pigs (Allan et al., 2000a). Harms et al. (2001) coinfected 3-week-old CDCD pigs at three weeks of age with PCV2 and PRRSV and showed that PCV2-infection increased the severity of PRRSV-induced interstitial pneumonia in CDCD pigs. Rovira et al. (2002) inoculated 5-week-old conventional pigs with PRRSV and seven days later with PCV2 and confirmed that PRRSV infection enhances PCV2 replication. A longer duration of PRRSV viremia and a higher proportion of viremic pigs were observed in the coinfected pigs compared to singular PRRSV infected pigs (Rovira et al., 2002). Chung et al. (2005) coinfected 10-week-old conventional pigs with PRRSV and PCV2 and injected a portion of the pigs with formalin-inactivated *Salmonella choleraesuis* and complete Freund’s adjuvant one week before inoculation. It was found that PRRSV was significantly upregulated by the Salmonella treatment whereas PCV2 was not (Chung et al., 2005). A recent *in vitro* study using swine alveolar macrophages found that PCV2-induced interferon-alpha reduced PRRSV infection and PRRSV-associated cytopathic effect (Chang et al., 2005). Subclinical infection of SPF pigs with PCV2 14 days before modified live PRRSV vaccination resulted in significantly (*P* > 0.05) increased PRRSV-induced macroscopic lung lesions after PRRSV challenge (Opriessnig and Halbur, 2005b). Evidence of interaction of PCV2 with the attenuated vaccine strain of PRRSV was lacking (Opriessnig and Halbur, 2005b).
c. *Mycoplasma hyopneumoniae*

Conventional pigs were inoculated intratracheally with *M. hyopneumoniae* at 4 weeks of age followed by intranasal inoculation with PCV2 at 6 weeks of age (Opriessnig et al., 2004c). Four of 17 (23.5%) dual-infected pigs had decreased growth rate and severe lymphoid depletion and granulomatous lymphadenitis associated with high amounts of PCV2-antigen consistent with PMWS. *M. hyopneumoniae* potentiated the severity of PCV2-associated lung and lymphoid lesions, increased the amount and prolonged the presence of PCV2-antigen, and increased the incidence of PMWS in pigs (Opriessnig et al., 2004c).

**Immunostimulation**

Studies have demonstrated that immune stimulation may trigger progression of PCV2 infection to disease and lesions characteristic of PMWS. Krakowka et al. (2001) reproduced PMWS in gnotobiotic pigs stimulated with KLH/ICFA and infected with PCV2. Allan et al. (2000c) observed PMWS in 21% of PCV2-infected and vaccinated (*M. hyopneumoniae* at 13 and 31 days of age, and APP at 47 days of age) colostrum-fed pigs. Opriessnig et al., (2003) demonstrated enhanced PCV2 replication manifest as significantly longer PCV2 viremia, a higher copy number of PCV2 genome in serum, a wider range of tissue distribution of PCV2 antigen, and an increased severity of lymphoid depletion in conventional pigs vaccinated with commercial APP and *M. hyopneumoniae* vaccines compared to PCV2-inoculated, unvaccinated pigs. A study testing the effect of different adjuvants on PCV2-associated lesions found that oil-in-water adjuvants increased the length of PCV2-viremia, increased the amount of PCV2 in serum and tissue, and increased the severity of lymphoid depletion compared to aqueous and aluminum hydroxide adjuvanted products (Hoogland et al., 2006). These experimental models suggest that immune stimulation in the form of adjuvanted vaccines may in some cases increase the incidence and severity of PMWS in PCV2-infected pigs. Field trials also support this hypothesis. Vaccination of piglets with a *M. hyopneumoniae* bacterin at 1 and 4 weeks of age resulted in a significant increase in losses associated with PMWS in the vaccinated pigs in two of five batches (Allan et al., 2001). Kyriakis et al. (2002) vaccinated a portion of the pigs in a herd experiencing a PMWS outbreak with a *M. hyopneumoniae* vaccine and observed PMWS in 43% of the vaccinated
pigs compared with 11% of the non-vaccinated pigs. Vanderstichel et al. (2003) conducted a field trial in herds affected by PMWS. The authors used different adjuvanted *M. hyopneumoniae* products (two oil-in-water adjuvanted *M. hyopneumoniae* bacterins and one aluminum hydroxide adjuvanted bacterin). It was found that a higher number of the pigs treated with aluminum hydroxide developed PMWS compared to pigs treated with saline or with the oil-in-water adjuvant. It was concluded that the immunostimulation from the aluminum hydroxide adjuvant may have facilitated the development of clinical disease (Vanderstichel et al., 2003).

In contrast, another recent study using conventional pigs failed to demonstrate an immune-stimulation dependent difference in pigs inoculated with PCV2 and stimulated with KLH/ICFA (Ladekjær-Mikkelsen, 2002). Resendes et al. (2004) used a control group, a vaccine adjuvant group, a PCV2-infected group, and a group infected with PCV2 and injected with a vaccine adjuvant. The authors found no differences between groups (Resendes et al., 2004). Harvey (2003) reported on observational studies on two farms known to be infected by PMWS and found no significant difference between vaccinated and unvaccinated groups in terms of overall mortality rates or development of clinical PMWS.

**Immunosuppression**

Krakowka et al. (2002) infected 12 gnotobiotic pigs with PCV2 at one day of age. In addition, 4 pigs received cyclosporine orally on a daily basis, and 4 pigs received corticosteroid (triamcinolone aceonide suspension) intramuscularly twice a week. The cyclosporine treatment but not the corticosteroid treatment resulted in increased PCV2 replication in tissues and promoted spread of PCV2 to hepatocytes. Inflammatory reactions typically of PMWS were absent although tissues contained high titer of virus (Krakowka et al. 2002). Kawashima et al. (2003) inoculated CDCD pigs intrasally and intraperitoneally with PCV2 (*n* = 7) and treated 3 of 7 PCV2-infected pigs with dexamethasone at 8 days of age. The authors were able to produce granulomatous lymphadentits in PCV2 and dexamethasone treated pigs but not in pigs infected with PCV2 alone suggesting that immunosuppression of the host has an effect on development of PMWS (Kawashima et al., 2003).
Age

PMWS is observed most often in pigs between 5 and 18 weeks of age with most cases occurring between 6 and 10 weeks of age. Reports of PMWS in adult pigs are rare. PCV2-associated lesions are typically observed from 14 to 35 days post PCV2 inoculation in experimental studies. This suggests that pigs in the field are most likely infected between 1 and 10 weeks of age. Studies investigating the influence of age on PCV2-infection are lacking. In experimental models described to date in the literature, pigs that were singularly-infected with PCV2 (no coinfection or immune stimulation) and developed clinical PMWS were between 1-21 days of age when inoculated. PMWS in older pigs was observed only in purebred Landrace pigs (35-39 days of age) (Opriessnig et al., 2006a) or with additional coinfections or immune stimulation (i.e. vaccination).

Gender

Corrége et al. (2001) collected data on PMWS over a 1-year period on a French pig farm and found that castrated male pigs were more susceptible to PMWS than females (38% versus 29%). In this study, the prevalence of PMWS was estimated by evaluating all dead pigs, recording clinical signs, and by regularly weighing the pigs (15-day-intervals during the first two month). Rodríguez-Arrioja et al. (2002) followed 250 3-week-old piglets (121 females, 106 castrated male, and 23 intact males) from a PMWS-affected farm up to 28 weeks of age and observed a difference in mortality between males and females. Males appeared to be at a higher risk of dying and it was concluded that this was likely due to an effect of castration associated with secondary infections.

Host susceptibility

Madec et al. (2000) found that 42% of the litters in PMWS herds were not affected but 16% of the litters accounted for 54% of the losses. This is indicative of a litter effect and increased susceptibility to PCV2-associated PMWS in some pigs.

A cohort study was conducted to investigate a suspected decreased susceptibility to PCV2-associated disease in Pietrain pigs by manipulating the genetics via artificial insemination on 4 PMWS-affected farms (Rose et al., 2004). Half of the sows were
inseminated with Pietrain semen whereas the remaining sows received the semen that was typically used on these farms. The PCV2-associated disease in the Pietrain-offspring did not differ from that observed in other pigs on these farms in terms of PCV2-seroconversion, morbidity, and mortality (Rose et al., 2004).

Allan et al. (2002a, 2003) reproduced PMWS in Northern Irish Large White/Landrace pigs experimentally infected with a porcine PCV2 isolate recovered from a clinically normal Swedish Yorkshire pig in 1993. The authors concluded that there is disease potential of PCV2 isolated from regions free of PMWS and that the status of the host and its environment may be an important factor in the development of PMWS (Allan et al., 2002a, 2003).

Duroc, Landrace, and Large White pigs were infected intranasally and intramuscularly at 5 to 7 weeks of age with PCV2 (Opriessnig et al., 2006a). Clinical disease compatible with PMWS was observed only in the Landrace pigs. Three of 19 Landrace pigs and none of the Duroc or Large White pigs developed severe lymphoid lesions associated with large amounts of intralesional PCV2-antigen typical of PMWS. In this experiment, 23 Duroc pigs (4 sires, 8 dams), 19 Landrace pigs (4 sires, 6 dams), and 21 Large White pigs (4 sires, 6 dams) were inoculated with PCV2. The results suggest a predisposition of the Landrace pigs used in this study to PCV2-induced disease and lesions (Opriessnig et al., 2006a).

McIntosh et al. (2005) tested 43 boars for presence of PCV2 DNA in semen. Duroc and Landrace boars were found to be positive for PCV2-DNA in semen whereas Large White and Meishan synthetic breeds were not observed to shed PCV2 DNA.

Zhou et al. (2006) looked at seroprevalence of PCV2 in 46 swine farms in Zhejian, China, and found that 44.83% of all Landrace sows and 64.28% of all Landrace piglets were positive for PCV2-antibodies. The seroprevalence of Landrace sows was higher than that of Yorkshire and Duroc sows.

López-Soria et al. (2004) compared the effect of 3 different genetic boar lines on 2 different Spanish farms on the outcome of general and PCV2-associated postweaning mortality. There was a significant effect of genetics on the expression of PCV2-associated diseases observed on both farms. Pigs with the paternal genetic backgrounds “A” (100% Pietrain) and “B” (50% Large White x 50% Pietrain) showed lower percentages of PCV2-
associated disease than those with the paternal genetic background “C” (25% Large White x 75% Duroc). However, due to the limited size of the study, it could not be concluded that the observed effect was due to a particular breed or line, or a particular boar (López-Soria et al., 2004).

Sibila et al. (2005) quantified and compared the PCV2 load in serum of naturally-infected pigs at different ages in two Spanish farms affected by PMWS with three different paternal genetic backgrounds (A=100% Pietrain; B=50% Large White x 50% Pietrain, and C=25% Large White x 75% Duroc). PMWS-associated mortality occurred mainly between 9-15 weeks of age and was related with the highest viral loads. A significant relationship between the paternal genetic background C and higher PCV2 viral loads in serum was observed in one farm (Sibila et al., 2005).

**Antibody levels**

In a field trial on a farm with a severe PMWS problem, the piglets of 8 sows were followed until 12 weeks of age. None of the piglets derived from the dams with highest PCV2-antibodies developed PMWS, and 60% of the piglets that did develop PMWS were derived from the dams with the lowest PCV2 serum antibody levels (Allan et al., 2002b). Another European field study investigated the effect of the sow on PMWS in a cohort study in 3 sow herds with a high postweaning mortality due to PMWS (Hassing et al, 2003). A total of 1,183 piglets derived from 125 sows were included in this study. It was found that the offspring from sows with high antibody levels against PCV2 at farrowing had a higher risk of dying after weaning. The risk of dying was also dependent on increasing IPMA titers against PCV2 from weaning until 4 weeks after weaning (Hassing et al., 2003).

Harms et al. (2002b) used a total of 124 pigs with highly variable PCV2-antibody titers and inoculated 51/124 with PCV2 and coinflculated 47/124 with PRRSV and PCV2 at different ages (3, 6 and 11 weeks of age). Piglets with a PCV2 ELISA S/P ratio of 0.6 were protected by PCV2 replication and development of PMWS (Harms et al., 2002b).

Allan et al. (2002b) published an experimental study in which ten piglets derived from 2 dams with high PCV2 titers (determined by IFA) and ten piglets derived from 2 dams with low PCV2 titers were weaned at 2 days of age and infected with PCV2 at 3 weeks of
age. Piglets derived from the low PCV2 titer dams had significantly reduced daily weight gain and a higher incidence of PCV2 antigen in lymphoid tissues as determined by IHC (Allan et al., 2002b).

Fenaux et al. (2004a) demonstrated that pigs vaccinated with PCV2 and challenged with the same strain were protected. McKeown et al. (2005) determined the effects of PCV2 maternal antibodies on the immune response to experimental PCV2 infection. Twenty-four piglets were divided into 4 groups on the basis of the ELISA titers of PCV2 maternal antibodies: group A (n = 6; negative), group B (n = 5; low antibody levels), group C (n = 8; high antibody levels), and group D (n = 5; high antibody levels). Piglets in groups A, B, and C were inoculated with PCV2 at 0 DPI and challenged with PCV2 at 42 DPI. Group D pigs were not exposed to PCV2 at 0 DPI but were challenged at 42 DPI. The authors concluded that protection against PCV2 infection conferred by maternal antibodies is titer dependent: higher titers are generally protective, but low titers are not (McKeown et al., 2005).

Pigs that have a lower birth and weaning weight are more frequently affected by PMWS (Corrégé et al., 2001) and 7 of 10 pigs in a PMWS affected herd with insufficient colostral transfer as defined by refractometer reading did not survive to weaning (Moll 2004). Unfortunately, PCV2 antibodies in the piglets or dams were not determined in those studies.

By using the gnotobiotic pig model, Meerts et al. (2005c) found that variation in the onset of adaptive immunity may account for variation in PCV2 replication in pigs. In particular, absence of PCV2 neutralizing antibodies may be an important factor in the development of increased virus replication (Meerts et al., 2005c).

**PCV2 isolates**

Molecular studies to determine the genetic variation of PCV2 found that minor branches of PCV2 were associated with geographic origin rather than differences in virulenceability (Fenaux et al., 2000; Mankertz et al., 2000). Meehan et al. (2001) investigated PCV2 isolates from cases of PDNS and abortions and found the isolates being closely related to a PMWS-associated PCV2 further establishing the apparent genetic stability of PCV2. Mahé et al. (2000) identified four dominant immunoreactive areas by PEPSCAN analysis within ORF2. Larochelle et al. (2002) sequenced 34 Eastern Canadian PCV2 isolates
recovered from 5- to 20-week-old pigs with various clinical conditions such as PMWS (15 isolates), PRRS (8 isolates), generalized tremors (5 isolates), erysipelas (1 isolate), gastric ulcer (1 isolate), nervous signs (1 isolate), arthritis (1 isolate), and no clinical signs (1 isolate) and compared the obtained sequences to 36 published sequences. Sequence analysis indicated that all the isolates were closely related. Three major regions of amino acid heterogeneity were identified among PCV2 isolates, and two of the regions corresponded to two of the immunoreactive areas described by Mahé et al. (2000). Comparison of three immunodominant regions however, revealed no link between capsid protein variation and pathogenicity of isolates (Larochelle et al., 2002).

Larochelle et al. (2003) compared PCV2 from PMWS-affected herds to PCV2 from healthy pigs from herds non-affected by PMWS and found closely related strains in 6 different herds (4 with PMWS and 2 without) sharing at least 99.4% of their nucleotide-sequence identity and more than 98.7% of their amino-acid identity for the capsid protein. One strain identified in a herd without PMWS was found to be 100.0% homologous to a PCV2 from a PMWS herd (Larochelle et al., 2003). De Boisséson et al. (2004) used 31 pigs originating from 13 PMWS-affected herds and 25 pigs from 10 PMWS free herds and sequenced 38 PCV2 isolates. All the isolates shared 94.2-100% nucleotide identity. A wider nucleotide diversity was observed in the PCV2 isolates originating from PMWS free herds compared with isolates from PMWS herds; however, residues found to be specific to non-PMWS strains were also found in PMWS strains and no molecular marker of virulence in PMWS strains could be identified (de Boisséson et al., 2004). Sequence analysis of a PCV1 associated with congenital tremor in 1960 and two PCV2 isolates associated with recent cases of congenital tremors demonstrated that the PCV2 isolates showed 99% sequence identity with each other and also with other PMWS-isolates (Choi et al., 2002b). No consistent genomic differences between PMWS and recent congenital tremor isolates were found. The 1960 PCV1 isolate showed 98% similarity to other PCV1 isolates (Choi et al., 2002b). In a case control study done by Pogranichniy et al. (2002) PCV2 was not only found in PMWS cases but also in 62.5% of the control cases. Sequencing and genetic comparison revealed no differences between 5 PMWS-associated PCV2 isolates and 4 PCV2 isolates recovered from cases not associated with PMWS (Pogranichniy et al., 2002).
In contrast, Wang et al. (2004) comparing the ORF2 of Taiwanese PCV2 isolates associated with PMWS (4 isolates), PDNS (2 isolates), nervous signs (1 isolate), abortion (1 isolate) found a small number of residue difference associated with the different clinical conditions. Farnham et al. (2003) further characterized 2 PCV2 isolates associated with abortions by ORF2 sequencing and found that the isolates were almost identical to each other and to other isolates associated with reproductive failure whereas there were at least 2 amino acid differences to PCV2 isolates associated with PMWS. Recently it has been demonstrated that two amino acid changes in the PCV2 capsid protein that occurred during serial in vitro passage enhanced the ability of PCV2 to grow in vitro and attenuated the virus in vivo (Fenaux et al., 2004b). Furthermore, we recently compared field isolates of PCV2 recovered from a case with moderate-to-severe PCV2-associated lesions to a PCV2 isolate from a case with no PCV2-associated lesions and demonstrated significant differences in incidence and severity of PCV2-induced lesions and in amount of PCV2 virus in the serum and tissues of experimentally-inoculated SPF pigs (Opriessnig and Halbur, 2005).

Delay et al. (2005) reported on markedly increased incidence and severity of porcine circovirus type 2 (PCV2)-associated disease in eastern Canada since January 2005. PCV2 strains isolated from these outbreaks appear to be different from the resident PCV2 strains that have been in the region for the last 10-15 years. There is a dramatic increase of restriction fragment length polymorphism (RFLP) type 321 strains, whereas the most common PCV2 strain had been type 422. Sequencing and comparison of the ORF2 gene of the recent 321 strains showed high homology with French isolates (99%) but only 95% sequence identity with pre-2005 strains from eastern Canada.

**Diagnosis**

**Detection of anti-PCV2 antibodies**

a. *Indirect immunofluorescence assay (IFA)*

This assay detects the ability of the antibody in the serum to bind to a fixed monolayer of virus-infected cells. The specific antibodies are labeled with fluorescein-conjugated anti-swine-IgG. IFA assays for PCV2 have been described in the literature (Allan et al., 1998b; Pogranichnyy et al., 2000; Tischer et al, 1995a). Recently, an IFA assay based
on an ORF2 protein has been described (Racine et al., 2004) and it was found that the regular whole PCV2-based IFA had only a 57.1% relative sensitivity compared to the ORF-2 protein based IFA. IFAs have also been described for antibodies against non-pathogenic PCV1 (Fenaux et al., 2003) and appear to be specific. Studies have shown that there is a low level of cross-reactivity between PCV1 and PCV2 on IFA test (Allan et al., 1998b, Pogranichnyy et al., 2000).

b. **Indirect immunoperoxidase monolayer assay (IPMA)**

The IPMA is similar to the IFA with the exception that the antibody used is a peroxidase-conjugated anti-swine IgG. IPMA for PCV2 is widely used (Ellis et al., 1998; Balasch et al., 1999). An interlaboratory testing comparing IFA and IPMA results on the same 20 serum samples performed in different laboratories in Europe and Canada found a wide variation in detected titers between laboratories (McNair et al., 2004). In general, IPMA gave higher titers than IFA, and paraformaldehyde used as fixative gave higher titers than did acetone or ethyl alcohol (McNair et al., 2004).

c. **Enzyme-linked immunosorbent assay (ELISA)**

There are several peer-reviewed articles describing PCV2 ELISA assays (Blanchard et al., 2003b; Liu et al., 2004; Nawagitgul et al., 2002). Recently, commercially available IgG and IgM PCV2-ELISA assays have been introduced in Europe (Ingezim PCV IgG® and Ingezim PCV IgM®, Ingenasa, Madrid, Spain). Combined IgG and IgM values might be useful to determine the timing of PCV2-infection: IgM values \( \geq \) IgG values: early active infection (within the first 21 DPI); IgM values < IgG values: active infection (approximately between 20 to 50 DPI); High IgG values and negative IgM values: late or resolving infection of convalescent (approximately 2 months after infection) (Segalés et al., 2005b). A variant of the regular ELISA assay is the competitive (blocking) ELISA (Walker et al., 2000). A competitive ELISA specific for PCV2-antibodies is available in Europe (Synbiotics, Europe, Lyon, France). An antibody-detection blocking ELISA to detect PCV2-specific antibodies on feces is also described (Lopez et al., 2005) and is commercially available in Europe (Synbiotics Europe, Lyon, France).

d. **Serum-virus neutralization (SVN) assay**

The basis of the SVN assay is that it detects the presence of antibodies that have the
ability to prevent virus from attaching to and/or infecting cells. Neutralizing antibody assays for PCV2 have been described in the literature (Meerts et al., 2006, 2005c; Pogranichnyy et al., 2000). Neutralizing antibodies were detected between 15 (Meerts et al., 2005c) and 28 (Pogranichnyy et al., 2000) days post PCV2-infection and were correlated with protection or clearance of PCV2 infection in gnotobiotic pigs (Meerts et al., 2005c).

Detection of PCV2 nucleic acids

a. Polymerase chain reaction (PCR)

There are several PCR assays for the detection of PCV2-specific nucleic acids described in the literature (Choi et al., 2000; Grierson et al., 2004b; Hamel et al., 2000; Morozov et al., 1998; Shibata et al., 2003). Variants of the regular PCR assay include the following:

Multiplex PCR. More than one target sequence is detected in a single PCR step. The following multiplex PCR assays have been described in the literature: PCV2/PCV1 (Pogranichnyy et al. 2000; Ouardani et al., 1999), PCV2/PPV (Kim et al., 2001a), and PCV2/pseudorabies virus/PPV (Huang et al., 2004).

Nested PCR. To increase the ability to detect very small amounts of the target sequence the PCR assay is done in two steps. The first step generates a product that serves as template for the second step (Kim and Chae, 2001b; Kim and Chae, 2004a, Larochelle et al., 2000).

Multiplex-nested PCR. This assay combines multiplex and nested PCR and is designed to detect very small amounts of several target sequences. Assays have been described for the simultaneous detection of PCV1/PCV2/PPV Kim et al., 2003c; Kim and Chae, 2003a) and PCV1/PCV2 (Kim et al., 2001b).

Quantitative real time PCR. Quantitative real-time PCR-assays have been developed and these assays allow for determination of the amount of PCV2 genomic copy numbers in the serum or tissues. PCR reaction and detection are combined in one step which decreases the turn-around time (Brunborg et al., 2004; Chung et al., 2005, Ladekjaer-Mikkelsen, 2002; Liu et al., 2000; Olvera et al., 2004; Opriessnig et al., 2003; Rovira et al., 2002)

Reverse transcriptase (RT-) PCR. This is used to detect PCV2 RNA which is only
present if PCV2 replicates (Yu et al., 2005). Reverse transcription of RNA is required to make a complementary DNA for further amplification.

Most pigs in the field (healthy or diseased pigs) are infected with PCV2 at some point in their lives. Therefore the use of PCR, which theoretically can detect one genomic copy, is considered by many to be too sensitive for most applications. One notable exception may be the detection of PCV2-nucleic acids in semen. PCR assays to detect PCV2 in semen have been described (Larochelle et al., 2000; Kim et al., 2003c; Kim et al., 2001b); however, the infection dose required to transmit PCV2 infection via semen is unknown.

The amount of PCV2 nucleic acids in serum and tissues has been demonstrated to be predictive of the clinical outcome which might be an application for quantitative real-time PCR on individual pigs (Brunborg et al., 2004; Olvera et al., 2004). Those pigs with $10^7$ or greater PCV2 genomic copies per milliliter of serum likely have PCV2-associated lymphoid lesions and disease (Brunborg et al., 2004).

b. In-situ-hybridization (ISH)

ISH for PCV2 uses a labeled DNA probe that corresponds to a specific portion of the PCV2-genome (Kim and Chae, 2003b; McNeilly et al., 1999; Rosell et al., 1999; Sirinarumitr et al., 2000). Hybridization is usually done over night and followed by a color reaction. This method allows localization of PCV2 but the method in general is time consuming (over night hybridization step) and the reagents are expensive. Several ISH assays that detect multiple viruses within the same tissue section have been described: PCV1/PCV2 (Kim and Chae, 2001a; Nawagtitgul et al., 2000b) PCV2/PRRSV (Choi and Chae, 2001; Sirinarumitr et al., 2001), and PCV2/PPV (Choi and Chae, 2000; Kim and Chae, 2002).

Detection of PCV2 virus or viral antigen

a. Immunohistochemistry (IHC)

IHC uses polyclonal antibody to detect PCV2 antigen in formalin-fixed, paraffin-embedded tissue sections (McNeilly et al., 1999; Sorden et al., 1999). This method allows localization of the antigen within a tissue section. It has been determined that an estimated minimum viral load of $10^8$ PCV2 genomes per 500 ng DNA was required to give visible
staining in IHC (Brunborg et al., 2004). A comparison of ISH and IHC on tissues from diseased pigs that were stored for up to 6 month in 10% neutral buffered formalin before being embedded in paraffin found that both techniques were able to detect antigen or nucleic acids in all tissues examined (McNeilly et al., 1999). ISH was found to be more specific than IHC especially when compared to IHC performed with polyclonal antibodies (Kim and Chae, 2004a).

b. **Virus isolation (VI)**

PK-15 cells support PCV2-replication *in vitro* and these cells can be inoculated with body fluids or homogenate from pigs suspected to be infected with PCV2 (Pogranichnyy et al., 2000). Glucosamine treatment of the cells has shown to be effective in increasing PCV2-replication (Tischer et al., 1987). A PCV2-induced cytopathic effect is typically not observed and in order to determine viral replication, immunofluorescent or immunoperoxidase staining has to be performed. VI is not routinely done for PCV2 because it is time consuming and not always efficient since viable virus is required and prolonged transit time and autolysis can have negative effects. Applications may include determining if PCV2 shed in semen is infectious. Another version of virus isolation is the quantitative virus isolation (McNeilly et al., 1999). For this assay, 10-fold dilutions of clinical specimens (serum, tissue homogenates) are inoculated on PK-15 cells. This test has been found useful in discriminating subclinical PCV2-infection from clinical PCV2-infection (McNeilly et al., 1999).

c. **Indirect and direct fluorescent antibody assays (IFA/FA) on tissue sections**

IFA/FA uses a monoclonal antibody or polyclonal antiserum to detect antigen(s) in frozen tissue sections (McNeilly et al., 2002). The assay is fast but antigen can not be confidently associated with lesions and the assay is relatively subjective. Studies using polyclonal antisera and monoclonal antibodies against PCV1 and PCV2 isolates on cells infected with either PCV1 or PCV2 have shown that there was no cross-reaction (Allan et al., 1999b).

d. **Antigen-capture ELISA**

Antigen-capture ELISA on tissue homogenates has been described and the results were found to be comparable to quantitative virus isolation and IHC (Allan et al., 2000b). An antigen detection capture ELISA optimized for fecal samples was also developed (Synbiotics
Europe, Lyon, France) and tested on samples with and without a history of PMWS (Lopez et al., 2005). The main advantage of this kit is that fecal samples are readily available. In contrast, PCV2 is not always detected in serum, bleeding pigs might be inconvenient, and PCR on large numbers of samples is expensive.

**e. Electron microscopy (EM)**

This method is used to demonstrate circovirus-like particles directly within a cell and to study the virus structure and size. Intracytoplastic inclusions in macrophages in affected lymph nodes have been shown to be electron dense, round to ovoid bodies with sharp margins. The matrix was described as being heterogenous, with different areas being granular, crystalline in a herringbone pattern or crystalline in cross-sectional arrays on non-enveloped, small, icosahedral-viral particles (Kiupel et al., 1998). EM is not routinely done in diagnostic labs because it is time consuming and expensive. The overall sensitivity is low and there has to be an abundance of virus present in the tissue in order to be detectable by EM.

**PCV2-associated microscopic lesions**

PCV2-associated disease diagnosis can not be done without evaluation of microscopic lesions. The hallmark lesions in growing pigs are lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues and granulomatus inflammation in a variety of organs such as liver, kidney, lungs, heart and intestines (Sorden, 2000). Chianini et al. (2003) described three stages in pigs with naturally occurring PMWS. Stage I is characterized by mild lymphoid depletion and mild histiocytic infiltration with few multinucleated giant cells mainly in germinal centers of follicular areas of lymphoid tissues. Stage II is characterized by moderate lymphoid depletion of follicles in lymphoid tissues with moderate histiocytic infiltration. Stage III is characterized by loss of follicles in lymphoid tissues and by marked histiocytic replacement of such areas (Chianini et al., 2003). Sarli et al. (2001) described the (1) initial stage: remnants of lymphoid follicles are present, (2) intermediate stage: absence of lymphoid follicles and a more extensive depletion of interfollicular tissue, and (3) end stage: absence of follicles, lymphoid cell depletion and medulla-like tissue prevalence over the lymphoid tissue. In aborted fetuses, the hallmark
lesion is necrotizing to necrotizing and lymphohistiocytic myocarditis. Determining if PCV2-associated microscopic lesions are present or not is critical for the diagnosis of PMWS (Sorden, 2000).

**Further characterization of the PCV2 isolate**

a. **Sequencing**

With sequence analysis it is possible to characterize the genetic information and compare isolates to each other (Choi et al., 2002b; De Boisséson et al., 2004; Fenaux et al., 2000; Grierson et al., 2004; Kim and Lyoo, 2002; Mankertz et al., 2000). To further investigate possible differences among PCV2 isolates it is possible to sequence the entire PCV2 genome or to sequence only ORF2. At this point in time, sequencing results may be a useful epidemiological tool but the knowledge to determine virulence based on sequence information is not yet available.

b. **Restriction fragment length polymorphism (RFLP)**

RFLP uses enzyme digestion of viral nucleic acid (partial or whole) which results in a specific cutting pattern that is visualized on a gel. If there are differences between viruses at the site of enzyme cutting, different patterns can be observed. This fingerprinting technique has been commonly used for DNA viruses. An ORF2 based PCR-RFLP assay described in 2000 using *Hinfl, HinP1I, KpnI, Msel*, and *RsaI* enzymes is able to distinguish among PCV2 isolates (PCV2A, B, C, D, and E) (Hamel et al., 2000). A PCR-RFLP assay using *NcoI* enzyme that differentiates between PCV1 and PCV2 was described in 2000 (Fenaux et al., 2000). An ORF2 based PCR-RFLP assay using *Sau3AI, BanII, NspI, XbaI*, and *CfrI* enzymes has been described recently and is able to distinguish 9 different PCV2 genotypes (Wen et al., 2005). PCV2 RFLP analysis showed that there was a significant change from RFLP type 422 to type 321 in 2005 in Ontario, Canada (Delay et al., 2005).

**Control of PCV2-associated Diseases**

**Good management practices**

Successful treatment and control of PMWS has primarily focused on assuring good production practices that minimize stress, eliminating coinfections or minimizing their effect
and eliminating potential triggering factors that induce immune stimulation. Madec et al. (2000) emphasized the role of the environment on development of PMWS within a herd. The focus on control of PCV2-associated diseases remains on improving pig comfort and minimizing the effect of those coinfections or other circumstances that trigger PCV2 infection to progress to PCV2-associated disease. Madec et al. (1999) proposed a 20-point plan against PMWS in severely affected farms. This plan includes recommendations for the farrowing units (1) application of strict all-in and all-out rules with thorough cleaning and disinfecting between batches, (2) sows should be washed and treated for parasites before farrowing, (3) cross-fostering should be limited; (4) post-weaning pens should be small and separated by solid partitions, (5) pits should be emptied, cleaned and disinfected on a regular basis, (6) the stock density should be lowered to 0.33 m$^2$ per pig, (7) the feeder space should be increased to more than 7 cm per pigs, (8) the air quality should be improved so that NH3 < 10 ppm, CCO < 0.1%, and RH < 85%, (9) the temperature should be controlled, (10) there shouldn’t be any mixing of batches; (11) grow/finish pens should be small and separated by solid partitions, (12) the pigs should be emptied, cleaned and disinfected on a regular basis and strict all-in, all-out rules should be applied, (13) there should be no mixing of pigs from the post-weaning pens, (14) there should be no remixing between finishing pens, (15) the stock density should be lowered to > 0.75 m$^2$ per pig, (16) the air quality and temperature should be improved. In addition, the following should be considered: (17) the vaccination program should be appropriate, (18) the air and animal flow within buildings should be carefully controlled, (19) strict hygiene should be applied (tail and teeth clipping, injections); and (20) sick pigs should be removed as soon as possible to a hospital room or should be euthanized. It is recommended that at least 16 of the points should be adopted in order for the plan to be effective (Madec et al., 1999). Usage of disinfectants in buildings and transport vehicles that have been demonstrated to be efficacious against PCV2 (Royer et al., 2001) is also recommended.

Rose et al. (2003) reported on the risk factors for PMWS in French farrow-to-finish herds and found that things such as PPV or PRRSV coinfection of finishers, large pen size versus small pen size for weaners, and increased levels of cross-fostering increased the risk for PMWS; whereas long empty periods in the pig flow, regular treatment against external
parasites, pen versus crated gestation, and internal versus external gilt replacement decreased the risk for PMWS. López-Soria et al. (2005) did an exploratory study on risk factors for PMWS involving 62 Spanish farms and found that vaccination of gilts against PRRSV increased the odds of PMWS expression and vaccination of sows against atrophic rhinitis decreased odds of the disease.

**Control of coinfections**

a. *M. hyopneumoniae*

Chlortetracycline (CTC)-treatment used in the *M. hyo*-PCV2-coinfection model provided evidence that the CTC treatment is highly efficacious in reducing lesions associated with PCV2 and *M. hyo* coinfection (Halbur et al., 2005). At 6 weeks of age, pigs were inoculated intratracheally with *M. hyopneumoniae*, followed by intranasal inoculation with PCV2 at 8 weeks of age. At 8 weeks of age, half of the pigs received a CTC feed additive at an approximate dose of 22 mg/kg. The CTC-treated and coinfected pigs had significantly less severe clinical disease, macroscopic and microscopic lung lesions compared to the non-treated coinfected pigs.

A recent study evaluated the losses or gains associated with the use of three different commercially available *M. hyopneumoniae* bacterins in pigs experimentally co-infected with *M. hyopneumoniae* and PCV2. Two hundred ninety-six *M. hyopneumoniae*-negative pigs were randomly assigned to one of four treatment groups. Three commercial vaccines, administered as per label direction, were tested: two bacterins containing an oil-based adjuvant and one bacterin containing an aqueous-based adjuvant. The *M. hyopneumoniae* challenge resulted in severe macroscopic and microscopic lesions in the non-vaccinated pigs. Pigs in all vaccine treatment groups had significantly higher mean body weight and average daily gain on 100 and 131 DPI, compared to the unvaccinated controls (Halbur et al., 2005).

Furthermore, to minimize the enhancing effect of adjuvanted *M. hyopneumoniae* vaccine products on PCV2-replication and PCV2-asscoiated lesions it has been shown that pigs should be vaccinated two to four weeks prior to expected PCV2 exposure (Opriessnig et al., 2006b).

b. Porcine parvovirus
Rodibaugh (2002) described changing a lepto-parvo-erysipelas program from weaning to pre-farrowing in a herd with approximately 7-8% mortality due to PMWS. After intervention, the mortality rate declined to 2-3% (Rodibaugh, 2002).

Conventional pigs were coinfected with PCV2 and PPV and the effect of PPV vaccination in reducing disease and lesions associated with PCV2/PPV coinfection was investigated (Opriessnig et al., 2004a). PPV vaccination was done 24 and 10 days before PCV2/PPV virus challenge with a killed PPV vaccine. Clinical signs consistent with PMWS (fever, respiratory disease, jaundice, weight loss) were seen in vaccinated and non-vaccinated PCV2/PPV coinfected pigs (Opriessnig et al., 2004a).

**PCV2-vaccines**

Vaccination of sows with an inactivated oil-adjuvanted PCV2-vaccine (CIRCOVAC®; Merial Inc.) in field conditions in Europe was beneficial in reducing the PCV2 circulation and shedding in the first weeks of life, but also in improving the pig health after an additional experimental PCV2 challenge at 3-4 weeks of age (Charreyre et al., 2005). Eleven gilts free of PCV2-antibodies were vaccinated with the PCV2-vaccine intramuscularly at 5 and 2 weeks before breeding and again at 2 weeks before farrowing. A group of 22 piglets born to 4 vaccinated gilts and a group of 22 piglets born to unvaccinated control gilts were inoculated with PCV2 intranasally at 3-4 weeks of age. Seroconversion was observed in piglets born to seronegative dams. PCV2 DNA in serum and mesenteric lymph nodes was significantly ($P = 0.00002$) lower in piglets born not vaccinated dams. In a field efficacy study, 3 groups of piglets were selected on farm and brought to the research facility. Group 1 pigs ($n = 12$) were born to unvaccinated sows, group 2 pigs ($n = 10$) were born to sows that had been vaccinated once with the PCV2-vaccine 2 weeks before farrowing, and group 3 pigs ($n = 11$) were from a different farm and free of antibodies to PCV2. All piglets were infected intranasally with PCV2 at 25-47 days of age. Piglets born to non-vaccinated sows had a rise in PCV2 antibodies, whereas the PCV2-antibodies decayed in the pigs born to vaccinated sows. Lymph nodes were grossly unremarkable in this group whereas the lymph nodes were enlarged in the two other groups. During field efficacy studies of the PCV2 vaccine conducted in Germany and France it was found that there was a rise in
PCV2 antibody level in the breeder herds concurrently with a decrease in PMWS rates in the pig originating from the farms (Charreyre et al., 2005).

Pogranichnyi et al. (2004) tested two inactivated US-PCV2 isolate preparations (ultraviolet irradiation or chemical inactivation) in the CDCD PCV2 PRRSV coinfection model and in the CDCD PCV2 KLH model using 57 piglets randomly assigned to 6 groups. Vaccination was done at 7 days of age and again 2 weeks later. PRRSV inoculation was done at 7 days of age. KLH with incomplete Freund’s adjuvant was infected at 21 and 27 days of age. At 24 days of age, the pigs were inoculated with PCV2. After PCV2 inoculation, the mortality in the vaccinated pigs was 20% whereas it was 70% in the non-vaccinated pigs implying that vaccination against PCV2 can be effective (Pogranichnyi et al., 2004).

It has been demonstrated that a chimeric PCV1-2 virus (with the immunogenic capsid gene of PCV2 cloned into the backbone of PCV1) induces an antibody response to PCV2 capsid protein and is attenuated in pigs (Fenaux et al., 2003). The attenuated chimeric PCV1-2 induced protective immunity to wild-type PCV2 challenge in pigs (Fenaux et al., 2004a). A total of 48 SPF pigs were randomly and equally assigned to 4 groups of 12 pigs each. Pigs in group 1-3 were vaccinated with the chimeric PCV1-2 and pigs in group 4 were not vaccinated and served as controls. At 42 days post vaccination, all pigs were challenged intranasally and intramuscularly with wild-type pathogenic PCV2. Mild-to-severe lymphoid depletion and histiocytic replacement were detected in lymphoid tissues in the majority of nonvaccinated group 4 pigs but in only a few vaccinated group 1-3 pigs (Fenaux et al., 2004a).

Blanchard et al. (2003a) found the ORF2 protein to be a major immunogen, inducing protection in a prime-boost protocol. Thirty-five, 25-day-old SPF pigs were divided into 5 groups of seven piglets. Groups 1-4 piglets received an intramuscular injection of DNA plasmid preparation followed by a second injection 2 weeks later. Pigs were challenged intratracheally and intramuscularly with PCV2 10 days after the second injection. As evaluated by growth parameters, clinical signs and seroconversion, the pigs were protected against a PCV2 challenge after vaccination. In a second trial, sixty-four 4-week-old SPF pigs were divided into eight groups with eight pigs in each group. Piglets received either in intramuscular DNA injection or a intramuscular infection of baculovirus-expressed protein...
followed by a booster injection 2 weeks later. The piglets were inoculated with PCV2 11 days after second injection. The results indicated that protection induced by a subunit vaccine was even better than the one induced by a DNA vaccine, since PCV2 replication was completely inhibited (Blanchard et al. 2003a).

Seven week old femal Balb/c mice were DNA vaccinated three times on the against PCV2 structural protein on 0, 30, and 52 days and seroconverted to PCV2 (Kamstrup et al., 2004). The authors cloned a 768 bp fragment of the capsid protein of a Danish PCV2 isolate into the vector pcDNA1.1/V5-His/TOPO. The plasmid DNA was coated onto gold particles and used for particle mediated DNA vaccination. After 2 vaccinations, alls mice had seroconverted to PCV2. A PCV2 challenge was not done in this study (Kamstrup et al., 2004).

**Serotherapy**

“Serotherapy” has been utilized extensively by some European practitioners to control and prevent PMWS; however, the literature reports of this technique are limited to abstracts describing uncontrolled field studies. In those trials, serum is obtained from healthy pigs from a group of pigs on the farm that went through PMWS 2-3 months prior. Recipient pigs are treated by subcutaneous (Ferreira et al., 2001) or intraperitoneal (Waddilove and Marco, 2002) injection of convalescent serum. With both protocols, a significant reduction of clinical disease and mortality was observed. Sick animals treated with serum had a significantly increased survival rate in Spain (48%) and in England (58%) compared with normal survival of below 10% on both farms (Waddilove and Marco, 2002). The overall herd mortality rate of growing pigs in three trials was reduced from 15-18% in untreated pigs to 2-5% in treated pigs (Ferreira et al., 2001).

A controlled study tested efficacy of serotherapy at minimizing PCV2-associated disease and lesions (Halbur et al., 2005). Serum from experimentally-infected pigs in the acute and convalescent stages of infection was used. In addition, serum from pigs with high levels of anti-PCV2 maternal antibodies was also used. These three serotherapy treatments were compared to a group that was vaccinated with an experimental PCV1-2 chimeric vaccine. All pigs were necropsied at 21 DPI. The group vaccinated with the chimeric vaccine
had significantly \((P < 0.05)\) lower levels of viremia than all other groups and four of the seven pigs in this group remained PCV2 negative by PCR for the duration of the project. Under the conditions of this study, neither the acute- or convalescent-serotherapy were effective practices for preventing PCV2 infection and PCV2-associated lesions. The apparent success of serotherapy reported in European field trials remains unexplained. The experimental PCV1-2 chimeric vaccine appears to be effective, safe, and superior to serotherapy in reducing PCV2 infection and viremia (Halbur et al., 2005).
CHAPTER 3. DERIVATION OF PORCINE CIRCOVIRUS TYPE 2-NEGATIVE PIGS FROM POSITIVE BREEDING HERDS

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**Summary**

**Objective:** To summarize procedures utilized to derive piglets negative both for porcine circovirus type 2 (PCV2) and for antibody to PCV2, from PCV2-positive breeding herds.

**Methods:** Sow herds were screened for antibodies to PCV2 by ELISA, with sample-to-positive (S:P) ratios ≥ 0.2 considered positive. Piglets were obtained by segregated early weaning (SEW) at 10 to 12 days of age. Maternal anti-PCV2 antibody was determined by testing weekly blood samples by ELISA and indirect immunoflorescence assay. Absence of PCV2 was determined by polymerase chain reaction (PCR) on weekly serum samples and by PCR and immunohistochemistry assays on lymphoid tissues collected at necropsy (8 to 15 weeks of age).

**Results:** Piglets negative for PCV2 by PCR and for anti-PCV2 antibody were derived from PCV2-positive breeding herds. Mean PCV2-antibody half-life in weanling piglets was estimated to be 19.0 days (95% confidence intervals, 17.6 and 20.3 days). Passively acquired PCV2-antibody present at 10 to 12 days of age declined to S:P ratios < 0.2 by 4.9 ± 1.2 weeks of age in piglets with S:P ratios < 0.2 and < 0.6; by 8.1 ± 1.9 weeks in piglets with S:P ratios ≥ 0.6 and < 1.0; and by 11.1 ± 2.5 weeks in piglets with S:P ratios ≥ 1.0. Pigs kept in isolation remained PCV2-negative by PCR and ELISA through 8 to 15 weeks of age.
Implications: SEW may be an effective technique to derive PCV2-negative piglets from positive breeding herds.

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a disease complex characterized by wasting and pneumonia in pigs 5 to 12 weeks old. The hallmark lesions of PMWS are lymphoid depletion and histiocytic replacement of follicles associated with porcine circovirus type 2 (PCV2) antigen. Transmission of PCV2 is thought to occur through direct contact via oronasal, fecal and urinary routes. Vertical transmission has been demonstrated in individual sows in the field and experimentally. There is experimental and field evidence that PCV2 may establish persistent infection in pigs.

Serological surveys in England and North America found that nearly all swine herds are infected with PCV2. However, detection of antibodies to PCV2 is not necessarily indicative of disease prevalence. Diseases associated with PCV2, such as PMWS, are seen in a relatively low percentage of herds infected with PCV2. It has been reported that pigs with sample-to-positive (S:P) ratios of at least 0.6 in an ELISA, using as antigen an open reading frame 2 (ORF2) PCV2 capsid protein, are protected against infection by PCV2 and development of PMWS.

The study described here indicates that, under research conditions, pigs negative both for PCV2 and antibodies against ORF2 of the virus can be derived from PCV2-positive breeding herds. This information may be extrapolated to the field for purposes of establishment of PCV2-free herds.

