RESEARCH REPORT
SWINE HEALTH

Title: Understanding if porcine circovirus type 2 strain differences explain the recent Canadian outbreak – **NPB #06-067**

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II. Industry Summary:

In 2004, a marked increase in the incidence and severity of porcine circovirus associated disease (PCVAD) was observed in eastern Canada. The severe outbreaks of PCVAD in Canada, followed by similar outbreaks in North Carolina and the Midwest United States raised concerns over introduction of a new and more virulent PCV2 variant into North America. Several research groups found the PCV2"b" cluster, previously not recognized in North America, to be associated with the majority of the recent severe PCVAD outbreaks. The first objective of this study was to compare the virulence of recent PCV2b isolates with well-characterized U.S. PCV2a isolates in the conventional specific pathogen free (SPF) pig model. The second objective of this study was to determine if infection with PCV2a isolates induces protective immunity against a recent PCV2b isolate. One-hundred and thirteen conventional SPF pigs were randomly assigned to treatment groups and rooms: pigs inoculated with PCV2a cluster isolates (ISU-40895 or ISU-4838), pigs inoculated with PCV2b cluster isolates (NC-16845 or Can-17639), and un-inoculated pigs. Necropsies were performed at 16 or 51 days post inoculation (p.i.). There were no significant differences in PCV2-associated lymphoid lesions between PCV2a and PCV2b clusters; however within the same cluster significant differences were found between isolates: ISU-4838 and Can-17639 inoculated pigs had significantly ($P < 0.05$) less severe lesions compared to ISU-40895 and NC-16845 inoculated pigs. To evaluate cross-protection, six pigs within each group were challenged on 35 days p.i. with an isolate from the heterologous cluster and were necropsied 51 days p.i. The severity of PCV2-associated lesions was reduced in pigs with prior exposure to an isolate from the heterologous cluster in comparison to singularly inoculated pigs. Results indicate that the virulence of PCV2a and PCV2b isolates is not different in the conventional SPF pig model; however, the virulence of isolates within the same cluster differs. Increased virulence as reported in the field associated with PCV2b isolates was not observed under the conditions of this study. Moreover, cross-protection between PCV2a and PCV2b exists.

In summary, convincing evidence that the recently identified PCV2b isolates are more virulent than PCV2a isolates that have been circulating in the pig population is lacking and thus the introduction of a new more virulent strain of PCV2 does not fully explain the re-emergence of severe PCVAD in North America in 2004. Another possible explanation for the recent devastating PCVAD outbreaks in North American could be the presence of more virulent known (i.e. PRRSV) or unknown concurrent infection that specifically enhances replication of PCV2 and thereby increases disease severity.

III. Scientific Abstract

Porcine circovirus type 2 (PCV2) is divided into two genetic clusters designated as PCV2a and PCV2b. The objectives of this study were to determine if isolates from different clusters vary in virulence and to determine if infection with PCV2a isolates induces protective immunity against subsequent infection with a recent PCV2b isolate. One-hundred and thirteen conventional SPF pigs were randomly assigned to treatment groups and rooms: pigs inoculated with PCV2a cluster isolates (ISU-40895 or ISU-4838), pigs inoculated with PCV2b cluster isolates (NC-16845 or Can-17639), and un-inoculated pigs. Necropsies were performed at 16 or 51 days post inoculation (p.i.). There were no significant differences in PCV2-associated lymphoid lesions between PCV2a and PCV2b clusters; however within the same cluster significant differences were found between isolates: ISU-4838 and Can-17639 inoculated pigs had significantly ($P < 0.05$) less severe lesions compared to ISU-40895 and NC-16845 inoculated pigs. To evaluate cross-protection, six pigs within each group were challenged on 35 days p.i. with an isolate from the heterologous cluster and were necropsied 51 days p.i. The severity of PCV2-associated lesions was reduced in pigs with prior exposure to an isolate from the heterologous cluster in comparison to singularly inoculated pigs. Results indicate that the virulence of PCV2a and PCV2b isolates is not different in the conventional SPF pig model; however, the virulence of isolates within the same cluster differs. Increased virulence as reported in the field associated with PCV2b isolates was not observed under the conditions of this study. Moreover, cross-protection between PCV2a and PCV2b exists.