Materials and Methods

Screening of breeding herds for PCV2 by ORF2 ELISA

Screening of blood samples for PCV2-specific antibodies was performed using an ELISA based on a recombinant ORF2 capsid protein as previously described. An S:P ratio of 0.2 was considered positive (sensitivity = 78.2%; specificity = 99.6%), and an S:P ratio of <0.12 was considered negative (sensitivity = 90.7%; specificity = 93.2%). Values between these S:P ratios were considered suspect for PCV2 antibody. For simplification, the 0.2 cutoff was used to separate seronegative and seropositive piglets in this study.
Six US breeding herds, designated as Herds A to F, were screened by testing the dams (sows and gilts) approximately 7 to 10 days prior to farrowing, except in the case of Herd B. In Herd B, only gilts were tested, and samples were collected 5 months prior to farrowing. None of the herds tested had a history of PMWS or PCV2-associated disease. The number of sows on each farm randomly selected for screening ranged from 12 to 43, depending on how many piglets were needed for our experiments and how many sows were in the batch ready to farrow at the time piglets were needed.

**Selection of dams for derivation of piglets**

Where parity information on the dams was available (Herd A, C, D, E, and F), ORF2 ELISA S:P ratios of the dams were compared by parity. Sows within herds were divided into three categories on the basis of prefarrowing ORF2 ELISA results. Category 1 included negative sows (S:P ratio <0.2), Category 2 included sows with S:P ratios 0.2 and < 0.6, and Category 3 included sows with S:P ratios 0.6. Dams were selected for derivation of piglets from either Category 1 or 2. No cross-fostering of piglets was allowed.

**Segregated early weaning**

The selected piglets were weaned at 10 to 12 days of age and moved to the Livestock Infectious Disease Isolation Facility at Iowa State University (Ames, Iowa) where they were housed. On the day of weaning, blood samples were collected from the anterior vena cava, using a single-use blood collection system (Vacutainer; Becton Dickinson, Franklin Lakes, New Jersey), and tested by ORF2 ELISA. Piglets were randomly assigned to groups and rooms independent of ORF2 ELISA S:P ratios. Handling and housing of the piglets was the same for all piglet groups. All people entering rooms were required to shower in, put on laundered coveralls, and wear latex gloves when touching piglets. The piglets were kept on raised wire decks, with feed and drinking water provided ad libitum. Airflow was unidirectional (positive pressure), with 10 to 15 air changes per hour.

**Monitoring PCV2 status through 15 weeks of age**

Randomly selected ELISA-negative samples from 10- to 12-day-old piglets were tested by indirect fluorescent antibody (IFA) assay to confirm results. Sera were tested at weekly intervals for PCV2-specific nucleic acids by regular polymerase chain reaction (PCR) and quantitative real-time PCR, using different primer annealing sites as described.
At the time of necropsy, performed when pigs were 8 to 15 weeks of age depending on experiment, lymphoid tissues (lymph nodes, spleen, tonsil, and thymus) were collected in 10% formalin, routinely processed, and tested for PCV2-antigen by immunohistochemistry (IHC) as previously described. In addition, blood was collected for ORF2 ELISA testing, and selected lymph nodes were collected, stored at -80°C, and tested by PCR for PCV2-specific nucleic acids.

**PCV2-antibody half-life calculation and monitoring of passive antibody decay**

The rate of maternal PCV2-antibody decay was determined as previously described. A linear regression curve was fitted to the natural logarithms (ln) of the ORF2 ELISA data of 199 randomly selected piglets. The equation \( h = \frac{-\ln(2)}{b} \) was used to estimate the antibody half-life, where \( h \) is the estimated half-life of antibody and \( b \) is the slope of the regression line. Antibody half-life for PCV2 antibodies was determined separately for each piglet, and the mean half-life was used as a point source estimate. The 95% confidence interval was calculated on the mean half-life.

For the purpose of following the passive antibody decay, the ELISA S:P ratios of the piglets at 10 to 12 days of age were separated into four categories. Category 1 included seronegative piglets (S:P ratio < 0.2), Category 2 included piglets with low levels of PCV2 antibodies (S:P ratio 0.2 and < 0.6), Category 3 included piglets with moderate levels of PCV2 antibodies (S:P ratio 0.6 and < 1.0), and Category 4 included piglets with high levels of PCV2 antibodies (S:P ratio 1.0). The mean category values were calculated, and passive PCV2-antibody levels were followed over time to determine antibody decay.

**Animals and experimental groups**

Individual groups of piglets were obtained from Herds A to F for use in several experimental PCV2-inoculation trials performed over a 2-year period. Details of the studies have been described previously. From the time of PCV2 inoculation until necropsy, this report focuses only on the non-infected negative control piglets in these experiments.

**Herd A:** A total of 98 piglets were obtained from Herd A and brought to the research facility in two batches 3 weeks apart. All piglets were serologically monitored until 6 weeks of age. Negative controls were monitored serologically each week until necropsy: 61 pigs were monitored to 10 weeks of age, 40 pigs to 12 weeks of age, and nine pigs to 15 weeks of
Herd B. Seventy-one piglets were obtained from Herd B. All piglets were serologically monitored until 6 weeks of age. Negative controls were monitored serologically each week until necropsy: 28 pigs were monitored to 10 weeks of age, and 14 pigs were monitored to 13 weeks of age.

Herd C. Seventy-eight piglets were obtained from Herd C. All piglets were serologically monitored until 8 weeks of age. Negative controls were monitored serologically each week until necropsy at 14 weeks of age (n=10).

Herd D. One hundred twenty-six piglets were obtained from Herd D. All piglets were serologically monitored until 9 weeks of age. Negative controls were monitored serologically each week until necropsy: 34 pigs were monitored to 9 weeks of age, and 16 pigs to 11 weeks of age.

Herd E. Ninety piglets were obtained from Herd E. All piglets were serologically monitored until 6 weeks of age. Necropsies were not performed on pigs of this group.

Herd F. Forty-one piglets were purchased from Herd F. All piglets were serologically monitored until 5 weeks of age. Negative controls were monitored serologically each week until necropsy: 16 pigs were monitored to 8 weeks of age, and 11 pigs to 11 weeks of age.

Statistical analysis

Summary statistics were calculated to assess the overall quality of the ORF2 ELISA S:P ratios. Welch analysis of variance (ANOVA) was used for parity comparison and for continuous data with unequal standard deviation. The rejection level for the null hypothesis was .05. The R-squared statistic was used to determine what proportions of the total S:P ratio variation of the piglets was explained by the S:P ratio of the dams with S:P ratios < 0.6.

Results

Sow herd PCV2 antibody profiles

Most breeding herds and most sows across parities within herds were seropositive for PCV2 antibodies by ORF2 ELISA (Table 1). There was no difference between high and low parity sows in terms of S:P ratios. The incidence of PCV2 ORF2 antibodies and the within-herd antibody distribution of S:P ratios in the six sow herds are summarized in Figure 1. In
the selected herds, 50 to 85% of dams had S:P ratios \( \geq 0.6 \), 15 to 42% had S:P ratios 0.2 and < 0.6, and 0 to 8% were seronegative. Among all breeding herds screened, only one herd (Herd B) was seronegative by ORF2 ELISA. In this case, all animals tested were gilts, and they were tested prior to breeding and not retested 7 to 10 days before farrowing as for sows from the other five herds.

**Transfer of passive antibodies from the dams to their offspring**

Similar passive antibody transfer data were obtained from all sow herds. Results for sows and piglets are summarized in Table 1. Within the same litter, some individual piglets had up to two to three times higher S:P ratios than their dams, whereas others were seronegative (Figure 2). The \( r^2 \) for sow S:P ratios and piglets S:P ratios was 0.13, ie, 13% of the variation in the piglets’ S:P ratios was explained by the sow S:P ratios (\( P < .001 \)). Overall, there was limited predictability of passive PCV2 antibody levels in piglets obtained from sows with S:P ratios < 0.6.

**Maternal antibody decay in piglets and PCV2 antibody half-life**

A total of 504 piglets were derived from herds A to F. Among all piglets, the PCV2 antibody levels at weaning (10 to 12 days of age) were below the ELISA cutoff in 175 piglets (34.7 %); within the lower S:P range (0.2 and < 0.6) in 202 piglets (40.1%); moderate (0.6 and < 1.0) in 93 piglets (18.5%); and high (1.0) in 34 piglets (6.7%). The individual distributions of S:P ratios of the piglets in each herd at weaning are summarized in Figure 3.

Within the observation periods, titers of passively acquired PCV2 antibodies declined in all piglets, and none of them seroconverted. The mean antibody half-life, based on declining ELISA S:P ratios in 199 pigs from Herds A to F, was 19.0 days. The 95% confidence intervals for the mean half-life were 17.6 and 20.3 days. In the group with S:P ratios 0.2 and < 0.6 at weaning (n=202), pigs became seronegative at 3 (n=7), 4 (n=96), 5 (n=33), 6 (n=44), 7 (n=13), and 8 weeks of age (n=9), respectively. Piglets with S:P ratios 0.6 and < 1.0 (n=93) became seronegative at 4 (n=9), 5 (n=2), 6 (n=9), 7 (n=1), 8 (n=32), 9 (n=18), 10 (n=13), and 11 weeks of age (n=9), respectively. Piglets with S:P ratios 1.0 (n=34) became seronegative at 5 (n=2), 9 (n=7), 10 (n=6), 11 (n=1), 12 (n=8), 13 (n=4), 14 (n=3), and 15 weeks of age (n=3), respectively. Antibody levels waned below cutoff levels
by 4.9 weeks of age for piglets with low levels of passive antibodies at weaning (95% confidence intervals, 4.8 and 5.1 weeks), at 8.1 weeks of age for piglets with moderate levels of passive antibodies at weaning (95% confidence intervals, 7.7 and 8.5 weeks), and at 11.1 weeks of age for piglets with high levels of passive antibodies at weaning (95% confidence intervals, 10.3 and 12.0 weeks). The antibody decay of 113 pigs is summarized in Figure 4 and represents a typical passive antibody decay curve.

A total of 149 piglets remained segregated and experimentally uninfected, and were necropsied at 8 (n=5), 9 (n=18), 10 (n=35), 11 (n=27), 12 (n=31), 13 (n=14), 14 (n=10), and 15 (n=9) weeks of age, respectively. All pigs were free of PCV2 antibody and virus on the basis of ELISA and IFA serologically testing, PCR on serum and selected lymph nodes, and IHC on lymphoid tissues.

Piglets that were experimentally infected with PCV2 (n=355) seroconverted within 2-4 weeks post challenge and developed PCV2-associated lymphoid depletion and histiocytic replacement of follicles characteristic of PMWS as previously described.\textsuperscript{16,19-22} PCV2-antigen was detected by IHC in lymphoid tissues associated with lesions, and PCV2-nucleic acids were detected by PCR on serum samples (data not shown).

**Discussion**

The objective of this report was to summarize the evidence from several experiments that piglets free of PCV2 and antibody to PCV2 can be derived from PCV2-positive breeding herds representative of US swine production. Recently, it has been demonstrated that PCV2-free pigs can be derived by cesarean-section and colostrum deprivation.\textsuperscript{23} The results of the study summarized here confirm that segregated early weaning (SEW) techniques may also be used for derivation of PCV2-free pigs.

In this study, for the purpose of deriving PCV2-free piglets for research trials, several breeding herds were screened for PCV2-antibodies, and most were positive. Only one breeding herd was seronegative, and these results were based on testing 12 gilts in one farrowing batch.

The use of SEW allowed us to obtain 504 PCV2-free piglets from six seropositive breeding herds with no history of PMWS or PCV2-associated disease. Passive PCV2
antibodies in the piglets, detected by the ORF2 ELISA, decayed to negative levels. The
PCV2-negative status of 149 of these pigs was confirmed by PCR on serum and selected
lymphoid tissues, as well as by IHC on lymphoid tissues. Most of the pigs were kept until 10
to 15 weeks of age, and none seroconverted during the observation period.

In contrast to reports of vertical intrauterine transfer of PCV2 resulting in viremic or
persistently infected piglets at birth,\textsuperscript{24} viremia was not detected in these piglets, which were
tested weekly by PCR on sera. It has been suggested that piglets can be infected in utero or
during the first days after birth.\textsuperscript{5,6} Vertical PCV2 infection may not always cause fetal death,
and virus, antibody, or both were detected in clinically normal piglets.\textsuperscript{25} It is possible that the
passively acquired antibody may suppress the PCV2 replication until maternal antibody
levels wane and the pigs become viremic. The data obtained in our repeated experiments do
not support this hypothesis, since all pigs remained seronegative after waning of maternal
antibodies.

Transmission routes for PCV2 have not yet been fully investigated. The absence of
virus in our pigs may have been due to the carefully controlled environment of the pigs, ie,
we can rule out possible transmission by means of semen, equipment, clothing, birds, or
rodents in these pigs.

We observed differences in the passive antibody levels within litters. These
differences may account for the temporal variability of onset and endemic manifestation of
PCV2-associated diseases in many herds. Direct comparison of dam and piglet PCV2 ORF2
ELISA results (Figure 2) suggested that sows with higher S:P ratios tended to have piglets
with higher passive antibody levels, and sows with lower S:P ratios tended to have piglets
with lower S:P ratios. However, testing was limited to selected dams in the categories with
lower S:P ratios. Besides the effect of the sow on the passively transferred antibodies, there
might also be a piglet effect, such as failure of colostrum uptake. This might in part explain
the lack of maternal antibodies in some piglets derived from seropositive dams (14.1%
derived from Herd C, and 8.7% derived from Herd D).

In the United States, clinical PMWS is most often observed in pigs that are 8 to 12
weeks of age, and, on the basis of experimental models,\textsuperscript{16,19-22} the piglets are probably
infected 7 to 21 days prior to this time. Our results indicate that in piglets weaned at 2 weeks
of age, PCV2 maternal antibody decays below ORF2 ELISA cutoff values by approximately 4.9 ± 1.2 weeks of age in piglets with low levels of antibody at weaning (S:P ratio < 0.6), by approximately 8.1 ± 1.9 weeks of age in piglets with moderate levels of antibody at weaning (S:P ratio 0.6 and < 1.0), and by approximately 11.1 ± 2.5 weeks of age in piglets with high levels of antibody at weaning (S:P ratio > 1.0).

It has been demonstrated that pigs with ELISA S:P ratios > 0.6 are protected from developing PMWS. Piglets with lower S:P ratios are therefore at a higher risk for PCV2 infection at an early age. In herds with PMWS, dams with high S:P ratios could be selected to obtain piglets passively protected for a longer time period. However, lack of maternal antibodies is certainly not the only important factor in the pathogenesis of PMWS. Colostrum-deprived, gnotobiotic pigs experimentally inoculated with PCV2 alone did not develop clinical disease. Other factors, such as coinfections or certain vaccination protocols, may enhance PCV2 infection and must also be considered.

The results of this study may be useful to veterinarians and producers planning to establish a PCV2-free herd. A uniform within-herd distribution of PCV2 antibody in dams might be achieved in the future with PCV2 vaccines, resulting in more uniform antibody levels in piglets and appropriate timing of vaccination in growing piglets.

**Implications**

Under the conditions of this study, in six breeding herds with no history of PCV2 infection or clinical PMWS, 50% or more of sows randomly tested by serum ELISA had moderate-to-high levels of antibody to PCV2. Individual sows with PCV2 ELISA S:P ratios close to the positive cutoff (< 0.2) were identified in every herd tested. Differences in amount of transfer of maternal anti-PCV2 antibody were observed among piglets within litter. Under the conditions of this study, the higher the PCV2 ELISA S:P ratio at weaning, the older the pig when the S:P ratio declined below the positive cutoff value. Under the conditions of this study, SEW-derived pigs remained free of PCV2-specific antigen and nucleic acids until at least 15 weeks of age.
SEW may be an effective technique to derive PCV2-free piglets from positive breeding herds for research or commercial production.

References


* Non-refereed references.
Table 1: Mean sample-to-positive (S:P) ratios ± SD of selected dams 7 to 10 days prefarrowing\(^1\) and their piglets at 10 to 12 days of age, tested by ELISA\(^2\) for porcine circovirus type 2 (PCV2)

<table>
<thead>
<tr>
<th>Herd</th>
<th>Total dams tested</th>
<th>Selected dams</th>
<th>Piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean S:P ratio</td>
<td>n</td>
</tr>
<tr>
<td>A</td>
<td>26</td>
<td>0.784 ± 0.57</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>0.016 ± 0.03</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>0.917 ± 0.28</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>1.001 ± 0.57</td>
<td>19</td>
</tr>
<tr>
<td>E</td>
<td>43</td>
<td>0.739 ± 0.34</td>
<td>12</td>
</tr>
<tr>
<td>F</td>
<td>43</td>
<td>0.939 ± 0.39</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^1\) In Herd B, all dams tested were gilts that were tested 5 month prior to farrowing.

\(^2\) ELISA using as antigen open reading frame 2 PCV2 capsid protein. An S:P ratio of ≥ 0.2 was considered positive.
Figure 1: Distribution of sample-to-positive (S:P) ratios in randomly selected sows tested by ELISA for porcine circovirus type 2 (PCV2) in six US swine breeding herds. Numbers of sows: Herd A, n=26; Herd B, n=12; Herd C, n=33; Herd D, n=40; Herd E, n=43; and Herd F, n=43. The ELISA used as antigen open reading frame 2 PCV2 capsid protein. An S:P ratio of ≥ 0.2 was considered positive. Dams in each herd were divided into three categories on the basis of the ELISA results: those with S:P ratios < 0.2, those with S:P ratios 0.2 and <0.6, and those with S:P ratios 0.6.
Figure 2: Sample-to-positive (S:P) ratios in 11 Herd E sows (shaded bars) tested 10 days postpartum and their piglets (unshaded bars) tested at 10 to 12 days of age by ELISA for porcine circovirus type 2 (PCV2). The ELISA used as antigen open reading frame 2 PCV2 capsid protein. An S:P ratio of 0.2 was considered positive.
Figure 3: Distribution of sample-to-positive (S:P) ratios in piglets derived from six different breeding herds, tested at weaning (10-12 days of age) by ELISA for porcine circovirus type 2 (PCV2). The ELISA used as antigen open reading frame 2 PCV2 capsid protein. An S:P ratio of ≥ 0.2 was considered positive. Numbers of piglets: Herd A, n=98; Herd B, n=71; Herd C, n=78; Herd D, n=126; Herd E, n=90; and Herd F, n=41. Piglets in each herd were divided into four categories on the basis of the ELISA results: those with S:P ratios < 0.2, those with S:P ratio 0.2 and < 0.6, those with S:P ratios 0.6 and < 1.0, and those with S:P ratios 1.0.
Figure 4. Decay of maternal porcine circovirus type 2 (PCV2) antibody in piglets tested weekly by ELISA, which used as antigen open reading frame 2 PCV2 capsid protein. A sample-to-positive ratio of $\geq 0.2$ was considered positive. Pigs were divided into four categories on the basis of ELISA results on samples collected at weaning (10-12 days of age). Group 1, S:P ratio $< 0.2$ (n=25); Group 2, S:P ratio $0.2$ and $< 0.6$ (n=48), Group 3, S:P ratio $0.6$ and $< 1.0$ (n=38); and Group 4, S:P ratio $1.0$ (n=9).
CHAPTER 4. EFFECT OF VACCINATION WITH SELECTIVE BACTERINS ON CONVENTIONAL PIGS INFECTED WITH TYPE 2 PORCINE CIRCOVIRUS

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Abstract. The objective of this study was to determine whether vaccination with bacterins commonly used in the USA, when administered at a time typical of US protocol, enhances porcine circovirus type 2 (PCV2) replication and the incidence and severity of clinical signs and lesions characteristic of postweaning multisystemic wasting syndrome (PMWS) in conventional pigs. Sixty-one pigs free of PCV2 were randomly assigned to four groups. Group 1 (n = 15) and group 2 (n = 15) pigs served as sham-inoculated negative controls. Groups 3 (n = 14) and 4 (n = 17) pigs were inoculated intralymphoid with PCV2 field isolate ISU-40895. Pigs in groups 2 and 4 were vaccinated with Actinobacillus pleuropneumoniae (APP) and Mycoplasma hyopneumoniae (M. hyopneumoniae) bacterins 21 days before and again 1 day before inoculation with PCV2. Mild transient respiratory disease and diarrhea were observed from 13 to 34 days postinoculation (DPI) in pigs in groups 3 and 4. Half the pigs from each group were necropsied at 22 and 34 DPI, respectively. Moderately enlarged, tan-colored lymph nodes were observed in the majority of pigs in groups 3 and 4. There was a significantly (P < 0.05) longer length of viremia (2.14 ± 0.26 versus 4.44 ± 0.23 weeks), a higher copy number of PCV2 genome in serum, a wider range of tissue distribution of PCV2 antigen, and an increased severity of lymphoid depletion in pigs vaccinated with commercial
APP and *M. hyopneumoniae* vaccines and inoculated with PCV2 compared with PCV2-inoculated unvaccinated pigs. Swine producers and veterinarians may need to consider changes in vaccination protocols in herds with recurrent PCV2-associated PMWS.

**Introduction**

Two types of porcine circovirus (PCV), referred to as type 1 PCV (PCV1) and type 2 PCV (PCV2), have been characterized in pigs.\(^5,24\) PCV is a very small, nonenveloped, single stranded DNA virus with a circular genome\(^33\) and belongs to the *Circoviridae* family.\(^23\) PCV1 was first recognized in 1974 as a cell culture contaminant.\(^35\) Experimental PCV1 infections have demonstrated no effect in pigs; thus, PCV1 is considered to be nonpathogenic.\(^3,34\) PCV2 was first recognized to be associated with postweaning multisystemic wasting syndrome (PMWS) in 1996.\(^12\) PMWS is characterized clinically by enlargement of the lymph nodes, chronic pneumonia, progressive weight loss, and less commonly by icterus, pallor, or diarrhea. Characteristic microscopic lesions include lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. Mild-to-severe granulomatous inflammation in lymphoid and other tissues also is commonly observed. Macrophages in affected lymphoid tissues may contain basophilic inclusion bodies.\(^1,31\)

Serological studies demonstrate that PCV2 is ubiquitous in the swine population, and essentially all herds are infected.\(^22,36\) In contrast, PMWS is manifested in a relatively low percentage of pigs in PCV2-infected herds.\(^1\) It has been demonstrated that other coinfections such as porcine reproductive and respiratory disease virus (PRRSV)\(^4,14,30\) or porcine parvovirus (PPV)\(^2,16,18\) may potentiate the development of PMWS in PCV2-infected pigs.

Recent studies have demonstrated that immune stimulation may trigger progression of PCV2 infection to disease and lesions characteristic of PMWS. Krakowka et al.\(^17\) reproduced PMWS in gnotobiotic pigs stimulated with keyhole limpet hemocyanin in Freund’s incomplete adjuvant (KLH-ICFA) and infected with PCV2. Allan et al.\(^6\) observed PMWS in 21% of PCV2-infected and vaccinated (*Mycoplasma hyopneumoniae* [*M. hyopneumoniae*] at 13 and 31 days of age, and *Actinobacillus pleuropneumoniae* [APP] at 47 days of age) colostrum-fed pigs. In contrast, another recent study using conventional pigs failed to
demonstrate an immune-stimulation-dependent difference in pigs inoculated with PCV2 and stimulated with KLH-ICFA.\textsuperscript{20} These experimental models suggest that immune stimulation may in some cases increase the incidence and severity of PMWS in PCV2-infected pigs.

Field trials further supported this hypothesis. Vaccination of piglets with a \textit{M. hyopneumoniae} bacterin at 1 and 4 weeks of age resulted in a significant increase in losses associated with PMWS in the vaccinated pigs in two of five batches.\textsuperscript{7} Kyriakis et al.\textsuperscript{19} vaccinated a portion of the pigs in a herd experiencing a PMWS outbreak with a \textit{M. hyopneumoniae} vaccine and observed PMWS in 43\% of the vaccinated pigs compared with 11\% of the non-vaccinated pigs.

The objective of this study was to determine whether vaccination with bacterins (APP and \textit{M. hyopneumoniae}) commonly used in the USA enhances the incidence and severity of PCV2-associated PMWS lesions in conventional pigs.

**Materials and Methods**

**Animal source**

Sixty-one pigs were weaned at 12-14 days of age and brought to the livestock infectious disease isolation facility at Iowa State University. All pigs were tested by enzyme linked immunosorbent assay (ELISA) and confirmed to be negative for antibodies to PCV2.\textsuperscript{28} The pigs were assigned randomly to rooms and pens and were housed in two rooms, with four pens in each room.

**Experiment design**

The experimental design is shown in Table 1. Pigs were 7-10 weeks of age when inoculated with PCV2. For the sham-inoculation, the pigs in groups 1 and 2 received a similar volume of a 0.9\% sodium chloride solution (Lot 65-181-DK; Abbot Laboratories, North Chicago, Illinois). Half of the pigs were necropsied at 22 days post-inoculation (DPI) and the remaining pigs at 34 DPI.

**Vaccines**

A \textit{Mycoplasma hyopneumoniae} (\textit{M. hyopneumoniae}) bacterin (M+PAC\textsuperscript{®}, Schering-Plough Animal Health, Omaha, Nebraska; Lot EX-0061-1108-01) and an APP bacterin (Serotypes 1, 5 and 7, PNEU PAC\textsuperscript{®}, Schering-Plough Animal Health, Lot NDC-0061-0260-
were used for immune stimulation. The APP vaccine was adjuvanted with emulsified paraffin and the *M. hyopneumoniae* bacterin contained Emunade®, a combination of aluminium hydroxide with oil-in-water dual-action adjuvant. The first dose of each vaccine was given 21 days before and the second dose was given 1 day before PCV2 inoculation. Each pig was vaccinated intramuscularly in the right neck with 2 ml of the APP and in the left neck with 1 ml of the *M. hyopneumoniae* bacterins according to the manufacturer’s directions.

**Virus inoculum**

The PCV2 isolate used in this study, field isolate ISU-40895, was originally obtained from a pig with PMWS in a herd in western Iowa with mild-to-moderate losses associated with PMWS. The virus preparation was performed on PK-15 cells free of PCV1 and PCV2, as previously described. Briefly, PK-15 cells were cultivated in T-25 culture flasks and transfected with the PCV2-infectious DNA clone. The transfected cells were harvested at 3 days posttransfection by freezing and thawing at -80°C three times. The infectious titer of the inoculum was determined by immunofluorescence assay as described previously. The virus inoculum was stored at -80°C until use. Each pig in groups 3 and 4 received 8 ml of isolate ISU-40895 with a titer of about 10³⁵ tissue culture infective dose intralymphoid. One half of the inoculum was injected in the right and the other half in the left superficial inguinal lymph node.

**Clinical evaluation**

The pigs were evaluated daily for clinical signs, including respiratory disease (score ranging from 0 [normal breathing] to 6 [severe dyspnea and abdominal breathing]). Nasal discharges and coughing were recorded by observers along with other changes such as lethargy. Rectal temperatures were recorded daily. The pigs were weighed at 20 and 3 days before inoculation and at 22 and 34 DPI.

**Serology**

Serum samples were taken before inoculation and at 7, 13, 21, 28, and 33 DPI. A PCV2-ELISA based on the recombinant open reading frame 2-capsid protein of PCV2 was performed on serum samples of all pigs as described previously. Samples with a sample-to-positive (S : P) ratio ≥ 0.2 were considered to be positive for antibodies to PCV2.
In addition, serum of all pigs was tested for the presence of specific antibodies to porcine parvovirus by hemagglutination inhibition (HI), and for the presence of PRRSV-specific antibodies by PRRSV ELISA (IDEXX Laboratories, Inc. Westbrook, MA) on 33 DPI.

**Gross pathology and histopathology**

At necropsy, macroscopic lung lesions (0-100% of the lung affected) and size of lymph nodes (score ranging from 0 [normal] to 3 [three times the normal size]) were determined. Bronchoalveolar lavage (BAL) for bacterial and virological examinations was collected as described previously. Tissue samples from lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), lung, tonsil, thymus, ileum, kidney, colon, skin, spleen, liver, stomach, bone marrow, and ribs were collected during necropsy and fixed in 10% formalin, and routinely processed for histologic examination. Microscopic lesions were evaluated blindly by a veterinary pathologist.

**Immunohistochemistry**

Immunohistochemistry (IHC) for detection of PCV2-specific antigen was performed on sections of paraffin blocks of selected tissue samples (lymph nodes, including superficial inguinal, mediastinal, tracheobronchial, and mesenteric, tonsil, and spleen) using a rabbit polyclonal antiserum as described previously. The amount and distribution of PCV2 antigen was compared by evaluation of IHC signals of each tissue section ranging from 0 (= no signal) to 3 (= strong positive signals) in a blinded fashion. The mean group score was determined for each lymphoid tissue and compared between groups.

**Polymerase chain reaction**

To confirm the presence of PCV2 infection and for the assessment of the length of PCV2 viremia, polymerase chain reaction (PCR) was performed on serum samples collected at different DPIs. Deoxyribonucleic acid (DNA) from weekly serum samples was extracted using a commercial DNA isolation kit (QIAamp® DNA Blood Mini Kit, Qiagen, Valencia, California). PCR was performed as described previously. As a positive control for DNA extraction and PCR, PCV2 isolate ISU-98-15237 was used.
Quantitative PCR

Sequence-specific oligonucleotide primers and a fluorescent probe for detection and relative quantification of PCV2 genomic DNA were designed using computer software (ABI Prism Primer Express, Version 1.5, PE Applied Biosystems, Foster City, California) according to the manufacturer’s suggestions and were engineered to be within a highly conserved region of the viral genome (Table 2). The PCR reaction consisted of 50 µl PCR mixtures that contained 25 µl of the commercially available master mix (TaqMan Universal PCR Master Mix, PE Applied Biosystems), 5 µl DNA extract from each pig serum, forward and reverse primers, and detection probe with concentrations of 400, 400, and 200 nM, respectively. All reactions were carried out in triplicate. In addition, each reaction included three replicates for each of the five progressive 1:10 dilutions of a known copy number of PCV2 genomic DNA excised from a purified PCV2 DNA clone that served to generate a standard curve. Each plate was run in the sequence detection system (GeneAmp 5700 Sequence Detection System, Applied Biosystems) under company-specified conditions that were optimal (2 minutes at 50 °C, 10 minutes at 95 °C followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C) for the Taq Master Mix used.

Bone marrow analysis

On both necropsy days, 22 and 34 DPI, bone marrow specimens were collected from the distal femur and were placed immediately into an ethylenediaminetetraacetic acid tube containing 0.5 ml sterile normal saline and 0.1 ml albumin and mixed to disperse cells. Two to three slides per animal were then prepared by cytopsin preparation, and three 500 cell-count differentials were performed on each animal.

Statistic analyses

Chi-square test was used to analyze the clinical scores. Daily rectal temperatures were analyzed with multiple analysis of covariance (MANCOVA), which accounts for the repeated measures on each pig and for baseline temperature. For the pigs that were necropsied at 22 DPI, all the data were analyzed from 0 to 21 DPI. For the remaining pigs, the data were analyzed from 0 to 31 DPI. If the time-by-group interaction was not significant, then the group effect was assessed. Otherwise, the data were analyzed cross-sectionally to determine at which time points the group means are different. MANCOVA also was chosen...
for analyzing the weight data, which accounted for the repeated measurements of each pig and for baseline weight at 20 days before inoculation. Analysis of ELISA results was done by using cross-sectional analysis (analysis of variance [ANOVA]), and differences between groups were evaluated with Tukey’s test. Non-repeated measures of necropsy and histopathology data were assessed using analysis of covariance (ANCOVA) or nonparametric ANOVA. If a nonparametric ANCOVA test was significant ($P < 0.5$), then Wilcoxon tests (with the Bonferroni correction) were used to assess the differences of pairs of groups. Fisher’s exact test was used to compare the incidence between groups.

Statistical analysis for real-time PCR was performed using the means of three replicate wells for each sample as compared to a standard curve generated with a purified known genome copy number of PCV2 genomic DNA ranging from $10^3$ to $10^7$ genomic copies per PCR plate well. Serial dilutions (1 : 10) between each standard resulted in a 5-point standard curve with a correlation of $>0.992$. The threshold cycle (threshold cycle $C_T$, is the cycle number at which a statistically significant increase in amplification above the threshold line [0.1 in all cases] is first detected) of triplicate unknowns were compared to $C_{T_s}$ of triplicate standards on a standard curve to determine the absolute number of viral genomes per ml of pig serum.

**Results**

**Clinical signs**

Pigs in the sham-inoculated control groups 1 and 2 showed no clinical signs of disease throughout the study. Pigs in PCV2-infected groups 3 and 4 exhibited intermittent diarrhea and mild respiratory disease from 13 DPI until the end of the study at 34 DPI. The respiratory disease was characterized by sneezing, clear nasal discharge, and occasionally coughing. Statistical analysis of the clinical scores (chi-square test) revealed that the clinical respiratory disease scores in pigs of groups 3 and 4 were statistically more severe than those of groups 1 and 2 pigs ($P < 0.0001$). There was no difference in clinical scores between pigs of groups 3 and 4 ($P = 0.6$). Statistical analysis of the daily rectal temperatures (0 to 33 DPI) showed neither a significant group-by-time interaction ($P = 0.14$) nor a significant group effect ($P = 0.08$). Statistical analysis of the weight data showed no statistically significant
difference in time-by-group effect ($P = 0.8$) or group effect ($P = 0.6$). At 34 DPI, the average weights appeared to differ (49.1 kg $\pm 5.4$ in group 1 pigs, 47.9 kg $\pm 4.2$ in group 2 pigs, 48.6 kg $\pm 7.5$ in group 3 pigs, and 45.6 kg $\pm 4.7$ in group 4 pigs) but there was substantial variability within each group. The average daily weight gain was 0.70 kg ($\pm 0.05$) for group 1 pigs, 0.67 kg ($\pm 0.05$) for group 2 pigs, 0.70 kg ($\pm 0.08$) for group 3 pigs, and 0.63 kg ($\pm 0.09$) for group 4 pigs.

**Gross lesions**

In sham-inoculated control groups 1 and 2, only 4 of the 30 pigs had visible gross lesions. The four pigs with lesions were necropsied at 34 DPI and had lymph node enlargement of approximately two times the normal size. There were significantly more pigs in groups 3 and 4 ($P < 0.05$) with enlarged lymph nodes (Table 3) compared with groups 1 and 2. In group 3, 12 of 14 pigs had enlargement of all lymph nodes from two to three times the normal size. In group 4, 15 of 17 pigs had lymph node enlargement of two to three times the normal size. The macroscopic evaluation of the lymph nodes sizes showed no statistical difference between groups 3 and 4 at the first ($P = 0.33$) or second ($P = 0.3$) necropsy.

**Microscopic lesions and IHC**

There were no microscopic lesions in the lymph nodes of pigs in groups 1 and 2. In groups 3 and 4, microscopic lesions characterized by lymphoid depletion of follicles and granulomatous lymphadenitis were observed in nearly all pigs: 12 of 14 in group 3 pigs, and 16 of 17 in group 4 pigs. At 34 DPI, there was a significant difference ($P = 0.02$) in severity of lymphoid depletion between groups 3 (mean = 0.86) and 4 (mean = 1.56). Mild to moderate lymphoplasmacytic interstitial nephritis was observed in 1 of 15 pigs in group 1 pigs, in 4 of 14 group 3 pigs, and in 5 of 17 group 4 pigs. Mild lymphohistiocytic hepatitis was observed in 7 of 14 pigs in group 3, and in 11 of 17 pigs in group 4. Mild to moderate lymphohistiocytic enteritis was observed in 6 of 14 pigs in group 3, and in 7 of 17 pigs in group 4 (Table 3).

PCV2 antigen was detected by IHC in a variety of tissues (lymph nodes, spleen, and tonsil) in groups 3 and 4. At 22 DPI, PCV2 antigen was detected in all pigs examined. There was no difference between the amount and distribution of PCV2 antigen between groups 3 and 4 at 22 DPI. At 34 DPI, PCV2 antigen was only detected in the lymph nodes in 4 of 7
group 3 pigs. In contrast, at DPI 34, PCV2 antigen was still detectable in the lymph nodes of all group 4 pigs (9 of 9) and the amount of PCV2 antigen in the lymph nodes was significantly ($P < 0.05$) higher in group 4 than in group 3 pigs at 34 DPI (Table 4).  

**Antibody response**

Pigs in groups 1 and 2 remained seronegative for PCV2 antibody throughout the study. Pigs in groups 3 and 4 seroconverted to PCV2 between 21 and 28 DPI. Cross-sectional analysis (using ANOVA) indicated that there were statistically different group means ($P < 0.0001$) at 21, 28, and 33 DPI. A Tukey’s test showed that for each day neither were groups 1 and 2 statistically different from each other nor were groups 3 and 4 different from each other. All serum samples tested were negative for presence of PRRSV- and PPV-specific antibodies at 33 DPI.

**PCV2 viremia**

In groups 1 and 2, all serum samples collected at different DPIs were negative for PCV2 DNA by PCR. Viremia in groups 3 and 4 pigs was detected at as early as 7 DPI in 24 of 31 pigs. All pigs in groups 3 and 4 were viremic at 13 DPI. By DPI 21, 11 of 14 pigs in group 3 and 16 of 17 pigs in group 4 remained positive with PCV2 DNA. By 28 DPI, 1 of 7 pigs in group 3 was positive for PCV2 DNA in sera, while 7 of 9 pigs in group 4 were positive. At 33 DPI, the day before necropsy, 7 of 9 pigs in group 4 were still positive for PCV2 DNA, whereas none of the pigs (0 of 7) in group 3 were positive. The PCR data were analyzed using nonparametric ANOVA as an omnibus test and Wilcoxon pairwise post hoc tests. The overall test was significant ($P < 0.001$), and post hoc tests indicated that groups 3 and 4 were different ($P = 0.03$) from each other and from groups 1 and 2 ($P < 0.0001$). To determine the length of viremia, only the animals that were alive at the end of study were analyzed. Pigs necropsied at 22 DPI were not included. The mean viremia length observed in group 3 pigs ($n = 7$) was 2.14 (±0.26) weeks, compared with 4.44 (±0.23) weeks in group 4 pigs ($n = 9$). The length of viremia in group 4 pigs was determined by the end of the study at 34 DPI. At this time point, 7 of 9 group 4 pigs were still viremic, whereas none of the nonvaccinated group 3 pigs were viremic. The difference in viremia length between groups 3 and 4 was highly significant ($P = 0.0003$).
**PCV2 genome titer in sera**

Analysis of the amount of PCV2 genomic DNA per milliliter of serum revealed major differences between groups 3 and 4 pigs. The mean copy number of PCV2 genomic DNA measured in nonvaccinated group 3 pigs was $1.3 \times 10^5$ genomic copies per milliliter serum at 7 DPI. On the following testing dates (13-33 DPI), the PCV2 genomic titer decreased in all pigs to $6.1 \times 10^3$ PCV2-copies per milliliter serum at 33 DPI. In contrast, the mean copy number of PCV2 genome in sera measured in vaccinated group 4 pigs was $2.7 \times 10^7$ copies per milliliter serum at 7 DPI. Furthermore, in the majority of group 4 pigs (7 of 9), the PCV2 genomic titer remained at a relatively high level (mean copy number per milliliter serum between $2.3 \times 10^6$ at 14 DPI and $1.7 \times 10^6$ at 33 DPI) compared with nonvaccinated group 3 pigs during the entire experiment. Instead of decreasing, PCV2 genomic titer continued to increase over time in some pigs. Figure 1 illustrates the comparison of group mean PCV2 genome titer. Differences in the amount of PCV2 genome copy number between both groups were significant ($P < 0.05$) on 13, 21, 28, and 32 DPI.

**Bacteriology**

There was no growth on cultures of the BAL-fluid in any of the pigs from groups 1 to 4.

**Bone marrow analysis**

All the variables were analyzed by using nonparametric ANOVA. There were no significant differences between the groups. Erythroid and myeloid cell series were present and followed a normal maturation sequence. M : E ratios were within reference intervals for this species. Megakaryocytes were observed in all specimens. One interesting feature was the presence of many eosinophils on marrow cytospin preparations; eosinophils are rarely observed on peripheral complete blood count differentials.

**Discussion**

The objective of this study was to determine whether *M. hyopneumoniae* and APP vaccination enhanced the incidence and severity of PMWS disease and lesions associated with PCV2 infection of conventional pigs. At least three European studies, one using an experimental model and two recent field studies, provide evidence that vaccine-induced
immune stimulation enhances PCV2 replication and increases the incidence of clinical PMWS. The results of these European studies are not ideally suited for application to the US field situation, where *M. hyopneumoniae* vaccines are typically used on older pigs. In the USA, PCV2-associated PMWS is most commonly seen in pigs aged 8-20 weeks,\(^{13,15}\) whereas in Europe it is observed in pigs aged 3-10 weeks.\(^1\) *M. hyopneumoniae* vaccination in the USA is typically done in nursery-age pigs from 3 to 10 weeks, whereas in Europe it is more commonly done on suckling pigs or on pigs in early nursery phase at 1-4 weeks of age. We attempted to mimic the immune stimulation approach of the European studies by vaccinating pigs with *M. hyopneumoniae* and APP; however, we vaccinated at 5 and 7 weeks of age using segregated early-weaned (12-14 days) pigs, consistent with current US production systems.

Diagnostic criteria of PMWS include clinical evidence of wasting, histological evidence of lymphoid depletion or granulomatous lymphadenitis, and the presence of PCV2 antigen or nucleic acids associated with the characteristic lymphoid lesions.\(^{31}\) PCV2-associated microscopic lesions consistent with PMWS were observed in both PCV2-inoculated groups in this study; however, we failed to reproduce clinical manifestation of PMWS. This might be due to removal of other key stressors not present in the clean experimental environment. Unlike most field situations, our pigs had no known concurrent viral or bacterial coinfections, which have been shown to increase the incidence and severity of PMWS in PCV2-infected pigs.\(^{2,4,14,16,18,30}\) The PCV2 inoculum was a pure and homogeneous infectious stock derived from an infectious DNA clone of PCV2 isolate ISU-40895, which was originally isolated from a pig with PMWS.\(^{9,10}\) Therefore, the disease and lesions reproduced in this study perhaps are more definitive than those using tissue homogenate or cell culture-propagated PCV2 inoculum, which might contain other common swine agents. The differences in environment and coinfection may at least partly explain the differences in results of different experiment models and differences in severity of PCV2-associated disease from farm to farm.

Pigs in the vaccinated and non-vaccinated PCV2-infected groups developed microscopic lesions consistent with PMWS. This observation in the nonvaccinated group 3 is in contrast to many PCV2 experimental models, where no or mild microscopic lesions typical
of PCV2-associated disease were observed.⁴,¹⁷,¹⁸,²⁹,³⁰ There are only a few reports to date which describe reproduction of severe microscopic lesions characteristic of PMWS with singular PCV2 inoculation.⁸,²⁰,²¹ Differences in experimental results could be explained by PCV2 strain variation, individual pig susceptibility differences, differences in age of inoculation, or type of animal housing. Typically, more severe lesions were observed in young gnotobiatic pigs,¹⁷,¹⁸ cesarean-derived-colostrum-deprived pigs,⁸,¹⁴ and colostrum-deprived pigs,²,⁴,¹⁶ compared with “conventional pigs".³⁰ The definition of conventional pigs also may vary. In our case, these are genetics typical of the current US swine industry; pigs free of several major pathogens (PRRSV, SIV, PCV2, APP, M. hyopneumoniae) and early weaned to a distant site where they are reared in all-in-all-out fashion.

The length of PCV2 viremia in the unvaccinated group 3 pigs (2.14 ± 0.26 weeks) was consistent with observations of other research groups.¹⁰,²⁹ PCV2-associated lymphoid lesions peaked around 22 DPI and were minimal or absent in the nonvaccinated pigs at the end of the study at 34 DPI. In contrast, pigs in the vaccinated group 4 had significantly longer length of PCV2 viremia (4.44 ± 0.23 weeks) and a longer duration of PCV2 antigen detection in various lymphoid tissues. Quantitative PCR results further confirmed the significantly ($P < 0.05$) higher amount of PCV2 genome titers in sera of vaccinated pigs compared with that of nonvaccinated pigs. However, this did not influence the clinical appearance of the pigs in this study. Only mild respiratory disease and mild diarrhea were observed. Nevertheless, the increased length of viremia may increase the window of opportunity for coinfecting agents or other cofactors to trigger progression of PCV2 infection to PMWS.

Most modern pig production systems utilize some types of vaccines, particularly in 2- to 12-weeks-old pigs. It has been speculated that both the use of vaccines and the type of vaccine may play an important role in the manifestation of clinical PMWS.⁶,¹⁹ In addition to the type of vaccine used, the timing of vaccination may also be important. This difference might be reflected in this current study, where none of the pigs vaccinated between 5 and 10 weeks of age developed clinical PMWS, whereas in a previous study⁶ with an earlier onset of vaccination (2 weeks of age), PMWS was developed in 21% of the pigs. Early vaccination, high virus prevalence in the environment, and low maternal antibody status may lead to
increased incidence and severity of PMWS typical of what is occurring in the UK, where the postweaning mortalities have increased by 5-40% in many affected herds.27

PMWS is a disease complex influenced by many factors. One of these factors may be commonly used vaccines. However, on a herd basis, the risk of elimination of effective vaccines may be greater than the risk of inducing PMWS in a low percentage of the population. Further work is warranted to determine whether there is an optimal time for vaccination and if certain types of adjuvants or antigens play a role in vaccine-induced enhancement of PMWS. Swine producers may need to consider changing vaccine products or regimens to minimize PCV2 replication and PCV2-associated diseases in affected herds.

Acknowledgements

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References


27 Muirhead M: Sources of information on PMWS/PDNS. Vet Rec 150:456, 2002


Table 1. Experimental design.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pigs</th>
<th>Vaccination*</th>
<th>Inoculation with PCV2</th>
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<tr>
<td></td>
<td></td>
<td>-21 DPI†</td>
<td>-1 DPI</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Vaccination with *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* bacterins.

†Days post inoculation.
Table 2. Sequences of the primer and TaqMan fluorescent probe used in fluorogenic real-time PCR for absolute quantification of PCV2 genomic DNA in serum samples. 6FAM = fluorescent reporter dye. TAMRA = fluorescent quencher dye.

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
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<td>P1570 (forward primer)</td>
<td>5’-TGGCCCGCAGTATTCTGATT-3’</td>
</tr>
<tr>
<td>P1642 (reverse primer)</td>
<td>5’-CAGCTGGGACAGCAGTTGAG-3’</td>
</tr>
<tr>
<td>P1591 (TaqMan probe)</td>
<td>5’-6FAM-CCAGCAATCAGACCCCCGTTGGAATG-TAMRA-3’</td>
</tr>
<tr>
<td>Group No.</td>
<td>Gross Lymphadenopathy (0-3)†</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Sham inoculated</td>
<td></td>
</tr>
<tr>
<td>1 (Nonvaccinated)</td>
<td>0/7 (0.0[0])§ ‖ 3/8 (2.0[2])A</td>
</tr>
<tr>
<td>2 (Vaccinated)</td>
<td>0/7 (0.0[0])A</td>
</tr>
<tr>
<td>PCV2 inoculated</td>
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<td>3 (Nonvaccinated)</td>
<td>6/7 (2.0[1-3])B</td>
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<tr>
<td>4 (Vaccinated)</td>
<td>6/8 (2.5[2-3])B</td>
</tr>
</tbody>
</table>

*Days postinoculation

†Score range (0 = normal; 1 = mild; 2 = moderate; 3 = severe).

‡Interstitial pneumonia score (0 = normal; 1 = mild multifocal; 2 = mild diffuse; 3 = moderate multifocal; 4 = moderate diffuse; 5 = severe multifocal; 6 = severe diffuse interstitial pneumonia).

§Incidence (mean severity for affected animals [range of severity for affected animals]).

‖Groups 1-4 were evaluated against each other in each column. Different superscripts (A-C) within each column indicate significantly ($P < 0.05$) different values in the overall mean group scores.
Table 3. (Continued).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Lymphohistiocytic Hepatitis</th>
<th>Lymphohistiocytic Enteritis</th>
<th>Interstitial Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 DPI (0-3)†</td>
<td>22 DPI (0-3)†</td>
<td>22 DPI (0-6)‡</td>
</tr>
<tr>
<td>Sham inoculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Nonvaccinated)</td>
<td>0/7 (0.0[0])A</td>
<td>0/7 (0.0[0])A</td>
<td>5/7 (1.0[1])A</td>
</tr>
<tr>
<td>2 (Vaccinated)</td>
<td>0/7 (0.0[0])A</td>
<td>0/7 (0.0[0])A</td>
<td>6/7 (1.2[1-2])A</td>
</tr>
<tr>
<td>PCV2 inoculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (Nonvaccinated)</td>
<td>3/7 (1.0[1])B</td>
<td>4/7 (1.3[1-2])B</td>
<td>6/7 (1.0[1])B</td>
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<tr>
<td>4 (Vaccinated)</td>
<td>4/8 (1.0[1])B</td>
<td>7/9 (1.3[1-2])B</td>
<td>7/9 (1.0[1])B</td>
</tr>
</tbody>
</table>

*Days postinoculation

†Score range (0 = normal; 1 = mild; 2 = moderate; 3 = severe).

‡Interstitial pneumonia score (0 = normal; 1 = mild multifocal; 2 = mild diffuse; 3 = moderate multifocal; 4 = moderate diffuse; 5 = severe multifocal; 6 = severe diffuse interstitial pneumonia).

§Incidence (mean severity for affected animals [range of severity for affected animals]).

¶Groups 1-4 were evaluated against each other in each column. Different superscripts (A-C) within each column indicate significantly ($P < 0.05$) different values in the overall mean group scores.
Table 4. Comparison of estimated amount of PCV2 antigen (ranging from 0 = not detectable to 3 = strong signal) in lymphoid tissues between groups 3 (PCV2 infected) and 4 pigs (PCV2 infected and vaccinated).

<table>
<thead>
<tr>
<th></th>
<th>Lymph nodes</th>
<th>Tonsil</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 DPI</td>
<td>34 DPI</td>
<td>22 DPI</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>7/7^A ‡ (1.7[1-3])^C †</td>
<td>4/7^A (1.0[1])^C</td>
<td>5/7^A (1.2[1-2])^C</td>
</tr>
<tr>
<td>Tonsil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>7/8^A (1.1[1-2])^C</td>
<td>9/9^A (1.4[1-2])^D</td>
<td>4/8^A (1.0[1])^C</td>
</tr>
</tbody>
</table>

*Days postinoculation.

†Incidence (mean estimated amount for affected animals [estimated range]).

‡Different superscripts within each column indicate significantly ($P < 0.05$) different values in incidence (A and B), or significantly ($P < 0.05$) different values in estimated overall mean group amount of PCV2 antigen (C and D).
Fig. 1. PCV2 genomic titers in sera measured by quantitative real-time PCR. The values are the mean group results for group 3 (nonvaccinated, \( n = 7 \)) and group 4 (vaccinated, \( n = 9 \)) pigs. Pigs that were necropsied at 22 DPI are not included. Error bars represent standard errors.
CHAPTER 5. EFFECTS OF TIMING OF THE ADMINISTRATION OF MYCOPLASMA HYOPNEUMONIAE BACTERIN ON THE DEVELOPMENT OF LESIONS ASSOCIATED WITH PORCINE CIRCOVIRUS TYPE 2

A paper published in the
Veterinary Record 158:149-154, 2006


Abstract

To determine whether there is an effect of the timing of vaccination on porcine circovirus type 2 (PCV-2) replication and PCV-2-associated lesions, 78 pigs were randomly assigned to eight groups: group 1 (10 pigs) was vaccinated with a commercial Mycoplasma hyopneumoniae vaccine at two and four weeks of age, group 2 (nine pigs) was vaccinated at four and six weeks of age, group 3 (10 pigs) at six and eight weeks of age and group 4 (10 pigs) at eight and 10 weeks of age; group 5 (nine pigs) was vaccinated once with a double dose at four weeks of age, and group 6 (10 pigs) was vaccinated once with a double dose at eight weeks of age. Groups 7 and 8, both of 10 pigs, were not vaccinated. At eight weeks of age, pigs in group 1 to 7 were inoculated with PCV-2. Fourteen days after they had been inoculated, the pigs in groups 1, 4 and 5 had significantly (P<0.05) more copies of the PCV-2 genome in their serum than the unvaccinated pigs. Microscopically, 14 of the 68 inoculated pigs had normal lymphoid tissues, 40 had mild PCV-2-associated lymphoid lesions and 14 had moderate lesions. The mean overall lymphoid lesions (lymphoid depletion, granulomatous inflammation, and quantity of PCV-2 antigen in spleen, tonsil, and five lymph nodes) were significantly (P<0.05) more severe in groups 4 and 5 than in groups 2, 3, 7 and 8.
Introduction

Porcine circovirus type 2 (PCV-2) was first described in association with postweaning multisystemic wasting syndrome (PMWS) in pigs in the mid-1990s (Harding and Clark 1997). The syndrome is characterised clinically by enlargement of the lymph nodes, chronic pneumonia and progressive weight loss, and less often by icterus, pallor or diarrhoea. The characteristic microscopic lesions include lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues; mild to severe granulomatous inflammation in lymphoid and other tissues is also commonly observed (Allan and Ellis 2000, Sorden 2000). The virus is now associated with several other disease manifestations in pigs, including reproductive failure (abortion), myocarditis, enteritis, and as a component of the porcine respiratory disease complex (PRDC) (Allan and Ellis 2000, Harms and others 2002, Opriessnig and others 2004b).

Immune stimulation by commonly used vaccines has been shown to enhance the replication of PCV-2 and increase the severity of PCV-2-associated lesions both experimentally (Allan and others 2000b) and in the field (Allan and others 2001, Kyriakis and others 2002). In a recent study, it was shown that the enhanced replication of PCV-2 manifests as significantly longer periods of PCV-2 viraemia, more copies of the PCV-2 genome in serum, a wider tissue distribution of PCV-2 antigen, and more severe lymphoid depletion in segregated early weaned pigs vaccinated with commercial Actinobacillus pleuropneumoniae and Mycoplasma hyopneumoniae vaccines than in unvaccinated, segregated early weaned pigs inoculated with PCV-2 (Opriessnig and others 2003).

The objective of this study was to investigate whether the timing of M hyopneumoniae vaccination in relation to infection with PCV-2 has an effect on the replication of the virus and the severity of the associated lesions.

Material and Methods

On the day they were weaned, 78 segregated early weaned, 10-to 12-day-old, crossbred pigs were randomly assigned to rooms and pens and housed in four equally lighted and power-ventilated rooms with two to eight pens in each room and four to five pigs in each pen.
Vaccination

The pigs were divided into eight groups (Table 1), six of which were vaccinated with M+PAC® (Schering-Plough Animal Health), a commercially available *M hyopneumoniae* bacterin. The vaccine is adjuvanted with Emunade (Schering-Plough Animal Health), a combination of aluminum hydroxide with an oil-in-water, dual-action adjuvant. According to the manufacturer’s directions, each pig in groups 1 to 4 was vaccinated intramuscularly in the right side of the neck with 1 ml of the bacterin and boosted two weeks later with a second 1 ml dose; the pigs in groups 5 and 6 each received a single injection in the form of 2 ml of the bacterin according to label directions for the single-dose regimen, and the pigs in groups 7 and 8 were not vaccinated. The pigs in each group were vaccinated at different intervals before all of them were inoculated with PCV-2 when they were eight weeks old (Table 1).

Inoculum

The PCV-2 inoculum was prepared on PK-15 cells free of porcine circovirus type 1 (PCV-1) and PCV-2 as described by Fenaux and others (2002). Before it was used, the inoculum was tested and shown to be negative for porcine parvovirus and PCV-1 by PCR. A dose of $10^{5.1}$ 50 per cent tissue culture infective doses (TCID$_{50}$) of PCV-2 field isolate ISU-40895 (Fenaux and others 2000), passage 6, in a 5 ml volume was administered to each pig intranasally by slowly dripping 2.5 ml of the inoculum into each nostril.

Clinical evaluation

The pigs were scored daily for clinical signs including sneezing (score range 0 No sneezing to 3 Severe sneezing), coughing (score range from 0 No coughing to 3 Severe coughing), and increased respiratory rate (score range from 0 Normal breathing to 6 Severe dyspnoea and abdominal breathing) as described by Halbur and others (1995) and Opriessnig and others (2003). Other clinical signs such as nasal discharge, lethargy, jaundice and rectal temperatures were recorded daily. The pigs were weighed at 42, 28, and 16 before they were inoculated, on the day they were inoculated, and seven, 14 and 42 days after they were inoculated.

Serology

Samples of blood were collected at weekly intervals, starting when the pigs were 10 to 12 days of age. The sera were tested for PCV-2-specific antibodies by ELISA (Nawagitgul
and others 2002); they were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or more.

In addition, serum samples collected from the pigs at 42 days after they were inoculated were tested for the presence of antibodies to porcine parovirus by haemagglutination inhibition (Mengeling and others 1988), and by ELISA for the presence of antibodies specific to porcine reproductive and respiratory syndrome virus (PRRSV) (IDEXX Laboratories) and *M hyopneumoniae* (DAKO).

**Detection and quantification of PCV-2 genomic DNA**

Real-time PCR was used to detect and measure PCV-2 genomic DNA in serum samples collected seven, 14, 21, 28, 35 and 42 days after the pigs were inoculated (Opriessnig and others 2003). The DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen).

**Gross pathology and histopathology**

All pigs were euthanased with an overdose of phenobarbital (Beuthanasia-D; Schering Plough Animal Health) at 14 weeks of age, 42 days after they had been inoculated. The gross lesions in the lungs and lymph nodes were scored at postmortem examination as described by Halbur and others (1995) and Opriessnig and others (2003). Samples of the lymph nodes, spleen, kidney, liver, tonsil, lung, thymus, heart, colon and ileum were collected in 10 per cent neutral-buffered formalin and processed for microscopic evaluation. The microscopic examination and scoring was performed in a blinded fashion. For the lungs the scoring system ranged from 0 for normal to 6 for severe diffuse for interstitial pneumonia; for the other organs the scores ranged from 0 for normal to 3 for severe lesions (Halbur and others 1995). Lymphoid tissues such as lymph nodes, tonsil, and spleen were evaluated for lymphoid depletion with scores ranging from 0 for normal to 3 for severe, and for the loss of lymphoid follicle structure and presence of inflammation with scores ranging from 0 for normal to 3 for severe histiocytic to granulomatous inflammation with replacement of follicles (Opriessnig and others 2004b).

**Immunohistochemistry**

Immunohistochemistry was used to detect PCV-2 antigen in lymph nodes, thymus, spleen, and tonsil as described by Sorden and others (1999). The amount and distribution of
PCV-2 antigen was scored blind, 0 for undetectable, 1 when less than 10 per cent of the lymphoid follicles contained cells with PCV-2 antigen staining, 2 for 10 to 50 per cent of the lymphoid follicles contained cells with PCV-2 antigen staining, and 3 when more than 50 per cent of the lymphoid follicles contain cells with PCV-2 antigen staining) in a blinded fashion (Opriessnig and others 2004b).

**Mean score for lymphoid lesions**

To evaluate the PCV-2-associated lesions for individual pigs, a combined scoring system was used for each lymphoid tissue that ranged from 0 to 9, with a lymphoid depletion score from 0 to 3, granulomatous inflammation score from 0 to 3 and an immunohistochemistry score from 0 to 3 (Opriessnig and others 2004b). The scores for the seven lymphoid tissues (five lymph nodes, spleen and tonsil) were added together and divided by 7. The lymph node pool consisted of tracheobronchial, superficial inguinal, external iliac, mediastinal and mesenteric lymph nodes. The mean lymphoid score of each treatment group was calculated and compared with the scores of the other groups. The pigs were grouped into four categories on the basis on the basis of their overall lymphoid lesion scores: I (normal; score 0), II (mild; score 1 to 3), III (moderate; score 4 to 6) and IV (severe; score 7 to 9). A pig was diagnosed with PMWS if it had a lower rate of weight gain or had lost weight, and if its mean lymphoid lesion score was in category IV.

**Bacteriology**

Selected lymphoid tissues were cultured for bacteria by routine methods, and selected formalin-fixed, paraffin embedded lymph nodes were stained for acid-fast bacteria.

**Statistical analysis**

Summary statistics were calculated for each group cross-sectionally to assess the overall quality of the data. The clinical scores were analyzed by response feature analysis followed by non-parametric Kruskal-Wallis analysis of variance (ANOVA) with a rejection level for the null hypothesis of P<0.05. If this non-parametric ANOVA test was significant (P<0.05), then Wilcoxon tests were used to assess the differences between pairs of groups. The repeated measurements of rectal temperature were analyzed by a multivariate ANOVA, and the continuous non-repeated measurements among the different treatment groups were also assessed using ANOVA. Non-repeated measurements from postmortem and the
histopathology data were assessed by non-parametric Kruskal-Wallis ANOVA followed by Wilcoxon tests. Differences in incidence were evaluated by Fisher’s exact test.

**Results**

**Clinical disease**

Some of the inoculated pigs in each of groups 1 to 7 showed signs of mild respiratory disease, characterised by sporadic sneezing, a clear nasal discharge, and mild dyspnoea, between six and 22 days after they had been inoculated; some of them had high temperatures between eight and 34 days, were lethargic and had a rough haircoat. There were no significant differences in the mean rectal temperatures or the mean respiratory scores between the groups. There were no significant differences between the average daily weight gains of the groups of pigs up to six weeks after they had been inoculated.

**Antibody response**

All pigs were negative for PCV-2-specific antibodies when they were inoculated at eight weeks of age, and the uninfected pigs in group 8 remained seronegative for PCV-2 antibodies throughout the study. All the pigs in groups 1 to 7 seroconverted to PCV-2 between 14 and 28 days after inoculation, and six weeks after inoculation there were no significant differences between the PCV-2 S/P ratios of the groups. Six weeks after they were inoculated, *M hyopneumoniae*-specific antibodies were detected in six of the 10 pigs in group 1, six of the nine pigs in group 2, nine of the 10 pigs in group 3, nine of the 10 pigs in group 4, four of the nine pigs in group 5, and eight of the 10 pigs in group 6. None of the unvaccinated pigs in groups 7 and 8 seroconverted to *M hyopneumoniae*. The average *M hyopneumoniae* S/P ratios were significantly (P<0.05) lower in groups 1 and 5 than in groups 2, 3, 4 and 6 pigs. Six weeks after the pigs had been inoculated, all serum samples were tested negative for PRRSV- and porcine parvovirus-specific antibodies.

**PCV2 genomic titers**

The mean numbers of copies of the PCV-2 genome and the mean viraemia recorded in the infected groups are summarized in Table 2. The mean period of viraemia did not differ significantly the groups. Fourteen days after they had been inoculated, there were significantly (P<0.05) more PCV2 copies of the PCV-2 genome in the sera of the pigs in
groups 1, 4 and 5 than in the unvaccinated pigs of group 7 pigs; at the other times there was a
trend towards higher numbers of copies PCV2 in the pigs in groups 1, 4 and 5 but the
increases were not significant.

**Macroscopic and microscopic lesions**

The gross lesions were limited to mildly to moderately enlarged lymph nodes in the
PCV-2-inoculated pigs, but there were no significant differences between the vaccinated or
unvaccinated inoculated pigs and the uninfected control pigs.

The microscopic lesions are summarized in Tables 3 and 4. The lesions in the lymph
nodes were characterised by mild to severe lymphoid depletion with loss of follicle definition
in the affected pigs in groups 1 and 5, and by mild to moderate lymphoid depletion in groups
2, 3, 4, 6 and 7. The overall mean (se) severity of the lymphoid depletion, including the
affected and unaffected pigs within the groups, was significantly (P<0.05) greater in groups 4
(1.5 [0.2]) 5 (1.6 [0.4]), and 6 (1.3 [0.2]) than in groups 7 (0.6 [0.2]) and 8 (0.0 [0.0]). There
was mild to moderate histiocytic to granulomatous infiltration of lymphoid tissues (Figs 1, 2)
and histiocytic replacement of follicles (Fig 3). Small numbers of macrophages and
multinucleated giant cells contained spherical, basophil, intracytoplasmic inclusion bodies. No
bacteria were isolated by routine culture, and acid-fast stains were negative.

There was mild to moderate depletion and mild to moderate histiocytic inflammation
in tonsils and spleen in the vaccinated and inoculated pigs in groups 1 to 6, and mild
lymphoid depletion and mild histiocytic inflammation in the unvaccinated, PCV-2-inoculated
group 7. Among the 68 inoculated pigs, PCV-2 antigen was detected by
immunohistochemistry in the lymph nodes of 32 (47.1 per cent), in the spleens of 12 (17.6
per cent) and in the tonsils of 16 (23.5 per cent).

The overall mean severity of the lymphoid lesions is summarised in Fig 4. Fourteen
of the 68 inoculated pigs (20.6 per cent) had no evidence of lymphoid lesions, 40 (58.8 per
cent) had mild lymphoid lesions, and 14 (20.6 per cent) had moderate PCV2-associated
lymphoid lesions; none of the pigs had the severe lymphoid lesions characteristic of PMWS
when they were examined postmortem, six weeks after they had been inoculated. None of the
unvaccinated, PCV-2-inoculated pigs in group 7 had more than mild lesions (Fig 4). The
group mean (se) lesion scores were significantly (P<0.05) higher in groups 4 (3.1 [0.4]) and 5 (3.3 [0.6]) than in groups 2 (1.7 [0.6]), 3 (1.5 [0.6]), and 7 (1.3 [0.3]) (Table 3).

The lung lesions were characterised by mild to moderate, multifocal peribronchial lymphoid hyperplasia and mild, focal to diffuse alveolar wall thickening by macrophages and lymphocytes. The mean (se) severity of interstitial pneumonia was significantly (P<0.05) higher in groups 1 (0.9 [0.2]), 4 (1.2 [0.1]), 5 (0.9 [0.1]) and 6 (1.2 [0.2]) than in groups 7 (0.3 [0.2]) and 8 (0.3 [0.2]).

Among the 68 PCV-2-infected pigs, 39 (57.4 per cent) had mild to moderate lymphohistiocytic hepatitis, 31 (45.6 per cent) had mild to moderate lymphohistiocytic interstitial nephritis, and 21 (30.9 per cent) had mild to moderate lymphohistiocytic myocarditis. The pigs in groups 5 and 6 had a significantly (P<0.05) higher incidence of lymphohistiocytic interstitial nephritis compared to group 8 pigs (Table 4). The pigs in group 4 pigs had a significantly (P<0.05) higher incidence of lymphohistiocytic myocarditis than the pigs in groups 3, 7 and 8 pigs (Table 4). The mean (se) severity of the lymphohistiocytic myocarditis was significantly (P<0.05) higher in the pigs in groups 4 (0.6 [0.2]), 5 (0.6 [0.2]), and 6 (0.5 [0.2]) than in the pigs in groups 7 (0.0 [0.0]) and 8 (0.0 [0.0]). The pigs in groups 1 and 4 had a significantly (P<0.05) higher incidence of lymphohistiocytic hepatitis than the pigs in group 8 (Table 4). The mean (se) severity of the lymphohistiocytic hepatitis was significantly (P<0.05) higher in groups 4 (0.9 [0.2]) and 5 (0.9 [0.2]) than in groups 7 (0.3 [0.2]) and 8 (0.0 [0.0]). Mild enteritis characterised by lymphohistiocytic infiltration of the lamina propria was observed in a few of the pigs in groups 1 to 6, but no group-specific differences or trends were observed.

**Discussion**

The experimental reproduction of PMWS in PCV-2-infected gnotobiotic pigs treated with keyhole limpet hemocyanin in incomplete Freund’s adjuvant (KLH/ICFA) suggested that activation of the immune system is a key component in the pathogenesis of PCV-2-induced disease (Krakowka and others 2001). Follow up studies using either KLH/ICFA (Ladekjaer-Mikkelsen and others 2002), commercial vaccine adjuvants (Resendes and others 2004), or complete commercial vaccines (Allan and others 2001, Kyriakis and others 2002,
Opriessnig and others 2003) in experimentally PCV-2-infected conventional pigs or field studies have given inconsistent results: PMWS in the stimulated and non-stimulated groups (Ladekjær-Mikkelsen and others 2002), no PCV2-associated lesions and no differences between the stimulated and unstimulated groups (Resendes and others 2004), and increased incidence of lesions and/or PMWS in the immunostimulated groups (Allan and others 2001, Kyriakis and others 2002, Opriessnig and others 2003). Although there have been field studies in which *M. hyopneumoniae* vaccine has been used as the only source of immune stimulation (Allan and others 2001, Kyriakis and others 2002), the present paper describes the first controlled study in which one commercially available product has been administered at different times relative to a challenge. In contrast with the vaccinated pigs, none of the unvaccinated pigs in group 7 had more than mild PCV-2-associated lesions. The study of the study show that vaccination with a commercial *M. hyopneumoniae* vaccine increases the severity of the lymphoid lesions and the quantity of PCV-2 genome in the serum of pigs vaccinated with two 1 ml doses of the vaccine at the time of, and two weeks after, the PCV-2-challenge (group 4), and in pigs vaccinated with a single 2 ml dose at four weeks of age (group 5).

At present, there are no commercially available PCV-2 vaccines and the recommendations for minimising losses associated with PMWS and other PCV-2-associated diseases are limited to the control of secondary infections and minimising stress by improving animal husbandry practices. *M. hyopneumoniae* vaccination has been shown to be economically beneficial by reducing the losses associated with respiratory disease (Maes and others 1996, Thacker and others 2000). Coinfections with *M. hyopneumoniae* and PCV-2 are commonly associated with the PRDC (Harms and others 2002). The experimental infection of pigs with both PCV-2 and *M. hyopneumoniae* results in significantly more severe lesions and an increased incidence of PMWS (Opriessnig and others 2004b). Evidence from this work and others (Allan and others 2000b, Allan and others 2001, Kyriakis and others 2002, Opriessnig and others 2003) suggests that the use of adjuvanted vaccines such as *M. hyopneumoniae* vaccines may be one of the triggers for the development of PMWS from an infection with PCV-2. It may be more appropriate to adjust the timing of vaccination rather
than to eliminate *M hyopneumoniae* vaccines and risk re-emergence in many herds of disease complexes associated with *M hyopneumoniae*.

The objective of the present study was to investigate the effect of timing of a commercial *M hyopneumoniae* vaccine on the replication of PCV-2 and the development of associated lesions. The experiment was designed to mimic some of the one- and two-dose *M hyopneumoniae* vaccination protocols that are used by pig producers. In a previous study, the authors observed that the PCV-2-associated lesions in vaccinated and unvaccinated pigs were essentially the same 21 days after inoculation, but were significantly different after 35 days (Opriessnig and others 2003). In the present experiment it was decided to euthanase all pigs at 42 days after inoculation in order to evaluate whether the lesions had resolved or progressed between 35 and 42 days after inoculation. At 42 days there were no lymphoid lesions in 20.6 per cent of the inoculated pigs, only minimal lesions in 58.8 per cent of the pigs and moderate lesions in the remaining 20.6 per cent. In general, the lesions appeared to be in the process of resolving because there was either no PCV-2 antigen, or only small amounts, associated with areas of inflammation in the lymphoid tissues, mainly restricted to a few macrophage-like or multinucleated giant cells in the centre of the lymphoid follicles (Figs 2, 3).

Vaccination of the pigs in group 4 at the time of, and two weeks after, PCV-2 challenge significantly (*P*<0.05) increased the severity of the lesions in lymphoid tissues, the numbers of copies of the PCV-2 genome in serum at 14 days after inoculation, the incidence of lymphohistiocytic myocarditis, and the severity of lymphohistiocytic myocarditis, lymphohistiocytic hepatitis, and interstitial pneumonia in comparison with the PCV-2-inoculated, unvaccinated pigs in group 7. Vaccination of the pigs in group 1 at two and four weeks of age also induced significantly (*P*<0.05) more copies of the PCV-2 genome in their serum at 14 days after inoculation and more severe interstitial pneumonia than in the pigs in group 7, even though the interval between their vaccination and challenge was longer than in the other groups. It is uncertain whether the lesions would have been even more severe if the pigs had been vaccinated at one and three weeks of age, as is commonly done in Europe, or if they had been challenged earlier.
Vaccination of the pigs in group 2 at four an six weeks of age (four and two weeks before they were challenged), and of the pigs in group 3 at six and eight weeks of age, had little influence on the PCV-2-associated lesions 42 days after they were challenged. The incidence and severity of the microscopic lesions in these groups were not different from those in the unvaccinated, PCV-2-inoculated group 7 or in the uninfected group 8. The results in group 3 differ from those observed in a previous study in which the same protocol induced significantly more severe lesions than were observed in unvaccinated pigs (Opriessnig and others 2003). One explanation for this difference might be that in the previous study two vaccines (against *A pleuropneumoniae* and *M hyopneumoniae*) were administered simultaneously. The products and adjuvant used in both studies were from the same manufacturer, but the *A pleuropneumoniae* vaccine contained a different adjuvant (paraffin) and probably other immune-modulating substances such as lipopolysaccharide. Acting together, the two vaccines might have provided a stronger immune stimulus than the *M hyopneumoniae* product alone.

Whether the single-dose (2 ml) regimens were administered at four weeks of age (group 5) or at eight weeks of age (group 6) made no difference to the severity of the lesions. Both single-dose regimens induced significantly (*P*<0.05) more severe lymphoid depletion, interstitial pneumonia and myocarditis, and a higher incidence of lymphohistiocytic nephritis than was observed in the unvaccinated pigs in group 7. The pigs in group 5 had significantly (*P*<0.05) more copies of the PCV-2 genome in serum 14 days after being challenged, and the lesions of lymphohistiocytic hepatitis and the overall PCV-2-associated lymphoid lesions were more severe compared to the unvaccinated pigs in group 7.

The control of PCV-2-associated disease remains a challenge for veterinarians. Several factors, including immune stimulation in the form of commonly used vaccines, or coinfections with porcine parvovirus (Allan and others 1999, Kennedy and others 2000, Krakowka and others 2000, Opriessnig and others 2004a), PRRSV (Allan and others 2000a, Harms and others 2001, Rovira and others 2002), or *M hyopneumoniae* (Opriessnig and others 2004b) have been shown to increase disease associated with PCV-2. Other factors, including the infectious dose, the type of vaccine adjuvant, the age of the pigs when they are infected and their genetic susceptibility, require further investigation. No clinical signs of
PMWS were reproduced during this study, suggesting that the *M. hyopneumoniae* bacterin used probably plays a relatively minor role in the pathogenesis of PCV-2-associated disease. However, the experiment used healthy, early-weaned pigs housed under strictly-controlled environment conditions without additional stressors or other infections, conditions that are quite different from those of field-based experiments. Nevertheless, most of the pigs developed the characteristic microscopic lesions of PMWS. Pig producers with herds that have suffered recurrent outbreaks of disease associated with PCV-2 should consider determining approximately when the pigs in the herd typically become infected with PCV-2, so that the effect of the timing of vaccination can be taken into account in trying to minimize the effects of PCV-2-associated disease. However, this study was carried out using only one commercial *M. hyopneumoniae* vaccine, and any inferences on the optimal timing of vaccine administration may be applicable to this vaccine only.

**Acknowledgements**

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**References**


CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Veterinary Pathology* **38**, 528-539


**TABLE 1.** Age in weeks at which the pigs in each group were vaccinated with a *Mycoplasma hyopneumoniae* bacterin and inoculated with porcine circovirus type 2 (PCV-2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of pigs</th>
<th>Age at first dose</th>
<th>Volume (ml)</th>
<th>Age at second dose</th>
<th>Volume (ml)</th>
<th>Age at inoculation with PCV-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>8</td>
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<td>5</td>
<td>9</td>
<td>4</td>
<td>2</td>
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<td>8</td>
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<tr>
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<td>10</td>
<td>8</td>
<td>2</td>
<td>-</td>
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<td>8</td>
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<tr>
<td>7</td>
<td>10</td>
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<td>8</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

* $10^{5.1}$ TCID$_{50}$ of field isolate ISU-40895

- None
TABLE 2. Mean (se) log porcine circovirus type 2 genome copy numbers/ml serum in the pigs of groups 1 to 7 at seven, 14, 21, 28, 35 and 42 days after they were inoculated, and the mean (se) period of viraemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Days after inoculation</th>
<th>Period of viraemia (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>5.0±0.3</td>
<td>6.0±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>3.8±0.8</td>
<td>5.4±0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>4.2±0.7</td>
<td>5.3±0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>4.9±0.3</td>
<td>6.1±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>4.3±0.7</td>
<td>6.2±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>4.9±0.6</td>
<td>5.6±0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>3.4±0.7</td>
<td>5.0±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Different superscripts within each column indicate significant (P<0.05) differences between groups
TABLE 3. Incidence (number affected/number examined) and mean severity of microscopic lesions in the lymphoid tissues of the pigs in groups 1 to 8, 42 days after they were inoculated with porcine circovirus type 2 (PCV-2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymph nodes</th>
<th></th>
<th></th>
<th></th>
<th>Tonsil</th>
<th></th>
<th></th>
<th>Spleen</th>
<th></th>
<th>Mean (se) lymphoid Score (0 to 9)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7/10 (1.0)</td>
<td>6/10 (0.9)</td>
<td>4/10 (0.4)</td>
<td>3/10 (0.4)</td>
<td>3/10 (0.3)</td>
<td>3/10 (0.3)</td>
<td>6/10 (0.6)</td>
<td>6/10 (0.8)</td>
<td>2/10 (0.2)</td>
<td>2.0±0.5\textsuperscript{a,b}</td>
</tr>
<tr>
<td>2</td>
<td>6/9 (0.9)</td>
<td>3/9 (0.4)</td>
<td>4/9 (0.7)</td>
<td>1/9 (0.1)</td>
<td>2/9 (0.2)</td>
<td>2/9 (0.4)</td>
<td>5/9 (0.7)</td>
<td>3/9 (0.3)</td>
<td>2/9 (0.2)</td>
<td>1.7±0.6\textsuperscript{b}</td>
</tr>
<tr>
<td>3</td>
<td>5/10 (0.7)</td>
<td>5/10 (0.6)</td>
<td>2/10 (0.3)</td>
<td>1/10 (0.2)</td>
<td>3/10 (0.4)</td>
<td>2/10 (0.3)</td>
<td>6/10 (0.6)</td>
<td>5/10 (0.7)</td>
<td>2/10 (0.2)</td>
<td>1.5±0.6\textsuperscript{b}</td>
</tr>
<tr>
<td>4</td>
<td>10/10 (1.5)</td>
<td>10/10 (1.2)</td>
<td>5/10 (0.9)</td>
<td>5/10 (0.5)</td>
<td>4/10 (0.4)</td>
<td>3/10 (0.5)</td>
<td>8/10 (1.2)</td>
<td>6/10 (0.7)</td>
<td>3/10 (0.4)</td>
<td>3.1±0.4\textsuperscript{a}</td>
</tr>
<tr>
<td>5</td>
<td>7/9 (1.6)</td>
<td>8/9 (1.3)</td>
<td>5/9 (1.0)</td>
<td>4/9 (0.4)</td>
<td>4/9 (0.6)</td>
<td>4/9 (0.6)</td>
<td>8/9 (1.1)</td>
<td>6/9 (0.8)</td>
<td>2/9 (0.2)</td>
<td>3.3±0.6\textsuperscript{a}</td>
</tr>
<tr>
<td>6</td>
<td>9/10 (1.3)</td>
<td>6/10 (0.8)</td>
<td>6/10 (0.7)</td>
<td>2/10 (0.2)</td>
<td>1/10 (0.1)</td>
<td>1/10 (0.1)</td>
<td>4/10 (0.5)</td>
<td>2/10 (0.2)</td>
<td>1/10 (0.1)</td>
<td>2.1±0.5\textsuperscript{a,b}</td>
</tr>
<tr>
<td>7</td>
<td>5/10 (0.6)</td>
<td>4/10 (0.5)</td>
<td>6/10 (0.6)</td>
<td>0/10 (0.0)</td>
<td>1/10 (0.1)</td>
<td>1/10 (0.1)</td>
<td>5/10 (0.5)</td>
<td>1/10 (0.1)</td>
<td>0/10 (0.0)</td>
<td>1.3±0.3\textsuperscript{b}</td>
</tr>
<tr>
<td>8</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0.0±0.0\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\*Mean (se) group score for depletion, histiocytic inflammation and PCV-2-antigen associated with the lesions as determined by immunohistochemistry (IHC) 0 Normal, 1 to 3 Mild, 4 to 6 Moderate, 7-9 Severe

\textsuperscript{a, b, c} Groups with different superscripts are significantly different
**TABLE 4.** Incidence (number affected/number examined) and mean severity of microscopic lesions in the lungs, heart, kidney and liver of the pigs in groups 1 to 8, 42 days after they were inoculated with porcine circovirus type 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Interstitial pneumonia</th>
<th>Lymphohistiocytic Myocarditis</th>
<th>Lymphohistiocytic interstitial nephritis</th>
<th>Lymphohistiocytic Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8/10 (0.9)</td>
<td>2/10 (0.2)</td>
<td>6/10 (1.1)</td>
<td>7/10 (0.7)</td>
</tr>
<tr>
<td>2</td>
<td>4/9 (0.4)</td>
<td>2/9 (0.2)</td>
<td>3/9 (0.6)</td>
<td>4/9 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>5/10 (0.7)</td>
<td>1/10 (0.2)</td>
<td>2/10 (0.3)</td>
<td>5/10 (0.5)</td>
</tr>
<tr>
<td>4</td>
<td>10/10 (1.2)</td>
<td>7/10 (0.6)</td>
<td>4/10 (0.6)</td>
<td>8/10 (0.9)</td>
</tr>
<tr>
<td>5</td>
<td>8/9 (0.9)</td>
<td>4/9 (0.6)</td>
<td>7/9 (1.1)</td>
<td>7/9 (0.9)</td>
</tr>
<tr>
<td>6</td>
<td>9/10 (1.2)</td>
<td>5/10 (0.5)</td>
<td>7/10 (0.8)</td>
<td>5/10 (0.7)</td>
</tr>
<tr>
<td>7</td>
<td>3/10 (0.3)</td>
<td>0/10 (0.0)</td>
<td>2/10 (0.2)</td>
<td>2/10 (0.3)</td>
</tr>
<tr>
<td>8</td>
<td>3/10 (0.3)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
<td>0/10 (0.0)</td>
</tr>
</tbody>
</table>
FIG 1: Mild histiocytic infiltration in a lymph node follicle of a pig from group 4. Haematoxylin and eosin. × 200. Inset: Moderate to large numbers of porcine circovirus type 2 (PCV-2) antigen-positive cells resemble macrophage-like cells. Immunohistochemical staining with anti-PCV-2 polyclonal antibody; streptavidin-biotin-peroxidase method counterstained with haematoxylin. × 500.
FIG 4: Percentages of the pigs in groups 1 to 7 that had zero lesion scores (normal), or mild, moderate or severe lesion scores for lymphoid depletion, histiocytic inflammation and porcine circovirus type 2 (PCV-2) antigen, 42 days after they had been inoculated with PCV-2
CHAPTER 6. PORCINE CIRCOVIRUS TYPE 2-INFECTION DECREASES THE EFFICACY OF A MODIFIED LIVE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VACCINE

A paper submitted to
Clinical and Vaccine Immunology

T. Opriessnig, N. E. McKeown, K. L. Harmon,
X. J. Meng, and P. G. Halbur

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV)-induced pneumonia is a major problem and vaccination is used to reduce losses associated with PRRSV. Porcine circovirus type 2 (PCV2) causes lymphoid depletion and there is concern this adversely affects the immune response. The objective of this study was to investigate the effect of PCV2 infection on efficacy of modified live virus (MLV) PRRSV vaccine. Sixty-nine, 2-week-old pigs were randomly assigned to one of seven groups of 9-10 pigs each. At 6 weeks of age, pigs in groups 4, 5 and 6 were inoculated intranasally with PCV2 ISU-40895. At 8 weeks of age, groups 3, 4, 6, and 7 were vaccinated with a MLV PRRSV vaccine. At 12 weeks of age, groups 2, 3, and 4 were challenged with PRRSV-SDSU73. All pigs were necropsied 14 days post PRRSV challenge. PCV2-infected, PRRSV-vaccinated and PRRSV-challenged pigs had significantly ($P < 0.05$) more severe macroscopic lung lesions compared to the PRRSV-vaccinated and PRRSV-challenged pigs that were not exposed to PCV2 prior to PRRSV vaccination. Non-vaccinated-PRRSV-infected pigs had significantly ($P < 0.001$) higher incidence of PRRSV-antigen in lungs compared to all other groups except the group infected with PCV2 prior to PRRSV vaccination and challenge. The nonvaccinated-PRRSV-
challenged group and the group challenged with PCV2 prior to PRRSV vaccination and challenge had significantly ($P < 0.001$) lower average daily weight gain compared to controls and the vaccinated groups. This work suggests that PCV2-infection has an adverse effect on development of protective immunity induced by PRRSV vaccine.

**INTRODUCTION**

Porcine circovirus type 2 (PCV2), a small, nonenveloped, single-stranded DNA virus with a circular genome (48), is ubiquitous in the global swine population (2). The hallmark microscopic lesions of PCV2-infection are lymphoid depletion and histiocytic replacement of lymphoid follicles in the lymphoid tissues and variable degrees of lymphohistiocytic inflammation in a variety of organs (45). PCV2 is associated with postweaning multisystemic wasting syndrome (PMWS) (1, 12) which is characterized by moderate to excessive loss of body condition often accompanied by respiratory disease in nursery and grow-to-finish pigs (17). The incidence of PCV2-associated disease in affected herds generally varies from 4% to up to 30% in individual farms (2, 17, 23). On most farms, PCV2-infection is widespread and subclinical.

Studies on pigs with naturally-acquired PMWS and PCV2-infection showed leukopenia characterized by decreased lymphocyte counts compared to healthy control pigs (10, 42, 43). Segalés et al. (42) found a significant decrease in the number of CD3+ and CD4+ cells in PMWS-affected pigs compared to clinically healthy pigs whereas no difference was found in the numbers of CD8+ cells. Darwich et al. (10) compared clinically PMWS-affected pigs that were infected by PCV2, wasted pigs that were not infected by PCV2, and healthy control pigs and found that, regardless of PCV2 infection status, wasted pigs in general had decreased CD4+ cells. However, only PMWS-affected and PCV2-infected pigs had decreased numbers of CD8+ and double-positive cells. The amount of PCV2 in lymphoid tissues was correlated to the degree of lymphoid depletion and to the decrease in IgM+ and CD8+ cells in peripheral blood (10). Further detailed assessment of PCV2-induced alteration of cells of the immune system in pigs experimentally-infected with PCV2 demonstrated a PCV2-induced lymphopenia present only in pigs that developed clinical PMWS but not in those that were subclinically-infected (29). The mean lymphocyte
levels of PCV2-infected pigs decreased below that of the control pigs which was most evident at 10 days post inoculation (DPI). A prominent lymphopenia was present in PMWS-affected pigs starting from DPI 14 until death of the pigs. All T-cell subpopulations were found to be susceptible to PCV2-induced lymphopenia (29).

Lymphoid depletion is a necessary and hallmark lesion of PCV2-associated PMWS (45); however, lymphoid depletion, to some degree, can also be observed in subclinically infected pigs (38). Studies on pigs with naturally acquired PMWS revealed that there was a reduction in number of interfollicular dendritic cells and a reduction or absence of B cells and CD4+ cells (40). Chianini et al. (8) graded lymphoid lesions in pigs with natural PMWS as mild, moderate, and severe and showed a reduction or loss of B and T lymphocytes. It has been suggested that PCV2-associated lymphoid depletion compromises the immune system of the pig. This is supported by case reports describing PCV2-infection associated with coinfecting pathogens that are typically indicative of an immunosuppressed stage of the host such as *Pneumocystis carinii* (9), *Chlamydia* spp, (6), pulmonary aspergillosis (44); and *Cryptosporidium parvum* (30).

Porcine reproductive and respiratory syndrome virus (PRRSV) is the major contributor to the porcine respiratory disease syndrome complex (PRDC) in the US (18) and is estimated to cost the US swine industry $560 million annually (28). PRRSV is an enveloped, positive sense, single-stranded RNA virus classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (7). Clinical signs of PRRSV-infection in growing pigs include fever, sneezing, dyspnea, tachypnea, anorexia, and decreased weight gain. Vaccination is a common procedure to minimize economic losses associated with this pathogen and vaccines have proven to be effective in experimental trials (33) and field studies (24, 47). However, PRRSV vaccine failures are not uncommon in the field and PRRSV continues to be the most common primary pathogen in cases of PRDC (18). PRRSV vaccine failure may be due to lack of cross-protection between the vaccine and field strain or failure of the pig to mount an effective response to PRRSV vaccine.

The effect of PCV2 infection on PRRSV vaccine efficacy has, to our knowledge, not been reported in the literature and the objective of this study was to investigate the effects of PCV2-infection on the efficacy of a modified-live-PRRSV (PRRS-MLV)-vaccine.
MATERIALS AND METHODS

Animals. Sixty-nine, segregated early weaned, specific-pathogen-free (SPF), crossbred pigs were brought to the research facility at Iowa State University at approximately 12-14 days of age. The pigs were purchased from a herd free of PRRSV based on regular serological testing. PCV2-associated disease was not observed in the source herd or the offspring of this herd.

Housing and feeding. On the day of arrival at the research facility, the pigs were randomly assigned to 7 groups and rooms with 9-10 pigs in each room. The pen size was 3 × 3.6 m and each pen contained a nipple waterer and one self feeder. The rooms were identical in size and environmental controls. The pigs were fed a complete, phased, corn- and soybean-based ration.

Experimental design, inoculations and vaccination. The experimental design is summarized in Table 1. Pigs were vaccinated with MLV-PRRSV vaccine at 14 days post PCV2-infection when PCV2-associated lymphoid depletion is typically most severe and the amount of PCV2 genomic copy numbers in serum is typically highest in this experimental SPF pig model (32, 34, 36).

The experimental protocols were approved by the Iowa State University Committee on Animal Care. After waning of the passively acquired antibodies to PCV2 at 6 weeks of age, pigs in groups 4, 5, and 6 were inoculated intranasally with 3 ml of the PCV2 ISU-40895. At 8 weeks of age, pigs in groups 3, 4, 6, and 7 were vaccinated intramuscularly in the right neck with 2 ml of the Ingelvac® PRRS ATP MLV vaccine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) according to the manufacturer’s recommendations. At 12 weeks of age, each pig in groups 2, 3, and 4 received 2 ml of PRRSV-SDSU73 intranasally.

PCV2 inoculum. The PCV2-virus stock was generated through direct transfection of PK-15 cells with an infectious DNA clone of PCV2 ISU-40895 as previously described (13). PCV2 isolate ISU-40895 was isolated in 1998 from a wasting pig with respiratory disease in Iowa. The pig came from a group of 300 pigs with the history of increased respiratory disease and lethargy with approximately 50% of the pigs affected (14). Inoculation was done with the PCV2-virus stock at a dose of $10^{5.2}$ 50% tissue culture infective dose (TCID$_{50}$) per
ml.

**PRRSV inoculum.** High virulent PRRSV-isolate SDSU73 was isolated from a sow herd with a high prevalence of abortions and higher than usual sow mortality in 1996 (26). Passage 2 of PRRSV-SDSU73 (Courtesy of Dr. M. Roof, BIVI) at a titer of $10^5$ TCID$_{50}$ per ml was used for inoculation of the pigs.

**Serology.** Blood samples were collected on arrival of the pigs followed by weekly blood collections. Sera collected at 2 (arrival), 8 (PRRSV-vaccination), 9, 10, 11, 12 (PRRSV-inoculation), 13, and during necropsy at 14 weeks of age were tested for the presence of PRRSV-specific antibodies by a commercial PRRSV ELISA (HerdChek PRRS virus antibody test kit; Idexx Laboratories Inc., Westbrook, MA, USA). The optical density at 405 nm greater or equal to 0.4 was set as the ELISA cut-off value. A fluorescent focus neutralization (FFN) assay to determine the amount of PRRSV-SDSU73 neutralizing antibodies was done on sera from all PRRSV vaccinated groups (3, 4, 6, and 7) collected on the day of vaccination, and at 7, 14, 21, and 28 days post vaccination according to protocols used at the Iowa State University Veterinary Diagnostic Laboratory.

Sera collected at 2 (arrival of the pigs), 6 (PCV2-inoculation), and at 14 weeks of age (necropsy) were tested for the presence of PCV2-specific antibodies by a PCV2-enzyme linked immunosorbent assay (ELISA) based on the recombinant ORF2 capsid protein of PCV2 (27). Samples were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater.

**Clinical evaluation.** The pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 = normal to 6= severe dyspnea and abdominal breathing (16). In addition, pigs were evaluated daily for clinical signs including sneezing and jaundice. Rectal temperatures, wasting, and behavioral changes such as lethargy were recorded daily. The pigs were weighed at weekly intervals.