IV. Introduction

Porcine circovirus (PCV) is a small, circular, non-enveloped, single-stranded DNA virus (Tischer *et al.*, 1982) that belongs to the genus *Circovirus* of the family *Circoviridae* (Todd *et al.*, 2005*)*. To date, two types of PCV have been recognized (Allan *et al.*, 1998; Hamel *et al.*, 1998; Morozov *et al.*, 1998) in pigs: the non-pathogenic PCV type 1 (PCV1) and the pathogenic PCV type 2 (PCV2) which is the etiological agent of porcine circovirus associated disease (PCVAD). Previously, phylogenetic analyses have shown that PCV2 isolates can be further divided into two main clusters (Larochelle *et al.*, 2002; Mankertz *et al.*, 2000; Olvera *et al.*, 2007) now commonly referred to as PCV2a and PCV2b (Gagnon *et al.*, 2007).

Systemic PCV2 infection, which is also known as postweaning multisystemic wasting syndrome (PMWS), is characterized clinically by wasting or decreased weight gain, enlarged lymph nodes, and dyspnea (Harding & Clark, 1997; Opriessnig *et al*., 2007). The hallmark microscopic lesions of systemic PCV2 infection are lymphoid depletion and granulomatous lymphadenitis associated with the presence of PCV2 antigen or nucleic acids (Sorden, 2000). Systemic PCV2 infection or PMWS was initially observed in a Canadian high health status herd in 1991 (Harding & Clark, 1997) and was later recognized worldwide (Allan & Ellis, 2000) and associated with major losses in Europe (Harding, 2004). PCVAD essentially became quiescent in Canada shortly after its initial recognition in the early 1990s except for sporadic case reports. **In 2004, a marked increase in the incidence and severity of PCVAD was observed in eastern Canada (Carman *et al.*, 2006; DeLay *et al.*, 2005). The severe outbreaks of PCVAD in Canada, followed by similar outbreaks in North Carolina and the Midwest United States, have raised concerns over introduction of a new and more virulent PCV2 variant into North America.** Several research groups found the PCV2b cluster, previously not recognized in North America, to be associated with the majority of the recent severe PCVAD outbreaks (Carman *et al*., 2006; Cheung *et al.*, 2007; DeLay *et al*., 2005; Gagnon *et al.*, 2007; Horlen *et al.*, 2007). Similar observations were made in Europe (Dupont *et al*., 2008; Grau-Roma *et al*., 2008).

It is still debatable if differences in virulence between PCV2 clusters and isolates exist. PCV2 has been demonstrated in both severely affected herds and clinically non-affected herds by PCR and ELISA. Interestingly, near identical PCV2 genomes have been found in healthy pigs and in diseased pigs from affected and non-affected herds (de Boisseson *et al.*, 2004; Larochelle *et al.*, 2002). In contrast, a PCV2a field isolate from a case with moderate-to-severe PCV2-associated lesions was compared to a PCV2a isolate from a case with no PCV2-associated lesions and significant differences in expression of lesions were seen in experimentally-inoculated pigs (Opriessnig *et al.*, 2006c). To our knowledge, with the exception of one small study done in germ free pigs (Lager *et al.*, 2007) and one study using conventional PCV2 seropositive pigs (Fort *et al*., 2008), a thorough head-to-head comparison of the virulence of PCV2a and PCV2b isolates has not yet

been done. **The first objective of this study was to compare the virulence of recent PCV2b isolates with well-characterized U.S. PCV2a isolates in the conventional specific pathogen free (SPF) pig model.**

Allan *et al.* (2002) demonstrated that piglets with passively acquired anti-PCV2-antibodies were protected from developing PCVAD after PCV2 challenge. Similarly, Fenaux *et al.* (2004a) demonstrated that pigs vaccinated with PCV2 and challenged with the same strain were protected. McKeown *et al.* (2005) found that protection against PCV2 infection conferred by maternal antibodies was titer-dependent. However, although PCV2 is widespread and essentially all pig herds have anti-PCV2-antibodies (Larochelle *et al.*, 2003; Liu *et al.*, 2002; Mankertz *et al.*, 2000; Walker *et al.*, 2000) in certain geographic regions such as England (Woodbine *et al.*, 2006), Canada (Carman *et al.*, 2006; DeLay *et al.*, 2005), Denmark (Vigre *et al.*, 2005), and the USA (Cheung *et al.*, 2007), PCVAD appears to have spread quickly implying that the antibodies present in the pigs were not protective. Nevertheless, it has been shown that the presence of PCV2-neutralizing antibodies correlates with decreased PCV2 replication and development of clinical PCVAD under experimental conditions (Meerts *et al.*, 2005; 2006) and in the field (Fort *et al.*, 2007). Interestingly, Lefebvre *et al.* (2008) identified differences in affinity and neutralization ability of monoclonal antibodies directed against the PCV2 capsid protein when tested against PCV2 isolates of different genetic or clinical backgrounds. **The second objective of this study was to determine if infection with PCV2a isolates induces protective immunity against a recent PCV2b isolate.**