**PCV2 DNA quantification.** DNA-extraction on sera collected at the day of PRRSV challenge and at 14 days post PRRSV challenge, and on bronchoalveolar lavage fluid collected during necropsy was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). DNA-extracts were used for quantification of the amount of PCV2 genomic DNA by real-time PCR as previously described (36).
**PRRSV RNA quantification.** RNA-extraction on sera collected 7 and 14 days post PRRSV challenge and on bronchoalveolar lung lavage fluid collected during necropsy was performed using the QIAamp® Viral RNA Mini Kit (Qiagen). Primer-probe combinations specific for the North American PRRSV [PRRSORF7F (TGTCAGATTACGGAGRA-TAAGTTAC), PRRSORF7R (ATCARGCGCACAGTRTGATGC), and PRRSORF7P (6FAM-TGTGGAGTTYAGTYTGCC)] and the European PRRSV [LELYRTF (GCTGAGATGACRTYCGGCA), LELYRTR (GCAGTYCCTGCGCCTTGAT), and LELYRTP (VIC-TGCAATCGATYCGGCA LELYRTP)] were used for the real time reverse-transcriptase PCR. Each PCR reaction consisted of 2.5µl of the RNA template and 22.5µl PCR master mix. The PCR master mix contained 0.35µl QuantiTect® Probe RT-PCR kit (Qiagen) with the magnesium chloride concentration adjusted to 6 mM. Forward and reverse primers and detection probes were used at concentrations of 800, 800 and 275 nM, and 400, 400, and 100 nM for the North American and European PRRSV, respectively. An additional 0.25 µl (1.25U) of HotStarTaq (Qiagen) was added to each reaction. Each reaction included five progressive 1:10 dilutions of a known copy number of PRRSV that served to generate a standard curve. Each plate was run in a sequence detection system (GeneAmp 7900, Applied Biosystem) under company-specific conditions (30 min at 50°C, 15 min at 95°C, followed by 35 cycles of 15 seconds at 94°C and 60 seconds at 60°C).

**Necropsy.** Necropsy was performed at 14 days post PRRSV challenge when the pigs were 14 weeks old. Pathologists were blinded to treatment group for evaluation of gross lesions. The total amount of lung affected by pneumonia (0-100% of the lung affected by grossly visible pneumonia) was recorded for each pig at necropsy in a blinded fashion as described (16). The scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each (16). The size of lymph nodes [score range from 0-3; 0 (normal), 1 (two times the normal size), 2 (three times the normal size), 3 (four times the normal size)] was estimated and recorded for each pig (36). Bronchoalveolar lavage (BAL) for bacterial and virologic examinations was collected as previously described using fifty milliliters of sterile...
phosphate-buffered saline for each lung (25). Sections of lungs, lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

**Histopathology.** Microscopic lesions were evaluated in a blinded fashion. Lung sections were scored for presence and severity of interstitial pneumonia ranging from 0 to 6 (0=normal; 1=mild multifocal; 2=mild diffuse; 3=moderate multifocal; 4=moderate diffuse; 5=severe multifocal; 6=severe diffuse) (16). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0=none to 3=severe (32). Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0=normal to 3=severe and histiocytic inflammation and replacement of follicles ranging from 0=normal to 3=severe (32).

**Immunohistochemistry (IHC) for detection of PRRSV and for PCV2 antigens.** IHC for detection of PRRSV specific antigen was performed on formalin-fixed and paraffin-embedded lung tissue sections as previously described (15). Similarly, IHC for detection of PCV2 specific antigen was performed on formalin-fixed and paraffin-embedded lung tissue sections using a rabbit polyclonal antiserum as previously described (46).

**Statistical analysis.** Prior to data analysis, descriptive statistics were performed to assess the overall quality of the data. Continuous data (weight, rectal temperature, macroscopic lung lesions and S:P ratios) were analyzed with a one-way analysis of variance (ANOVA). If a one-way ANOVA was significant ($P < 0.05$), pairwise testing using the Tukey’s adjustment was performed. Discrete data (microscopic lesions, clinical observations) were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA. If the nonparametric ANOVA was significant ($P < 0.05$), Wilcoxon tests were used for pairwise testing. Response feature analysis was performed to account for clinical observations. The observations obtained after PRRSV-challenge were combined and average for each pig, and differences among groups were compared by using a nonparametric Kruskal-Wallis ANOVA. Fisher’s exact test was used to evaluate differences in incidence.
RESULTS

**Clinical disease.** Clinical disease was characterized by increased rectal temperatures, by respiratory disease with labored breathing and sneezing, and by reduced average daily weight gain after PRRSV challenge. The NV-PRRSV, the PCV2-V-PRRSV, and V-PRRSV groups had increased rectal temperatures after PRRSV challenge. The temperatures ranged from 40.1 to 41.8°C with peak temperatures at 5 to 7 days post PRRSV challenge with 80% of the pigs febrile. After challenge there were significant ($P < 0.001$) differences in mean rectal temperatures between groups. The NV-PRRSV pigs had highest rectal temperatures, and all PRRSV challenged groups had significantly higher rectal temperatures compared to non-PRRSV inoculated groups.

After PRRSV challenge, the highest average group respiratory scores were observed in the NV-PRRSV pigs (mean score 1.6), in the PCV2-V-PRRSV pigs (mean score 1.2) and in the V-PRRSV pigs (mean score 1.1) which were all significantly ($P < 0.0001$) higher compared to the other groups but not different from each other (Data not shown).

The average daily weight gain is summarized in Table 2. From the day of PRRSV vaccination to the day of PRRSV-challenge, the average daily gain was not significantly ($P = 0.24$) different between the groups. From the day of PRRSV challenge to the day of necropsy, NV-PRRSV pigs had significantly ($P < 0.0001$) lower average daily gain compared to all other groups. The average weight gain in the V-PRRSV and the PCV2-V-PRRSV pigs was not different from each other but were significantly ($P < 0.001$) lower compared to the CONTROLS, PCV2, PCV2-V, and V groups. When the total average weight gain for the period from the day of PRRSV vaccination until necropsy was evaluated, the NV-PRRSV and PCV2-V-PRRSV pigs had significantly ($P < 0.001$) lower average daily gain compared to CONTROLS and the V groups.

**Antibody response.** All pigs were free of PRRSV-specific antibodies at arrival in the research facility. The V-PRRSV, PCV2-V-PRRSV, and PCV2-V pigs seroconverted to PRRSV between 7 to 21 days post vaccination. The mean group S/P ratios were numerically but not significantly lower in the PCV2-V-PRRSV group and in the V-PCV2 group compared to the V-PRRSV group. The non-vaccinated and non-PRRSV challenged groups (CONTROLS, PCV2) remained seronegative until the termination of the experiment. The
NV-PRRSV pigs seroconverted within 2 weeks post PRRSV challenge. Neutralizing antibodies to SDSU-73 were not detected in any of the vaccinated pigs prior to PRRSV challenge.

At arrival, the majority of the pigs had maternal antibodies to PCV2, which waned over the following weeks. On the day of PCV2 inoculation, all pigs were negative for PCV2-specific antibodies. Seroconversion to PCV2 was observed at the termination of the study in 10/10 PCV2-V-PRRSV pigs, 9/10 PCV2-V pigs, and 7/10 PCV2 pigs. The mean S/P ratios were significantly ($P < 0.001$) higher in the PCV2-V-PRRSV (0.87 ± 0.08) group compared to PCV2 (0.41 ± 0.09) and PCV2-V (0.49 ± 0.05) groups.

**Amount and incidence of PRRSV-RNA detection.** The CONTROLS and PCV2 groups were negative for PRRSV-RNA in serum and lung lavage fluid (Table 3). The incidence of PRRSV-viremic pigs was 9/9 for NV-PRRSV, 9/10 for V-PRRSV, 10/10 for PCV2-V-PRRSV, 3/10 for PCV2-V, and 2/10 for the V group on DPI 7. The incidence of PRRSV-RNA-positive pigs was 8/9 for NV-PRRSV, 6/10 for V-PRRSV, 5/10 for PCV2-V-PRRSV, 0/10 for PCV2-V, and was 0/10 for V group on DPI 14. The amount of PRRSV-RNA in serum was significantly ($P < 0.001$) higher in the NV-PRRSV group compared to that of the V-PRRSV and PCV2-V-PRRSV groups at 7 and at 14 days post PRRSV-infection (Figure 1). The incidence of PRRSV-RNA-positive lavage fluids is summarized in Table 3. There was a significantly ($P < 0.05$) higher incidence of PRRSV-RNA positive BAL in the NV-PRRSV, V-PRRSV, PCV2-V-PRRSV, and PCV2-V pigs compared to the V pigs. There was no significant ($P > 0.05$) difference in group mean amount of PRRSV-RNA in BAL fluids between NV-PRRSV, V-PRRSV, and PCV2-V-PRRSV pigs.

**Amount and incidence of PCV2-DNA detection.** At the day of PRRSV challenge, PCV2-DNA were detected in 8/10 PCV2-V-PRRSV pigs, in 7/10 PCV2-V pigs, and in 6/10 PCV2 pigs. At the day of necropsy, PCV2-DNA were detected in 9/10 PCV2-V-PRRSV pigs, in 7/10 PCV2-V pigs, and in 5/10 PCV2 pigs. All PCV2-infected pigs were positive for PCV2-DNA on BAL (Table 3). The amount of PCV2-antigen was not different ($P > 0.05$) among the three groups.

**Macroscopic lesions.** PRRSV-induced macroscopic lung lesions were characterized by failure of the lungs to collapse and by focal-to-diffuse, mottled-tan, well-to-poorly
demarcated areas of pneumonia. There were significant differences among groups in the mean percentage of lung macroscopically affected by pneumonia as summarized in Table 3.

**Microscopic lesions.** PRRSV challenge induced moderate-to-severe lung lesions characterized by type 2 pneumocyte hypertrophy and hyperplasia, septal infiltration with mononuclear cells and increased amounts of necrotic alveolar exudate. No or mild lung lesions characterized by septal infiltration with mononuclear cells and mild peribronchiolar fibroplasia and histiocytic infiltrates were observed in singular PCV2-inoculated pigs in this study consistent with our previous studies (32, 36).

**PRRSV and PCV2-antigen in lungs.** The incidence of PRRSV and PCV2-antigen detection in lungs as determined by IHC stains is summarized in Table 3. NV-PRRSV pigs had a significantly ($P < 0.001$) higher incidence of PRRSV-antigen detection compared to all other groups except PCV2-V-PRRSV.

**DISCUSSION**

PCV2 infection is widespread and PCV2 is now commonly associated with varying conditions in pigs such as PMWS, enteritis, abortion, and respiratory disease. Another important impact of PCV2 may actually be its adverse effect on the immune system in the form of compromising the development of a protective immune response to commercial vaccines commonly used on pigs around the time of PCV2 infection. PCV2 infection commonly occurs in pigs at 6 to 12 weeks of age in the US (37). Vaccines commonly used at that time include *Mycoplasma hyopneumoniae*, swine influenza virus, and PRRSV. Porcine respiratory disease complex (PRDC) is a major problem in the pig industry and PRRSV is the most commonly associated primary pathogen in PRDC. Coinfections with PRRSV and PCV2 are commonly observed in the field (37, 49).

The overall goal of this study was to investigate the effect of PCV2 infection on the efficacy of the PRRS MLV vaccine to protect pigs against PRRSV-induced clinical disease and lesions. The vaccination protocol used in this study has been shown previously to be effective in reducing clinical disease and lesions associated with PRRSV SDSU73-infection (33).

The effect of other immunomodulating pathogens such as PRRSV (11, 22) or
Trypanosma evansi (T. evansi) (20) on vaccine efficacy has been investigated. Pigs were inoculated with a European PRRSV strain, vaccinated with a pseudorabies virus (PRV) vaccine 2 weeks later, and challenged with PRV 10 weeks after PRRSV-inoculation. The results of that study indicated that the lymphoproliferative response was quicker and longer in pigs that were not previously infected by PRRSV. However, PRRSV infection did not inhibit the vaccine efficacy (11). Another study investigated the effect of PRRSV infection on the antibody response to classical swine fever virus (CSFV) vaccination (22). In that study, pigs were vaccinated 2 days after PRRSV inoculation and it was found that the PRRSV-inoculated pigs had significantly lower levels of anti-CSFV-antibodies compared to non-PRRSV-inoculated pigs within 3-5 weeks after vaccination. Pigs infected with T. evansi and vaccinated four weeks later against CSFV and challenged 12 weeks after T. evansi infection with CSFV had significantly reduced antibody response compared to non-infected control pigs. However, there was no effect on growth performance or feed conversion. (20).

In this study, differences in IgG antibody response or in presence of neutralizing antibodies against PRRSV were not observed between V-PRRSV and PCV2-V-PRRSV pigs. However, we did observe a significant ($P < 0.05$) increase in PRRSV-associated macroscopic lung lesions, a higher incidence of PRRSV-antigen in lung sections, and more severe microscopic PRRSV-associated lung lesions in pigs that were subclinically infected by PCV2 at the time of PRRSV-vaccination compared to pigs that were not infected with PCV2.

Previous studies have investigated the interaction of PCV2 and PRRSV in experimental models using colostrum-deprived pigs, cesarean-derived/colostrum-deprived (CDCD) pigs, or conventional pigs. In all these models, PCV2 and PRRSV inoculations were done within a narrow time window (same day, or within one week). Allan et al. (4) inoculated 1-2 day old colostrum-deprived pigs with PCV2 and PRRSV and observed upregulation of PCV2 in coinfected pigs. The replication and distribution of PRRSV in concurrently infected pigs was not enhanced compared to single PRRSV infected pigs (4). Harms et al. (19) coinfected 3-week-old CDCD pigs with PCV2 and PRRSV and showed that PCV2-infection increased the severity of PRRSV-induced interstitial pneumonia in CDCD pigs. Rovira et al. (39) inoculated 5-week-old conventional pigs with PRRSV and seven days later with PCV2 and confirmed that PRRSV infection enhances PCV2
replication. A longer duration of PRRSV viremia and a higher proportion of viremic pigs were observed in the coinfected pigs compared to singular PRRSV infected pigs (39). In the current study, PCV2 infection and PRRSV challenge were done 6 weeks apart since we were most interested in the PCV2-PRRSV MLV-vaccine interaction. We did not expect to observe a PRRSV-PCV2 interaction since we know from past PCV2 studies that PCV2-associated lesions typically peak at 2-3 weeks post inoculation and usually become minimal or resolved by 4-6 weeks post inoculation (34, 35).

The interval of 2 weeks between PCV2 inoculation and PRRSV MLV vaccination was chosen because at this time we frequently observe high amounts of PCV2-genomic copies in serum in our pig model. A relation between PCV2 DNA load and amount of PCV2 antigen in lymphoid lesions has been demonstrated (31). In addition, Nielsen et al. (29) found that the white blood cell count and the mean lymphocyte counts were significantly decreased at 10 DPI following experimental PCV2 infection compared to uninfected controls. Rovira et al. (39) found maximal PCV2 DNA loads in serum of experimentally PCV2-infected pigs at 21 DPI. In contrast, Allan et al. (3) observed minimal amounts of PCV2 antigen in mesenteric lymph nodes starting at 10 DPI with increasing density and distribution of PCV2 antigen at 14, 17, 21, and 26 DPI.

Krakowka et al. (21) reported evidence that strong immunostimulation can upregulate PCV2 infection towards manifestation of clinical PMWS in the gnotobiotic pig model. Similar results were observed in the conventional pig model using commercially available adjuvanted, killed vaccines (5, 36). To our knowledge, PRRSV MLV vaccines have not yet been tested for possible interaction with PCV2 infection. In the current study we found no evidence of vaccine-induced enhancement of PCV2-associated disease. None of the PCV2-V pigs developed clinical disease and lungs sections were negative for the presence of PCV2-antigen by IHC. Mild interstitial pneumonia lesions were present in a portion of the PCV2-V pigs; however, this was not different from CONTROLS, PCV2, or V pigs. Interestingly, there was a significantly ($P < 0.05$) higher incidence of PRRSV-RNA positive bronchoalveolar lavage samples in PCV2-V compared to V. However, PRRSV-antigen as determined by IHC was not detected in any of the PCV2-V or V pigs. We concluded that the use of PRRSV MLV-vaccines in subclinically PCV2-infected pigs appeared to be safe under
the conditions of this study. This is in contrast to the findings that PRRSV MLV vaccine predisposed pigs to disease associated with *Streptococcus suis* infection (41).

In this study, we found that subclinical infection of SPF pigs with PCV2 prior to PRRSV MLV vaccination and subsequent PRRSV challenge resulted in increased PRRSV-induced macroscopic and microscopic lung lesions. The adverse effect of PCV2 infection on the development of protective immunity against PRRSV and other respiratory pathogen vaccines may be an important factor in controlling PRDC and other diseases in growing pigs.

**ACKNOWLEDGEMENTS**

This study was funded in part by a grant from the Healthy Livestock Initiative and by Fort Dodge Animal Health, Inc. The vaccine and the PRRSV-inoculum for this study were kindly provided by Boehringer Ingelheim Vetmedica, Inc.

**REFERENCES**


TABLE 1. Experimental design to determine the effect of porcine circovirus type 2 (PCV2) infection on the efficacy of porcine reproductive and respiratory syndrome virus (PRRSV) vaccine

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>6 weeks of age</th>
<th>8 weeks of age</th>
<th>12 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 10)</td>
<td>CONTROL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td>NV-PRRSV</td>
<td>-</td>
<td>PRRSV</td>
</tr>
<tr>
<td>3 (n = 10)</td>
<td>V-PRRSV</td>
<td>-</td>
<td>Vaccination&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 (n = 10)</td>
<td>PCV2-PRRSV</td>
<td>PCV2</td>
<td>Vaccination&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 (n = 10)</td>
<td>PCV2</td>
<td>PCV2</td>
<td>-</td>
</tr>
<tr>
<td>6 (n = 10)</td>
<td>PCV2-V</td>
<td>PCV2</td>
<td>Vaccination&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 (n = 10)</td>
<td>V</td>
<td>-</td>
<td>Vaccination&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> NV, non-vaccinated; V, vaccinated.

<sup>b</sup> Ingelvac PRRS ATP (Boehringer Ingelheim Vetmedica Inc, St. Joseph, Missouri, USA.)
TABLE 2. Average daily weight gain (ADG) (group mean ± SE) for the period from vaccination to prior to inoculation with porcine reproductive and respiratory syndrome virus (PRRSV) (ADG 1), post PRRSV challenge (ADG 2), and the entire period from vaccination to necropsy (AGD 3)

<table>
<thead>
<tr>
<th>Group</th>
<th>ADG 1 (g)</th>
<th>ADG 2 (g)</th>
<th>ADG 3 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>774.9 ± 28.8</td>
<td>1277.4 ± 33.3</td>
<td>875.4 ± 29.1</td>
</tr>
<tr>
<td>NV-PRRSV</td>
<td>793.2 ± 36.1</td>
<td>388.2 ± 108.0</td>
<td>694.9 ± 36.1</td>
</tr>
<tr>
<td>V-PRRSV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>813.5 ± 23.6</td>
<td>783.3 ± 47.9</td>
<td>807.5 ± 20.8</td>
</tr>
<tr>
<td>PCV2-V-PRRSV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>715.0 ± 34.0</td>
<td>711.0 ± 37.2</td>
<td>714.2 ± 31.0</td>
</tr>
<tr>
<td>PCV2</td>
<td>732.2 ± 38.8</td>
<td>1099.3 ± 32.3</td>
<td>805.6 ± 33.1</td>
</tr>
<tr>
<td>PCV2-V&lt;sup&gt;b&lt;/sup&gt;</td>
<td>739.6 ± 26.8</td>
<td>1044.3 ± 41.1</td>
<td>800.6 ± 22.5</td>
</tr>
<tr>
<td>V&lt;sup&gt;b&lt;/sup&gt;</td>
<td>797.3 ± 36.5</td>
<td>1138.2 ± 46.8</td>
<td>865.5 ± 35.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values within a column with no common superscript are significantly (P < 0.0001) different.

<sup>b</sup> Groups vaccinated with Ingelvac PRRS ATP modified live virus vaccine (Boehringer Ingelheim Vetmedica Inc, St. Joseph, Missouri).
Table 3: Incidence and severity of macroscopic and microscopic lung lesions and presence of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) nucleic acids and antigens in lungs detected by PCR on bronchoalveolar lavage (BAL) and immunohistochemistry (IHC) at 14 days post PRRSV challenge. Data presented as incidence and group mean ± SE

<table>
<thead>
<tr>
<th>Group</th>
<th>Macroscopic lung lesions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Microscopic lung lesions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PRRSV BAL PCR</th>
<th>PRRSV IHC</th>
<th>PCV2 BAL PCR</th>
<th>PCV2 IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>1/10 (0.3±0.3)&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>10/10 (1.2±0.1)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0/10</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NV-PRRSV</td>
<td>9/9 (50.1±3.1)&lt;sup&gt;B&lt;/sup&gt;</td>
<td>9/9 (4.6±0.4)&lt;sup&gt;B&lt;/sup&gt;</td>
<td>9/9</td>
<td>8/9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>V-PRRSVe</td>
<td>10/10 (13.2±2.6)&lt;sup&gt;C&lt;/sup&gt;</td>
<td>10/10 (2.5±0.3)&lt;sup&gt;A,C&lt;/sup&gt;</td>
<td>10/10</td>
<td>0/10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PCV2-V-PRRSVe&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10/10 (31.7±5.0)&lt;sup&gt;D&lt;/sup&gt;</td>
<td>10/10 (3.3±0.5)&lt;sup&gt;B,C&lt;/sup&gt;</td>
<td>10/10</td>
<td>4/10</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>PCV2</td>
<td>4/10 (1.0±0.6)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>10/10 (1.2±0.1)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0/10</td>
<td>NA</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>PCV2-V&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3/10 (2.2±1.3)&lt;sup&gt;A,C&lt;/sup&gt;</td>
<td>10/10 (1.8±0.2)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>8/10</td>
<td>0/10</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>V&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0/10 (0.0±0.0)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>8/10 (1.2±0.3)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2/10</td>
<td>0/10</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of lung grossly affected by lesions ranging from 0 to 100%.

<sup>b</sup> Interstitial pneumonia score ranging from 0 (normal) to 6 (severe, diffuse).

<sup>c</sup> Values within a column with no common superscript represent significantly (<i>P</i> < 0.05) different group means.

<sup>d</sup> Not applicable.

<sup>e</sup> Groups vaccinated with Ingelvac PRRS ATP modified live virus vaccine (Boehringer Ingelheim Vetmedica Inc, St. Joseph, Missouri).
FIG 1: Mean log transformed group porcine reproductive and respiratory syndrome virus (PRRSV) genomic copies. Error bars represent standard errors. * = significant ($P < 0.001$) differences between groups.
CHAPTER 7. EFFECT OF PORCINE PARVOVIRUS VACCINATION ON THE
DEVELOPMENT OF PMWS IN SEGREGATED EARLY WEANED PIGS
COINFECTED WITH TYPE 2 PORCINE CIRCOVIRUS AND PORCINE
PARVOVIRUS

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Thacker, K.M. Lager, X.J Meng, P.G. Halbur

Abstract
The objectives of this study were to determine if coinfection of segregated early
weaned (SEW) pigs with porcine circovirus type 2 (PCV2) and porcine parvovirus (PPV)
induces an increase in the incidence of postweaning multisystemic wasting syndrome
(PMWS) compared to singular PCV2 infection, and to determine if vaccination against PPV
protects pigs against PMWS associated with PCV2/PPV coinfection in SEW pigs. Seventy,
3-week-old, SEW pigs were randomly assigned to one of five groups. Pigs in group 1 ($n = 14$) served as the negative controls, group 2 pigs ($n = 14$) were inoculated with PCV2, group 3 pigs ($n = 12$) were inoculated with PPV, group 4 ($n = 16$) and 5 ($n = 14$) pigs were
inoculated with both PCV2 and PPV. Pigs in groups 1-3 and 5 were vaccinated with two
doses of a killed parvovirus-leptospira-erysipelothrix (PLE) vaccine prior to inoculation. The
PCV2/PPV-coinfected pigs (groups 4 and 5) had significantly ($P < 0.05$) higher and more
persistent fevers than the singular PCV2-infected pigs. One pig in each of the coinfected
groups developed clinical disease (fever, respiratory disease, jaundice, weight loss)
consistent with PMWS. Lymphoid depletion was significantly ($P < 0.05$) more severe in the
dually-infected pigs at 42 days post inoculation (DPI). Vaccinated, coinfected pigs (group 5) remained viremic significantly ($P < 0.05$) longer and had higher copy numbers of genomic PCV2 DNA in sera at 28, 35, and 42 DPI compared to the unvaccinated coinfected pigs (group 4). PPV-viremia was detected only in the unvaccinated group 4 pigs. PLE-vaccination prevented PPV-viremia but did not prevent clinical PMWS or reduce the severity of lymphoid depletion in PCV2/PPV-coinfected pigs. Evidence of increased incidence of clinical PMWS due to vaccination was not observed in this model.

1. Introduction

Post-weaning multisystemic wasting syndrome (PMWS) is an emerging disease associated with porcine circovirus type 2 (PCV2). Mortality associated with PMWS ranges from 2 to 30% or more (Harding and Clark, 1997; Allan and Ellis, 2000). Typical clinical signs of PMWS are progressive weight loss, enlarged lymph nodes, and pneumonia. Less common signs of PMWS include diarrhea, icterus, and pallor. Characteristic microscopic lesions include lymphoid depletion, histiocytic replacement of follicles in lymphoid tissues, and mild-to-severe granulomatous inflammation in lymphoid and other organs (Allan and Ellis, 2000; Sorden, 2000).

Single PCV2 infection is generally not associated with clinical disease in pigs and causes only mild microscopic lesions (Allan et al., 2000b; Krakowka et al., 2000, 2001; Rovira et al., 2002), whereas a high percentage of PCV2 inoculated pigs coinfected with other swine pathogens developed more severe disease (Allan et al., 1999, 2000b; Ellis et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000; Harms et al., 2001; Rovira et al., 2002; Kim et al., 2003). Several groups have investigated the interaction of PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV) in experimental models. Harms et al. (2001) induced significantly more severe disease and higher mortality in colostrum-deprived, caesarean-derived (CDCD) pigs coinfected with PRRSV and PCV2 compared to singular infection. Experimental dual infection of colostrum-deprived (CD) pigs (Allan et al., 2000b) or conventional pigs (Rovira et al., 2002) with PCV2 and PRRSV potentiated the replication and distribution of PCV2.

Porcine parvovirus (PPV) has also been shown to enhance disease associated with
PCV2. Ellis et al. (1999) reproduced most of the lesions typical of PMWS in gnotobiotic pigs inoculated with filtered cell culture material and filtered lymphoid tissues from pigs with naturally acquired PMWS. Both PCV2 and PPV and antibodies to these viruses were detected in the experimentally inoculated pigs. Since then, several groups have demonstrated that CD pigs dually-inoculated with PCV2 and PPV develop more severe disease and lesions than pigs with singular PCV2 infection (Allan et al., 1999; Kennedy et al., 2000). Krakowka et al. (2000) further confirmed the synergistic effect of PCV2 and PPV in gnotobiotic pigs by reproducing clinical disease and lesions typical of PMWS in coinfected pigs but not in pigs infected with PCV2 or PPV alone. To our knowledge, this synergistic effect has not been demonstrated in segregated early weaned (SEW) pigs which are typical of current US production techniques.

PPV is widespread in the swine population. Incoming gilts and less often sows are routinely vaccinated to protect against PPV-associated reproductive losses. There is evidence that different PPV-isolates vary in pathogenicity (Mengeling and Cutlip, 1976; Oraveerakul et al., 1993). PPV-isolates have been classified as non-pathogenic (Cutlip and Mengeling, 1975; Paul and Mengeling, 1980), pathogenic to non-immunocompetent fetuses leading to death (Mengling et al., 1979), pathogenic to immunocompetent fetuses and inducing dermatitis (Choi et al., 1987; Kresse et al., 1985; Lager et al., 1992; Lager and Mengeling, 1994), and enteric PPV-strains (Dea et al., 1985, Duhamel et al., 1991). In general, infection of growing pigs with PPV alone is not associated with clinical disease (Brown et al., 1980; Allan et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000).

PPV is similar to PCV in many ways. Both viruses are small, non-enveloped DNA viruses. Both have a strong cellular tropism for mitotically active tissues like lymph nodes or heart muscle (Oraveerakul et al., 1993; Allan and Ellis, 2000). It is thought that stimulation of host DNA to enter the S-phase of the cell cycle by PPV promotes PCV2-replication (Krakowka et al., 2000). One potential way to minimize the effect of PPV-associated enhancement of replication of PCV2 and induction of PMWS may be by PPV-vaccination of growing pigs in PCV2/PPV-coinfected herds. Attempts to control PMWS with PPV vaccine on finishing sites with confirmed PPV circulation have been repeatedly successful (Halbur, 2000, 2001).
The objectives of this study were to determine if coinfection of SEW pigs with PCV2 and PPV induces an increase in the incidence of PMWS compared to singular PCV2 infection, and to determine if vaccination against PPV protects pigs against PMWS associated with PCV2/PPV coinfection in SEW pigs.

2. Materials and methods

2.1. Animal source

Seventy, 12-14 day-old pigs were transported to the livestock infectious disease isolation facility (LIDIF) at Iowa State University, Ames, IA. Prior to purchase, sera from the dams were tested and confirmed to be negative for the presence of antibodies to PPV and PCV2 by hemagglutination inhibition (HI) (Mengeling et al., 1988) and ELISA (Nawagitgul et al., 2002), respectively.

2.2. Experimental design

The experimental design is summarized in Table 1. All groups were housed in one room until 1 day prior to inoculation at which time pigs from groups 1-3 were removed and housed separately. Pigs in groups 4 and 5 remained in one room together. Necropsy was performed on half of the pigs at 21 days post-inoculation (DPI), and the remaining pigs at DPI 42.

2.3. Virus inocula

PCV2 isolate ISU-40895 was obtained via direct transfection of PK-15 cells with an infectious clone of PCV2 (Fenaux et al., 2002). Passage number 4 of the virus at a titer of $10^4$ TCID$_{50}$ was used for inoculation of the pigs. Each pig received 2 ml of the PCV2-inoculum intranasally, and 0.5 ml intralymphoid into left and 0.5 ml into the right superficial inguinal lymph node (Fenaux et al., 2002). PPV-strain NADL-8 was isolated from fetal porcine kidney cells from a naturally infected pig in 1977 in Perry, Iowa (Mengeling et al., 1979). In this study, PPV-passage 4 (10% tissue homogenate suspension containing lungs of aborted fetuses and minimal essential medium) from the year 1982 was used. Each pig received 2 ml of the PPV-inoculum with a titer of about $10^{4.9}$ TCID$_{50}$ intranasally. Prior to inoculation, the PPV-inoculum was tested and confirmed to be negative for the presence of viable PCV2 or PCV2-specific nucleic acids by virus isolation on PK-15 cells and by PCR (Fenaux et al.,
2. Sham inoculation for pigs in groups 1 and 3 was done with sterile water (Abbott Laboratories, North Chicago, IL). The pigs were 6-7 weeks old at the time of inoculation.

2.4. Vaccine

The vaccine used in this study was a commercial parvovirus-leptospira-erysipelo... (Lot# A133712), which contained killed PPV, *Leptospira* (serovar bratislava, canicola, grippotyphosa, leptospira, hardjo, icterohaemorrhagiae, and pomona) antigens, and *Erysipelothrix rhusiopathiae*, and was adjuvanted with Amphigen®. Fifty-five pigs (groups 1, 2, 3, and 5) were vaccinated with the PLE-vaccine at three weeks of age. A booster dose was given two weeks later at -10 days prior to inoculation. Each pig was vaccinated intramuscularly in the left neck with 5 ml of the vaccine according to the manufacturer’s instructions.

2.5. Clinical evaluation

Following challenge, the pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 to 6 (0 = normal; 6 = severe) as previously described (Halbur et al., 1995). Rectal temperatures were recorded daily. One day prior to inoculation, and at 14, 21, 28, 35, and 42 DPI the pigs were weighed.

2.6. Serology

Blood samples were taken -30 and -4 days prior to, on the day of inoculation, and at 7, 14, 21, 28, 35, and 42 DPI. A PCV2-ELISA based on the recombinant ORF2 capsid protein of PCV2 was performed on all serum samples as previously described (Nawagitgul et al., 2002). A hemagglutination inhibition (HI) assay for detection of PPV-specific antibodies was performed on all serum samples as previously described (Mengeling et al., 1988). In addition, 42 DPI sera of all pigs were tested for the presence of specific antibodies to PRRSV by PRRSV-ELISA (IDEXX Laboratories, Inc. Westbrook, MA).

2.7. Polymerase chain reaction (PCR) and quantification of PCV2 genomic DNA

Sera collected on DPI 0, 7, 14, 21, 28, 35, and 42 were tested by PCR for PCV2 and PPV-specific nucleic acids by PCR. DNA-extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). A multiplex assay for both viruses was performed simultaneously as previously described (Kim et al., 2001). DNA-extracts were also used for quantification of the amount of PCV2 genomic DNA by real-time PCR as previously
described (Opriessnig et al., 2003).

2.8. Gross pathology and histopathology

At necropsy, the lungs were given a score for the estimated percentage of lung affected by pneumonia (0-100%) (Halbur et al., 1995). The size of five lymph nodes (tracheobronchial, external inguinal, internal iliac, mediastinal, and mesenteric lymph nodes) from each pig were scored individually (0 = normal to 3 = three times the normal size). Tissue samples (lymph nodes, spleen, kidney, liver, brain, tonsil, pancreas, lung, thymus, heart, stomach, colon, and ileum) were collected in 10% neutral buffered formalin, and processed for microscopic evaluation. The microscopic examination and scoring was performed in a blinded fashion. The scoring system used values ranging from 0 to 6 (0 = normal; 6 = severe diffuse interstitial pneumonia) for lung sections and values of 0 to 3 (0 = normal; 3 = severe inflammation) for all other organs (Halbur et al., 1995).

2.9. Immunohistochemistry (IHC) for PCV2 and PPV

Immunohistochemistry for PCV2 was performed on selected lymphoid tissues (lymph nodes, thymus, spleen, and tonsil) as previously described (Sorden et al., 1999). The amount and distribution of PCV2 was scored ranging from 0 (=undetectable) to 3 (=large amount of antigen) in a blinded fashion. To evaluate the amount of PPV in lymphoid tissues, an IHC method for PPV was developed by using monoclonal PPV-antibodies (VMRD, Pullman, WA) at a dilution of 1:300. The amount of PPV was scored from 0 to 3 as was done for PCV2.

2.10. Statistical Analysis

Descriptive statistics for all variables were computed to assess the distributions of the data and identify potential spurious observations. For the different types of data, several inferential methods were used to assess experimental group differences. Continuous repeated measured data were assessed with the conservative multivariate analysis of variance method (MANOVA). In each MANOVA analysis, the group by time interaction had to be non-significant \( (P > 0.05) \) before the group effect was considered. If the group by time interaction was significant, then cross sectional analysis was used to determine the significant time points. Ordinal repeated measures data were assessed with response feature analysis (Everitt, 1995). Non-repeated measures were assessed using analysis of covariance (ANOVA) or non-
parametric ANOVA. If an ANOVA test was significant \((P < 0.05)\), then pair wise tests with Tukey adjustment were used to assess specific group differences. If a non-parametric ANOVA test was significant, then Wilcoxon tests with Bonferroni correction were used to assess the differences of pairs of groups.

3. Results

3.1. Clinical signs

Pigs in the sham-inoculated control group 1 had no signs of clinical disease at any time during the experiment. Three of 12 pigs in the PPV-inoculated group 3 had fevers ranging from 40.4°C to 40.7°C at 1 DPI, and were clinically normal thereafter.

The PCV2-infected group 2 pigs and the PCV2/PPV-coinfected group 4 and 5 pigs showed intermittent fevers between 1 and 21 DPI (Fig. 1). On DPI 6, 10 and 13, the mean daily rectal temperatures in group 4 pigs were significantly \((P < 0.05)\) higher compared to those in group 5 pigs. Group 4 pigs had significantly \((P < 0.05)\) higher mean temperatures compared to those in group 2 pigs from 8 to 21 DPI. Group 5 pigs had significantly \((P < 0.05)\) higher mean temperatures than group 2 pigs on DPI 8, 9, 12, 13, and 17-19.

The clinical manifestation in the coinfected group 4 and 5 pigs was further characterized by sneezing starting from 2 DPI through 21 DPI. Beginning at 13 DPI, sporadic coughing was also observed. Based on the assessment of the weekly body weight data, two wasting pigs were identified in the coinfected groups. One group 4 pig lost 2.7 kg between DPI 14 to DPI 20, and one group 5 pig lost 1.4 kg between 14 and 21 DPI. Both pigs developed severe respiratory disease, jaundice, and depression and had to be euthanized at DPI 20 and DPI 21, respectively. After 21 DPI, clinical disease began to resolve in the coinfected groups and most pigs returned to normal by 28 DPI.

The mean daily weight gain for group 1 pigs was 0.77 kg, for group 2 pigs was 0.70 kg, for group 3 pigs was 0.74 kg, for group 4 pigs was 0.71 kg, and for group 5 pigs was 0.74 kg. Statistically, there was no difference in daily weight gain between the groups \((P = 0.4335)\).

3.2. Serology

Prior to PPV-vaccination, 9/14 group 1 pigs, 8/14 group 2 pigs, and 3/12 group 3 pigs
had residual passive antibody titers to PPV ranging from 1:32 to 1:256. Group specific PPV-antibody titer trends are summarized in Fig. 2. There was a group by time trend at 7, 14, 21, 28, 35, and 42 DPI. PPV-titers in group 1 and 2 (PPV-vaccinated, non-PPV-infected) were not different from each other at any time of the experiment. This was also the case for PPV-titers in groups 3 and 5 (PPV-vaccinated, PPV-infected), which were not different from each other during the experiment. Groups 1 and 2 pigs (PPV-vaccinated) had significantly ($P < 0.05$) lower PPV-titers compared to groups 3 and 5 pigs (PPV-vaccinated, PPV-infected). PPV-titers in group 4 pigs (non PPV-vaccinated, PPV-infected) were significantly ($P < 0.05$) higher than those in all other groups at 7, 14, 35, and 42 DPI.

Pigs in groups 1 and 3 remained free of PCV2-specific antibodies at the end of the experiment at DPI 42 whereas pigs in groups 2, 4, and 5 seroconverted to PCV2 between 21 and 42 DPI. There was no significant difference in serum PCV2-antibody levels in groups 2, 4, and 5. At 42 DPI, all pigs were free of antibodies to PRRSV.

3.3 PCR

All pigs in groups 1-3, and 5 were negative by PCR for PPV-nucleic acids in serum. In group 4, 9/16 pigs were PPV-PCR positive at 7 DPI, and 3/16 pigs were PPV-PCR positive at 14 DPI. The mean PPV-viremia length was determined to be 0.75 ($\pm 0.11$) weeks.

PCR testing for the presence of PCV2-specific nucleic acids in serum revealed no PCR-positive pigs in groups 1 and 3. The majority of the PCV2-infected group 2, 4 and 5 pigs (40/44) were positive for PCV2-specific nucleic acids in sera starting at 7 DPI. The mean PCV2-viremia length was determined by PCV2-PCR results of the pigs that remained until the end of the study. Viremia length was 4.9 $\pm 0.63$ weeks for group 2 pigs, 4.0 $\pm 0.69$ weeks for group 4 pigs, and 6.0 $\pm 0.00$ weeks for group 5 pigs. Groups 2 and 4 ($P = 0.45$), and groups 2 and 5 ($P = 0.06$) were not significantly different from each other, but there was a significant difference in mean PCV2-viremia length between groups 4 and 5 ($P = 0.025$).

3.4. PCV2 genome titer in sera

The mean PCV2 genome copy numbers in sera measured at 7, 14, 21, 28, 35, and 42 DPI were not different between group 2 and 4 pigs, nor was there a difference between group 2 and 5 pigs. Group 5 pigs had significantly ($P < 0.05$) higher PCV2 genome copy numbers in sera at 28, 35, and 42 DPI compared to group 4 pigs (Fig. 3). The highest genomic copy
levels measured in the entire experiment ($1.53 \times 10^9$ and $5.07 \times 10^9$ PCV2 copies/ml serum) were found in sera obtained from the two PMWS-affected pigs from group 4 and 5 at 20 and 21 DPI, respectively. In addition, another pig in group 5 had relatively high PCV2 titers at 14 DPI ($1.06 \times 10^9$ PCV2 copies/ml serum), but the titer decreased over time to $3.8 \times 10^5$ PCV2 copies/ml of serum at DPI 42.

3.5. Macroscopic lesions

At 21 and 42 DPI necropsies, group 1 and 3 pigs had no remarkable gross lesions. In contrast, most of the pigs in groups 2, 4, and 5 had enlargement of all lymph nodes evaluated (Table 2).

At 21 DPI, the two wasting pigs (one in group 4 and one in group 5) were also icteric, and had severe chronic bleeding gastric ulcers of the pars esophagea. In addition, the group 4 wasting pig had moderate edema of the gastric wall, a pneumonia score of 21%, and serous atrophy of fat on the heart. The group 5 wasting pig had a pneumonia score of 15% and had well demarcated, purple lesions especially in the cranioventral part of the lung. This pig also had approximately 200 ml icteric fluid in the abdomen.

At 42 DPI necropsy, three pigs (one group 4 pig and two group 5 pigs) had enlarged tan kidneys with multifocal white spots and streaks.

3.6 Microscopic lesions

Microscopic lesions are summarized in Tables 2 and 3. Mild-to-severe interstitial pneumonia and lymphoplasmacytic hepatitis were observed in pigs in groups 2, 4 and 5 (Table 2). At 21 DPI, there were significantly more severe lymphoplasmacytic interstitial nephritis ($P < 0.05$) and lymphoplasmacytic interstitial hepatitis ($P < 0.05$) lesions in group 4 pigs than in group 2 pigs. Also, the incidence of interstitial nephritis was significantly ($P < 0.05$) higher in group 4 pigs compared to that of group 2 pigs (Table 2). Pigs in groups 2, 4 and 5 had lymphoplasmacytic myocarditis and lymphoplasmacytic meningoencephalitis lesions but there were no differences between the groups in incidence or severity (data not shown). Mild lymphocytic depletion of thymus was observed in one group 5 pig (data not shown). At 42 DPI, the lymphoid depletion in lymph nodes was significantly ($P < 0.05$) more severe in groups 4 and 5 compared to group 2 pigs (Table 3). There was no difference in severity of lymphoid depletion between group 4 and 5.
3.7 Immunohistochemistry for PCV2 and PPV

The immunohistochemical distribution of PCV2 and PPV-antigen in lymph nodes, spleens, and tonsils of all pigs is summarized in Table 3. Between groups there was no difference in amount or incidence of PCV2 antigen in thymus. Individual pigs in groups 2, 4 and 5 pigs had a few PCV2 antigen positive cells at 21 DPI (data not shown).

4. Discussion

Current recommendations for minimizing losses from PCV2-associated PMWS include identifying and minimizing the effect of concurrent infections. In addition to more well established pathogens such as PRRSV, swine influenza virus, and *Mycoplasma hyopneumoniae*, there is now interest in eliminating or minimizing the potential effect of PPV on triggering PCV2 infection to progress to clinical PMWS manifest as progressive wasting, pallor and/or jaundice, and respiratory disease. This is largely based on literature suggesting that characteristic PMWS disease and lesions can be induced in CD or gnotobiotic pigs by PCV2/PPV-coinfection (Allan et al., 1999, 2000c; Kennedy et al., 2000; Krakowka et al., 2000; Kim and Chae, 2002; Kim et al., 2003). We attempted to mimic the field situation in the U.S. by coinfection of conventional 6-7-week-old SEW pigs with PCV2 and PPV. Furthermore, we attempted to decrease the synergistic effect between these two viruses by vaccinating pigs against PPV prior to coinfection.

In this study, we were able to demonstrate the development of PMWS in conventional SEW pigs coinfectected with PCV2 and PPV. The combined mortality in both PCV2/PPV-infected groups was 6.7%, which is similar to PMWS-associated mortality in affected herds in the US. One of the two PMWS-affected pigs in the current experiment was PPV-vaccinated prior to PPV/PCV2 inoculation, while the other pig was not. PCV2 infection, either as single infection or in combination with PPV-infection, induced typical PCV2-associated macroscopic and microscopic lesions. The most obvious difference between the PCV2-single-infected pigs compared to the pigs in both of the PCV2/PPV-coinfectected groups was the clinical manifestation of disease. The coinfectected pigs had a mild-to-severe clinical disease characterized by fevers, sneezing, coughing, and wasting, whereas the singular PCV2-infected pigs remained clinically normal throughout the study. Despite their
vaccination status, the two coinfected groups (PPV-vaccinated as well as non-vaccinated) were similar in terms of clinical presentation, macroscopic, and microscopic lesions.

The PPV-vaccine regime used in this study was not able to reduce clinical PMWS or lymphoid lesions characteristic of PMWS in PCV2/PPV-coinfected pigs. Although there was no measurable PPV-viremia by PCR in the PPV-vaccinated and PPV-infected pigs, the IHC and the anamnestic serological response suggested that PPV-replication in tissues was not completely inhibited by vaccine-induced PPV-antibodies. Low-to-high amounts of PPV were detected by IHC in lymphoid tissues of the majority of PPV-infected pigs, whether or not they were PPV-vaccinated. PPV-infection in pigs other than fetuses is usually not associated with disease. There are some exceptions such as the sporadic observations of dermatitis (Kresse et al., 1985), non-suppurative myocarditis (Bolt et al., 1997), and enteritis (Dea et al., 1985; Duhamel et al., 1991) in young pigs. Brown et al., (1980) experimentally inoculated 6-week-old conventional pigs with PPV and demonstrated PPV-distribution mainly in lymphoid tissues without observation of clinical signs, macroscopic or microscopic lesions. Nevertheless, PPV has been identified as an important potential trigger for PCV2 infections to progress to PMWS in CD and gnotobiotic pigs. Our work further supports that synergism occurs in SEW pigs typical of the current US field production. However, the particular PPV vaccine used in this study did not fully inhibit PPV-replication and did not decrease the incidence of PMWS- or PCV2-associated lesions.

It is difficult to measure the efficacy of PPV vaccine in growing pigs since the strain used in this study by itself does not appear to induce disease or lesions. Explanations for the failure of the vaccine to completely prevent PPV-infection in this study could include inappropriate timing of the PPV-vaccination in terms of age of the pigs at vaccination time, and timing of PPV-vaccination in relation to PPV-infection. Although the presence of passively acquired PPV-antibodies in pigs at the time of vaccination may also play a role, a negative influence on vaccine efficacy in the current study can be excluded. None of the PPV-vaccinated and coinfected group 5 pigs had passively acquired PPV-antibodies prior to vaccination. Investigations carried out in gilts suggested that low PPV-titers at the time of PPV-vaccination did not interfere with immunization, whereas high PPV-titers blocked the response to vaccination; however, after a second dose there was an anamnestic response
Effective control of acute PPV infection is believed to depend on humoral immunity (Ladekjær-Mikkelsen and Nielsen, 2002). All PPV-vaccinated pigs in this study reached PPV-antibody levels higher than 1:128 by the time of PPV-challenge. There are different opinions as to how high the PPV-titers need to be for protection since the definition of “protection” varies. Some groups evaluated the efficacy of the PPV-vaccine based on reduction or prevention of transplacental transfer of PPV (Mengeling et al., 1979), while others define protection as lack of any evidence of PPV-replication in the dam (Suzuki and Fujisaki, 1976). In the current study, there was no detectable PPV-viremia in the PPV-vaccinated and PPV-inoculated pigs; however, four-fold increasing HI-titers and demonstration of PPV-antigen by IHC staining in lymphoid tissues suggests that PPV-replication occurred in the PPV-vaccinated pigs. At DPI 21 and 42, moderate-to-large amounts of PPV antigen were not only present in pigs with “low” serum antibody titers (1:256) at inoculation time, but also in pigs with vaccination titers between 1:512 and 1:1024. There may also be some degree of antigenic variation between the vaccine strain and the challenge strain, though previous studies have failed to confirm antigenic differences among PPV-isolates based on hemagglutination (Molitor and Joo, 1990).

It is not known if different strains of PPV vary in their synergistic effect with PCV2. From the four PCV2/PPV coinfection studies published to date, all the PPV-isolates were from recent field-cases of PMWS. Three of the studies were done with the same Canadian PPV-isolate (Allan et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000) on the basis of an 800-bp sequence described as similar to the NADL and the Kresse strain of PPV (Allan et al., 2000a,b), and one with a Korean PPV-isolate (Kim et al., 2003). The isolate we used is over 25 years old and has been well characterized (Mengeling et al., 1979).

Recent studies suggest that the immunostimulatory effects of bacterins increase PCV2 replication (Allan et al., 2000a, 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). Based on the level of mortality and morbidity in the current study, the PLE-vaccine regime did not increase the severity or incidence of PMWS in the coinfected groups. There was a significantly longer length of PCV2-viremia and higher numbers of PCV2-genomic copies in sera of vaccinated coinfected pigs compared to non-vaccinated coinfected pigs. Although we were not able to experimentally prove a positive effect of PPV-
vaccination in reducing the incidence of clinical PMWS in PCV2/PPV-coinfected pigs, reduction in morbidity and mortality of grow-finish pigs affected by PMWS and respiratory disease has been repeatedly achieved in the field with the use of PPV vaccines (Halbur, 2000, 2001). Another approach to reduce the losses in weaning pigs due to coinfection with PCV2 and PPV could be through hyperimmunization of the breeding herd against PPV. A direct correlation between the antibody titer of the sow’s colostrum and her litter’s average antibody titer has been demonstrated (Paul and Mengeling, 1982; Damm et al., 2002). Sows with higher PPV-antibodies in sera have piglets with higher maternal PPV-antibody titers that decay over a longer period of time. As supported by a recent study (Rodibaugh, 2002), vaccination of sows may even be more practical than vaccinating growing pigs, and the potential adverse vaccine dependent-effect on PCV2 replication in growing pigs could be avoided.

**Acknowledgements**

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**References**


Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., Allan, G.M., 2000. Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone and in


with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. 76, 3232-3239.


Table 1
Experimental design

<table>
<thead>
<tr>
<th>Group number</th>
<th>Number of pigs</th>
<th>Vaccination</th>
<th>Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>+</td>
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<td>PPV</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>+</td>
<td>PPV</td>
</tr>
</tbody>
</table>

*Parvovirus-leptospira-erysipelothrix vaccine.*
Table 2. Macroscopic and microscopic lesions in group 1 (sham-inoculated, PPV-vaccinated), 2 (PCV2-inoculated, PPV-vaccinated), 3 (PPV-inoculated, PPV-vaccinated), 4 (PCV2/PPV-inoculated, non-vaccinated), and 5 (PCV2/PPV-inoculated, PPV-vaccinated) pigs at 21 and 42 days post inoculation

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphadenopathy (0-3(^a))</th>
<th>Interstitial pneumonia (0-6(^b))</th>
<th>Lymphoplasmacytic interstitial nephritis (0-3(^c))</th>
<th>Lymphoplasmacytic hepatitis (0-3(^d))</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>0/7 (0.0[0.0]) A</td>
<td>0/7 (0.0[0.0]) A</td>
</tr>
<tr>
<td>2</td>
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<td>7/7 (1.1[1.1]) B</td>
<td>1/7 (0.1[1.0]) BC</td>
<td>4/7 (0.6[1.0]) B</td>
</tr>
<tr>
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<td>0/6 (0.0[0.0]) A</td>
<td>3/6 (0.4[1.0]) A</td>
<td>0/6 (0.0[0.0]) AB</td>
<td>0/6 (0.0[0.0]) A</td>
</tr>
<tr>
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<td>8/8 (1.6[1.6]) B</td>
<td>6/8 (0.9[1.2]) D</td>
<td>8/8 (1.3[1.3]) C</td>
</tr>
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<td>7/7 (2.0[2.0]) B</td>
<td>4/7 (0.6[1.0]) CD</td>
<td>6/7 (1.3[1.5]) BC</td>
</tr>
<tr>
<td>42 DPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
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<td>1/6 (0.1[1]) AB</td>
<td>0/6 (0.0[0.0]) AC</td>
<td>0/6 (0.0[0.0]) A</td>
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<td>8/8 (2.1[2.1]) C</td>
<td>8/8 (1.5[1.5]) C</td>
<td>4/8 (0.8[1.5]) BC</td>
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</tr>
<tr>
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<td>7/7 (1.1[1.5]) C</td>
<td>3/7 (0.7[1.7]) AB</td>
<td>7/7 (1.0[1.0]) B</td>
</tr>
</tbody>
</table>

\(^a\)Score range (0 = normal; 3 = three times the normal gross size).

\(^b\)Microscopic interstitial pneumonia score (0 = normal; 6 = severe diffuse interstitial pneumonia).

\(^c\)Score range (0 = normal; 3 = severe).

\(^d\)Incidence (mean group score [mean severity for affected animals]).

\(^e\)Different letters indicate significantly (\(P < 0.05\)) different mean group values within columns for 21 or 42 DPI.
Table 3
Lymphoid depletion and amount of PCV2 and PPV-specific antigen demonstrated by immunohistochemistry in lymphoid tissues in group 1 (sham-inoculated, PPV-vaccinated), 2 (PCV2-inoculated, PPV-vaccinated), 3 (PPV-inoculated, PPV-vaccinated), 4 (PCV2/PPV-inoculated, non-vaccinated), and 5 (PCV2/PPV-inoculated, PPV-vaccinated) pigs at 21 and 42 days post-inoculation

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymph nodes</th>
<th>Spleen</th>
<th>Tonsil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depletiona</td>
<td>IHCb PCV2</td>
<td>IHC PPV</td>
</tr>
<tr>
<td>21 DPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0/7 (0.0[0.0]) A</td>
<td>0/7 (0.0[0.0]) A</td>
<td>0/7 (0.0[0.0]) A</td>
</tr>
<tr>
<td>2</td>
<td>7/7 [1.0(1.0)] B</td>
<td>7/7 (1.4[1.4]) B</td>
<td>0/7 (0.0[0.0]) A</td>
</tr>
<tr>
<td>3</td>
<td>0/6 (0.0[0.0]) A</td>
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<td>3/6 (0.5[1.0]) A</td>
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<tr>
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<td>8/8 (1.5[1.5]) B</td>
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<td>6/7 (0.86[1.0]) B</td>
<td>5/7 (0.7[1.0]) B</td>
<td>0/7 (0.0[0.0]) A</td>
</tr>
<tr>
<td>3</td>
<td>0/6 (0.0[0.0]) A</td>
<td>0/6 (0.0[0.0]) A</td>
<td>5/6 (1.0[1.2] BC</td>
</tr>
<tr>
<td>4</td>
<td>8/8 (1.6[1.6]) C</td>
<td>7/8 (1.0[1.1] BC</td>
<td>5/8 (0.8[1.2]) B</td>
</tr>
<tr>
<td>5</td>
<td>7/7 (1.8[1.8]) C</td>
<td>6/7 (1.6[1.8]) C</td>
<td>6/7 (1.4[1.6] C</td>
</tr>
</tbody>
</table>

aScore range (0 = normal; 3 = severe lymphoid depletion of follicles).
bScore range (0 = undetectable; 3 = large amount of antigen).
cIncidence (average value within group [mean severity for affected animals]).
dDifferent letters within columns represent significantly ($P < 0.05$) different mean group values within columns for each DPI.
Fig. 1. Percentage of pigs in groups 2 (PCV2-inoculated, PPV-vaccinated), 4 (PCV2/PPV-inoculated, non-vaccinated), and 5 (PCV2/PPV-inoculated, PPV-vaccinated) with rectal temperatures higher than 40.2°C from 1 to 21 days post-inoculation.
Fig. 2. Mean group PPV-antibody titers in serum of pigs in groups 1 (sham-inoculated, PPV-vaccinated), 2 (PCV2-inoculated, PPV-vaccinated), 3 (PPV-inoculated, PPV-vaccinated), 4 (PCV2/PPV-inoculated, non-vaccinated), and 5 (PCV2/PPV-inoculated, PPV-vaccinated). Error bars represent standard errors.
Fig. 3. Mean group PCV2 genomic copy numbers in sera obtained from groups 2 (PPV-vaccinated, PCV2-infected, $n = 7$), 4 (non-vaccinated, PCV2/PPV-coinfected, $n = 8$), and 5 pigs (PPV-vaccinated, PCV2/PPV-coinfected, $n = 7$). Error bars represent standard errors.
CHAPTER 8: EXPERIMENTAL REPRODUCTION OF POSTWEANING MULTISYSTEMIC WASTING SYNDROME IN PIGS BY DUAL-INFECTION WITH *MYCOPLASMA HYOPNEUMONIAE* AND PORCINE CIRCOVIRUS TYPE 2

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**Abstract.** The objectives of this study were to investigate the interactions between *Mycoplasma hyopneumoniae* and porcine circovirus type 2 (PCV2) and to establish a model for studying the pathogenesis of and testing intervention strategies for the control of PCV2-associated porcine respiratory disease complex (PRDC). Sixty-seven pigs were randomly assigned to four groups. Group 1 (*n* = 17) pigs served as controls, group 2 (*n* = 17) pigs were inoculated with *M. hyopneumoniae*, group 3 (*n* = 17) pigs were dual infected with *M. hyopneumoniae* and PCV2, and group 4 (*n* = 16) pigs were inoculated with PCV2. Pigs were inoculated intratracheally with *M. hyopneumoniae* at 4 weeks of age followed by intranasal inoculation with PCV2 at 6 weeks of age. Dual-infected pigs had moderate dyspnea, lethargy, and reduced weight gain. The overall severity of macroscopic lung lesions, PCV2-associated microscopic lesions in lung and lymphoid tissues, and the amount of PCV2-antigen associated with these lesions were significantly (*P* < 0.05) higher in dual-infected pigs compared with all other groups. Four of 17 (23.5%) dual-infected pigs had decreased growth rate and severe lymphoid depletion and granulomatous lymphadenitis associated with high amounts of PCV2-antigen consistent with postweaning multisystemic wasting syndrome (PMWS). PCV2-antigen in lung tissue was most often associated with *M. hyopneumoniae*
induced peribronchial lymphoid hyperplasia suggesting that this is an important site for PCV2 replication in the lung. This study indicates that *M. hyopneumoniae* potentiates the severity of PCV2-associated lung and lymphoid lesions, increases the amount and prolongs the presence of PCV2-antigen, and increases the incidence of PMWS in pigs.

**Introduction**

Porcine circovirus type 2 (PCV2) is associated with several disease manifestations in swine including postweaning multisystemic wasting syndrome (PMWS).\(^1,20,21,33\) PMWS is characterized clinically by wasting, decreased weight gain, enlarged lymph nodes and dyspnea.\(^20,49\) The hallmark microscopic lesions of PMWS are lymphoid depletion or granulomatous lymphadenitis (or both) and the presence of PCV2 antigen or nucleic acids associated with the lymphoid lesions.\(^49\)

PCV2 alone is limited in its ability to induce the full spectrum of disease and lesions associated with PMWS in pigs\(^3,14,30,41,42,47\) and is considered by some to be a ubiquitous opportunist.\(^49\) Porcine reproductive and respiratory syndrome virus (PRRSV)-induced potentiation of PCV2-associated PMWS has been experimentally confirmed.\(^3,24,47\) Porcine parvovirus (PPV) has also been found to potentiate the progression of PCV2-infection to clinical PMWS.\(^2,25,26,30,41\) The authors are not aware of previous reports of bacterial potentiation of PCV2 infection.

Investigations carried out in the field further support the important role of coinfections in the pathogenesis of PCV2-associated disease. A retrospective analysis of PMWS cases in the Midwest United States revealed that PCV2 alone was found in only 1.9% of all cases investigated.\(^44\) *Mycoplasma hyopneumoniae* was found in combination with PCV2 in 35.5% of the cases, second only to PRRSV/PCV2 coinfection in 51.9% of the cases.\(^44\)

*M. hyopneumoniae* is associated with porcine enzootic pneumonia,\(^18,36\) a disease characterized by high morbidity but low mortality in affected herds. In the early stages of infection, *M. hyopneumoniae* colonizes the luminal surface of bronchial and bronchiolar epithelial cells without invading epithelial cells.\(^5,31,48\) Attachment to the cilia leads to progressive loss of cilia, exfoliation of epithelial cells, and accumulation of inflammatory
cells in airway lumina and around airways. The hallmark lesion associated with \textit{M. hyopneumoniae} is hyperplasia of the bronchus-associated lymphoid tissue (BALT); however, \textit{M. hyopneumoniae} antigen/nucleic acids are not detectable within the hyperplasic BALT. Lymphoid hyperplasia of BALT may lead to obliteration of bronchioles and atelectasis of surrounding alveoli. The authors have commonly observed the presence of abundant PCV2 antigen in the hyperplasic BALT of lungs from field cases confirmed to have \textit{M. hyopneumoniae}/PCV2 coinfection by immunohistochemistry (IHC).

Porcine respiratory disease complex (PRDC) is a condition observed mainly in 8-26-week-old pigs and associated with multiple respiratory pathogens including PRRSV, swine influenza virus (SIV), and \textit{M. hyopneumoniae}. PRDC is characterized by decreased rate of growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea. Recent field investigations suggest that PCV2 may be playing an important role in some cases of PRDC. Studies investigating the relationship of PCV2/\textit{M. hyopneumoniae} coinfection are lacking in the literature. The objectives of this study were to investigate the interactions between \textit{M. hyopneumoniae} and PCV2 and to establish a model for studying the pathogenesis of and testing intervention strategies for the control of PCV2-associated PRDC.

\section*{Materials and Methods}

\subsection*{Animals}

Sixty-seven colostrum-fed, crossbred piglets were purchased from a herd with no history of PCV2-associated diseases, free of \textit{M. hyopneumoniae} and PRRSV based on monthly serological testing of the breeding herd, and routinely using PPV and SIV vaccination of the breeding animals. The pigs were early-weaned at 12 days of age and transported to the livestock infectious disease isolation facility at Iowa State University, Ames, Iowa. The pigs were randomly assigned to four groups of 16-17 pigs each and separated into 4 rooms with 3 pens each and 5-6 pigs per pen.

\subsection*{Experimental design, \textit{Mycoplasma hyopneumoniae}, and PCV2 inocula}

The experimental design is summarized in Table 1. At 4 weeks of age, a tissue homogenate containing strain 232, a derivative of \textit{M. hyopneumoniae} strain 11 challenge inoculum (10^5 color changing units/ml), was administered intratracheally to each pig in
groups 2 and 3 in a dilution of 1 : 100 in 10 ml Friis medium. Before inoculation, the Friis medium and *M. hyopneumoniae* inoculum were tested and confirmed negative for the presence of PCV2-, PCV1-, and PPV-specific nucleic acids by polymerase chain reaction (PCR). 

PCV2 isolate ISU-40895 was obtained through direct transfection of PK-15 cells with an infectious clone of PCV2. Passage four of the virus at a titer of $10^{4.8}$ 50% tissue culture infective dose (TCID$_{50}$) was used for inoculation of the pigs. Each pig in groups 3 and 4 received 6 ml of the PCV2-inoculum intranasally at 6 weeks of age. In addition, each pig in groups 1 and 2 was sham inoculated at 6 weeks of age by administration of 6 ml PK15-cell-supernatant intranasally.

**Clinical evaluation**

Following PCV2 challenge, the pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 to 6 (0 = normal; 1 = mild dyspnea or tachypnea or both when stressed; 2 = mild dyspnea or tachypnea or both when at rest; 3 = moderate dyspnea or tachypnea or both when stressed; 4 = moderate dyspnea or tachypnea or both when at rest; 5 = severe dyspnea or tachypnea or both when stressed; 6 = severe dyspnea or tachypnea or both when at rest). In addition, pigs were evaluated daily for clinical signs including sneezing (score range from 0 [no sneezing] to 3 [severe persistent sneezing]), coughing (score range from 0 [no coughing] to 3 [severe persistent coughing]), and jaundice (score range from 0 [normal] to 3 [severe icterus]). Rectal temperatures, wasting, and behavioral changes such as lethargy were recorded daily. The pigs were weighed on the day of arrival, -14 days before PCV2 inoculation, at the day of PCV2 inoculation and at 7, 14, 21, and 35 days postinoculation (DPI).

**Serology**

Blood samples were collected at -24 and -14 days before, on the day of PCV2 inoculation, and at 7, 14, 21, 28, and 35 DPI. A PCV2-enzyme linked immunosorbent assay (ELISA) based on the recombinant ORF2 capsid protein of PCV2 was performed on -24, -14, 0, 21, and 35 DPI sera samples. Samples were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater.
M. hyopneumoniae titers were determined by ELISA on -14, 21, and 35 DPI sera.\textsuperscript{8} Known positive and negative sera were included on every plate. Readings more than 2 standard deviations above the mean value of the negative control were considered positive.

In addition, -14, 0, and 35 DPI sera from 8 pigs in each group were tested for the presence of antibodies to PRRSV by PRRSV-ELISA (IDEXX Laboratories, Inc. Westbrook, MA), antibodies to SIV subtypes H1N1 and H3N2 by hemagglutination inhibition (HI) assay (protocol used at the Iowa State University Veterinary Diagnostic Laboratory), and for the presence of antibodies to PPV by HI assay.\textsuperscript{38}

**PCV2 quantification**

DNA extraction on sera collected on the day of PCV2 inoculation and at 7, 14, 21, 28, and 35 DPI was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). DNA-extracts were used for quantification of the amount of PCV2 genomic DNA by real-time PCR.\textsuperscript{42}

**Necropsy and histopathology**

Necropsies were performed on half of the pigs in each group at 21 DPI and the remainder at 35 DPI. Lung lesions consistent with mycoplasmal pneumonia (well demarcated, dark red-to-purple consolidated areas in the cranioventral regions) were sketched on a standard diagram and assessed for the proportion of lung surface with lesions using a Zeiss SEM-IPS image analyzing system.\textsuperscript{46} In addition, the total amount of macroscopic lung lesions (0-100% of the lung affected by grossly visible pneumonia) was estimated and recorded for each pig.\textsuperscript{19} The scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each.\textsuperscript{19} The mean size of several lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, mesenteric) was estimated (score range from 0 [normal] to 3 [three times the normal size]) and recorded for each pig.\textsuperscript{42}

Tracheobronchiolar swabs and bronchoalveolar lavage for bacterial and virologic examinations were collected at 21 and 35 DPI.\textsuperscript{37} Fifty milliliters of sterile phosphate-buffered saline (PBS) was used to lavage each lung. Lungs collected at 21 DPI were insufflated with
fixative until the natural contour of the lung was reestablished, and then the right bronchus was clamped off and the right lung was submerged in fixative. Sections of lung from each lobe were collected at 35 DPI and submerged in 10% neutral-buffered formalin. Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral buffered formalin and routinely processed for histologic examination. Microscopic lesions were evaluated in a blinded fashion by two veterinary pathologists (T. Opriessnig and P.G. Halbur). Lung sections were scored for presence and severity of type 2 pneumocyte hypertrophy and hyperplasia, alveolar septal infiltration with inflammatory cells, peribronchial lymphoid hyperplasia , amount of alveolar exudate, and amount of inflammation in the lamina propria of bronchi and bronchioles ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse).

Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 to 3 (0, normal; 1, mild lymphoid depletion with loss of overall cellularity; 2, moderate lymphoid depletion; 3, severe lymphoid depletion with loss of lymphoid follicle structure) and presence of inflammation ranging from 0 to 3 (0, normal; 1, mild histiocytic-to-granulomatous inflammation, 2, moderate histiocytic-to-granulomatous inflammation, 3, severe histiocytic-to-granulomatous inflammation with replacement of follicles).

In addition, Warthin-Starry (WS) silver staining for the identification of cilia-associated respiratory bacillus was done on lung tissues (three sections for each pig) on all pigs necropsied at 21 DPI (n = 35).

**IHC for detection of PCV2 and image analysis for quantification of PCV2-specific staining**

Immunohistochemical detection of PCV2-specific antigen was performed on sections of formalin-fixed, paraffin-embedded blocks of selected tissue samples (lungs [three sections], lymph nodes [one section each of superficial inguinal, external iliac, mediastinal,
tracheobronchial, and mesenteric], tonsil [one section], thymus [one section] and spleen [one section]) using a streptavidin-biotin-peroxidase complex method (DAKO, Carpinteria, CA). The fixed sections were pretreated with 0.05% protease (Sigma, St. Louis, MO), and a rabbit polyclonal antiserum (Identification number ISU-31) was used in a dilution of 1:1000. Assessment of staining for PCV2 antigen was done in a blinded fashion and scores ranged from 0 to 3 (0, negative; 1, less than 10% of the lymphoid follicles have cells with PCV2 antigen staining; 2, 10-50% of the lymphoid follicles contain cells with PCV2 antigen staining; 3, more than 50% of the lymphoid follicles contain cells with PCV2 antigen staining). The mean group score was determined for each tissue and compared among groups.

Image analysis was done on ten randomly selected fields of tracheobronchial lymph node (one section per pig) labeled with anti-PCV2 antibody by IHC from all singular PCV2 and dual-infected pigs. Images were captured with a Sony DXC-S500 color digital camera (Sony Electronics, Inc., Park Ridge, NJ) mounted on a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and processed using KS400 image analysis software (Carl Zeiss). A 20× objective and a 1.25× optivar were used, resulting in a final magnification of 25×. The images were converted from RGB to HLS mode and the red and green color planes added together to isolate stained areas. The areas were then interactively discriminated from the surrounding tissue and measured. The software was calibrated to measure in microns. The total area for each field was 227,319.2 square microns, and the percentage of the mean PCV2-specific stained area compared to the total area was calculated for each pig.

**IHC for detection of *M. hyopneumoniae***

Immunohistochemical detection of *M. hyopneumoniae*-specific antigen on selected lung tissues (three sections for each pig) was done using the heat-induced epitope retrieval (HIER) technique. Paraffin-embedded tissue sections were dewaxed and rehydrated, covered with 1 : 10 ethylenediaminetetraacetic acid butter solution, pH 6.0 (Richard Allan Scientific, Kalamazoo, MI), placed in a microwave and boiled for 5 minutes. After cooling for 20 minutes, the slides were rinsed, and *M. hyopneumoniae* monoclonal antibody (Identification number D79DI-7; Dr. Richard Ross, Iowa State University) was applied in a 1 : 500 dilution for 2 hours at room temperature and further processed by using a labeled streptavidin-biotin
detection kit (DAKO). Specificity of the *M. hyopneumoniae* IHC procedure was evaluated by testing sections from formalin-fixed paraffin-embedded blocks from pigs known positive for other pathogens (SIV, PRRSV, PCV2, *Mycoplasma flocculare*). Specificity was determined to be 100%. There was no evidence of cross-reaction with any of the pathogens tested. Sensitivity was evaluated by comparing the IHC results to those obtained with *M. hyopneumoniae* culture, which is considered the gold standard for *M. hyopneumoniae* detection. The sensitivity of the IHC procedure was determined to be 71% (24/34). Known *M. hyopneumoniae*-positive tissue sections as well as known *M. hyopneumoniae*-negative tissue sections were used as controls for each IHC run. Slides were scored ranging from 0 to 3 (0, no signal detectable; 1, weak labeling lining the ciliated epithelium of at least one airway; 2, weak-to-moderate labeling on the surface of a low number of airways; 3, intense labeling on the surface of several airways).

**Isolation of *M. hyopneumoniae* and other bacteria from lungs**

Isolation of *M. hyopneumoniae* was performed on tracheobronchial swabs that were inoculated into Friis medium. Tracheobronchial and lung airway swabs were also used for routine bacterial culture on MacConkey and sheep blood agar plates.

**Definition and diagnosis of PMWS in this study**

Criteria required for the diagnosis of PMWS in this experiment include weight loss or decreased average daily weight gain compared to negative controls, severe lymphoid depletion and histiocytic inflammation in the majority of lymphoid tissues evaluated, and association of the lymphoid lesions with PCV2-antigen. To evaluate pigs in our experiment we used a scoring system for each lymphoid tissue ranging from 0 to 9 (lymphoid depletion score 0-3; granulomatous inflammation score 0-3; PCV2-IHC score 0-3). The scores (lesions and PCV2-IHC) of the seven lymphoid tissues ([lymph node pool] × 4, tracheobronchial lymph node, spleen, and tonsil) were added together and divided by 7. The lymph node pool consisted of superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes. The mean treatment group lymphoid score was calculated and compared between groups. Pigs were grouped into four categories on the basis of overall microscopic lymphoid lesion scores: I (normal; score = 0), II (mild; score = 1-3), III (moderate; score = 4-6) and IV
(severe; score = 7-9). A pig was diagnosed with PMWS if it had decreased weight gain or weight loss and if the mean lymphoid microscopic lesion severity score was in category IV.

**Statistical analysis**

Summary statistics were calculated for each group cross-sectionally to assess the overall quality of the data. Analysis of variance (ANOVA) was used for cross-sectional assessment of the average daily weight gain and non-repeated continuous measures. The rejection level for the null hypothesis was 0.05 followed by pairwise testing using the Tukey Kramer adjustment to identify the groups that were different. The chi-square test was used to analyze clinical scores and daily rectal temperature data were analyzed with multivariate ANOVA. Nonrepeated measures of necropsy and histopathology data were assessed using nonparametric Kruskal-Wallis ANOVA. If a nonparametric ANOVA test was significant ($P < 0.05$), then Wilcoxon tests were used to assess the differences of pairs of groups. Differences in incidence were evaluated by using Fisher’s exact test. Kendall’s tau was used for nonparametric correlations.

**Results**

**Clinical disease**

Singular *M. hyopneumoniae*- and dual-infected pigs exhibited respiratory disease which was mild-to-moderate in the singular *M. hyopneumoniae*-infected pigs and mild-to-severe in the dual-infected pigs and was characterized mainly by increased respiratory rates, lethargy, coughing, and occasionally sneezing. Coughing in dual-infected pigs was significantly ($P = 0.01$) more severe compared to pigs infected singularly with *M. hyopneumoniae*. Coughing was not observed in control pigs or singular PCV2-infected pigs. Sneezing was occasionally observed in singular PCV2-infected pigs. Singular *M. hyopneumoniae*- and dual-infected pigs had significantly ($P < 0.05$) higher respiratory disease scores compared to all other groups. Dual-infected pigs had significantly ($P < 0.05$) higher respiratory scores compared to singular *M. hyopneumoniae*-infected pigs except in the 1st week after PCV2-inoculation, when the two groups were not different from each other. The majority of the dual-infected pigs had rough hair coats and appeared to be falling behind their cohorts in the other treatment groups.
Individual pigs within all groups had periodically elevated temperatures. None of the pigs developed persistent fever. Assessment of the mean rectal temperatures showed no significant ($P = 0.06$) differences between groups.

There was no difference in mean group weight on the day of $M.\ hyopneumoniae$ inoculation ($P = 0.318$) or at the day of PCV2 inoculation ($P = 0.207$), and there was no difference ($P = 0.162$) in average daily weight gain in the 2 weeks before PCV2 inoculation. The average daily weight gain in the 1st 3 weeks after PCV2 inoculation was $721.7 \pm 16.4$ grams for the control pigs, $662.2 \pm 23.8$ grams for the singular $M.\ hyopneumoniae$-infected pigs, $576.6 \pm 26.2$ grams for the dual-infected pigs, and $678.8 \pm 32.5$ grams for the singular PCV2-infected pigs. During this time period, the average daily weight gain was significantly ($P < 0.02$) decreased in the dual-infected pigs compared to all other groups. The average daily weight gain from 21 to 35 DPI was not different ($P = 0.605$) among the groups.

**Serology**

All pigs were serologically negative for $M.\ hyopneumoniae$-specific antibodies before $M.\ hyopneumoniae$ challenge. Singular $M.\ hyopneumoniae$- and dual-infected pigs seroconverted to $M.\ hyopneumoniae$ between 21 and 35 DPI. At 21 DPI, 12/17 dual-infected pigs were positive, 1/17 dual-infected pigs were in the suspect positive category, and 4/17 dual-infected pigs were negative for $M.\ hyopneumoniae$-specific antibodies. There were 5/17 singular $M.\ hyopneumoniae$-infected pigs that were positive, 7/17 singular $M.\ hyopneumoniae$-infected pigs were suspect, and 5/17 singular $M.\ hyopneumoniae$-infected pigs were negative for $M.\ hyopneumoniae$ specific antibodies at 21 DPI. By 35 DPI, 8/8 dual-infected and 7/8 singular $M.\ hyopneumoniae$-infected pigs had seroconverted to $M.\ hyopneumoniae$. Dual-infected pigs had significantly ($P < 0.05$) higher $M.\ hyopneumoniae$ S/P ratios compared to singular $M.\ hyopneumoniae$-infected pigs at 21 and at 35 DPI. Control and singular PCV2-infected pigs remained negative for $M.\ hyopneumoniae$-specific antibodies.

At arrival, the pigs had maternal antibodies to PCV2, which decayed below ELISA cutoff levels (S/P ratio < 0.2) before PCV2 inoculation. Control and singular $M.\ hyopneumoniae$-infected pigs remained PCV2 antibody negative. Singular PCV2 and dual-infected pigs seroconverted to PCV2 between 21 and 35 DPI. There were 11/17 dual-infected
pigs and 6/16 singular PCV2-infected pigs positive for PCV2-specific antibodies by 21 DPI. By 35 DPI, all singular PCV2- and dual-infected pigs had seroconverted to PCV2. At 21 and at 35 DPI, S/P ratios were not different between the two PCV2-infected groups.

All pigs tested were negative for PRRSV as determined by ELISA at -14, 0, and 35 DPI. Some pigs had low maternal antibody titers to SIV and PPV which decreased over time as expected in pigs from a vaccinated breeding herd.

**PCV2 viremia length and genomic copy numbers**

Control and singular *M. hyopneumoniae*-infected pigs were negative by PCR for the presence of PCV2 nucleic acids in sera throughout the study. At 7 DPI, PCV2 viremia was detected in 11/17 dual-infected pigs and in 4/16 singular PCV2-infected pigs. All dual and singular PCV2-infected pigs were viremic by 14 DPI. The duration of the PCV2-viremia was based on results from the eight dual-infected and eight singular PCV2-infected pigs that remained in the study for the entire 35 days. PCV2-viremia was determined to be 4.6 ± 0.26 weeks in the dual-infected pigs compared with 3.9 ± 0.40 weeks in the singular PCV2-infected group (*P* = 0.138). The mean copy number of PCV2 genomic DNA in the dual-infected pigs was significantly higher at 14 (*P* < 0.01) and 21 (*P* < 0.05) DPI compared to the singular PCV2-infected pigs (Fig. 1). At 7, 28, and 35 DPI, there was no significant difference in the amount of PCV2 genomic DNA in the sera, although dual-infected pigs had higher values.

**Macroscopic lesions**

Macroscopic lesions are summarized in Table 2. Mean percentages of the lung tissue with grossly visible pneumonia in singular *M. hyopneumoniae*-infected pigs and dual-infected pigs were most severe at 21 DPI and generally appeared to be resolving by 35 DPI. The severity (%) of *M. hyopneumoniae*-associated lung lesions did not differ between singular *M. hyopneumoniae*- and dual-infected pigs, although the total gross lung lesion scores were significantly (*P* < 0.05) more severe in dual-infected pigs at 21 DPI compared with all other groups and at 35 DPI compared to singular PCV2-infected pigs and controls (Fig. 2-5). Lymph nodes in dual- and singular PCV2-infected pigs were two to three times normal size on both necropsy days, whereas control and singular *M. hyopneumoniae*-infected
pigs had essentially normal-sized lymph nodes.

**Microscopic lesions**

Microscopic lesions are summarized in Tables 3-5. All treatment groups except controls (Fig. 6) had interstitial pneumonia characterized by type 2 pneumocyte hypertrophy and hyperplasia and alveolar wall thickening by macrophages and lymphocytes (Table 3). If present, these lesions were mild in the singular *M. hyopneumoniae*-infected pigs, mild-to-moderate in the singular PCV2-infected pigs and mild-to-severe in the dual-infected pigs (Fig. 7). Lungs from singular *M. hyopneumoniae*- and dual-infected pigs also had moderate-to-severe peribronchiolar and perivascular lymphohistiocytic cuffing and nodule formation, moderate-to-severe increase in alveolar exudate and eosinophilic fluid, lymphohistiocytic inflammation in the lamina propria of airways, and mixed inflammation in the lumina of the airways (Fig. 8, 9). There was clumping and loss of cilia, sloughing of epithelial cells into the airway lumina, mild necrosis and ulceration of epithelium over hyperplastic peribronchiolar lymphoid nodules, and infiltration of mononuclear cells in the lamina propria in the *M. hyopneumoniae*-infected groups. In addition, some PCV2- and dual-infected pigs had mild peribronchial fibroplasia. Multinucleated giant cells were infrequently observed in peribronchial areas of lymphoid hyperplasia.

Lesions in the lymphoid tissues are summarized in Tables 4 and 5. Control and singular *M. hyopneumoniae*-infected pigs had essentially normal lymphoid tissues. PCV2-associated lesions in lymphoid tissues were characterized by mild-to-severe lymphoid depletion and mild-to-severe histiocytic-to-granulomatous inflammation with low-to-moderate numbers of multinucleated giant cells. Low-to-moderate numbers of macrophages and multinucleated giant cells contained several, variable-sized, spherical, basophilic intracytoplasmic inclusion bodies. If present (31/34 singular PCV2- and dual-infected pigs), the lymphoid lesions were restricted to one or two of the seven lymphoid tissues examined in 16/31 (51.8%) of the pigs. Two of the singular PCV2-infected pigs had severe lymphoid depletion and moderate histiocytic inflammation; however, this was restricted to individual lymph nodes, was not associated with high amounts of PCV2 antigen (PCV2 IHC score 0 and 1, respectively), and was not observed in tonsil or spleen. The individual scores for overall severity of microscopic lymphoid lesions associated with PCV2 antigen in dual and
singular PCV2-infected pigs are summarized in Fig. 10. The mean group severity score for lymphoid lesions was 4.8 ± 0.8 for the dual-infected pigs and 2.1 ± 0.6 for the singular PCV2-infected pigs at 21 DPI, 3.1 ± 0.7 for the dual-infected pigs and 1.8 ± 0.5 for the singular PCV2-infected pigs at 35 DPI. Four dual-infected pigs were in category IV (three were necropsied at 21 DPI, and one was necropsied at 35 DPI) and had decreased rate of weight gain and moderate respiratory disease and fulfilled our definition for PMWS.

Individual pigs in all groups had mild-to-moderate lymphohistiocytic myocarditis, mild-to-moderate lymphohistiocytic hepatitis, mild-to-moderate lymphohistiocytic interstitial nephritis, and mild lymphohistiocytic enteritis. Dual-infected pigs had significantly \((P < 0.05)\) more severe lymphohistiocytic myocarditis compared with control pigs and \textit{M. hyopneumoniae}-infected pigs but not compared with the singular PCV2-infected group at DPI 21 and 35. The four pigs that were diagnosed with PMWS had moderate lymphohistiocytic myocarditis and hepatitis, moderate lymphohistiocytic-to-granulomatous nephritis, and mild-to-moderate histiocytic-to-granulomatous inflammation of Peyer’s patches.

**IHC, image analysis, and \textit{M. hyopneumoniae} isolation**

The estimated mean amount of PCV2 antigen in lung and lymphoid tissues by groups as determined by IHC is summarized in Tables 3-5. PCV2-antigen in lung tissue was most often associated with cells that had morphological features consistent with macrophages or dendritic cells in areas of \textit{M. hyopneumoniae}-induced peribronchial lymphoid hyperplasia (Fig. 11), within the expanded lamina propria beneath areas of \textit{M. hyopneumoniae} colonization and damage to epithelial cells (Fig. 12), within alveolar macrophages and in cells morphologically consistent with septal fibroblasts. In these cells, abundant staining was observed mainly in the cytoplasm but also in few nuclei (Fig. 7 inset). Compared with singular PCV2-infected pigs, dual-infected pigs had significantly \((P < 0.05)\) more PCV2 antigen in lymph nodes at 21 (Fig. 13) and 35 DPI, in spleen at 21 DPI, and in the lung at 35 DPI. Two dual-infected pigs and one PCV2-infected pig had a few PCV2 positive cells in the thymus (data not shown).

Image analysis on tracheobronchial lymph nodes demonstrated a significant \((P < 0.05)\) difference between the singular PCV2- and the dual-infected pigs at both 21 and 35
DPI. Because of their distribution, the data are presented as medians with a 2.5% to 97.5% interval that contains 95% of the data. There was a median stained area of 1,173.1 (0.0; 15,883.2) square microns (0.516% of the total area) in the dual-infected pigs and 20.9 (0.0; 732.8) square microns (0.009% of the total area) in the singular PCV2-infected pigs at 21 DPI. There was a median stained area of 238.6 (2.1; 2,241.4) square microns (0.105% of the total area) in the dual-infected pigs and 5.5 (0.0; 174.2) square microns (0.002% of the total area) in the singular PCV2-infected pigs at 35 DPI. The correlation between IHC scoring and image analysis data was high (0.78) and statistically significant ($P < 0.0001$). Those pigs with IHC scores of 0 (n = 13), 1 (n = 10), 2 (n = 7), and 3 (n = 3) had mean stained areas of 11.8 ± 7.2 (0.01% of the total area), 122.0 ± 69.5 (0.05% of the total area), 1,207.3 ± 342.5 (0.53% of the total area), and 9,638.1 ± 3,368.3 (4.24% of the total area) square microns, respectively.

The IHC results for *M. hyopneumoniae* are summarized in Table 3. *M. hyopneumoniae* antigen was associated with the surface of epithelial cells of bronchi and bronchioli (Fig. 14). At 35 DPI, there was significantly ($P < 0.05$) more *M. hyopneumoniae* antigen in dual-infected pigs compared with singular *M. hyopneumoniae*-infected pigs, but there was no difference ($P = 0.315$) in incidence between the two groups. *M. hyopneumoniae* was isolated from 0/17 control pigs, from 17/17 singular *M. hyopneumoniae*-infected pigs, and from 17/17 dual-infected pigs. *M. hyopneumoniae* was also isolated from 1/16 singular PCV2-infected pigs. This pig was necropsied at 35 DPI and had no lesions characteristic for *M. hyopneumoniae* infection (peribronchial lymphoid hyperplasia score = 0). IHC staining for *M. hyopneumoniae* on this pig was negative and the pig was seronegative for antibodies to *M. hyopneumoniae* at necropsy. Therefore, this likely is a case of contamination at some point in sample handling and processing.

**Bacteriology**

Cultures of tracheobronchial and lung swabs of all pigs were negative for common swine respiratory pathogens including *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Streptococcus suis*, *Salmonella* sp., *Actinobacillus* sp., and others.
Organisms morphologically consistent with cilia-associated respiratory bacillus were not detected in any of the lung sections of the 35 pigs tested as determined by WS silver staining.

Discussion

PCV2 is now considered an important contributor to the PRDC. The overall goal of this study was to investigate the potential interaction between PCV2 and *M. hyopneumoniae*. We demonstrated that dual-infection of pigs with *M. hyopneumoniae* and PCV2 results in increased severity of pneumonia and increased incidence of PMWS. Four of 17 (23.5%) of the dual-infected pigs had clinical signs and microscopic lesions consistent with PMWS, whereas none of the singular PCV2-infected pigs developed PMWS. This is to our knowledge the first report of bacterial potentiation of PCV2-infection resulting in PMWS in experimentally infected pigs. As in the results reported here, *M. hyopneumoniae* significantly prolonged and increased the severity of PRRSV-induced pneumonia; however, *M. hyopneumoniae* had no effect on concurrent SIV infection. Neither those studies nor the work described here demonstrated a virus-dependent enhancement of mycoplasmal pneumonia.

*M. hyopneumoniae* infection increased the amount of PCV2 genomic copy numbers in the sera, prolonged the presence of PCV2 antigen in lymphoid and lung tissues and increased the amount of PCV2 antigen in lung tissues in dual-infected pigs. PCV2-associated lesions and the amount of PCV2 antigen associated with the lesions were enhanced by concurrent infection by viruses (PRRSV, PPV) that replicate in cells of the monocyte/macrophage lineage. Two recent in vitro studies reported that monocytes, macrophages, and dendritic cells were successfully infected with PCV2; however, PCV2 was not found to replicate in vitro in these cell types under culture conditions. PCV2 replication depends on the availability of host cell polymerase which is active in regularly dividing cells. Although tissue macrophages are capable of cell division, usually less than 5% of the resident macrophage population arise de novo. Unlike PCV2, PPV and PRRSV both readily replicate in the monocyte lineage in vitro. Because both
PPV and PRRSV have been shown to enhance PCV2 replication in vivo, it is possible that these viruses activate the macrophages in a way that supports PCV2 replication.

*M. hyopneumoniae* infection induces an infiltration of inflammatory cells in BALT areas, and PCV2 antigen in dual-infected pigs was demonstrated mainly in the cytoplasm and also in a few nuclei of cells with morphologic features consistent with macrophages, dendritic-like cells, and fibroblasts in areas associated with *M. hyopneumoniae* colonization. In situ hybridization and IHC have failed to demonstrate *M. hyopneumoniae* in these cells; however, the immune response induced by *M. hyopneumoniae* is complex with reports of both immunostimulation and immunosuppression, nonspecific mitogenic effects on swine lymphocytes, immunogenic mycoplasmal proteins, or cytokines secreted by activated macrophages. Hence, the mitogenic activity of *M. hyopneumoniae* could upregulate the macrophage proliferation in the lung, thereby supporting PCV2 replication at the site of infection.

It has been shown that PCV2 is not degraded upon uptake by bone marrow- or blood monocyte-derived dendritic cells in vitro, suggesting that these cells provide potentially important vehicle for transport of the virus throughout the body and tissues. Macrophages and dendritic cells are upregulated by *M. hyopneumoniae*, and thus *M. hyopneumoniae* coinfection might promote systemic distribution of PCV2. This could explain how localized *M. hyopneumoniae* infection could trigger systemic PCV2-infection to progress to PMWS as shown in this model.

It has been proposed that the diagnosis of PMWS requires a pig to exhibit characteristic clinical signs (wasting), histological lesions (lymphoid depletion and granulomatous inflammation), and PCV2 antigen associated with these lesions. Under field conditions, many other factors in addition to PCV2 infection can cause a pig to exhibit signs of wasting as described in the context of the current definition of PMWS. It is also not clear whether wasting actually means weight loss or decrease in rate of average daily gain (and the appearance of wasting) compared to healthy cohorts. Furthermore, the current PMWS definition neither accounts for distribution or severity of lesions nor for relative amount of PCV2 antigen in association with the lesions. Lymphoid depletion and granulomatous inflammation can be present in various degrees and diffusely distributed throughout all
lymphoid tissues or restricted to one or two lymph nodes in individual pigs. It is unknown what consequences these variable manifestations of lymphoid lesions have for individual pigs. It seems likely that even the pigs with mild lymphoid depletion may be immunologically compromised to some degree and perhaps more susceptible to secondary infections and more at risk for vaccination failures. To address this problem, and to properly distinguish between PCV2 infection (subclinically or associated with other diseases) and PMWS, an overall severity score for microscopic lymphoid lesions that accounted equally for distribution (lymph nodes, spleen, and tonsil), severity of lymphoid depletion, severity of inflammation, and amount of PCV2 antigen was calculated. Microscopic lesions (mild, moderate, and severe PCV2-associated lymphoid depletion and granulomatous inflammation) have been staged on cases of naturally occurring PMWS.\textsuperscript{10,45} We believe that the mean group severity score for lymphoid lesions as developed and used in this model allows for a more uniform understanding and interpretation of PCV2-associated lesions.

Some dual-infected pigs in this study had severe dyspnea (persistent coughing and moderate-to-severe respiratory score) in combination with decreased weight gain and macroscopic (more than 40% of the lung affected by lesions) and microscopic lung lesions (moderate-to-severe bronchointerstitial pneumonia) consistent with PRDC. The macroscopic and microscopic lung lesions of these pigs were remarkably similar to those observed with PRRSV/PCV2 coinfection.\textsuperscript{24} These pigs also had lymphoid depletion, histiocytic inflammation of lymph nodes, and PCV2 antigen associated with the lesions. However, we found that the lymphoid lesions lacked the overall severity and distribution to clearly classify them as cases of PMWS. We believe that PCV2-associated pneumonia and PCV2-associated lymphoid depletion are more descriptive terms in such cases.