V. Objectives

1. To compare the virulence of a recent Canadian and a recent North Carolina PCV2 isolate with well characterized U.S. isolates.
2. To determine if infection with a U.S. isolate induces immunity against a recent Canadian isolate.

VI. Materials and Methods

PCV2 isolates. Isolate ISU-40895 (cluster a) (GenBank accession number AF264042) was recovered from an Iowa farm in 1998 (Fenaux *et al.*, 2000) and has been well characterized genetically (Fenaux *et al.*, 2000) and in the conventional SPF pig model (Fenaux *et al.*, 2002; 2003; 2004a; 2004b; Opriessnig *et al.*, 2003; 2004a; 2004b; 2006a; 2006b). Isolate ISU-40895 was found to be capable of inducing characteristic PCV2-associated microscopic lesions and clinical disease under experimental conditions (Opriessnig *et al.*, 2004a; 2004b; 2006a).

Isolate ISU-4838 (cluster a) (GenBank accession number DQ397521) was recovered from a subclinically infected pig on an Iowa farm in 2003 and is of low virulence based on experimental inoculations (Opriessnig *et al.*, 2006c).

Isolate Can-17639 (cluster b) was recovered from a clinically affected pig with systemic PCV2-infection during the PCVAD outbreak in 2006 in Canada. Affected pigs had clinical signs of wasting and coughing, with bronchointerstitial pneumonia and histiocytic lymphadenitis and were found to be negative for swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae*. Abundant PCV2 antigen was found by immunohistochemistry (IHC) in tonsil and lymph nodes.

PCV2-isolate NC-16845 (cluster b) was recovered from a clinically affected 9-week-old pig with systemic PCV2-infection from a group of pigs with a history of severe respiratory disease in 50% of the pigs and approximately 20% mortality in the group. The pigs had severe necrotizing lymphadenitis with lymphoid depletion of follicles, severe bronchointerstitial pneumonia, and were positive for PCV2 (abundant staining) by IHC and for PRRSV by PCR.

Amplification, genetic and phylogenetic analyses of the complete genomic sequence of the PCV2 isolates. PCV2a isolates ISU-40895 and ISU-4838 were sequenced in previous studies (Fenaux *et al.*, 2000; Opriessnig *et al.*, 2006c). To determine the complete genomic sequence of the PCV2b isolates NC-16845 and Can-17639, DNA was extracted from pooled lung and lymph node homogenates according to the protocol of the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). Primers F-PCV2SacII and R-PCV2SacII were used as previously described (Opriessnig *et al.*, 2006c) to amplify the entire genome of both isolates. The PCR products of approximately 1.7 kb length were purified by gel extraction (Qia Quick Gel extraction kit,

Qiagen) and ligated into plasmid pSKII+ (Stratagene, California, USA) after SacII digestion of both plasmid and inserts. The pSKII+/PCV2b constructs were sequenced as described (Opriessnig *et al.*, 2006c). Sequence assembly was carried out using Lasergene6 (DNASTAR Inc.). Sequences were analyzed and the percentage identities were determined using the Clustal alignment program in the MacVector computer software package. Phylogenetic analysis was conducted with the PAUP program using the maximum-parsimony method with 100 bootstrap replicates (David L. Swofford, Smithsonian Institution, Washington, D.C.; distributed by Sinauer Associates, Inc., Sunderland, Mass.). The heuristic search protocol with bootstrap analysis was used to generate a phylogenetic tree.