It is of interest that at 21 and at 35 DPI, the dual-infected pigs had a significantly ($P < 0.05$) higher serum antibody response to \textit{M. hyopneumoniae} compared with the singular \textit{M. hyopneumoniae}-infected pigs. There was also a trend to a higher antibody response to PCV2 in dual-infected pigs compared to the singular PCV2-infected pigs at 35 DPI ($P = 0.055$). Similar results have been reported with PPV antibody response in PCV2/PPV dual-infected pigs.\textsuperscript{41} The three pigs that were diagnosed with PMWS in the current study did not seroconvert to \textit{M. hyopneumoniae} by 21 DPI. The one PMWS pig that remained in the study
through 35 DPI was negative at 21 DPI and had a weak positive antibody response (near ELISA cutoff value) to *M. hyopneumoniae* by 35 DPI. Three of the four PMWS pigs seroconverted to PCV2 at 21 DPI but had lower S/P ratios than the other pigs in this group. Similarly, the antibody response in PCV2-infected, immunostimulated, PMWS-affected pigs was markedly reduced. The dual-infected pigs in this study certainly had more stimulus in the form of inflammation than did the singular-infected pigs. Thus, in the context of additional stimulation (coinfection or nonspecific immunostimulation), the B-cell response in PCV2-infected non-PMWS pigs appeared to be enhanced due to PCV2 infection, whereas it is severely diminished in pigs that progressed to PMWS. Up-regulation of certain cytokines during the course of inflammation can lead to enhanced proliferation of T lymphocytes and B lymphocytes inducing B cell- and plasma cell-dependent antibody production which could explain the better response in the non-PMWS pigs. It appears that if the inflammatory stimulation reaches a certain level, it has more of a counterproductive effect subsequently leading to PMWS. However, the quality of the enhanced B-cell response, the presence of an enhanced T-cell response, and inflammatory aspects in the dual-infected pigs were not evaluated in this study, thus, making any conclusions about possible alterations of the immune system speculative. This coinfection model should allow us to further explore those questions.

The PCV2/*M. hyopneumoniae* coinfection model presented here demonstrated that, by themselves, PCV2 and *M. hyopneumoniae* induce mild transient respiratory disease and lesions but in concurrent infection induce severe respiratory disease and lesions consistent with PMWS as well as PRDC. The sequence of infection (*M. hyopneumoniae* followed 2 weeks later by PCV2) of this dual-infection model was designed to address our hypothesis that *M. hyopneumoniae* lesions are present first and create an ideal environment and cell-type accumulation in the lung for PCV2 replication. This may or may not be typical of the sequence of infection in the field. After weaning and mixing of pigs at 2-4 weeks of age, *M. hyopneumoniae* is commonly spread slowly throughout the population. PCV2 infection likely coincides with waning of maternal antibodies over a large window of time from 5-11 weeks of age. The exact timing and sequence of infection of pigs with these two pathogens likely
differs from pig to pig and from herd to herd and these different scenarios should be further explored to see if there are similar effects as observed in this model.

This dual-infection model will be very useful for testing vaccine and antimicrobial intervention strategies. Experimental PCV2 vaccines have been shown to protect PCV2-infected pigs from developing the characteristic PCV2-associated lymphoid lesions.\textsuperscript{15,16} \textit{M. hyopneumoniae} vaccines are well established in the field and have been shown to be economically beneficial in reducing losses associated with respiratory disease.\textsuperscript{35} However, recent field studies have indicated that \textit{M. hyopneumoniae} vaccination in PMWS-affected herds actually increased the incidence of PMWS.\textsuperscript{4,32} This information has led some practitioners to discontinue the use of \textit{M. hyopneumoniae} vaccination programs in affected herds. Based on the PCV2/\textit{M. hyopneumoniae} model, the detrimental effects from lack of \textit{M. hyopneumoniae} control may be more costly than the detrimental effects of vaccine induced enhancement of PCV2 replication. This risk and benefit associated with \textit{M. hyopneumoniae} vaccination should be thoroughly assessed. It also needs to be determined what vaccine regimen (singular PCV2 or \textit{M. hyopneumoniae} or a combination of both) is most efficient in reducing PRDC and PMWS in pigs from herds that are at a high risk of dual-infection by \textit{M. hyopneumoniae} and PCV2.

**Acknowledgements**

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**References**


29 Kishima M, Ross RF: Suppressive effect of nonviable Mycoplasma hyopneumoniae on


40 Nawagitgul P, Harms PA, Morozov I, Thacker BJ, Sorden SD, Lekcharoensuk C, Paul
PS: Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin Diagn Lab Immunol 9:33-40, 2002


the bronchus-associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Vet Pathol **40**:395-404, 2003

49 Sorden SD: Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). J Swine Health Prod **8**:133-136, 2000


55 Thacker EL, Thacker BJ, Young TF, Halbur PG: Effect of vaccination on the potentiation of porcine reproductive and respiratory syndrome virus (PRRSV)-induced pneumonia by *Mycoplasma hyopneumoniae*. Vaccine **18**:1244-1252, 2000


58 Vincent IE, Carrasco CP, Herrmann B, Meehan BM, Allan GM, Summerfield A, McCullough KC: Dendritic cells harbor infectious porcine circovirus type 2 in the
**Table 1.** Experimental design.

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>No. of Pigs Necropsied at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Weeks* -14 Days before PCV2 inoculation</td>
<td>6 Weeks DPI 0† 9 Weeks DPI 21 11 Weeks DPI 35</td>
</tr>
<tr>
<td>1 (n=17)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2 (n=17)</td>
<td><em>Mycoplasma hyopneumoniae</em></td>
<td>None</td>
</tr>
<tr>
<td>3 (n=17)</td>
<td><em>Mycoplasma hyopneumoniae</em></td>
<td>PCV2</td>
</tr>
<tr>
<td>4 (n=16)</td>
<td>None</td>
<td>PCV2</td>
</tr>
</tbody>
</table>

*Age of the pigs.
†Days post inoculation (DPI) with porcine circovirus type 2 (PCV2).
Table 2. Macroscopic lesions at 21 and 35 days after PCV2 inoculation. Data presented as group mean ± SE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>Percentage of Lung with Pneumonia</th>
<th>Gross</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total†</td>
<td>M. hyopneumoniae Lesions‡</td>
<td>Lymphadenopathy*</td>
</tr>
<tr>
<td>21 after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>Sham inoculation</td>
<td>0.00±0.00^A§</td>
<td>0.07±0.03^A</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td>M. hyopneumoniae</td>
<td>16.55±3.18^B</td>
<td>12.60±3.43^B</td>
</tr>
<tr>
<td>3 (n = 9)</td>
<td>M. hyopneumoniae and PCV2</td>
<td>33.22±6.64^C</td>
<td>22.53±6.35^B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>0.63±0.50^A</td>
<td>0.45±0.29^A</td>
</tr>
<tr>
<td>35 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 8)</td>
<td>Sham inoculation</td>
<td>0.00±0.00^A</td>
<td>0.12±0.07^A</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>M. hyopneumoniae</td>
<td>10.50±3.27^B</td>
<td>6.05±1.68^B</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>M. hyopneumoniae and PCV2</td>
<td>23.88±5.91^B</td>
<td>6.60±1.85^B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>1.00±0.87^A</td>
<td>0.67±0.43^A</td>
</tr>
</tbody>
</table>

*Score range from 0 (normal) to 3 (three times the normal size).
†Total amount (0-100%) of the entire lung affected by any type of pneumonia lesion.
‡Well-demarcated cranioventral purple-tan lesions suggestive of *M. hyopneumoniae* infection.
§Different superscripts (A, B, C) within each column indicate significantly (*P* < 0.001) different values in mean score among treatment groups at 21 or 35 DPI.
Table 3. Microscopic lung lesions in pigs in group 1 (sham-inoculated), 2 (singular *Mycoplasma hyopneumoniae*-infected), 3 (*M. hyopneumoniae* and PCV2 dual infected), and 4 (singular PCV2 infected) at 21 and 35 days after PCV2 inoculation. Data presented as incidence (group mean [mean severity score for affected animals]).

<table>
<thead>
<tr>
<th>Group</th>
<th>Type 2 Pneumocyte Hypertrophy</th>
<th>Septal Infiltration</th>
<th>Peribronchiolar Lymphoid Hyperplasia</th>
<th>Alveolar Exudate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6*</td>
<td>0-6*</td>
<td>0-6*</td>
<td>0-6*</td>
</tr>
<tr>
<td>21 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>1/9 (0.1[1.0])^A^‡</td>
<td>4/9 (0.4[1.0])^A</td>
<td>1/9 (0.1[1.0])^A</td>
<td>2/9 (0.2[1.0])^A</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td>9/9 (1.9[1.9])^B</td>
<td>9/9 (1.3[1.3])^B</td>
<td>9/9 (3.7[3.7])^B</td>
<td>8/9 (3.7[4.1])^B</td>
</tr>
<tr>
<td>3 (n = 9)</td>
<td>9/9 (3.0[3.0])^C</td>
<td>8/8 (2.6[2.6])^C</td>
<td>9/9 (2.9[2.9])^B</td>
<td>9/9 (4.1[4.1])^B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>8/8 (1.4[1.4])^B</td>
<td>8/8 (1.4[1.4])^B</td>
<td>4/8 (0.6[1.3])^A</td>
<td>8/8 (1.6[1.6])^C</td>
</tr>
<tr>
<td>35 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 8)</td>
<td>0/8 (0.0[0.0])^A</td>
<td>8/8 (1.1[1.1])^A</td>
<td>3/8 (0.4[1.0])^A</td>
<td>3/8 (0.4[1.0])^A</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>6/8 (1.0[1.3])^B</td>
<td>8/8 (1.4[1.4])^B</td>
<td>8/8 (4.3[4.3])^B</td>
<td>6/8 (2.9[3.8])^B</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>8/8 (2.4[2.4])^C</td>
<td>8/8 (3.2[3.2])^C</td>
<td>8/8 (4.5[4.5])^B</td>
<td>8/8 (3.5[3.5])^B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>7/8 (1.3[1.4])^B</td>
<td>8/8 (2.0[2.0])^B</td>
<td>5/8 (1.1[1.8])^A</td>
<td>7/8 (1.4[1.6])^C</td>
</tr>
</tbody>
</table>

*Score range from 0 (normal) to 6 (severe diffuse).
†Score range as determined by immunohistochemistry (0 = undetectable; 3 = large amount of antigen).
‡Different superscripts (A, B, C) indicate significantly (*P* < 0.05) different mean group severity scores within columns for each DPI.
<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation of Lamina Propria</th>
<th>Necrotizing Bronchiolitis</th>
<th>Amount of PCV2 Antigen in Lung Tissue</th>
<th>Amount of ( M. ) hyopneumoniae Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6*</td>
<td>0-6*</td>
<td>0-3†</td>
<td>0-3†</td>
</tr>
<tr>
<td>21 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>0/9 (0.0[0.0])(^A)</td>
<td>0/9 (0.0[0.0])(^A)</td>
<td>0/9 (0.0[0.0])(^A)</td>
<td>0/9 0.0[0.0](^A)</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td>9/9 (2.6[2.6])(^B)</td>
<td>1/9 (0.1[1.0])(^A)</td>
<td>0/9 (0.0[0.0])(^A)</td>
<td>8/9 1.7[1.9](^B)</td>
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<tr>
<td>3 (n = 9)</td>
<td>9/9 (3.0[3.0])(^B)</td>
<td>7/9 (1.0[1.2])(^B)</td>
<td>6/9 (0.8[1.2])(^B)</td>
<td>7/9 1.4[1.9](^B)</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>5/8 (0.9[1.4])(^C)</td>
<td>2/8 (0.6[2.5])(^A,B)</td>
<td>2/8 (0.3[1.0])(^A,B)</td>
<td>0/8 0.0[0.0](^A)</td>
</tr>
<tr>
<td>35 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 8)</td>
<td>2/8 (0.3[1.0])(^A)</td>
<td>0/8 (0.0[0.0])(^A)</td>
<td>0/8 (0.0[0.0])(^A)</td>
<td>0/8 0.0[0.0](^A)</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>7/8 (1.4[1.6])(^B)</td>
<td>0/8 (0.0[0.0])(^A)</td>
<td>0/8 (0.0[0.0])(^A)</td>
<td>3/8 0.4[1.0](^A)</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>8/8 (2.8[2.8])(^C)</td>
<td>6/8 (1.3[1.7])(^B)</td>
<td>6/8 (0.8[1.0])(^B)</td>
<td>6/8 1.4[1.8](^B)</td>
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<tr>
<td>4 (n = 8)</td>
<td>5/8 (0.8[1.2])(^B)</td>
<td>2/8 (0.3[1.0])(^A)</td>
<td>0/8 (0.0[0.0])(^A)</td>
<td>0/8 (0.0[0.0])(^A)</td>
</tr>
</tbody>
</table>

*Score range from 0 (normal) to 6 (severe diffuse).
†Score range as determined by immunohistochemistry (0 = undetectable; 3 = large amount of antigen).
‡Different superscripts (A, B, C) indicate significantly \( P < 0.05 \) different mean group severity scores within columns for each DPI.
Table 4. Microscopic lesions (lymphoid depletion and inflammation) and amount of PCV2-specific antigen demonstrated by immunohistochemistry (IHC) in lymph nodes at 21 and 35 days after PCV2 inoculation. Data presented as incidence (mean group severity score [mean severity for affected animals]).

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>Lymph node pool*</th>
<th>Tracheobronchial lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Depletion†</td>
<td>Inflammation‡</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>21 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>Sham inoculation</td>
<td>0/9 (0.0[0.0])A</td>
<td>0/9 (0.0[0.0])A</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td><em>Mycoplasma hyopneumoniae</em></td>
<td>0/9 (0.0[0.0])A</td>
<td>0/9 (0.0[0.0])A</td>
</tr>
<tr>
<td>3 (n = 9)</td>
<td><em>M. hyopneumoniae</em> and PCV2</td>
<td>9/9 (2.3[2.3])B</td>
<td>9/9 (1.8[1.8])B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>6/8 (1.4[1.8])C</td>
<td>5/8 (1.0[1.6])B</td>
</tr>
<tr>
<td>35 days after PCV2 inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 8)</td>
<td>Sham inoculation</td>
<td>0/8 (0.0[0.0])A</td>
<td>0/8 (0.0[0.0])A</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td><em>M. hyopneumoniae</em></td>
<td>2/8 (0.3[1.0])A</td>
<td>1/8 (0.1[1.0])A,B</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td><em>M. hyopneumoniae</em> and PCV2</td>
<td>8/8 (1.9[1.9])B</td>
<td>5/8 (0.9[1.4])C</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>7/8 (1.3[1.4])B</td>
<td>4/8 (0.6[1.3])B,C</td>
</tr>
</tbody>
</table>

*Superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes.
†Score range 0 (normal) to 3 (severe lymphoid depletion).
‡Score range 0 (normal) to 3 (severe histiocytic-to-granulomatous inflammation with replacement of follicles).
§Score range 0 (undetectable) to 3 (large amount of PCV2 antigen).

Different superscripts within columns (A, B, C) represent significantly (P < 0.05) different mean group severity scores within columns for each DPI.
Table 5. Microscopic lesions (lymphoid depletion and inflammation) and amount of PCV2-specific antigen demonstrated by immunohistochemistry (IHC) in tonsil and spleen at 21 and 35 days after PCV2 inoculation. Data presented as incidence (mean group severity score [mean severity for affected animals]).

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>Spleen</th>
<th>Tonsil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Depletion*</td>
<td>Inflammation†</td>
</tr>
<tr>
<td>21 days after PCV2-inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 9)</td>
<td>Sham-inoculation</td>
<td>0/9 (0.0[0.0])§</td>
<td>0/9 (0.0[0.0])‡</td>
</tr>
<tr>
<td>2 (n = 9)</td>
<td>M. hyopneumoniae</td>
<td>0/9 (0.0[0.0])</td>
<td>0/9 (0.0[0.0])</td>
</tr>
<tr>
<td>3 (n = 9)</td>
<td>M. hyopneumoniae and PCV2</td>
<td>7/9 (1.1[1.4])B</td>
<td>5/9 (1.0[1.8])B</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>4/8 (0.5[1.0])B</td>
<td>2/8 (0.4[1.5])A,B</td>
</tr>
<tr>
<td>35 days after PCV2-inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 8)</td>
<td>Sham-inoculation</td>
<td>0/8 (0.0[0.0])A</td>
<td>0/8 (0.0[0.0])A</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>M. hyopneumoniae</td>
<td>0/8 (0.0[0.0])</td>
<td>0/8 (0.0[0.0])</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>M. hyopneumoniae and PCV2</td>
<td>4/8 (0.5[1.0])B</td>
<td>2/8 (0.4[1.5])A</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>PCV2</td>
<td>2/8 (0.4[1.5])A,B</td>
<td>2/8 (0.3[1.0])A</td>
</tr>
</tbody>
</table>

*Score range 0 (normal) to 3 (severe lymphoid depletion).
†Score range 0 (normal) to 3 (severe histiocytic-to-granulomatous inflammation with replacement of follicles).
‡Score range 0 (undetectable) to 3 (large amount of PCV2 antigen).
§Different superscripts within columns (A, B, C) represent significantly (P < 0.05) different mean group severity scores within columns for each DPI.
Fig. 1. Mean group PCV2-genomic copy numbers obtained from singular PCV2 and dual *Mycoplasma hyopneumoniae*/PCV2-infected pigs. Error bars represent standard errors. * = significant \((P < 0.05)\) differences between groups.
Fig. 2. Lung; pig, 35 days after inoculation with PCV2. No gross lesions were observed.

Fig. 3. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae*. The lung has well-demarcated, dark red areas of pneumonia in the cranioventral region.

Fig. 4. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. The lung failed to collapse, has a diffuse mottled-tan appearance and severe multifocal dark-purple-to-tan consolidation.

Fig. 5. Lung; pig, 49 days after inoculation with *Mycoplasma hyopneumoniae* and 35 days after inoculation with PCV2. The lung is diffusely mottled-tan and has well-demarcated, dark-red tan areas of consolidation.
Fig. 6. Lung; control pig. Normal. HE. Bar = 250 μm.

Fig. 7. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. There is thickening of the alveolar septa by marked type 2 pneumocyte hypertrophy and hyperplasia and infiltration of alveolar space by moderate numbers of macrophages and low numbers of neutrophils. HE. Bar = 75 μm. *Inset:* Intense nuclear and cytoplasmic staining for PCV2 antigen in a macrophage-like cell. Immunohistochemical staining with anti-PCV2 polyclonal antibody. Streptavidin-biotin-peroxidase complex method counterstained with hematoxylin. Bar = 20 μm.
**Fig. 8.** Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae*. There is peribronchiolar lymphoid hyperplasia typical of *M. hyopneumoniae* infection. HE. Bar = 250 µm.

**Fig. 9.** Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. There is peribronchiolar lymphoid hyperplasia typical of *M. hyopneumoniae* infection. The interstitial pneumonia, peribronchial fibroplasia, and infiltration of the lamina propria with macrophages and lymphocytes (arrows) is typical of PCV2-infection. HE. Bar = 250 µm.
Fig 10. Distribution of individual pig lymphoid lesion (lymphoid depletion and inflammation and PCV2-antigen) scores in pigs in singular PCV2 ($n = 16$) and dual *M. hyopneumoniae*/PCV2 ($n = 17$) infected groups. Score ranges from 0 to 9.
Fig 11. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. PCV2 antigen-positive cells (arrows) are associated with the peribronchial lymphoid hyperplasia. Immunohistochemical staining with anti-PCV2 polyclonal antibody. Streptavidin-biotin-peroxidase complex method counterstained with hematoxylin. Bar = 120 µm. *Inset:* Detail of the area of follicular lymphoid hyperplasia with several PCV2 antigen-labeled macrophage- and dendritic-like cells. Bar = 35 µm.

Fig 12. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. PCV2 antigen-positive cells (arrows) in the lamina propria and submucosa of a bronchus. Immunohistochemical staining with anti-PCV2 polyclonal antibody. Streptavidin-biotin-peroxidase complex method counterstained with hematoxylin. Bar = 80 µm. *Inset:* Detail of the area with macrophage-like cells with numerous variable-sized PCV2 antigen-positive intracytoplasmic spherical bodies as well as nuclear staining. Bar = 30 µm.
Fig 13. Tracheobronchial lymph node; pig. Fig. 13A. small amount of PCV2 antigen (arrows) in the follicle of a singular PCV2-infected pig 21 days after inoculation with PCV2, Fig. 13B. large amount of PCV2 antigen (arrows) in a follicle of a dual-infected pig 35 days after inoculation with *M. hyopneumoniae* and 21 days after inoculation with PCV2. These pigs were the individual pigs with the highest amount of PCV2 antigen in each treatment group based on scoring and image analysis results. Immunohistochemical staining with anti-PCV2 polyclonal antibody. Streptavidin-biotin-peroxidase complex method counterstained with hematoxylin. Bar = 30 µm.

Fig 14. Lung; pig, 35 days after inoculation with *Mycoplasma hyopneumoniae* and 21 days after inoculation with PCV2. *M. hyopneumoniae*-specific staining (arrow) at the luminal surface of the bronchus epithelial cells. Immunohistochemical staining with anti-*M. hyopneumoniae* monoclonal antibody. Streptavidin-biotin complex method counterstained with hematoxylin. Bar = 200 µm. *Inset:* The epithelial cell layer has intense staining for *M. hyopneumoniae* antigen on the surface. Bar = 30 µm.
CHAPTER 9. EVIDENCE OF BREED-DEPENDENT DIFFERENCES IN SUSCEPTIBILITY TO PORCINE CIRCOVIRUS TYPE-2-ASSOCIATED DISEASE AND LESIONS

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M. F. ROTHSCHILD, X. J. MENG, AND P. G. HALBUR

Abstract. Porcine circovirus type 2 (PCV2) has been confirmed as the primary cause of postweaning multisystemic wasting syndrome (PMWS). However, in the field, PMWS is seen only in a small percentage of pigs infected with PCV2. The overall objective of this study reported here was to determine whether host genetic differences in the susceptibility to PCV2-associated disease exist among selected breeds of pigs. This study included Duroc \((n = 23)\), Landrace \((n = 19)\), and Large White \((n = 21)\) pigs. The pigs were infected intranasally and intramuscularly at 5-7 weeks of age with PCV2. A portion of the pigs \((31/63; 30.2\%)\) had low passively acquired PCV2 antibodies at the time of infection. There were no differences in mean weight gain, rectal temperature, or respiratory score. Clinical disease compatible with PMWS was observed only in the Landrace pigs. Most of the PCV2-infected pigs had enlarged lymph nodes, and individual Duroc and Landrace pigs had mottled tan lungs. PCV2-associated lymphoid depletion and granulomatous inflammation were observed in pigs of all breeds. Three of 19 Landrace pigs and none of the Duroc or Large White pigs developed severe lymphoid lesions associated with large amounts of intralesional PCV2-antigen typical of PMWS. Compared with seronegative Landrace pigs, Landrace pigs that had low maternal antibodies at the time of PCV2 inoculation had significantly \((P < 0.05)\)
less-severe PCV2-associated lesions. The results suggest a predisposition of the Landrace pigs used in this study to PCV2-induced disease and lesions, and that low levels of passively acquired antibodies are protective.

**Introduction**

Porcine circovirus (PCV) was first detected as a contaminant of a continuous porcine kidney cell line in 1974 and was further described in 1982. In the late 1990s, PCV was associated with postweaning multisystemic wasting syndrome (PMWS). Further analysis of the virus documented two distinct types of PCV: nonpathogenic type 1 (PCV1) and pathogenic and PMWS-associated type 2 (PCV2). Subsequently, PCV2 has been associated with several other swine diseases including porcine respiratory disease complex, reproductive disorders characterized by abortions with mummified fetuses, enteritis, and porcine dermatitis and nephropathy syndrome. PMWS was first observed in a Canadian herd in 1991 and is now recognized worldwide. The syndrome is characterized by a clinical history of wasting or poor performance in weaned pigs and by severe lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. Detection of PCV2 antigen or nucleic acids within characteristic microscopic lesions is required for the diagnosis of PMWS.

Serological surveys indicate a high prevalence of PCV2 in the swine population; however, the prevalence of PCV2-associated disease seems to vary considerably from farm to farm and country to country. Morbidity attributable to PMWS reportedly varies from as little as 4% to as high as 30%, and mortality in affected pigs is typically 70-80%. However, most reports to date lack important additional information regarding breed, production style, and environment of the farm, or description of other diseases present in the pigs that may have influenced expression and outcome of PCV2-associated diseases. Genetic analyses to date have not been able to document major differences between PCV2 isolates recovered from affected pigs and those from nonaffected pigs. Analysis and comparison of PCV2 isolates from severely affected countries like Spain or France with those from countries that are less affected like the United States have failed to explain differences in clinical manifestations of PCV2-associated diseases. All
isolates sequenced and compared so far indicated a nucleic acid sequence homology >94%.\textsuperscript{20,45}

Experimental PCV2 infection models indicate that PCV2 is an opportunist, depending on immunostimulation\textsuperscript{3,39} or coinfecting agents like porcine parvovirus (PPV),\textsuperscript{7,36,40,52} porcine reproductive and respiratory syndrome virus (PRRSV),\textsuperscript{2,32,64} or Mycoplasma hyopneumoniae\textsuperscript{54} for PCV2 infection to progress to clinical PMWS. Although most research groups where not able to induce clinical disease in pigs inoculated singularly with PCV2, some groups have succeeded in this regard.\textsuperscript{8,32} PMWS was recently induced in conventional colostrum-fed pigs singularly-infected with PCV2.\textsuperscript{41} The exact reasons for the different outcomes in experimental PCV2 infection models are not known, but there are several possible explanations including differences in environmental conditions and stressors, age of the pigs at infection, or differences in type and dose and routes of the PCV2-inoculation.

One other aspect that, to the authors’ knowledge, has not been investigated is the influence of host genetics on PCV2 infection. Differences in genetic susceptibility of populations or breeds to parasites, bacteria, and viruses have been reported.\textsuperscript{9,10,30,68} Evidence exists for pig breed-dependent differences in disease resistance for a variety of pathogens including pseudorabies virus,\textsuperscript{59} PRRSV,\textsuperscript{27} Escherichia coli,\textsuperscript{15,49} and Sarcocystis miescheriana.\textsuperscript{60} Pigs of all breeds seem to be susceptible to PCV2 infection, and PMWS has been observed within a large variety of purebred and crossbred pigs submitted to a US diagnostic laboratory (P.G. Halbur, unpublished data). Recently, PMWS has been reported in Eurasian wild boars in Canada\textsuperscript{19} and Spain.\textsuperscript{71} Retrospective studies indicate that naturally acquired PCV2-infections in pig herds date back for at least 20 years prior to the first observation of PMWS\textsuperscript{25,44,65,72}; however, PCV2-associated diseases emerged in the late 1990s. Allan et al.\textsuperscript{5} reported experimental induction of PMWS in colostrum-deprived Northern Ireland Landrace/Large White crossbred pigs inoculated with a PCV2 isolate recovered in 1993 from a subclinically infected Swedish Yorkshire pig, implying host and/or environmental dependant factors associated with the development of PMWS. A recent in \textit{vitro} study investigating the replication patterns of PCV2 in pulmonary alveolar macrophages indicated clear differences among macrophages derived from different
conventional crossbred pigs that were suggestive of differences in susceptibility to PCV2-associated disease. The overall objective of the study reported here was to determine whether there is evidence for host genetic differences in susceptibility to PCV2-associated disease among selected breeds including Duroc, Landrace, and Large White. These breeds were selected because they could be purchased from a single source.

**Materials and Methods**

**Animal source**

Seventy-five purebred (n = 26 Duroc; n = 25 Landrace; and n = 24 Large White), colostrum-fed, female pigs raised under identical conditions on the same farm and in the same barn were purchased from a herd that is routinely tested for major swine pathogens and is free of PRRSV and *M. hyopneumoniae*. There was no evidence of PCV2-associated disease in the source herd or in the multiplier herds supplied by this source. The source herd is routinely vaccinated against PPV infection and leptospirosis. The pigs were born within a range of 23 days, randomly selected on farm after blocking by sire and litter, weaned at 10-12 days of age, and transported in two groups of 39 (n = 12 Duroc; n = 14 Landrace; and n = 13 Large White) and 36 (n = 14 Duroc; n = 11 Landrace; and n = 11 Large White) pigs 2 weeks apart to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, Iowa. The pigs from the various breeds were housed in two rooms until inoculation.

**Serologic testing**

Blood samples were collected at arrival of the pigs, followed by blood collections at 2-week intervals until the day of PCV2 inoculation. Thereafter, blood was collected on postinoculation day (PID) 7, 14, 21, 28, and 35. Preinoculation sera (from arrival up to PID 0) and serum samples obtained on PID 21 and 35 were tested by a PCV2-enzyme linked immunosorbent assay (ELISA) that is based on the recombinant ORF2 capsid protein of PCV2. Results were considered positive if the calculated sample-to-positive ratio (S : P) ratio was ≥0.2.

The serum samples obtained on arrival of the pigs at the research facility and the serum samples obtained at necropsy from 11 randomly selected PCV2-infected pigs of each
of the three breeds were tested for the presence of antibodies to PRRSV by use of a PRRSV-ELISA (IDEXX Laboratories, Inc. Westbrook, MA), and for the presence of antibodies to PPV by hemagglutination-inhibition assay.

**Experimental design and PCV2 inoculation**

Twelve pigs (three Duroc, six Landrace, and three Large White pigs) were kept as sham-inoculated control pigs and housed together in a separate room. These pigs were procedural controls, selected on the basis of presence of maternal antibodies to PCV2. Experimentally induced PCV2 in the remaining 63 pigs (23 Duroc, 19 Landrace, and 21 Large White pigs) was initiated after waning of the PCV2 maternal antibody levels below the negative cutoff (S : P ratio <0.2). All of the PCV2-infected pigs were housed in three rooms and randomly distributed into two to seven pens of five to six pigs per pen. The procedural controls were housed separately in a fourth room. To account for and minimize sire and litter effects, a minimum of four sires were represented within each breed and two to five piglets were chosen from each dam. The sire and dam distribution in the PCV2-inoculated pigs was as follows: Duroc pigs (n = 23) represented four sires and eight dams, and the Landrace (n = 19) and the Large White (n = 21) pigs represented four sires and six dams, respectively.

PCV2 isolate ISU-40895 was obtained via direct transfection of PK-15 cells with an infectious clone of PCV2 as previously described. Passage 4 of the virus at a titer of 10^4.2 50% tissue culture infective doses was used for inoculation of the pigs. The inoculum tested negative for the presence of porcine parvovirus and PCV1 nucleic acids on the basis of results of polymerase chain reaction (PCR) analysis. After production, the PCV2 inoculum was stored in individual 50-ml tubes at -80ºC and thawed only once immediately prior to inoculation. To better ensure infection of all pigs at the time of inoculation, each pig received 1 ml of the PCV2 inoculum intranasally and 0.5 ml intramuscularly in the right side of the neck as described. On the day of PCV2 inoculation, the pigs were 5-8 weeks old. Mean ± SE age was 43.5 ± 1.8 days for the Duroc, 48.6 ± 2.0 days for the Landrace, and 46.0 ± 2.0 days for the Large White pigs.

**Clinical evaluation**

After PCV2 inoculation, the pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 to 6 (0 = normal and 6 = severe dyspnea and
abdominal breathing). In addition, pigs were evaluated daily for clinical signs including sneezing (score range from 0 [no sneezing] to 3 [severe persistent sneezing]), and jaundice. Rectal temperatures, wasting, and behavioral changes such as lethargy were recorded daily. The pigs were weighed on the day of PCV2 inoculation and on PID 7, 14, 21, 28, and 35.

**Quantification of PCV2**

Extraction of DNA from sera obtained on the day of PCV2 inoculation and on PID 7, 14, 21, 28, and 35 was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The DNA extracts were used for quantification of the amount of PCV2 genomic DNA by real-time PCR analysis.

**Necropsy**

Necropsies were performed on half of the PCV2-infected pigs of each breed at PID 21 (n = 12 Duroc, n = 11 Landrace, and n = 11 Large White) and on the remaining pigs at PID 35 (n = 11 Duroc, n = 8 Landrace, n = 10 Large White). In addition, 7 of 12 control pigs (two Duroc, three Landrace, and two Large White pigs) were necropsied on PID 21, and 5 of 12 (one Duroc, three Landrace, and one Large White pig) were necropsied on PID 35. The total amount of macroscopic lung lesions (0-100% of the lung affected), and size of lymph nodes (score range from 0-3; 0 [normal], 1 [two times the normal size], 2 [three times the normal size], 3 [four times the normal size]) were estimated.

Lungs were insufflated with fixative as described. Sections of lymph nodes (superficial inguinal, external iliac, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and processed in routine manner for histologic examination.

**Histologic examination and immunohistochemical analysis**

Microscopic lesions were evaluated in a blinded manner. Lung sections were scored for presence and severity of interstitial pneumonia ranging from 0 to 6 (0=normal; 6=severe diffuse). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and were scored from 0 = none to 3 = severe. Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 = normal to 3 = severe histiocytic inflammation and replacement of follicles ranging from 0 = normal to 3 = severe.
Immunohistochemical detection of PCV2-specific antigen was performed on formalin-fixed and paraffin-embedded lymphoid tissue sections (lymph nodes [superficial inguinal, external iliac, mediastinal, tracheobronchial, and mesenteric], tonsil, spleen, and thymus) of all pigs and sections of intestine from selected pigs using a rabbit polyclonal antiserum. Scoring for PCV2 antigen was done in blinded manner, and scores ranged from 0 = no signal to 3 = >50% of the lymphoid follicles containing cells with PCV2 antigen staining. Mean group score was determined for each tissue and compared among groups.

The overall PCV2-associated lesions were evaluated as described. A combined scoring system for each lymphoid tissue that ranged from 0 to 9 (lymphoid depletion score 0-3; granulomatous inflammation score 0-3; PCV2-IHC score 0-3) was used. The scores (lesions and PCV2-IHC) of the seven lymphoid tissues ([lymph node pool multiplied by five] + [spleen] + [tonsil]) were added together and divided by seven. The lymph node pool consisted of one section each of tracheobronchial, superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes. Pigs were grouped into four categories on the basis of overall microscopic lymphoid lesion scores: normal (score = 0), mild (score = 1-3), moderate (score = 4-6) and severe (score = 7-9). The mean treatment group lymphoid score was calculated and compared among groups. A pig was diagnosed as having microscopic lesions typical of PMWS if the mean lymphoid microscopic lesion severity score was severe (score of 7-9) and if there was evidence of lymphohistiocytic inflammation in other organ systems.

**Statistical analysis**

The main outcome of interest in this study was evaluation differences in PCV2 susceptibility among breeds. Therefore, owing to the low number of sham-inoculated control pigs, statistical analyses were limited to PCV2-infected pigs. Summary statistics were calculated for all PCV2-infected pigs within each breed to assess the overall quality of the data. Analysis of variance (ANOVA) was used for cross-sectional assessment of the average daily weight gain and nonrepeated continuous measures. The significance level was $P < 0.05$ followed by pairwise testing using the Tukey-Kramer adjustment to identify the groups that were different. Effect of sire or dam on the outcome was tested by using the model $y = \text{breeds + sires (breeds) + dams (sires, breeds)}$. In order to summarize and simplify the clinical
observations, response feature analysis and chi-square testing was used. The clinical scores for each pig were reduced to one weekly mean score, and the resulting values were subject to statistical analysis. Daily rectal temperature data were analyzed with multivariate ANOVA. Nonrepeated measures of necropsy and histopathology data were assessed using nonparametric Kruskal-Wallis ANOVA. If a nonparametric ANOVA results was significant ($P < 0.05$), Wilcoxon tests were used to assess the differences of pairs of groups. Differences in incidence were evaluated by using Fisher’s exact test.

A portion of the pigs within each breed had low maternal antibodies at the time of PCV2 infection. To evaluate influence of the antibody status on the results regarding PCV2-associated lesions, maternal-antibody-negative and maternal-antibody-positive pigs were compared separately among breeds, and pairwise Wilcoxon tests on outcomes of antibody negative (S : P ratio<0.2) and antibody positive pigs (S : P ratio≥0.2) within each breed were done.

**Results**

**Clinical disease**

From PID 7 to 14, PCV2 infection was clinically characterized by mild respiratory distress and, occasionally, by sneezing. Mean respiratory scores were higher in the Landrace pigs for PID 7-14, but were not significantly different ($P > 0.05$) among breeds. Individual pigs of all three breeds had periodically increased temperatures; however, there was no difference during the 21 days ($P = 0.189$) or from PID 21 to PID 35 ($P = 0.372$) in mean body temperature between breeds nor was there a time-by-group interaction.

From the day of PCV2 inoculation onwards to PID 35, mean ± SE weight gain was 633.7 ± 32.8 g for Duroc pigs, 763.8 ± 49.5 g for Landrace pigs, and 746.4 ± 36.8 g for Large White pigs. The weekly weight gain (data not shown) was not significantly different ($P > 0.05$) among the breeds.

One Landrace pig developed persistent fever >41.1 C from PID 14 onwards and had to be euthanized at PID 21, although it had been randomly selected for the PID 35 necropsy group. This pig gained only 6 g between PID 14 and 21, which is consistent with wasting and clinical PMWS in a growing pig. One other Landrace pig had decreased average daily weight
gain, compared with the other Landrace pigs.

Serologic test results

At arrival, the majority of the pigs (65/75) had maternal antibodies to PCV2, with mean PCV2-ELISA S : P ratios of 0.46 ± 0.08 for Duroc pigs, 0.57 ± 0.08 for Landrace pigs, and 0.43 ± 0.07 for Large White pigs. In the majority of the pigs (18/23 Duroc, 10/19 Landrace, 16/21 Large White), maternal antibodies waned below the ELISA cutoff (S : P ratio <0.2) prior to PCV2-inoculation (PID-0 seronegative pigs). At the time of PCV2 inoculation, 5 of 23 Duroc, 9 of 19 Landrace, and 5 of 21 Large White pigs had low maternal antibody levels (PID-0 seropositive pigs) with mean S : P ratios of 0.22 ± 0.00 for Duroc, 0.23 ± 0.01 for Landrace, and 0.24 ± 0.01 for Large White pigs. As expected, the passively acquired antibodies in the noninfected control pigs decayed over time. None of the control pigs seroconverted to PCV2 during the study. Most of the PCV2-infected pigs seroconverted to PCV2 between PID 21 and PID 35I. At PID 35, 7 of 11 Duroc, 6 of 8 Landrace, and 6 of 10 Large White pigs were positive for PCV2-specific antibodies.

All pigs tested negative for PRRSV-specific antibodies. The pigs had maternal antibodies to PPV that decayed over time as expected. Indications of seroconversion to PRRSV or PPV were lacking.

PCV2 viremia duration and genomic copy numbers

All serum samples obtained from the noninfected control pigs were negative for PCV2-specific nucleic acids, as tested by real-time PCR analysis. In the PCV2-infected pigs, PCV2 viremia was detected first at PID 7 in 13 of 23 Duroc, 14 of 19 Landrace, and in 9 of 21 Large White pigs. Landrace pigs had significantly (P < 0.05) higher incidence of viremic pigs at PID 7, compared with that for the Large White pigs. The majority of the PCV2-infected pigs were viremic by PID 14 (21/23 Duroc, 17/19 Landrace, and 21/21 Large White). The duration of the PCV2 viremia was based on results from the PCV2-infected pigs (n = 11 Duroc, n = 8 Landrace, and n = 10 Large White) that remained in the study for the entire 35 days. PCV2 viremia was determined to be 4.6 ± 0.2 weeks in Duroc pigs (n = 11), 4.3 ± 0.6 weeks in Landrace pigs (n = 8), and 4.2 ± 0.4 weeks in Large White pigs (n = 10), and was not significantly different (P = 0.669) among breeds. Mean copy number of PCV2
genomic DNA in serum was not different ($P > 0.05$) among the breeds at PID 7, 14, 21, 28, and 35.

**Macroscopic lesions**

There were no gross lesions in the noninfected control pigs at the PID-21 or the PID-35 necropsy dates. At PID 21, 9 of 12 PCV2-inoculated Duroc, 10 of 11 PCV2-inoculated Landrace, and 11 of 11 PCV2-inoculated Large White pigs had gross lymph node enlargement scores of $1.4 \pm 0.3$, $2.0 \pm 0.3$, and $1.6 \pm 0.2$, respectively. In addition, three Landrace pigs and one Duroc pig had mottled-tan lungs that failed to collapse. The Landrace pig that had to be euthanized at PID 21 (prior to scheduled necropsy at PID 35) was emaciated, had a pale liver, and had moderate edema throughout the stomach wall. At PID 35, gross lesions in the PCV2-inoculated pigs were limited to enlargement of lymph nodes in 9 of 11 Duroc (mean $1.8 \pm 0.3$), 5 of 8 Landrace (mean $1.4 \pm 0.4$), and 5 of 10 Large White (mean $0.9 \pm 0.3$) pigs.

**Microscopic lesions and amount of PCV2-antigen**

There were no microscopic lesions in any of the control pigs (data not shown). A total of 7 of 63 PCV2-infected pigs had no PCV2-associated lymphoid lesions (overall score, 0) and 35 of 63 had mild PCV2-associated lymphoid lesions (overall score, 1-3). In 18 of 63 PCV2-infected pigs, lymphoid lesions were moderate (overall score, 4-6), and 3 of 63 pigs had severe lymphoid lesions consistent with PMWS (overall score, 7-9) (Table 1). If present, PCV2-associated lesions were characterized by mild-to-severe lymphoid depletion and mild-to-severe granulomatous inflammation in lymphoid tissues associated with low to high amounts of PCV2-antigen (Tables 2,3). Landrace pigs had significantly ($P < 0.05$) more severe lymphoid depletion scores in tonsil at PID 21 (Table 2) and in the lymph node pool at PID 35 compared with those for Duroc and Large White pigs (Table 3).

At PID 21, lymphohistiocytic hepatitis was mild to moderate in 3 of 12 Duroc and 6 of 11 Landrace pigs, and mild in 6 of 11 Large White pigs. Lymphohistiocytic myocarditis was moderate in 1 of 12 Duroc, mild to moderate in 4 of 11 Landrace, and mild in 2 of 11 Large White pigs. Lymphohistiocytic nephritis was mild in 3 of 12 Duroc, mild to moderate in 3 of 11 Landrace and 3 of 11 Large White pigs.
At PID 35, lymphohistiocytic hepatitis was mild to moderate in 4 of 11 Duroc pigs, and was mild in 4 of 8 Landrace pigs and 1 of 10 Large White pigs. Lymphohistiocytic myocarditis was mild in 1 of 11 Duroc, 2 of 8 Landrace, and 0 of 10 Large White pigs. Mild lymphohistiocytic nephritis was observed in 5 of 11 Duroc, in three of eight Landrace, and 3 of 10 Large White pigs.

Lung lesions were characterized by mild, focal-to-diffuse, lymphohistiocytic interstitial pneumonia with type-2 pneumocyte hypertrophy and hyperplasia in 5 of 12 Duroc and 4 of 11 Large White pigs. Lymphohistiocytic interstitial pneumonia was mild to moderate, focal to diffuse in 8 of 11 Landrace pigs at PID 21. At PID 35, mild, focal-to-diffuse, lymphohistiocytic interstitial pneumonia was present in 6 of 11 Duroc, 5 of 8 Landrace, and 5 of 10 Large White pigs.

Three Landrace pigs had microscopic lesions consistent with PMWS, with severe lymphoid depletion characterized by complete loss of the follicular structure of all lymphoid tissues evaluated, severe histiocytic replacement of lymphoid follicles, and high amounts of PCV2 antigen associated with the lesions. These pigs also had mild to moderate lymphohistiocytic inflammation in other organ systems including heart, kidney, liver, and thymus. There also was moderate to severe granulomatous enteritis with moderate numbers of multinucleated giant cells present in Peyer’s patches of the ileum associated with moderate to high amounts of PCV2-antigen.

Evaluation of the influence of passively acquired antibodies on PCV2-associated lesions and viremia among breeds

There was a total of 5 of 23 PID-0 seropositive Duroc, 9 of 19 PID 0 seropositive Landrace, and 5 of 21 PID 0 seropositive Large White pigs on the day of PCV2-inoculation. To delineate any possible influence of low amounts of passively acquired anti-PCV2-antibodies, the outcomes of the PID-0 seronegative pigs of all breeds were compared with each other. Similarly, the outcomes of the PID-0 seropositive pigs among all breeds were compared with each other. Separation of the weekly weight gain data into PID-0 serongetive and PID-0 seropositive pigs did not result in major changes, compared with the weight gain data that included PID-0 seronegative and PID-0 seropositive pigs and was not significantly different \( (P > 0.05) \) among the breeds (data not shown). Mean PCV2-genomic copy numbers
for the PID-0 seronegative pigs was not significantly \((P > 0.5)\) different among the breeds at PID 7, 14, 21, 28, and 35. Likewise, no significant difference \((P > 0.05)\) was observed among breeds in mean PCV2 copy number for the PID-0 seropositive pigs. At PID 21, PID-0 seronegative Landrace pigs had a significantly \((P = 0.01)\) higher mean group overall lymphoid severity score compared with PID-0 seronegative Duroc pigs (Table 1). The PID-0 seronegative Landrace pigs had significantly \((P < 0.05)\) more severe depletion of the lymph nodes at PID 21 and 35 (Table 3), and significantly \((P < 0.05)\) more severe lymphoid depletion, inflammation, and PCV2 antigen associated with these lesions in tonsils at PID 21, compared with that in PID-0 seronegative Duroc and Large White pigs (Table 2). There was a higher incidence of microscopic lung, liver, heart and kidney lesions in PID-0 seronegative pigs compared with PID-0 seropositive pigs across breeds (data not shown), but this difference was not significant \((P > 0.5)\).

In this study, the total incidence of microscopic lesions consistent with PMWS was 15.8\% (3 of 19) for Landrace pigs, whereas none of the Duroc and Large White pigs developed lesions consistent with PMWS. When separated into PID-0 seronegative and PID-0 seropositive, the PMWS incidence increased to 30\% (3 of 10) in PID-0 seronegative Landrace pigs.

**Within breed influence of passively acquired antibodies on PCV2-associated lesions and viremia**

To further explore the possible influence of the presence of the amounts of passively acquired PCV2 antibodies at the time of PCV2 inoculation on PCV2 infection, outcomes of PID-0 seropositive and PID-0 seronegative pigs within each breed were compared with each other. At PID 14 and 21, PID-0 seronegative Landrace pigs \((n = 10)\) had significantly \((P < 0.01)\) higher amounts of PCV2 nucleic acids in serum compared with those in PID-0 seropositive Landrace pigs \((n = 9)\). At PID-21 necropsy, mean overall lymphoid severity score was significantly \((P < 0.002)\) higher in PID-0 seronegative Landrace, compared with PID-0 seropositive Landrace pigs (mean score 6.2 ± 1.0 in PID-0 seronegative pigs \([n = 6]\) versus 1.1 ± 0.3 in PID-0 seropositive pigs \([n = 5]\)) (Table 1). There were no significant differences in Duroc or Large White pigs between PID-0 seronegative and PID-0 seropositive pigs, although PID-0 seronegative pigs had higher values (Table 1).
Evaluation of the littermates of the PMWS affected pigs

There was no litter or sire effect on the development of PCV2-associated lesions. The three Landrace pigs with microscopic lesions typical of PMWS were from three litters, and two sires and were housed in two rooms and three pens. Comparison of data (serologic, overall lymphoid lesions score) among the three Landrace pigs with lesions consistent with PMWS and their littermates is summarized in Table 4.

Discussion

The overall goal of this study was to conduct a controlled experiment to investigate the influence of host genetics on susceptibility to PCV2. We utilized Duroc, Landrace, and Large White pigs because they were available from a single source. Today, all three of these breeds are used extensively worldwide in purebred and crossbred breeding programs.

A limited number of peer-reviewed manuscripts have compared disease susceptibility in various pig breeds. The muscle load of Sarcocystis miescheriana bradyzoites after oral exposure was found to be 20 times higher in Pietrain, compared with Meishan pigs.\(^{60}\) Compared with Meishan pigs, Large White pigs were found to be more susceptible to infection with pseudorabies virus\(^{59}\) or K88+ Escherichia coli.\(^{15}\) Compared with Duroc and Meishan pigs, Hampshire pigs developed significantly less severe macroscopic lung lesions after experimental PRRSV-inoculation.\(^{27}\) Meishan pigs were found to be highly resistant to K88ac+ E. coli-induced disease, whereas there were both susceptible and disease-resistant pigs within the Fengjjing and Minzhu breeds.\(^{49}\)

A cohort study was conducted on four PMWS-affected farms to investigate a suspected decreased susceptibility to PCV2-associated disease in Pietrain pigs by manipulating the genetics via artificial insemination. Half of the sows were inseminated with Pietrain semen, whereas the remaining sows received the semen that was typically used on these farms.\(^{62}\) The PCV2-associated disease in the Pietrain offspring did not differ from that observed in other pigs on these farms in terms of PCV2 seroconversion, morbidity, and mortality. We did not include Pietrain pigs in the study reported here because the farm from which we purchased our purebred pigs did not raise Pietrain and we believed it important to minimize confounding factors (differences in environment, production style, coinfection
status) typical in pigs derived from various sources.

All breeds in the current study appeared to be equally susceptible to PCV2 infection. There were no significant differences in viremia duration, amount of PCV2 genomic DNA copies, or anti-PCV2-antibody production among the breeds. However, clinical disease and microscopic lesions consistent with PMWS were only observed in the Landrace pigs. The results indicate that the Landrace pigs of this study were predisposed to progress from subclinical PCV2-infection to clinical PMWS.

To the best of our knowledge, all other confounding factors were similar across breeds in this study. In the field, PMWS often resembles an end-stage disease complex and multiple pathogens in addition to PCV2 are often detected. Experimental coinfection of pigs with PCV2 and other viruses such as PPV\textsuperscript{7,36,40,52} or PRRSV,\textsuperscript{2,32,64} or bacteria such as \textit{M. hyopneumoniae}\textsuperscript{54} has been documented to enhance the amount of PCV2 genomic copies and PCV2-associated lesions and to increase the incidence of PMWS. There may be a variety of other known and unknown pathogenic and non-pathogenic organisms that are able to trigger progression of PCV2 infection to PMWS. To account for this, we believed it important that all pigs came from the same farm and, farrowed in the same barn, and were transported together to our research facilities, thereby equalizing the microbiological flora across breeds as much as possible.

Sex can be a confounding factor as well. There are at least two studies that concluded male pigs were more susceptible to development of PMWS than were female pigs, and the authors thought this might be attributable to castration and associated secondary infections.\textsuperscript{11,61} Thus, in our study, we used only female pigs to control for sex.

To our knowledge, all breeding herds are infected with PCV2. We usually derive PCV2-negative crossbred pigs for our experimental PCV2-inoculation trials by screening the entire batch of dams scheduled to farrow during the desired timeframe. We then select piglets from those sows in the batch that have the lowest anti-PCV2-antibody titers.\textsuperscript{56} Due to litter and sire restrictions and to the low number of purebred sows within the same farrowing batch, such was not possible in this study. We, therefore, did not screen sows prior to purchase of the pigs, and a portion of the pigs had high levels of maternal antibodies at time of arrival. Compared with that in the other two breeds, the Landrace pigs of this study had
higher levels of PCV2-antibodies at weaning and at the time of PCV2 inoculation, but the difference was not statistically significant ($P > 0.5$). Because of the higher incidence and titer of maternal PCV2 antibodies, we increased the number of procedural control Landrace pigs (six rather than three), which decreased the number of experimentally infected pigs within this breed. Because of budget and room restrictions, experimental infection of the pigs was started prior to complete waning of the maternal antibody titers in these pigs.

We were also concerned about a possible but unknown host age effect on PCV2-infection. At 5 to 8 weeks of age, 19 of 63 pigs (30.1%) still had low titer of passively acquired antibodies to PCV2 at the time of PCV2 inoculation. There were 47.4% (9 of 19) of the experimentally infected Landrace pigs that had low levels of PCV2-specific antibodies at the time of PCV2-inoculation, compared with 21.8% (5 of 23) of the Duroc and 23.8% (5 of 19) of the Large White pigs. Landrace piglets that had low but measurable maternal antibodies at the time of PCV2-inoculation had significantly ($P < 0.05$) reduced overall PCV2-associated lesions, compared with Landrace piglets that were seronegative at the time of PCV2-inoculation. All three Landrace pigs that developed lesions consistent with PMWS were seronegative at the time of PCV2 inoculation, as were the 18 Duroc, 16 Large White, and seven additional Landrace pigs that did not develop PMWS. The presence of anti-PCV2-antibodies has been documented to protect pigs from developing severe PCV2-associated lesions.$^{4,22}$ Our experimental results support this conclusion and indicate further that only a portion of the anti-PCV2-negative pigs are susceptible to PCV2-associated diseases. It has been suggested, that a PCV2-ELISA S : P of 0.6 prevents PCV2 replication and development of PMWS.$^{33}$ On the basis of the results of our study, even lower levels of passively acquired antibodies are protective against development of severe PCV2-associated lesions. This suggests that PCV2 vaccination of breeding herds resulting in high passively transferred antibody levels may be one way to control PCV2-associated disease in growing pigs.

We were able to induce lesions consistent with PMWS in 3 of 19 (15.8%) Landrace pigs, whereas we were not able to induce PMWS in any of the Duroc or Large White pigs. This is comparable to what is seen in commercial crossbred swine production in the field where the incidence of PMWS in affected herds usually peaks with around 10% of the herd affected, but may vary from as little as 4% to as high as 30% in individual farms.$^{1,28,43,61}$
In the current study we used a genetically-well-characterized PCV2-isolate\textsuperscript{20} that has been used extensively in pig models. Previously, singular PCV2 infection with the same isolate at a similar cell culture passage and in approximately the same dose as used in the purebred pigs in the current study did not result in expression of clinical disease or presence of microscopic lesions consistent with PMWS in 264 crossbreed pigs that were part of nine different studies.\textsuperscript{21-24,35,52-55} This further supports our conclusion that the purebred Landrace pigs used in this experiment were more susceptible to PCV2-associated diseases.

It appears to be a common belief among some producers and veterinarians that Landrace pigs are more susceptible to infectious diseases in general; however, publications describing experimental results that substantiate this clinical impression are lacking to our knowledge. On the basis of field observations, it has been suggested that Landrace pigs in the UK appeared to be less affected by progressive atrophic rhinitis than are Large White pigs.\textsuperscript{13} Landrace pigs are known to be predisposed to type III congenital tremor, which is attributable to a monogenic sex-linked factor resulting in a deficiency of oligodendrocytes in the spinal cord in male Landrace pigs.\textsuperscript{29} Danish Landrace pigs are thought to be the origin of dermatosis vegetans which is caused by a semilethal autosomal recessive factor.\textsuperscript{14}

More studies including larger numbers of Landrace pigs from several different lines and different geographic locations are warranted to further investigate a possible predisposition to PCV2-associated PMWS within this breed. Genetic resistance may be associated with a single gene, but is usually associated with multiple genes. Another approach may be to further study PMWS-affected pigs and non-affected littermates (who share at least 50\% of their genes in common) by gene array analysis to possibly identify gene loci with differences in expression that may be responsible for increased susceptibility to PCV2-associated diseases. In conclusion, despite its limited size, this work adds to the body of evidence to explain differences in clinical expression of PCV2-associated diseases from farm to farm.
Acknowledgements

This study was funded in part by a grant from the Iowa Livestock Health Advisory Council, support from Genetiporc, and by the Iowa Agriculture and Home Economics Experiment Station, Hatch and State of Iowa funding.

References

8. Bolin SR, Stoffregen WC, Nayar GPS, Hamel AL: Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-


35. Hoogland MJ, Opriessnig T, Halbur PG: Comparison of the effect of different adjuvants on porcine circovirus type 2-associated lesions. J Swine Health Prod. accepted for publication


66. Sorden SD: Update on porcine circovirus and postweaning multisystemic wasting
Table 1. Comparison of mean group lymphoid lesions (lymphoid depletion, inflammation, and porcine circovirus type 2 [PCV2] antigen) in PCV2-inoculated pigs at postinoculation day (PID) 21 and 35 in pigs of various breeds and with various antibody status at the time of PCV2 infection. Score ranges from 0 to 9.

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* Seronegative pigs based on ELISA sample-to-positive ratio <0.2 on the day of PCV2-inoculation.
† Seropositive pigs based on ELISA sample-to-positive ratio ranging from 0.2 to 0.3 on the day of PCV2-inoculation.
‡ Mean group score ± SE.
§ NA = not applicable.
**Table 2.** Microscopic lesions (lymphoid depletion and granulomatous inflammation) and amount of PCV2-antigen as determined by immunohistochemistry (IHC) in tonsil of PCV2-infected Duroc, Landrace, and Large White pigs with different antibody status at the time of PCV2 inoculation. Data presented as incidence (mean group severity score [mean severity for affected animals]).

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<tr>
<th>Breed</th>
<th>Depletion (0=normal, 3=severe)</th>
<th>Inflammation (0=normal, 3=severe)</th>
<th>PCV2-antigen (0=undetectable, 3=large amount)</th>
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*Seronegative pigs based on ELISA sample-to-positive ratio <0.2 on the day of PCV2-inoculation.
† Seropositive pigs based on ELISA sample-to-positive ratio ranging from 0.2 to 0.3 on the day of PCV2-inoculation.
‡ Different superscripts (I and II) within columns represent significantly ($P < 0.05$) different mean group severity scores within columns for each day after inoculation.
§ NA=not applicable.
Table 3. Microscopic lesions (lymphoid depletion and granulomatous inflammation) and amount of PCV2-antigen as determined by IHC in the lymph node pool (tracheobronchial, superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes) in PCV2-infected Duroc, Landrace, and Large White pigs with different antibody status at the time of PCV2 inoculation. Data presented as incidence (mean group severity score [mean severity for affected animals]).

<table>
<thead>
<tr>
<th>Breed</th>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Large White</td>
<td>9/10 (0.9[1.0])‡</td>
<td>9/10 (0.9[1.0])‡</td>
<td>NA§</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Seronegative pigs based on ELISA sample-to-positive ratio <0.2 on the day of PCV2-inoculation.
† Seropositive pigs based on ELISA sample-to-positive ratio ranging from 0.2 to 0.3 on the day of PCV2-inoculation.
‡ Different superscripts (I and II) within columns represent significantly (p<0.05) different mean group severity scores within columns for each day after inoculation.
§ NA=not applicable.
Table 4. Comparison of the Landrace pigs with lesions consistent with postweaning multisystemic wasting syndrome (PMWS) and their littermates.

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>PMWS</th>
<th>Sire</th>
<th>Dam</th>
<th>S : P ratio* at 10 Days of Age</th>
<th>S : P ratio* at Time of PCV2 Infection</th>
<th>Age at PCV2 Infection</th>
<th>Overall Lymphoid Severity Score (Classification)†</th>
<th>Necropsy (PID)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>A</td>
<td>1</td>
<td>0.025</td>
<td>0.032</td>
<td>51 days</td>
<td>8 (Severe)</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>A</td>
<td>1</td>
<td>0.011</td>
<td>0.009</td>
<td>51 days</td>
<td>5 (Moderate)</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>A</td>
<td>1</td>
<td>\textbf{0.731}</td>
<td>\textbf{0.265}</td>
<td>51 days</td>
<td>1 (Mild)</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>A</td>
<td>2</td>
<td>\textbf{0.255}</td>
<td>0.175</td>
<td>48 days</td>
<td>8 (Severe)</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>No</td>
<td>A</td>
<td>2</td>
<td>\textbf{0.333}</td>
<td>\textbf{0.215}</td>
<td>48 days</td>
<td>3 (Mild)</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>B</td>
<td>3</td>
<td>\textbf{0.568}</td>
<td>0.192</td>
<td>48 days</td>
<td>9 (Severe)</td>
<td>21§</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>B</td>
<td>3</td>
<td>\textbf{0.480}</td>
<td>\textbf{0.211}</td>
<td>48 days</td>
<td>2 (Mild)</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>No</td>
<td>B</td>
<td>3</td>
<td>\textbf{0.567}</td>
<td>0.173</td>
<td>48 days</td>
<td>3 (Mild)</td>
<td>21</td>
</tr>
</tbody>
</table>

* PCV2 ELISA sample-to-positive ratio (S: P ratio ≥0.2 is considered positive [positive values are in bold-face type]).

† Mean value from seven lymphoid tissues (tonsil, spleen, five lymph nodes) ranging from 0-9 (lymphoid depletion 0-3; inflammation 0-3; amount of PCV2 0-3); normal, score = 0; mild score = 1-3, moderate score = 4-6, severe score = 7-9.

‡ Postinoculation day

§ Originally selected for PID-35 necropsy.
CHAPTER 10. GENETIC AND EXPERIMENTAL COMPARISON OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) ISOLATES FROM CASES WITH AND WITHOUT PCV2-ASSOCIATED LESIONS PROVIDES EVIDENCE FOR DIFFERENCES IN VIRULENCE

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T. Opriessnig, N. E. McKeown, E.-M. Zhou, X. J. Meng, and P. G. Halbur

SUMMARY

There are marked differences in clinical expression of diseases associated with porcine circovirus type 2 (PCV2) in the field. The objective of this study was to compare the sequences and pathogenicity of PCV2 isolates from field cases with and without PCV2-associated lesions. Forty-two specific-pathogen-free (SPF) pigs were randomly assigned to three groups of 14 pigs each. At seven weeks of age, group 1 pigs were sham-inoculated with saline, group 2 pigs were each inoculated with PCV2-4838 (isolated from a pig with no evidence of PCV2-associated lymphoid lesions), and group 3 pigs were each inoculated with PCV2-40895 (isolated from a pig with PCV2-associated lymphoid lesions and disease). The PCV2-4838 and PCV2-40895 isolates shared approximately 98.9% of their nucleotide sequence identity across the entire genome. A total of 9 amino acid changes in ORF2 and 2 amino acid changes in ORF1 were identified between the two isolates. The PCV2-4838 inoculated pigs had significantly ($P < 0.0001$) more genomic copy numbers of PCV2 in sera at seven days post inoculation (DPI) and significantly ($P < 0.05$) fewer genomic copy numbers at 14, 21, and 28 DPI compared to pigs inoculated with PCV2-40895 isolate.
Microscopic lesions in lymphoid tissues were significantly \((P < 0.05)\) less severe and the amount of PCV2-antigen associated with these lesions was significantly lower \((P < 0.05)\) in pigs inoculated with PCV2-4838. The results of this study suggested that PCV2-isolates differ in virulence in a SPF pig model typical of modern production systems in North America.

**INTRODUCTION**

Porcine circovirus (PCV) is an approximately 1.76 kb, single-stranded, non-enveloped DNA virus containing a circular genome which was first recognized in 1974 as a contaminant of a continuous porcine kidney cell line (PK-15) (Tischer et al., 1974) and was further characterized in 1982 (Tischer et al., 1982). Until the late 1990’s, PCV was thought to be non-pathogenic for pigs. In 1991, a new disease syndrome in swine was recognized in western Canadian high health herds and, based on the clinical presentation, was described as “postweaning multisystemic wasting syndrome (PMWS)” (Harding & Clark, 1997). In 1998, researchers first reported on the association of PCV with PMWS (Allan et al., 1998; Ellis et al., 1998; Morozov et al., 1998). In order to distinguish the non-pathogenic PCV from the virus associated with PMWS, the non-pathogenic PCV is referred to as PCV1 and the PMWS-associated PCV as PCV2.

PMWS is characterized by a clinical history of wasting or poor performance in weaned pigs and by lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. The detection of PCV2-antigen or DNA within characteristic microscopic lesions is required for the diagnosis of PMWS (Sorden, 2000). In addition to PMWS, PCV2 is also associated with sporadic reproductive failure (West et al., 1999), enteritis (Kim et al., 2004), porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000), and is considered an important part of the porcine respiratory disease complex in North America (Harms et al., 2002; Opriessnig et al., 2004a).

The non-pathogenic PCV1 and the PMWS-associated PCV2 share approximately 75-76% nucleotide sequence identity (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998). The lengths of the genomes of PCV1 isolates range from 1,758 to 1,760 bp (Fenaux et al., 2000), whereas the genomes of PCV2 isolates range from 1,767 to 1,768 bp in length.
PCV2 isolates from pigs in different countries share approximately 94.6-99% nucleotide sequence identities (De Boisséson et al., 2004; Fenaux et al., 2000). The two main viral genes are ORF1 and ORF2 which are oriented in opposite directions and represent approximately 93% of the PCV2 genome. ORF1 encodes the Rep proteins involved in virus replication, and is highly conserved among isolates (Mankertz et al., 1998). The ORF2 gene encodes for the capsid protein (Nawagitgul et al., 2000, 2002) and shares approximately 91-100% nucleotide sequence identity and 90-100% amino acid sequence identity among PCV2 isolates (De Boisséson et al., 2004; Fenaux et al., 2000). In addition to ORF1 and ORF2, there are several other potential ORFs with coding capacity of larger than 5kDa (Meehan et al., 1998). Recently, the ORF3 protein has been characterized in vitro and shown to be not essential for PCV2 replication but is involved in PCV2-induced apoptosis by activating caspase-8 and caspase-3 pathways (Liu et al., 2005).

During productive infection of PK-15 cells, nine RNAs, capsid RNA, five Rep-associated RNAs (Rep, Rep’, Rep3a, Rep3b, and Rep3c), and three NS-associated RNAs (NS515, NS672, and NS0) are synthesized by PCV2 (Cheung, 2003c), and it was demonstrated that Rep and Rep’ are essential for PCV2 replication (Cheung, 2003b). PCV1 and PCV2 were found to differ from each other in expression levels of NS- and Rep3c-associated RNAs (Cheung 2003a).

Several groups attempted to investigate the potential correlation between PCV2 genomic sequences and pathogenicity. Mahé et al. (2000) identified four dominant immunoreactive epitopes in ORF2 by PEPSCAN analysis. Larochelle et al. (2002) identified three major regions of amino acid heterogeneity among PCV2 isolates, and two of the regions corresponded to two of the immunoreactive epitopes described by Mahé et al. (2000). Comparison of three immunodominant regions, however, revealed no link between capsid protein sequence variation and pathogenicity of PCV2 isolates (Larochelle et al., 2002). There was no distinct difference in genomic sequences among PCV2 isolates recovered from healthy pigs and diseased pigs (Choi et al., 2002; De Boisséson et al., 2004; Grierson et al., 2004; Larochelle et al., 2002, 2003; Pogranichnyi et al., 2002). These studies led to the assumption that there is no difference in pathogenicity among PCV2 isolates.

However, recently it has been demonstrated that two amino acid changes in the PCV2 capsid
protein that occurred during serial in vitro passage enhanced the ability of PCV2 to grow in vitro and attenuated the virus in vivo (Fenaux et al., 2004b).

The overall objectives of this study were to determine if there is a difference in PCV2 virulence by experimentally inoculating specific-pathogen-free (SPF) pigs with PCV2 isolates recovered from pigs with and without the hallmark PCV2-associated lymphoid lesions, and to determine if there are genetic changes in the PCV2 genome associated with the difference in virulence.

**METHODS**

**PCV2 isolates.** The PMWS-associated PCV2-40895 isolate (GenBank accession number AF264042) was recovered from a 40 kg pig that was diagnosed with PMWS in 1998 in an Iowa farm. The pig had severe lymphoid depletion, moderate histiocytic replacement of follicles in lymphoid tissues and high amounts of PCV2-antigen associated with the lesions as determined by immunohistochemistry (IHC) (Sorden et al., 1999). PCV2 isolate 40895 has been well characterized genetically (Fenaux et al., 2000) and in the SPF pig model (Fenaux et al., 2002, 2003 2004a, b; Opriessnig et al., 2003, 2004a, b, 2006a, b). The non-PMWS-associated PCV2 isolate 4838 was recovered from a 45 kg pig that died from a respiratory disease in 2003 in an Iowa farm. *Streptococcus suis* and *Pasteurella multocida* type A were isolated from the lungs of the pig by routine culture and *Mycoplasma hyopneumoniae* antigen was also detected by routine fluorescent antibody (FA) test on frozen lung sections. In addition, swine influenza virus (SIV) antigen was detected in the lungs of this pig by IHC (Haines et al., 1993). The pig had been vaccinated against *M. hyopneumoniae*, SIV, *Erysipelothrix rhusiopathiae*, and *Lawsonia intracellularis*. There was no evidence of PCV2-associated lymphoid lesions in the pig from which PCV2 isolate 4838 was recovered and IHC staining for PCV2 antigens was negative in lymphoid tissues and lungs. However, PCR for PCV2 DNA (Fenaux et al., 2000) was positive on pooled lymphoid tissues of this pig.

**Amplification and genetic analysis of the complete genomic sequence of PCV2 isolate 4838.** The PCV2 isolate 40895 from a pig with PMWS was sequenced in a previous study (Fenaux et al., 2000). To determine the complete genomic sequence of PCV2 isolate...
4838 which was not associated with PMWS, DNA was extracted from pooled lymph node homogenates of pig 4838 according to the protocol of the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The complete genomic DNA of PCV2 4838 was amplified by PCR with PCV2-specific primers F-PCV2SacII and R-PCV2SacII as previously described (Fenaux et al., 2002) with the following modifications: The extracted DNA was amplified by PCR with Platinum® PCR SuperMix High Fidelity (Invitrogen Inc., Carlsbad, CA). The PCR consisted of an initial enzyme activation step at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50 °C for 1 minute, and extension at 68°C for 2 minutes, and a final extension at 68°C for 30 minutes. The amplified PCR product of PCV2 isolate 4838 genome was excised from the gel, purified and subsequently cloned into the vector pCR 2.1 using the Original TA Cloning Kit (Invitrogen Inc.). The TA plasmid containing PCV2 isolate 4838 was subsequently sequenced using the M13 forward and reverse primers, as well as several PCV2-specific primers including AORF2 (5’-GCCCT-GAATTCCATATGAAATAATTA-3’), BORF2 (5’-CCGAAGTGCGCTGGTAATCTTAC-3’), UP (5’ GGTAACGCCTCCTTGAGATACTTGTC 3’) and LP (5’ GGGCCAAAA-AAGGTACAGTCC 3’). The sequences were assembled using AssemblyLIGN (Accelrys, San Diego, CA). The complete sequence of the PCV2 isolate 4838 was then aligned and compared with that of PCV2 isolate 40895. The percentage of nucleotide sequence and amino acid sequence identities among PCV2 isolates 4838 and 40895 were determined using the MacVector computer program (Accelrys).

**Production of virus inocula.** To generate an infectious stock of PCV2 isolate 4838 for animal studies, the PCV2 isolate 4838 virus genome cloned in the TA vector was first digested with the SacII enzyme to release the complete viral genome. The expected virus genome band of approximately 1.7 kb was excised from the agarose gel and purified using the GeneClean Spin Kit (Q-biogene Inc., Morgan Irvine, CA). The concentration of the viral genomic DNA was determined on a 1% agarose gel using the molecular weight ladder, Hyperladder I (Bioline USA Inc., Randolph, MA). The purified viral genomic DNA was concatemerized using T4 DNA ligase (Stratagene Coop., La Jolla, CA) in an overnight ligation reaction at 4°C. To generate an infectious PCV2 stock, approximately 12 µg of the concatemerized PCV2 genomic DNA was transfected into each of seven T-25 flasks of 70%
confluent PK-15 cells using Lipofectamine and Plus Reagent (Invitrogen Inc.) essentially as previously described (Fenaux et al., 2002). Three days after transfection, the virus was harvested by freezing and thawing the transfected PK-15 cells three times. The infectivity titer of the PCV2 isolate 4383 virus stock was determined by inoculation of PK-15 cells with serially-diluted virus stock followed by immunofluorescence assay (IFA) with PCV2-specific antibodies as described by Fenaux et al. (2002). The infectivity titer for PCV2 isolate 4838 virus stock was $0.5 \times 10^{4.5}$ 50% tissue culture infective dose (TCID$_{50}$) per ml.

For PCV2 isolate 40895, the viral genome was excised from the PCV2-40895 infectious DNA clone (Fenaux et al., 2002) using the SacII restriction enzyme. The purified viral genome was concatemerized, and subsequently transfected into each of fifteen T-25 flasks of 70% confluent PK-15 cells using Lipofectamine and Plus Reagent as previously described (Fenaux et al., 2002). Three days after transfection, the virus stock was harvested and its infectivity titer was determined essentially the same as described for the PCV2 isolate 4838 and as previously described (Fenaux et al., 2002). The infectivity titer of PCV2 isolate 40895 virus stock was $0.5 \times 10^{5.8}$ TCID$_{50}$ per ml.

**Experimental characterization of PCV2-4838 and PCV2-40895.** Forty-two colostrum-fed, crossbred, SPF pigs were purchased from a herd that is routinely tested for major swine pathogens and known to be free of porcine reproductive and respiratory syndrome virus (PRRSV) and *M. hyopneumoniae*. The pigs were weaned at two weeks of age and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, Iowa. At the time of inoculation, seven of the 42 pigs had evidence of low levels of passively acquired antibodies to PCV2 as determined by PCV2 enzyme linked immunosorbent assay (ELISA) based on the recombinant PCV2 capsid protein for PCV2-specific IgG antibodies (Nawagitgul et al., 2002). Those pigs were assigned to the negative control group 1. The remaining pigs were randomly assigned either to the control group or to one of the other two groups. The groups were housed in three separate rooms on raised wire decks. The rooms were identical in environmental controls and size. At seven weeks of age, group 1 pigs ($n = 14$) were sham-inoculated with 1.7 ml saline intramuscularly and 1.8 ml saline intranasally and served as the control group. Group 2 pigs ($n = 14$) were each inoculated with PCV2 isolate 4838: each pig received 1.7 ml intramuscularly and 1.8 ml
intranasally (approximately $10^{4.7}$ TCID$_{50}$ per pig). Group 3 pigs ($n = 14$) were each inoculated with PCV2 isolate 40895: each pig received 1 ml virus and 0.7 ml saline intramuscularly and 1 ml virus and 0.8 ml saline intranasally (approximately $10^{4.7}$ TCID$_{50}$ per pig).

**Serology.** Blood samples were collected at arrival of the pigs in the research facility at 36 days and again at 27 and 5 days before PCV2 inoculation and sham-inoculation and at 7, 14, 21, and 28 days post inoculation (DPI) and tested by PCV2 ELISA (Nawagitgul et al., 2002). Samples were considered positive if the calculated sample-to-positive (S:P) ratio was 0.2 or greater. We also used the same ORF2 capsid antigen to detect PCV2-specific IgM antibodies. Corrected optical density (OD) values were generated from the OD values against recombinant baculovirus carrying the ORF2 gene of PCV2 (AcMNPV.ORF2) subtracted from that against wild type baculovirus (AcMNPV.wt) which served as control antigen (Nawagitgul et al., 2002).

The serum samples from 3 randomly selected pigs in each group taken on arrival at the research facility and taken at necropsy were tested for the presence of antibodies to PRRSV by PRRSV-ELISA (IDEXX Laboratories, Inc. Westbrook, MA), to porcine parvovirus (PPV) by hemagglutination inhibition (HI) assay (Mengeling et al., 1988), to *M. hyopneumoniae* by ELISA as described (Bereiter et al., 1990), and to H1N1 SIV and H3N2 SIV by HI assays according to the protocol used at the Veterinary Diagnostic Laboratory at Iowa State University.

**Clinical evaluation.** Following PCV2-inoculation, the pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). In addition, pigs were evaluated daily for clinical signs including sneezing ranging from 0 (no sneezing) to 3 (severe persistent sneezing), and jaundice. Rectal temperatures, wasting, and behavioral changes such as lethargy were recorded daily. The pigs were weighed on the day of PCV2 inoculation and at seven, 14, 21 and 28 DPI.

**PCV2 DNA quantification.** DNA-extractions on sera collected five days prior to PCV2 inoculation and at 7, 14, 21 and 28 DPI were performed using the QIAamp® DNA Mini Kit (Qiagen). DNA-extracts were used for quantification of the copy number of PCV2
genomic DNA by real-time PCR as described previously (Opriessnig et al., 2003).

**Necropsy.** Necropsies were performed on half of the pigs at 14 DPI and the remainder at 28 DPI. The total amount of macroscopic lung lesions ranging from 0 to 100% of the lung affected, and size of lymph nodes ranging from 0 (normal) to 3 (four times the normal size) were estimated (Halbur et al., 1995; Opriessnig et al., 2004a).

Lungs were insufflated with fixative as previously described (Halbur et al., 1995). Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

**Histopathology.** Microscopic lesions were evaluated in a blinded fashion. Lung sections were scored for presence and severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe diffuse) (20). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2004a).

**Immunohistochemistry.** IHC for detection of PCV2-specific antigen was performed on selected formalin-fixed and paraffin-embedded section of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, and thymus using a rabbit polyclonal antiserum (Sorden et al., 1999). PCV2-antigen scoring was done in a blinded fashion and scores ranged from 0 (no signal) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2-antigen staining) (Opriessnig et al., 2004a). The mean group score was determined for each tissue and compared among groups.

**PCR and sequencing.** PCR products amplified from virus recovered from three selected pigs at 28 DPI in each inoculation group were sequenced and compared to the respective inoculum to confirm the origin of the infecting virus. A nested PCR was used to amplify the entire ORF2 gene for sequencing and sequence comparison. The PCR reaction for a 50-µl reaction (first and second rounds) contained 1 × *Taq* buffer (Promega, Madison, WI), 2 mM MgCl₂ (Promega), 0.2 mM deoxynucleoside triphosphates, 0.20 µM of the primer
(outer or nested pair), 1.5 U Taq polymerase (Promega), and 8 μl of extracted DNA template or first-round product. The external primer pair for the first round PCR was N1ORF2 (5’-GGAACTGTACCTTTTTTGCCCG-3’) and N2ORF2 (5’-GAAGGATTATTACGCGTG-AACACCC-3’) and the internal primer pair for the nested PCR was AORF2 (5’-GCCCTG-AATTCCATATGAAATAAATTA-3’) and BORF2 (5’-CCGAAGTGCGCTGTAATA-CTTAC-3’). The first round PCR consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; 7 min final extension at 72°C; and a 4°C hold. The second round PCR consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; 7 min final extension at 72°C; and a 4°C hold.

The PCR products were run on a 1% agarose gel and the expected 820 bp bands were excised, purified and sequenced at the Virginia Bioinformatics Institute at Virginia Tech using an Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA). The sequences were analyzed with the MacVector computer program and compared to the sequences of the original virus inocula.

Statistical analysis. Summary statistics were calculated for all groups to assess the overall quality of the data. Continuous data were analyzed using analysis of variance (ANOVA). If an ANOVA was significant (P < 0.05), pairwise testing using the Tukey’s adjustment was performed to assess specific group differences. Differences between the PCV2 infected groups were compared by t-tests. In order to summarize and simplify the clinical observations, response feature analysis and chi-square test was used. The clinical scores for each pig were reduced to one weekly mean score and the resulting values were subject to statistical analysis. Daily rectal temperature data were analyzed with response feature analysis on average weekly temperature. Non-repeated measures of necropsy and histopathology data were assessed using non-parametric Kruskal-Wallis one-way ANOVA. If this non-parametric ANOVA test was significant (P < 0.05), then pairwise Wilcoxon tests were used to assess differences between groups.

Nucleotide sequence accession number. The complete genomic sequence of PCV2 isolate 4838 has been deposited with the GenBank database under accession number
DQ397521. The sequence of PCV2 isolate 40895 has been reported previously (GenBank accession number AF264042) (Fenaux et al., 2000).

RESULTS

Genetic characterization of PCV2 isolates 4838 and 40895. The complete genomes of PCV2-4838 and PCV2-40895 were both 1,768 bp in length. The nucleotide sequence identity between PCV2-4838 and PCV2-40895 across the entire genome was 98.9% with a total of 19 nucleotide differences between the two isolates. In ORF1, PCV2 isolates 40895 and 4838 share approximately 99.5% nucleotide and 99.4% amino acid sequence identities. There were a total of 5 nucleotide differences between PMWS-associated PCV2 isolate 40895 and the non-PMWS associated PCV2 isolate 4838. Two of the five nucleotide differences were non-silent and resulted in amino acid changes: at position 280, PCV2-4385 had a serine compared to a phenylalanine in PCV2-40895, and at position 302, PCV2-4838 had a proline compared to a serine in PCV2-40895.

The ORF2 capsid gene of PCV2 isolates 40895 and 4838 share approximately 98.0% nucleotide and 96.1% amino acid identities with a total of 14 nucleotide differences between the two isolates. Eleven of the 14 nucleotide differences resulted in 9 amino acid changes between PCV2-4838 and PCV2-40895. The amino acid differences were found at positions 59 (arginine vs alanine), 63 (threonine vs arginine), 75 (lysine vs asparagine), 76 (leucine vs isoleucine) 131 (proline vs threonine), 134 (proline vs threonine), 185 (leucine vs methionine), 206 (lysine vs isoleucine), and 232 (asparagine vs lysine) for PCV2-4838 vs PCV2-40895, respectively.

In ORF3, PCV2 isolates 40895 and 4838 share approximately 99.0% nucleotide and 99.7% amino acid sequence identities. There was only one nucleotide difference resulting in an amino acid change: at position 46 of the ORF3, PCV2-4838 had a phenyalanine whereas PCV2-40895 had a leucine.

Antibody responses of PCV2-4838 and PCV2-40895 infections in pigs. Both PCV2-infected groups had significantly ($P < 0.0001$) higher PCV2-IgM corrected OD-values compared to negative control pigs by 14 DPI. PCV2-specific IgM antibodies peaked by 21
DPI in PCV2-40895 infected pigs (significantly \([P < 0.0001]\) higher than in the PCV2-4838 inoculated pigs) and waned by 28 DPI (Fig. 1).

In the PCV2-4838 inoculated pigs, four of the 14 pigs seroconverted by 14 DPI, two of seven pigs were positive for PCV2-specific IgG by 21 DPI, and three of the seven pigs were positive for PCV2-specific IgG by 28 DPI. In the PCV2-40895 inoculated pigs, one of the 14 pigs, six of the 14 pigs, three of the seven pigs, and four of the seven pigs were positive for PCV2-specific IgG by 7, 14, 21, and 28 DPI, respectively. There were no significant differences in mean anti-PCV2-IgG S:P ratios (Fig. 2).

**Comparative pathogenicity of PCV2-4838 and PCV2-40895 in SPF pigs.** To compare the virulence of the PMWS-associated PCV2 isolate 40895 and the non-PMWS PCV2 isolate 4838, various clinical, pathological, virological and serological parameters were compared.

**Clinical disease.** There was clinical evidence of mild respiratory disease characterized by sporadic sneezing observed in both of the PCV2-inoculated groups from five to seven DPI. None of the pigs developed rectal temperatures above 40°C and there was no difference in mean rectal temperatures between the two PCV2-infected groups. The average daily weight gain was not different between groups.

**PCV2 viremia length and genomic copy numbers.** Viremia was detected by PCR in 12/14, 10/14, 5/7, and 6/7 pigs inoculated with PCV2-4848; and in 6/14, 13/14, 7/7, and 7/7 pigs inoculated with PCV2 40895 at 7, 14, 21, and 28 DPI, respectively. The mean numbers of PCV2 genomic copies in serum are summarized in Fig. 3. At 7 DPI, the pigs inoculated with PCV2-4848 had significantly \((P < 0.001)\) higher numbers of PCV2 genomic copies in sera compared to the pigs inoculated with PCV2-40895. At 14, 21, and 28 DPI, the number of PCV2 genomic copies in sera was significantly \((P < 0.01, P < 0.01, \text{and} P < 0.05, \text{respectively})\) higher in the pigs inoculated with PCV2-40895 compared to the pigs inoculated with PCV2-4838.

**Macroscopic lesions.** At 14 DPI, one pig inoculated with PCV2-40895 had mottled-tan lungs that failed to collapse. Lymph node enlargement was 1.4 ± 0.3 for the PCV2-4838-inoculated pigs and it was 2.7 ± 0.2 for the PCV2-40895-inoculated group, which was significantly \((P < 0.01)\) different. At 28 DPI, one pig inoculated with PCV2-40895 had renal
lymph nodes that were approximately five times the normal size and the kidneys had severe interstitial nephritis and multifocal renal infarcts. The average enlargement of the lymph nodes at 28 DPI was 0.1 ± 0.1 for the PCV2-4838 inoculated pigs and 1.7 ± 0.4 for the PCV2-40895 inoculated pigs, which again was a significantly (P < 0.01) different.

**Microscopic lesions and amount of intralesional PCV2-antigen.** The PCV2-40895 inoculated pigs developed moderate-to-severe, generalized lymphoid lesions with lymphoid depletion and histiocytic infiltration of depleted lymphoid follicles. The mean lymphoid depletion scores in the PCV2-40895 inoculated pigs were significantly (P < 0.05) more severe than those observed in the PCV2-4838-inoculated pigs (Table 1). It should also be noted that when lymphoid lesions were observed in the PCV2-4383 inoculated pigs they were often confined to one lymph node. The IHC results are summarized in Table 2. In the majority of the tissues evaluated, the PCV2-40895 inoculated pigs had significantly (P < 0.05) higher amounts of PCV2-antigen compared to the PCV2-4838 inoculated pigs. In addition to the PCV2-associated lymphoid lesions, the PCV2-40895 inoculated pigs also had significantly (P < 0.02) more severe interstitial pneumonia which was associated with significantly (P < 0.01) higher amounts of PCV2-antigen at 14 DPI. There was also significantly (P < 0.001) more severe lymphohistiocytic hepatitis and significantly (P < 0.02) more severe lymphohistiocytic interstitial nephritis in the PCV2-40895 pigs at 14 DPI. At DPI 28, there was severe lymphohistiocytic interstitial nephritis associated with PCV2-antigen in one of the PCV2-40895 inoculated pigs.

**The pathological lesions observed in the PCV2 infected pigs were solely attributable to the respective PCV2 inoculum.** Several experiments were performed to confirm that the pathological lesions observed in inoculated pigs were indeed induced by respective PCV2 inoculum.

**Sequencing confirmation.** The sequences recovered from three selected PCV2-4838 inoculated pigs and from three selected PCV2-40895 inoculated pigs are identical to the respective sequences from the original virus inoculum.

**Negative PCV2 status of the source pigs.** The passively acquired antibodies to PCV2 in the seven control pigs decayed over the 14 days following arrival and all control pigs remained negative for PCV2-specific antibodies through the termination of the experiment.
All negative control pigs were negative for PCV2 DNA in serum for the entire duration of the study. Lymphoid tissues and lungs from the control pigs were negative for PCV2 antigen as determined by IHC (Table 2). PCV2-associated microscopic lymphoid lesions were not present in the control pigs (Table 1).

Absence of other infectious agents in the pigs. All pigs tested were negative for PRRSV and *M. hyopneumoniae*-specific antibodies. The pigs had maternal antibodies to PPV, H1N1 SIV and H3N2 SIV which all decayed over time. Indication of seroconversion to PRRSV, PPV, H1N1 SIV or H3N2 SIV was lacking. All tissues from the control pigs were normal and there were no lesions suggestive of an infectious disease.

**DISCUSSION**

Most pig herds are infected with PCV2 and most pigs within herds are exposed to and seroconvert to PCV2 at some time during the growing period; however, many of the herds report no evidence of PCV2-associated disease or PMWS while others report high PMWS incidence and high mortality. Differences in virulence of PCV2 isolates could be one logical explanation for these observed differences in disease manifestation.

The overall goals of this study were to determine if there is evidence of differences in virulence among PCV2 isolates that may at least in part explain differences in severity of clinical manifestation of PCV2-associated disease in the field, and to determine if there is a genetic basis associated with the difference in virulence. To our knowledge, a carefully controlled study comparing PCV2 isolates in experimentally-infected SPF pigs typical of those used in commercial production has not been done.

Molecular studies to determine the genetic variations of PCV2 revealed that minor branches of PCV2 were associated with geographic origin rather than with differences in virulence (Fenaux *et al*., 2000; Hamel *et al*., 1998; Mankertz *et al*., 2000; Meehan *et al*., 2001). Overall there were no interpretable differences between PMWS-associated isolates and PCV2 isolates recovered from cases not associated with PMWS (De Boisséson *et al*., 2004; Larochelle *et al*., 2002, 2003; Grierson *et al*., 2004) and it was concluded that differences in primary nucleotide sequence do not explain the differences in virulence seen in the field. However, when seven different PCV2 strains were compared in PK-15 cells, it was...
found that abortion-associated strains (GeneBank accession numbers AJ293867 and AJ293868) had different replication kinetics compared to PMWS or PDNS-associated PCV2 strains (Meerts et al., 2005). A higher number of infected cells were observed at 24 hours post inoculation and the percentage of infected cells with nuclear localized antigens was lower compared to that of the other strains (Meerts et al., 2005). Wang et al. (2004) compared the ORF2 of Taiwanese PCV2 isolates recovered from four PMWS-associated cases, two PDNS-associated cases, one case with nervous signs, and one abortion-associated case, and found a small number of amino acid differences associated with the different clinical conditions. Farnham et al. (2003) further characterized two PCV2 isolates associated with abortions by ORF2 sequencing and found that the isolates were almost identical to each other and to other isolates associated with reproductive failure whereas there were at least two amino acids differences compared to PCV2 isolates associated with PMWS.

The results of the present study indicate that the two PCV2 isolates 4838 and 40895 differ significantly in several parameters in vivo including serum antibody profiles, amount of PCV2 genome in serum, amount of PCV2 antigen in lymphoid tissues, and severity of PCV2-associated gross and microscopic lesions. PCV2 isolate 4838 was less virulent compared to PCV2 isolate 40895 in this study. The two PCV2 isolates have an overall nucleotide sequence identity of 98.9%. Within the ORF2 capsid gene, a total of 11 non-silent nucleotide differences, resulting in 9 amino acid differences, were identified between these two isolates. Larochelle et al. (2002) identified three immunogenic regions in ORF2 at residues 59-80, residues 121-136, and at residues 180-191. Seven of the nine amino acid differences between PCV2-4838 and PCV2-40895 were located in these immunogenic regions. The T134P difference in PCV2-4838 was reported in a Canadian isolate recovered from a non-PMWS pig with generalized tremors (CAN-97V19; 27). De Boisséson et al. (2004) compared French PCV2-isolates and determined the most frequent mutations in PCV2 genomes. Five of the nine ORF2 amino acid differences between the two isolates observed in the present study (A59R, T63R, L185M, K206I, and N232K) corresponded to these most frequent mutations whereas the two ORF1 amino acid differences S280F and P302S were not among the most frequently reported ORF1 mutations (De Boisséson et al., 2004). Moreover, comparison of the PCV2-4838 ORF1 change with 44 sequences from the
GeneBank (data not shown) did not result in any matches at position 302, thus it appears that this amino acid in ORF1-PCV2-4838 is unique. A chimeric virus created by substitution of the PCV2-ORF1 with that of PCV1 in the genomic backbone of PCV2 has shown to be attenuated in pigs (Fenaux et al., 2003).

The sequence analysis does show that the two PCV2 isolates in this study, with apparent differences in virulence in the field and under experimental conditions, also differ genetically which is in contrast to other studies. At least two studies (De Boisséson et al., 2004; Grierson et al., 2004) have found identical PCV2 sequences in clinically healthy and diseased pigs. More than one PCV2 sequence can be found in the same pig under field conditions (unpublished personal observation) and a possible explanation for finding identical PCV2 sequences in healthy and diseased pigs is that a copy of a rare variant in the population rather than the causative PCV2 was sequenced. This emphasizes the importance of in vivo comparison of isolates.

It has been reported that differences in virulence of attenuated chicken anemia virus (CAV), another member of the Circoviridae family, obtained after a large number of passages in cell culture, are associated with a few nucleic acid changes in the CAV genome (Todd et al., 2002; Yamaguchi et al., 2001). Recently, we reported that two amino acid changes in the PCV2 capsid protein that occurred during serial in vitro passage slightly enhanced the ability of PCV2 to grow in vitro and attenuated the virus in vivo (Fenaux et al., 2004b). None of the two previously described ORF2 amino acid mutations, P110A and R191S, were found in PCV2 isolate 4838. However, the current study is different in that isolate 4838 is a true field isolate and its genetic determinant(s) for virulence could be different from the ones identified during serial in vitro passages. In addition, other factors may also contribute to viral virulence. It is possible that the observed amino acid differences in the present study in ORF1 and ORF2 either alone or collectively may be responsible for the observed difference in virulence, and clearly further studies are warranted to fine-map the critical mutation(s) responsible for virulence.