Generation of infectious DNA clones of PCV2a and PCV2b, and production of the virus inocula. The construction of the infectious DNA clones for the PCV2a isolates ISU-40895 and ISU-4838 has been reported (Fenaux *et al.*, 2002; Opriessnig *et al*., 2006c). The infectious DNA clones of the two PCV2b isolates (NC-16845 and Can-17639) were constructed essentially as previously described (Fenaux *et al.*, 2000; Opriessnig *et al.*, 2006c). The infectivity of all infectious DNA clones was confirmed by transfection of PK-15 cells followed by immunfluorescence detection of virus capsid antigen and the generation of infectious stock of the PCV2 isolates was carried out as described (Fenaux *et al.*, 2002; 2003; 2004a; 2004b; Opriessnig *et al.*, 2006c) with a minor modification. DNA quantification for transfection was carried out using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA). For comparison purpose, the infectious titers of all four viral stocks were adjusted to the same titer, $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml. Each pig received 5ml of the respective PCV2 inoculum intranasally (3 ml) and intramuscularly (2 ml).

Animals and housing. One-hundred-and-thirteen, crossbred, 21-day-old, conventional specific-pathogen-free (SPF) pigs were purchased from a herd that is free of PCV2, PRRSV, and SIV. Blood samples taken from a portion of pigs on the day of arrival and at 11 weeks of age were negative for porcine parvovirus (PPV), PRRSV, H1N1 SIV, H3N2 SIV, and *Mycoplasma hyopneumoniae.* On the day of arrival, the pigs were randomly assigned to one of thirteen rooms (Table 1). Rooms contained 2.5 × 3.6 m raised wire decks and were equipped with one nipple drinker and a self-feeder per pen. All groups were feed *ad libidum* a balanced, pelleted, complete feed ration free of animal proteins and antibiotics (Nature's Made, Heartland Coop, Iowa, USA).

Experimental design. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. The experiment was divided into two parts as summarized in Table 1.

In vivo characterization and virulence comparison of PCV2a and PCV2b isolates. This part of the experiment compared the PCV2a and PCV2b isolates in an established conventional SPF pig model (Opriessnig *et al.*, 2006c). Pigs were inoculated with ISU-40895, ISU-4838, Can-17639, or NC-16845 at 4 or at 9 weeks of age (Table 1). Six pigs per group were necropsied 16 days post inoculation (p.i) and 6-8 pigs per group were necropsied at 51 days p.i. The amount of PCV2 DNA in serum samples, the levels of anti-PCV2-antibodies, and the average scores of the overall PCV2-associated lymphoid lesions were compared at 16 and 51 days p.i.

Evaluation of cross-protection between PCV2a and PCV2b isolates. Pigs were inoculated with ISU-40895, ISU-4838, or NC-16845 at 4 weeks of age followed by challenge with the heterologous cluster at 9 weeks of age (Table 1). All pigs were necropsied 16 days after the challenge (51 days after initial inoculation). The amount of PCV2 DNA in serum samples, the levels of anti-PCV2-antibodies, and the average scores of overall lymphoid lesions in heterologously-challenged pigs were compared to pigs singularly inoculated with either PCV2a (ISU-40895 and ISU-4838) or PCV2b (NC-16845) at 9 weeks of age. Presence and amount of neutralizing PCV2-antibodies in heterologously-challenged pigs were compared to pigs singularly inoculated with either PCV2a or PCV2b at 4 weeks of age.

Serology. Blood samples were collected at the arrival of the pigs to the research facility and weekly thereafter until necropsy. *Anti-PCV2-IgG-antibodies.* The serum samples were tested by an ORF2-PCV2 IgG ELISA as previously described (Nawagitgul *et al.*, 2002). Samples were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater. *Neutralizing PCV2 antibodies.* A fluorescence focus

neutralization assay was done on serum samples collected on 0 (inoculation 1), 35 (inoculation 2), and 49 days p.i. in order to determine the presence of neutralizing antibodies against PCV2 according to the Iowa State University Veterinary Diagnostic Laboratory standard operating protocol (Pogranichnyy *et al*., 2000). The assay was performed twice with PCV2a isolate ISU-98-15237 and with PCV2b isolate ISU-07-6594. *Additional serology.* The serum samples from 1 randomly selected pig in each group taken on arrival at the research facility and at necropsy were tested for the presence of antibodies to PRRSV by PRRSV-ELISA (IDEXX Laboratories, Inc. Westbrook, MA), PPV by hemagglutination inhibition (HI) assay (Mengeling *et al.*, 1988), *Mycoplasma hyopneumoniae* by ELISA (Bereiter *et al.*, 1990), and 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 evaluated daily for clinical signs including wasting, behavioral changes such as lethargy, and inappetence.