It has been demonstrated experimentally that PCV2 replication is enhanced and PCV2-associated lesions are more severe in pigs that are coinfectected with pathogens such as PPV (Allan et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000; Opriessnig et al.,
2004), PRRSV (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002), or M. hyopneumoniae (Opriessnig et al., 2004a). Several groups have also demonstrated that immunostimulation in the form of adjuvanted vaccines (Allan et al., 2000b; Opriessnig et al., 2003) may also enhance the severity of PCV2-associated disease and lesions. We knew from previous studies that our isolate PCV2-40895 was of moderate-to-high virulence (Fenaux et al., 2002, 2003, 2004a, b; Opriessnig et al., 2003, 2004a, b, 2006a, b). We decided to use isolate PCV2-4838 from a field case where there was no evidence of PCV2-associated disease or lesions despite having multiple coinfections and use of adjuvanted vaccines in the growing pigs. Our reasoning was that the number and combination of other cofactors were such that conditions were optimum for this isolate to have expressed its virulence.

Based on the parameters measured in this study, there are significant differences in virulence between the PCV2-40895 and PCV2-4838 isolates in experimentally infected SPF pigs. This knowledge does help explain differences observed in the field. Most herds are infected with PCV2 but relatively few have major losses associated with PCV2 infection, suggesting that perhaps the majority of the PCV2 isolates are non- or low-virulent. To further substantiate that these low virulent PCV2 isolates are not a relevant problem would require demonstrating that known cofactors such as concurrent infections or use of immune stimulants, such as adjuvanted vaccines, do not trigger progression of PCV2 infection to PMWS as has been demonstrated with the PCV2-40895 isolate (Opriessnig et al., 2004a, b). These low virulent isolates such as PCV2-4838 should also be inoculated into pigs known to have increased susceptibility to PCV2-associated disease, such as certain lines of Landrace pigs (Opriessnig et al., 2006a).

To our knowledge, this is the first report to demonstrate a clear difference in virulence between two well-characterized PCV2 isolates in a controlled experimental setting using a SPF pig model typical of modern pork production. Since only a few genetic differences were found between the two isolates compared in this study, these two isolates and the identified genetic difference could serve as a launching pad for experimentally determining which genetic differences are important for virulence differences in the future. The availability of infectious PCV2 DNA clones and a well-defined pig model will position
scientists now to further understand the molecular basis of pathogenesis of PCV2-associated diseases, develop effective vaccines, and improved diagnostic assays.

ACKNOWLEDGEMENTS

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REFERENCES


7. **Cheung, A. K. (2003b).** The essential and nonessential transcription units for viral


TABLE 1. Comparison of microscopic lymphoid lesions in pigs inoculated with porcine circovirus type 2 (PCV2)-4838, in pigs inoculated with PCV2-40895, and in sham-inoculated control pigs at 14 and 28 days post inoculation. Score ranges from 0 (no lesions) to 3 (severe) for lymphoid depletion (LD) and histiocytic replacement (HR) of lymphoid follicles. Data presented as incidence (mean group score ± SE)

<table>
<thead>
<tr>
<th></th>
<th>Tracheobronchial Lymph Node</th>
<th>Lymph Nodes Pool</th>
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<th>Tonsil</th>
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<tr>
<td></td>
<td>LD</td>
<td>HR</td>
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<td>HR</td>
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<tr>
<td>14 days post PCV2 inoculation</td>
<td></td>
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<tr>
<td>PCV2-4838 (n = 7)</td>
<td>4/7 (0.9±0.3)A(^b)</td>
<td>3/7 (0.6±0.3)A(^b)</td>
<td>4/7 (0.7±0.3)A</td>
<td>3/7 (0.4±0.2)A</td>
</tr>
<tr>
<td>PCV2-40895 (n = 7)</td>
<td>7/7 (2.0±0.2)B</td>
<td>7/7 (1.3±0.2)A</td>
<td>7/7 (2.4±0.2)B</td>
<td>7/7 (1.9±0.3)B</td>
</tr>
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<td>Controls (n = 7)</td>
<td>3/7 (0.4±0.2)A</td>
<td>0/7 (0.0±0.0)B</td>
<td>3/7 (0.4±0.2)A</td>
<td>0/7 (0.0±0.0)A</td>
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<td>28 days post PCV2 inoculation</td>
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<tr>
<td>PCV2-4838 (n = 7)</td>
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<tr>
<td>PCV2-40895 (n = 7)</td>
<td>6/7 (1.3±0.4)</td>
<td>4/7 (0.9±0.4)</td>
<td>7/7 (1.3±0.4)B</td>
<td>5/7 (1.3±0.4)B</td>
</tr>
<tr>
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<td>0/7 (0.0±0.0)</td>
<td>4/7 (0.6±0.2)A</td>
<td>0/7 (0.0±0.0)A</td>
</tr>
</tbody>
</table>

a Superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes.

b Different superscripts within columns (A, B, C) represent significantly (P < 0.05) different mean group scores within columns for each day post inoculation.
TABLE 2. Comparison of amount of porcine circovirus type 2 (PCV2)-antigen in tissues demonstrated by immunohistochemistry (IHC) in pigs inoculated with PCV2-4838, in pigs inoculated with PCV2-40895, and in sham-inoculated control pigs at 14 and 28 days post inoculation. Score ranges from 0 (undetectable) to 3 (large amount of PCV2 antigen). Data presented as incidence (mean group score ± SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung</th>
<th>Tracheobronchial Lymph Nodes</th>
<th>Lymph Node Pool</th>
<th>Spleen</th>
<th>Tonsil</th>
<th>Thymus</th>
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<tr>
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<td>2/7 (0.4±0.3)\textsuperscript{A}</td>
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<tr>
<td>PCV2-40895 (n = 7)</td>
<td>6/7 (1.2±0.4)\textsuperscript{B}</td>
<td>7/7 (2.1±0.3)\textsuperscript{B}</td>
<td>7/7 (2.4±0.3)\textsuperscript{B}</td>
<td>7/7 (1.7±0.4)\textsuperscript{B}</td>
<td>4/7 (0.9±0.4)\textsuperscript{B}</td>
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<tr>
<td>Controls (n = 7)</td>
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<td>6/7 (1.4±0.4)\textsuperscript{B}</td>
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<td>Controls (n = 7)</td>
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\textsuperscript{a} Superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes.

\textsuperscript{b} Different superscripts within columns (A, B) represent significantly (p<0.001 for all tissues except thymus: p<0.05) different mean group scores within columns for each DPI.
FIG. 1. Mean group IgM (Corrected optical density values) antibody response to porcine circovirus type 2 (PCV2)-4838 and PCV2-40895 at the day of inoculation and at 7, 14, 21, and 28 days post PCV2 inoculation (DPI). * = significant ($P < 0.05$) differences between groups.
FIG. 2. Mean group IgG (Serum-to-positive ratios) antibody response to porcine circovirus type 2 (PCV2)-4838 and PCV2-40895 at the day of inoculation and at 7, 14, 21, and 28 days post PCV2 inoculation (DPI). A = Residual passively acquired antibodies in 7 of 14 of the control pigs. * = significant ($P < 0.05$) differences between groups.
FIG. 3. Mean group porcine circovirus type 2 (PCV2) genomic copy numbers obtained from pigs inoculated with PCV2-4838 and obtained from pigs inoculated with PCV2-40895 pigs at 7, 14, 21, and 28 days post inoculation (DPI). Error bars represent standard errors. * = significant ($P < 0.05$) differences between groups.
CHAPTER 11. GENERAL CONCLUSIONS

Porcine circovirus type 2 (PCV2) is considered to be the third most important viral pathogen in the US pig population (Halbur et al., 2005). Its ubiquitous distribution in the pig population and its presence in healthy as well as in diseased herds distinguishes PCV2 from most classical swine pathogens. It is well known that the presence of PCV2 doesn’t necessarily equate to PCV2-associated disease. The reasons for this need to be better understood and our work described in this thesis has contributed substantially to the base of knowledge that is beginning to clarify the pathogenesis and epidemiology of PCV2-associated diseases.

PCV2 has been linked to “postweaning multisystemic wasting syndrome” or “PMWS” (Harding and Clark, 1997), and it has been proposed by Sorden (2000) that a pig has to show characteristic clinical signs and the hallmark microscopic lesions in lymphoid tissues associated with PCV2-antigen. Soon it became clear, that PMWS may not have been the best choice of names. Many common diseases can cause wasting and the name wasting causes confusion with the transmissible spongiform encephalopathies observed in cervidae. On the other hand, large amounts of PCV2 antigen can be found in older pigs or even fetuses and neonates that are not primarily affected by wasting. This has led to the creation of the new term “PCV2-associated diseases” (PCVD). PCV2-associated diseases are “multifactorial” i.e. there is involvement of more than one factor or pathogen but the presence of PCV2 is one necessity.

This work describes our efforts to identify factors that contribute to PCV2-associated diseases in order to give researchers, diagnosticians, practitioners and pig producers tools to better understand and implement best practices for control of PCV2-associated problems. We looked at co-factors such as vaccine induced immunostimulation and concurrent infections, we investigated host-specific factors such as differences in pig genetics, and finally, we assessed virus-specific factors such as differences in PCV2-isolates.

The lack of PCV2 negative breeding herds is a major disadvantage for researchers focused on PCV2-related research. The specific-pathogen-free pig model is more suitable for application of research findings into field-situations than are the gnotobiotic pig model, the
cesarean-derived and colostrum-deprived (CDCD) pig model, or the colostrum-deprived (CD) pig model. We were successful in deriving pigs free of PCV2 and anti-PCV2 antibodies from commercial U.S. breeding herds by segregated early weaning and have documented this protocol (Opriessnig et al., 2004d). In rare cases, PCV2 has been shown to be transmitted vertically resulting in the birth of PCV2 positive piglets (Harms et al., 1999; Jolie et al., 2000). In assuming that dams that shed PCV2 intermittently likely have high anti-PCV2-antibody levels, we attempted to bypass the risk of bringing PCV2 positive piglets into our research facility by selecting piglets only from sows with negative or low levels of PCV2 antibodies. In following the same protocol for several projects, we were always successful in deriving PCV2 negative pigs and we found our “restricted” segregated early weaning method useful (Opriessnig et al., 2004d).

Evidence from European studies suggested that commercial vaccines can enhance PCV2-associated diseases in pigs (Allan et al., 2000c; Allan et al., 2002b, Kyriakis et al., 2002). We confirmed that this is also true for the US and we found that vaccination with adjuvanted bacterins enhanced PCV2 replication and the incidence and severity of the hallmark microscopic lesions associated with PCV2-infection in conventional pigs (Opriessnig et al., 2003). It is assumed that the underlying mechanism of vaccine-induced enhancement of PCV2-associated diseases is the stimulating effect of adjuvants on triggering proliferation of cells of the immune system. The major cell type that supports PCV2 replication in vivo has not been identified yet but the most likely scenario is that PCV2 replicated in many different cell types including macrophages and lymphocytes (Shan Yu, unpublished). In a follow up study we found that oil-in-water adjuvanted vaccines are more likely to enhance PCV2-associated lesions than aqueous-carbopol or aluminum hydroxide adjuvanted vaccines are (Hoogland et al., 2006). A similar observation was also made by Krakowka et al. (2006) who infected gnotobiotic pigs at 1 day of age with PCV2 and vaccinated them at 1 or 1 and 3 weeks of age with commercially available bacterins. Krakowka clearly showed a detrimental effect of a particular oil-in-water product (Amphigen®, Pfizer Animal Health, Inc.) resulting in clinical PMWS in 7/11 piglets, whereas piglets vaccinated with other products (MetaStim®, Fort Dodge Animal Health, Inc; Impran®, Boehringer Ingelheim, Ltd.) did not develop disease (Krakowka et al., 2006).
It would appear that timing of vaccination is important and we found a trend towards no to minimal PCV2-associated lesions when pigs are vaccinated with a *M. hyopneumoniae* bacterin 2 to 4 weeks prior to PCV2 exposure (Opriessnig et al., 2006b). Based on the research data available to date, our recommendation for herds with PCV2-associated problems is to determine the time of PCV2 exposure by means of serology and PCR and to place vaccinations, especially those with oil-in-water adjuvanted products, as far away as possible from this time. If the time of exposure to PCV2 can not be determined, it might be beneficial to change the product used and to switch to a bacterin that is not adjuvanted with an oil-in-water-based product.

By vaccinating pigs at the time of PCV2-infection with a modified-live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine and by challenging the pigs with PRRSV to evaluate vaccine efficacy we found an adverse effect of PCV2 infection on development of protective immunity induced by the PRRSV vaccine (Opriessnig and Halbur, 2005b). PCV2-associated lymphoid depletion is seen to some degree even in subclinically-infected pigs and it has been found that there is a correlation of the degree of lymphoid depletion and the amount of PCV2 in lymphoid tissues (Darwich et al., 2002). In this regard, a decrease of cells of the immune system has been described (Chianini et al., 2003; Darwich et al., 2002; Nielsen et al., 2004; Segales et al., 2000; Segales et al., 2002). Segales et al. (2000) reported of lower mean lymphocyte percentages in PMWS-affected pigs compared to non-affected pigs. Further analysis revealed that pigs suffering from PMWS had lower proportions of CD4+ and IgM+ cells in blood than clinically healthy pigs (Segalés et al., 2001). Darwich et al. (2002) found significantly decreased numbers of CD8+ and double-positive cells in PMWS-pigs (Darwich et al., 2002). Nielsen et al. (2003) found primarily lymphopenia with all T lymphocyte subsets involved in experimentally PCV2-inoculated pigs. Future work needs to investigate if this PCV2-induced decrease in development of an effective immune response to PRRSV vaccine can be extrapolated to other vaccines (swine influenza virus, *M. hyopneumoniae, Salmonella* sp.) commonly used on PCV2-infected pigs. The adverse effect of PCV2 infection on the development of protective immunity against PRRSV and other respiratory pathogens may be an important, and perhaps an underappreciated factor in controlling porcine respiratory disease complex (PRDC) and other
diseases in pigs. It will be interesting to see what impact the soon-to-be-released commercial PCV2 vaccines have on control of PRDC which continues to be the most important disease problem facing the U.S. swine industry.

In coinfecting pigs with porcine parvovirus (PPV) and PCV2 we confirmed that this coinfection results in clinical disease in conventional pigs (Opriessnig et al., 2004a). This work showed the importance of PPV in the PCV2-disease complex in conventional pigs. PPV is in many ways similar to PCV2. It is a small, non-enveloped DNA virus that is ubiquitously distributed in the pig population without being associated with disease except in pregnant females. Similar to PCV2, PPV has a strong cellular tropism for mitotically active tissues such as lymph nodes or heart muscle (Oraveerakul et al., 1993; Allan and Ellis, 2000). It is thought that stimulation of the host DNA to enter the S-phase of the cell cycle by PPV promotes PCV2 replication (Krakowka et al., 2000). A recent in vitro study found that PCV2 replication is enhanced by both type I interferon (IFN-α) and type II IFN (IFN-γ) which are strong antiviral cytokines produced to control viral infections (Meertz et al., 2005b). Moreover, Chang et al. (2005) infected swine alveolar macrophages with PCV2 and PRRSV and found that PCV2 induced upregulation of IFN-α production. Taken together, these findings imply that concurrent viral infections indirectly enhance PCV2 replication by enhancing interferon production. Further work needs to establish if this mechanism is also true in vivo.

In our work we have shown that the combination PPV and PCV2 can negatively impact a herd (Opriessnig et al., 2004a), and whatever the true mechanism of PPV-enhancement on PCV2 replication is, this highlighted the need for PPV diagnosis in growing pigs. PPV diagnosis is typically done by immunofluorescence assays on frozen tissue sections of aborted fetuses. In all other growing stages, serology is the test of choice to confirm PPV infection. In our work we developed an immunohistochemistry assay for PPV which we have validated and adapted for use in diagnostic cases and have found it to be useful for detecting PPV-antigen in formal-fixed and paraffin-embedded tissues from field cases. This test has been especially useful on cases that have lymphohistiocytic liver or heart lesions. We recently reported cases where both PPV and PCV2 antigen were associated with mycocarditis lesions in growing pigs (Opriessnig et al., 2006c). Both pathogens are not
typically observed in heart tissues in growing pigs. Maybe this is due to a lack of awareness and lack of tools to look rather than due to a true low incidence. We now are aware that this occurs and have the tools to confirm or rule out PCV2/PPV infection or coinfection.

The discovery that adjuvanted vaccines, and particularly *M. hyopneumoniae* vaccines, may enhance PCV2 replication and severity and incidence of PCV2-associated diseases has led some producers to discontinue successful *M. hyopneumoniae* vaccination programs. We found that *M. hyopneumoniae* potentiates the severity of PCV2-associated lung and lymphoid lesions, increases the amount and prolongs the presence of PCV2-antigen, and increases the incidence of PMWS in pigs (Opriessnig et al., 2004c). This model indicates that it is critical to control *M. hyopneumoniae* in herds where PCV2 and *M. hyopneumoniae* coinfection occurs and reducing the effect of *M. hyopneumoniae* by either vaccination or chlortetracycline treatment has shown to be beneficial (Halbur et al., 2005). The potentiating mechanism of *M. hyopneumoniae* infection on PCV2 is not known. We speculate that induction of peribronchial lymphoid hyperplasia and the mitogenic activity of *M. hyopneumoniae* on alveolar macrophage proliferation enhances PCV2 replication at the site of infection. In an attempt to further characterize the effect of dual *M. hyopneumoniae* and PCV2 infection on a pig, we also recently characterized the cytokine gene expression profiles in tracheobronchial lymph nodes (unpublished). We found that single PCV2 infection was characterized by significantly increased IFN-γ, IL-8 and chemokine gene expression. Dual-infection with *M. hyopneumoniae* resulted in a significant increase of IL1β, IL-8 (CXCL-8), CCL5 (RANTES), and CXCL10 (IP-10), and weak stimulation of IFN-β and IL-6. IL-13 and IFN-α were significantly down regulated; ICAM1 was slightly down regulated. The most significant increase in IFN-γ gene expression was observed with the PCV2/*M. hyopneumoniae* coinfection, in contrast to previous findings of decreased IFN-γ mRNA in TBLN of pigs with recent naturally acquired PMWS (Darwich et al., 2003a). The increase in IFN-γ, as proposed by Meerts et al. (2005b) as being one way to enhance PCV2 *in vitro*, the increase in chemokines, and the decrease in IFN-α, in our study was correlated with increases in severity of microscopic lesions and amount of PCV2-antigen. Thus, in the context of *M. hyopneumoniae* coinfection, up-regulation of certain cytokines and chemokines during the course of inflammation above a certain level has more of a counterproductive effect on PCV2
subsequently leading to PMWS.

In the field, PMWS is seen only in a small percentage of pigs infected with PCV2. We investigated the effect of host genetics in the susceptibility to PCV2 replication and PCV2-associated lesions and disease among pig breeds by comparing PCV2-associated disease and lesions in purebred Duroc, Landrace, and Large White in our SPF pig model. Clinical disease and microscopic lesions compatible with PMWS were only observed in the Landrace pigs suggesting a predisposition of the Landrace pigs used in this study (Opriessnig et al., 2006a). Although evidence from the field suggested there were differences in host susceptibility, this was the first experimental confirmation of differences among breeds in susceptibility to PCV2. This study was conducted with a small number of pigs from one farm. Some producers have taken this preliminary research data and evidence from the field and made decisions to change breeding programs on farms suffering major losses associated with PCV2. More solid data needs to be generated to support such major decisions.

Anecdotally, it has been suggested that Pietrains are less susceptible to PCV2-associated diseases. Rose et al. (2004) conducted a cohort study on 4 PMWS affected farms to investigate susceptibility to PCV2-associated diseases by manipulating the genetics via artificial insemination. Half of the sows were inseminated with Pietrain semen, whereas the remaining sows received the semen that was typically used on the farms. The PCV2-associated disease did not differ in the Pietrain offspring compared to that observed in the offspring from the other breeds (Rose et al., 2004). Nevertheless, France changed 80% of boar genetics towards Pietrain and the importance of PCV2-associated diseases decreased in this country (R. Desrosiers, PMWS Listening Session at the 2006 AASV Meeting, Kansas City, MI). Belgium, which appears less affected by PCV2-asscoiated diseases (Segalés, 2006), uses 100% Pietrain semen for synthetic pig production. Comparing Landrace and Pietrain pigs in the same experimental model is a logical next step since this has not been done before and evidence from the field supports differences among these two breeds.

Essentially nothing is known about the mechanism of susceptibility to PCV2-associated diseases. Once the results are confirmed in a larger number of pigs, researchers need to start looking closer at genes from pigs affected by PCV2-asscociated disease and their healthy littermates that share at least 50% of their genes. Genetic resistance may be associated with a
single gene, but usually it is associated with multiple genes and to investigate gene regulation by means of gene array analysis might be one way to continue this work. Using genetically engineered animals might be another approach.

The recent increased incidence of PCV2-associated diseases combined with high mortality in eastern Canada and North Carolina has raised concerns over the emergence of a new, more virulent PCV2 strain. The international PCV2 research community in general continues to argue against differences in virulence among PCV2 strains. This is mainly based on genetic comparisons of sequences rather than on *in vivo* studies. We attempted to compare the pathogenicity of PCV2 isolates obtained from field cases with and without PCV2-associated lymphoid lesions and disease in a controlled experimental trial. The two PCV2 isolates that we used shared 98.9% of their nucleic acid sequence. Microscopic evaluation of tissues and comparison of amount of PCV2 antigen and nucleic acids by IHC and quantitative PCR indicated significant differences in incidence and severity of microscopic lesions and amount of PCV2 between the 2 isolates tested (Opriessnig et al., 2005a). This was the first evidence that highly homologous PCV2-isolates may differ significantly in virulence. This knowledge helps explain at least in part the differences in clinical manifestation of PCV2 in the field and has stimulated great interest in further characterization of PCV2 isolates. Based on ORF2 sequencing, our isolates are less than 95% homologous to recent eastern Canadian isolates. Future studies need to focus on comparing these possibly more virulent isolates to our moderate and low virulent PCV2 isolates. The virulence regions in PCV2 have to be determined to allow further testing including sequencing to distinguish virulent from non-virulent isolates. Meerts et al. (2005b) found that type I and type II IFNs did not influence PCV1 replication in PK-15 cells whereas both cytokines increased PCV2-replication *in vitro*. One possible next approach would be to measure if our two PCV2 isolates differ in their interaction with IFNs *in vitro* and also *in vivo*. The low virulent PCV2 isolate that we used in our study was isolated from a field case coinfected with several pathogens and a reduced interaction with IFN would explain the limited expression of PCV2 in this case.

Overall, the information that there are differences in virulence among PCV2 isolates will impact the industry in that producers need to understand the risk of introducing new
PCV2 strains into a herd and that appropriate biosecurity protocols have to be established. Since most herds are already infected with PCV2 it will also be important to understand if infection with a resident strain is likely to confer immunity against a different stains. If so, concern about introduction of a different PCV2 strain is reduced and also commercial PCV2 vaccines are more likely to be cross protective. If not, vaccine manufacturers may need to explore development of multi-valent PCV2 vaccines and producers and veterinarians will need to develop appropriate biosecurity and testing to minimize the risk of introducing new strains of PCV2 to pig farms. Differences in virulence of PCV2 isolated may also impact international trade of pigs and pork products in that countries less affected by PCV2-associated diseases such as Australia may chose to close their trading borders to countries that have documented severe PCV2-associated disease outbreaks such as Europe and Canada.

Many people still question the role of PCV2 in disease. However, there is plenty of evidence in favor of an important role for PCV2 in pig diseases. High amounts of PCV2 antigen are usually correlated with increased severity of microscopic lesions. The hallmark microscopic lymphoid lesions associated with PCV2 have been reproduced in specific-pathogen-free pigs infected with a pure infectious PCV2 DNA clone (Fenaux et al., 2002). Finally, several research groups have succeeded in reproducing clinical disease in pigs experimentally infected with only PCV2 (Bolin et al., 2001, Harms et al., 2001, Okuda et al., 2003, Ladekjær Mikkelsen et al., 2002, Opriessnig et al., 2006a). Trends of PCV2-associated diseases submitted to diagnostic laboratories show that PCV2-associated diseases are increasing and most likely will continue to gain importance in the future (Halbur et al., 2005).

This work has provided substantial new knowledge that will better enable researchers and veterinary practitioners to understand the pathogenesis and epidemiology of PCV2-associated diseases and make important decisions on the most effective intervention strategies. We showed that PCV2-free pigs can be derived from positive breeding herds. We also showed that vaccination with commonly used bacterins can enhance PCV2-associated lesions but that this effect can be reduced by optimal timing of vaccination. We found indication of reduced vaccine efficacy when pigs are in the acute stages of PCV2 infection at the time they were vaccinated with a MLV PRRSV vaccine. We showed that PCV2 and PPV
coinfection results in clinical PMWS in a conventional pig model. Similarly, we showed that PCV2 and *M. hyopneumoniae* coinfection results in clinical PMWS in a conventional pig model. We found evidence that a certain line of Landrace pigs are more susceptible to develop PCV2-associated disease compared to Duroc and Large White pigs. Finally, we documented evidence for differences in virulence between PCV2 isolates. These new discoveries have impacted pork producers and shaped the direction of future research on PCV2-associated diseases.
APPENDIX A. CARDIOVASCULAR LESIONS IN PIGS NATURALLY OR EXPERIMENTALLY INFECTED WITH PORCINE CIRCOVIRUS TYPE 2

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Abstract
Abundant intracytoplasmic porcine circovirus type 2 (PCV2) was associated with myocardocyte swelling or necrosis, or myocardial fibrosis (or both) in three naturally-infected pigs aged 4-7 weeks from three different farms. One 6 week old pig from a fourth farm had severe diffuse segmental to circumferential lymphohistiocytic and plasmacytic periarteritis and endarteritis in several organs, PCV2 antigen was demonstrated in endothelial cells, and inflammatory cells in the arterial walls. In three pigs experimentally infected with PCV2, viral antigen was also associated with obliterated blood vessels in areas of granulomatous and necrotizing lymphadenitis. Together these findings suggest that the cardiovascular system in general and endothelial cells in particular play an important role in the pathogenesis of PCV2-associated diseases.

Summary of the Short Communication
Porcine circovirus type 2 (PCV2) infection of growing pigs is characterized by depletion of lymphoid follicles and histiocytic-to-granulomatous inflammation of varying degrees in lymphoid tissues and certain organs (Sorden, 2000). If the lymphoid lesions are severe and accompanied by high amounts of intralesional PCV2 antigen or nucleic acids a diagnosis of
PCV2-associated postweaning multisystemic wasting syndrome (PMWS) (Sorden, 2000) can be confirmed. The pathogenesis of PCV2 infection and the major cell types that support PCV2 replication are poorly understood. High amounts of PCV2 antigen or nucleic acids are often detected in the cytoplasm of macrophages and dendritic cells by immunohistochemistry (IHC) or in-situ-hybridization (ISH). To a lesser extent, PCV2 antigen is also found in epithelial cells in lungs and kidneys, in smooth muscle cells, and in endothelial cells in several tissues in pigs experimentally infected with PCV2 (Kennedy et al., 2000) as well as in pigs with naturally occurring PCV2-associated PMWS (McNeilly et al., 1999; Rosell et al., 1999). This report describes field and experimental cases in which PCV2 was associated with severe cardiovascular lesions in growing pigs.

Pig 1. A previously healthy pig aged 4 weeks was found dead and submitted to the laboratory. On initial examination nutritional myocardiopathy was suspected. Histopathological changes included severe myocardial fibrosis and mixed leucocytic infiltration throughout the myocardium (Fig. 1a), mild multifocal chronic suppurative and histiocytic pericarditis, mild suppurative and lymphohistiocytic interstitial nephritis and hepatitis, and mild suppurative and lymphohistiocytic interstitial pneumonia. Routine immunohistochemistry (IHC) for porcine reproductive and respiratory syndrome virus (PRRSV), as described by Halbur et al. (1995), gave negative results on sections of heart and lung tissue. Similar IHC results were given by examination of these tissues with a pseudorabies virus (PRV) monoclonal antibody (VMRD, Pullman, WA, USA), diluted 1 in 1000, overnight at 4°C, with protease pretreatment. Abundant PCV2 antigen was detected by IHC with a polyclonal antibody (Sorden et al., 1999) in the cytoplasm and nuclei of myocardiocytes, within vascular endothelial cells in the myocardium and in areas of myocardial fibrosis (Fig. 1b). IHC for porcine parvovirus (PPV), as described by Opriessnig et al. (2004), revealed strong labelling within inflammatory cells in areas of inflammation in the myocardium (Fig. 1c). IHC as carried out by Haines et al. (1992) gave negative results for bovine virus diarrhoea virus (BVDV) in the heart.

Although there was no bacterial growth in culture, the lesions in this pig were consistent with chronic bacterial septicaemia and with PCV2- and PPV-associated myocarditis and
heart failure. Experimentally, PPV has been shown to enhance progression of PCV2-infection towards clinical PMWS (Allan et al., 1999; Opriessnig et al., 2004); however, this pig had no PCV2-associated lymphoid lesions and died from heart failure rather than from PMWS. PCV2-induced myocarditis is typically observed in fetuses and newborn piglets (West et al., 1999). PPV has sporadically been associated with myocarditis in growing pigs (Bolt et al., 1997).

**Pigs 2 and 3.** Both animals came from herds with a history of previously healthy animals being found dead. Pigs 2 (aged 5 weeks) and 3 (aged 7 weeks) came from herds of 1100 and 4000, respectively. Both animals had severe transmural cardiac haemorrhages and the lungs were inflamed and oedematous, and nutritional myocardiopathy was initially suspected. In addition, pig 2 had fibrin tags adherent to the liver, and pig 3 had an enlarged spleen and pulmonary consolidation (bilateral, cranioventral, tan coloured) affecting 5-10% of the lung tissue. Microscopical examination revealed severe hepatic and pulmonary congestion and severe multifocal myocardial haemorrhage with swelling and necrosis of myofibers (Fig. 2a) in both pigs. No bacteria were cultured from the tissues. IHC gave negative results for PRRSV and PRV but strongly positive results for PCV2 in the hearts of both animals. PCV2 antigen was detected within the cytoplasm of myocardiocytes and within vascular endothelial cells in the myocardium (Fig. 2b). Abundant PCV2 antigen also was demonstrated within Kupffer cells and in lesser amounts within hepatocytes in the liver. IHC for PPV and BVDV gave negative results on tissues from both pigs.

Liver and heart vitamin E concentrations (Stahr, 1991) were both 2.1 ppm (reference range 1-3 ppm) in pig 2; and in this animal the selenium concentrations were 580 ppb and 240 ppb (reference range 400-1200 ppb) in the liver and heart, respectively. Pig 3 had 2.6 ppm vitamin E and 410 ppb selenium in the liver. From a retrospective study of 77 pigs with nutritional myocardiopathy, Pallarés et al. (2002) concluded that vitamin E concentrations of less than 2 ppm were deficient; all the pigs had adequate concentrations of liver selenium. In the present study, the vitamin E and selenium concentrations in the liver of pigs 2 and 3 were within the normal range.

As with pig 1, pigs 2 and 3 showed abundant PCV2-antigen associated with myocardial lesions but no evidence of PRRSV- or PRV-associated myocarditis. The possibility that
PCV2 may have predisposed the pigs to heart failure should be borne in mind. Alternatively, PCV2 might have been an opportunistic invader of damaged myocardial cells. An increased incidence of heart failure in association with PCV2 has been reported by Marco (2002), who observed mild myocarditis with and without myofibrilar degeneration. In considering differential diagnosis it is relevant that pigs 2 and 3, like pig 1, came from an area free of foot-and-mouth disease. The infectious porcine myocarditis syndrome recently recognized in Australia (McOrist et al., 2004) is thought to be due to a virus, as yet undefined but known not to be PCV2.

**Pig 4.** A 6-week-old pig was submitted from a herd with a history of an increased incidence of sudden death in fast-growing pigs and an increased incidence of respiratory disease. Microscopical examination revealed small arterioles with diffuse, segmental to circumferential, moderate to severe, lymphohistiocytic and plasmacytic periarteritis and endarteritis, with segmental panarteritis and multifocal apoptosis of cells of the tunica intima and media. The affected arterioles were observed in the myocardium (Fig. 3a), meninges, lungs, kidneys (medulla and pelvis) and intestinal mesentery. Also observed were: lymphohistiocytic cuffing around small vessels in the grey matter of the brain; mild lymphoid depletion and histiocytic replacement of lymphoid follicles in the lymph nodes, tonsil and spleen; mild diffuse lymphohistiocytic interstitial pneumonia; and mild multifocal lymphohistiocytic hepatitis. *Haemophilus parasuis* was isolated from the lungs. Abundant PCV2 antigen was detected by IHC in the cytoplasm and occasionally in the nuclei of endothelial-like cells and smooth muscle-like cells (Fig. 3b) and to a lesser extent in infiltrating inflammatory cells in the walls of arterioles. In the liver, PCV2 antigen was demonstrated in the cytoplasm and nuclei of the majority of endothelial cells lining sinusoids, and occasionally in hepatocytes. PCV2 antigen was also demonstrated in the cytoplasm of myocardiocytes. Lymphoid tissues were negative for PCV2 antigen. Severe polyarteritis, such as that observed in pig 4, is not a frequent observation in pigs submitted to diagnostic laboratories. Differential diagnosis should include PRRSV, African swine fever, classical swine fever, and BVDV infection in pigs (Terpstra and Wensvoort, 1988; Paton and Done, 1994). Neither African nor classical swine fever is present in the US, and IHC for PRRSV and BVDV was negative.
Pigs 5-7. Two 14-week-old pigs (nos. 5 and 6) (Opriessnig et al., 2005) and one 13-week-old pig (no. 7) from two different PCV2 inoculation experiments had generalized lymphadenopathy at 42 days post-inoculation. There was multifocal accumulation of high numbers of macrophages and multinucleated giant cells and multifocal to coalescing necrotic areas with central mineralization (Fig. 4a) in the lymph nodes. PCV2 antigen was demonstrated in the outer zone of the necrotic areas by IHC (Fig. 4b). The necrotic areas were positive by IHC for (1) smooth muscle actin by incubation for 30 min at room temperature with a monoclonal anti-human antibody (BioGenex, San Ramon, CA, USA) diluted 1 in 200 (Fig. 4c), and (2) endothelial cell marker, after pretreatment with protease, by incubation overnight at 4°C with an anti-Factor VIII-related antigen polyclonal antibody (Zymed® Laboratories, South San Francisco, CA, USA) diluted 1 in 20 (Fig. 4d). Masson’s trichrome staining (Sheehan and Hrapchak, 1980) demonstrated that many of the foci of necrosis had an outer layer of collagen. These findings suggest that the necrotic areas were associated with damaged vessels.

Routine bacterial cultures on tissues of these pigs were negative. Similarly, Ziehl-Nielsen, periodic acid-Schiff (PAS), and Brown and Hopp’s gram stain (Sheehan and Hrapchak, 1980) gave negative results. Serum was negative for PRRSV and PPV specific antibodies by enzyme-linked immunosorbent assay (ELISA) (HerdChek® PRRS virus antibody test kit; IDEXX Laboratories, Westbrook, MA, USA) and haemagglutination inhibition (HI) assay (Mengeling et al., 1988), respectively.

Rodríguez-Arrioja et al. (1999) and Segalés et al. (2004) described necrotizing lesions in lymph nodes of pigs with naturally occurring PMWS with and without concurrent PRV infection. Pigs 5-7 were all negative for antibodies to PRV and the lymphoid tissues were negative for PRV antigen. Pigs with septicemic salmonellosis also may have necrotizing lymphadenitis, but such infection was excluded by culture. The pigs had seroconverted by ELISA (Nawagtitgul et al., 2002) to PCV2 with sample-to-positive (S:P) ratios >1.0 (“cut-off” <0.20) in the two older pigs and an S:P ratio of 0.24 in the younger pig. A recent case-control study on pigs with porcine dermatitis and nephropathy syndrome (PDNS) found a relation between PDNS and high amounts of PCV2-specific antibodies, consistent with a hypersensitivity type III-like reaction (Wellenberg et al., 2004). It is possible that primary
PCV2-associated damage occurred in endothelial cells of the central lymph follicle vessel which, due to altered cytokine and chemokine signalling, initiated an inflammatory response that subsequently interfered with homing of lymphocytes, resulting in the pattern of lymphoid depletion and granulomatous lymphadenitis observed in these pigs.

This report describes distinctive PCV2-associated lesions in growing pigs naturally or experimentally infected with PCV2. The infection was associated with heart failure due to acute necrotizing or chronic fibrosing myocarditis, and with chronic vasculitis in heart, kidney and lymphoid tissues. Together these findings suggest that the cardiovascular system in general and endothelial cells in particular play an important role in the pathogenesis of PCV2-associated diseases.

References


Kennedy, S., Moffett, D., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S. and Allan, G. M. (2000). Reproduction of lesions of postweaning multisystemic wasting syndrome by infection of conventional pigs with porcine circovirus type 2 alone or in combination


Fig. 1 a-c. Section of ventricle (myocardium) from pig 1. (a) There is mild-to-moderate, multifocal fibrosis and necrosis and moderate lymphohistiocytic inflammation in the heart. HE. Bar, 100 µm. (b) PCV2-antigen positive cells include fibroblast-like cells (arrow) and inflammatory cells resembling macrophages or lymphocytes. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 40 µm. (c) There is abundant PPV labelling in many macrophage- and lymphocyte-like cells in areas of myocardial fibrosis. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 40 µm.
Fig. 2 a, b. Section of ventricle (myocardium) from pig 2. (a) There is myocardial haemorrhage and acute necrosis of myocardiocytes. HE. Bar, 130 µm. (b) Many myocardiocytes and endothelial-like cells (arrows) have PCV2-antigen within their cytoplasm. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 40 µm.
Fig. 3 a, b. Section of ventricle (myocardium and epicardium) from pig 4. (a) There is moderate lymphohistiocytic and plasmacytic periarteritis and endarteritis with focal apoptosis within the tunica intima and media. HE. Bar, 80 µm. (b) PCV2-antigen positive cells are mainly in the intima and media. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 80 µm.
Fig. 4 a-d. Section of lymph node from pig 5, aged 14 weeks, experimentally infected with PCV2 42 days previously. (a) There is focal necrosis of a lymph node follicle with central mineralization surrounded by granulomatous inflammation and fibrosis. HE. Bar, 150 µm. (b) In the area adjacent to the necrotic lymph node follicle there are many PCV2-positive cells (arrows). IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 150 µm. (c) There is abundant labeling of smooth muscle actin in the zone around the necrotic lymph node follicle. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 150 µm. (d) There are many Factor VIII-positive cells in the area around the necrotic lymph node follicle. IHC (streptavidin-biotin peroxidase complex method, haematoxylin counterstain). Bar, 150 µm.
REFERENCES CITED


Chang HW, Jeng CR, Liu JJ, Lin TL, Chang CC, Chia MY, Tsai YC, Pang VF: Reduction of porcine reproductive and respiratory syndrome virus (PRRSV) infection


**Cheung AK:** Comparative analysis of the transcriptional patterns of pathogenic and nonpathogenic porcine circoviruses. Virology 310:41-49, 2003a

**Cheung AK:** The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology 313:452-459, 2003b

**Cheung AK:** Transcriptional analysis of porcine circovirus type 2. Virology 305:168-180, 2003c


**Choi C, Chae C:** Distribution of porcine parvovirus in porcine circovirus 2-infected pigs with postweaning multisystemic wasting syndrome as shown by in-situ hybridization. J Comp Pathol. 123:302-305, 2000

**Choi C, Chae C:** Colocalization of porcine reproductive and respiratory syndrome virus and porcine circovirus 2 in porcine dermatitis and nephrology syndrome by double-labeling technique. Vet Pathol. 38:436-441, 2001


**Choi C, Kim J, Kang IJ, Chae C:** Concurrent outbreak of PMWS and PDNS in a herd of pigs in Korea. Vet Rec. 151:484-485, 2002a

**Choi J, Stevenson GW, Kiupel M, Harrach B, Anothayanontha L, Kanitz CL, Mittal SK:** Sequence analysis of old and new strains of porcine circovirus associated with


Darwich L, Pie S, Rovira A, Segales J, Domingo M, Oswald IP, Mateu E: Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. J Gen Virol. 84:2117-2125, 2003b

Darwich L, Segalés J, Domingo M, Mateu E: Changes in CD4(+), CD8(+), CD4(+) CD8(+), and immunoglobulin M-positive peripheral blood mononuclear cells of postweaning multisystemic wasting syndrome-affected pigs and age-matched uninfected wasted and healthy pigs correlate with lesions and porcine circovirus type 2 load in lymphoid tissues. Clin Diagn Lab Immunol. 9:236-242, 2002

De Boisséson C, Béven V, Bigarré L, Thiéry R, Rose N, Eveno E, Madec F, Jestin A:


Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ: A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol. 78:6297-6303, 2004a


Gibbs MJ, Weiller GF: Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc Natl Acad Sci USA. 96:8022-8027, 1999


Harding JCS, Clark EG: Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 5:201-203, 1997


Harvey, RE: The effect of vaccination against Mycoplasma hyopneumoniae on the mortality rate of growing pigs affected with post-weaning multisystemic wasting syndrome. Pig J. 52:201-205, 2003


syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine
circovirus type 2. Vet Microbiol. 106:49-60, 2005

Hattermann K, Roedner C, Schmitt C, Finsterbusch T, Steinfeldt T, Mankertz A:
Infection studies on human cell lines with porcine circovirus type 1 and porcine
circovirus type 2. Xenotransplantation. 11:284-294, 2004

Hines RK, Lukert PD: Porcine circovirus as a cause of congenital tremors in newborn pigs.

Hines RK, Lukert PD: Porcine circovirus: a serological survey of swine in the United
States. Swine Health Prod. 3:71-73, 1995

experimentally inoculated with tissue homogenates from pigs with postweaning

Hoogland MJ, Opriessnig T, Halbur PG: Effects of different adjuvants on porcine

Horner G: Pig circovirus antibodies present in New Zealand pigs. *Surveil Wellington* 18:23,
1991

Huang C, Hung JJ, Wu CY, Chien MS: Multiplex PCR for rapid detection of pseudorabies

Jolie R, Runnels P, McGavin D: Post-weaning multisystemic wasting syndrome in a group
of caesarian derived colostrums deprived pigs. *In: Proc Int Conf Pig Vet Soc.* 16:639,
2000

Johnson CS, Joo HS, Direksin K, Yoon KJ, Choi YK: Experimental in utero inoculation
of late-term swine fetuses with porcine circovirus type 2. *J Vet Diagn Invest.* 14:507-
512, 2002

Jung K, Ha Y, Ha SK, Kim J, Choi C, Park HK, Kim SH, Chae C: Identification of
porcine circovirus type 2 in retrospective cases of pigs naturally infected with porcine

Kamstrup S, Barfoed AM, Frimann TH, Ladekjær-Mikkelsen AS, Bøtner A:
Immunisation against PCV2 structural protein by DNA vaccination of mice. *Vaccine.*
22:1358-1361, 2004


Kim J, Chae C: Expression of monocyte chemoattractant protein-1 but not interleukin-8 in granulomatous lesions in lymph nodes from pigs with naturally occurring postweaning multisystemic wasting syndrome. Vet Pathol. 40:181-186, 2003c


Kim J, Choi C, Han DU, Chae C: Simultaneous detection of porcine circovirus type 2 and porcine parvovirus in pigs with PMWS by multiplex PCR. Vet Rec. 149:304-305, 2001a


Niagro FD, Forsthoefel AN, Lawther RP, Kamalanathan L, Ritchie BW, Latimer KS, Lukert PD: Beak and feather disease virus and porcine circovirus genomes:


Opriessnig T, Janke BH, Halbur PG: Cardiovascular lesions in pigs naturally or experimentally infected with porcine circovirus type 2. J Comp Pathol. 134:105-110, 2006c


Oraveerakul K, Choi CS, Molitor TW: Tissue tropisms of porcine parvovirus in swine.


Quintana J, Balasch M, Segalés J, Calsamiglia M, Rodríguez-Arríoja GM, Plana-Durán J, Domingo M: Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. Vet Res. 33:229-237, 2002


Iberian pigs naturally infected with *Lawsonia intracellularis*. Vet Pathol. 38:343-346, 2001b


Sipos W, Duvineau JC, Pietschmann P, Schilcher F, Hofbauer G, Hartl RT, Schmoll F: Porcine dermatitis and nephropathy syndrome (PDNS) is associated with a
systemic cytokine expression profile indicative of proinflammation and a Th1 bias.
Vet Immunol Immunopathol. 107:303-313, 2005


Sorden SD: Update on porcine circovirus and postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 8:133-136, 2000


Vicente J, Segalés J, Höfle U, Balasch M, Plana-Durán J, Domingo M, Gortázar C:
  Epidemiological study on porcine circovirus type 2 (PCV2) infection in the European
  wild boar (Sus scrofa). Vet Res. 35:243-253, 2004

Vincent IE, Carrasco CP, Guzylack-Piriou L, Herrmann B, McNeilly F, Allan GM,
  Summerfield A, McCullough KC: Subset-dependent modulation of dendritic cell
  activity by circovirus type 2. Immunology. 115:388-398, 2005

Vincent IE, Carrasco CP, Herrmann B, Meehan BM, Allan GM, Summerfield A,
  McCullough KC: Dendritic cells harbor infectious porcine circovirus type 2 in the
  absence of apparent cell modulation or replication of the virus. J Virol. 77:13288-
  13300, 2003

Walker IW, Konoby CA, Jewhurst VA, McNair I, McNeilly F, Meehan BM, Cottrell
  TS, Ellis JA, Allan GM: Development and application of a competitive enzyme-
  linked immunosorbent assay for the detection of serum antibodies to porcine

Waddilove AEJ, Marco E: Assessing serotherapeutic control of PMWS in the field. In:

Wang C, Huang TS, Huang CC, Tu C, Jong MH, Lin SY, Lai SS: Characterization of

  86:281-293, 2002

Wellenberg GJ, Stockhfe-Zurwieden N, Boersma WJ, De Jong MF, Elbers AR: The
  presence of co-infections in pigs with clinical signs of PMWS in The Netherlands: a

Wellenberg GJ, Stockhfe-Zurwieden N, De Jong MF, Boersma WJA, Elbers ARW: Excessive
  porcine circovirus type 2 antibody titres may trigger the development of
  99:203-214, 2004b

Wen L, Guo X, Yang H: Genotyping of porcine circovirus type 2 from a variety of clinical
  conditions in China. Vet Microbiol. 110:141-146, 2005


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