PCV2 DNA quantification. DNA-extraction on serum samples collected on 0, 7, 14, 21, 28, 35, 42, and 49 days p.i. was performed using the QIAamp® DNA Mini Kit (Qiagen). DNA-extracts were used for quantification of PCV2 genomic DNA copy numbers by real-time PCR (Opriessnig *et al.*, 2003).

Necropsy. Necropsies were performed on randomly selected pigs on 16 days p.i. and the remainder on 51 days p.i. The total amounts of macroscopic lung lesions were estimated in a blinded fashion as described (Opriessnig *et al.*, 2004b). Sections of lung, 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 in lungs, heart, liver, kidney, brain, ileum and colon were evaluated in a blinded fashion as described (Opriessnig *et al.*, 2004b). 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.*, 2004b).

Immunohistochemistry. IHC for detection of PCV2-specific antigen was performed on formalin-fixed and paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, Peyer's patches, 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.*, 2004b).

Overall lymphoid lesion score. The overall microscopic lymphoid lesions scores which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues were calculated for each pig as previously described (Opriessnig *et al.*, 2004b) and ranged from 0=normal to 9=severe.

Sequencing. PCR products amplified from virus recovered from one randomly selected pig at 49 days p.i. in each inoculation group were sequenced and compared to the respective inoculum. Nested PCR was used to amplify a fragment of the PCV2 genome including the entire ORF2 gene for sequencing and sequence comparison (Opriessnig *et al.*, 2006c). For the PCR reaction, Invitrogen Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, California, USA) was used. The PCR products were run on a 1% agarose gel and the expected 820bp products were excised, purified and sequenced at the Virginia Bioinformatics Institute at Virginia Tech using an Automated DNA Sequencer (Applied Biosystems Inc., Foster City, California, USA). The sequences were analyzed with the MacVector computer program and compared to the sequences of the original virus inocula.

Differential PCR. Serum samples obtained in singular and re-inoculated pigs obtained on 42 and 49 days p.i. were tested for PCV2a and PCV2b by a differential quantitative real-time PCR (Veterinary Diagnostic

Laboratory at Kansas State University). The PCR was performed according to laboratory specific protocols and was able to detect and differentiate between the PCV2a and PCV2b cluster isolates used in this study.

Statistical analysis. Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. Analysis of variance (ANOVA) was used for cross sectional assessment of continuous measures. The rejection level for the null hypothesis was 0.05 followed by pairwise testing using the Tukey Kramer adjustment. In order to summarize and simplify the clinical observations, response feature analysis and a chi-square test was used. Non-repeated measures of necropsy and histopathology data were assessed using non-parametric Kruskal-Wallis one-way ANOVA. If this was significant ($P < 0.05$), then pairwise Wilcoxon tests were used to assess differences between groups. All data were analyzed for clusters (PCV2a versus PCV2b) followed by analysis for isolates. The data were summarized for pigs inoculated at 4 and at 9 weeks of age if the effect of age was non-significant in an initial ANOVA testing.

Nucleotide sequence accession numbers. The complete genomic sequences of PCV2 isolate Can-17639 and PCV2 isolate NC-16845 have been deposited in the GenBank database under accession numbers EU340257 and EU340258.

VII. Results

Genetic characterization of the PCV2a and PCV2b isolates

Comparison of the entire genome sequences of the PCV2a and PCV2b isolates revealed an average identity of 95.7%. The two PCV2b isolates were similar and differed by just two base pairs, resulting in 99.9% nucleotide sequence identity. The capsid proteins of both PCV2b isolates were 100% identical although there was a one base pair difference in the DNA sequence. The replicase genes of the PCV2b isolates had a single amino acid change at position 41 wherein the amino acid asparagine in isolate NC-16845 had mutated to aspartic acid in isolate Can-17639. In addition, there was an average nucleotide sequence identity of 92.2% and amino acid sequence identity of 93% between the PCV2a and PCV2b capsid genes. Alternatively, the replicase genes were less variable with 98.5% amino acid sequence identity. The genome length of the PCV2b isolates was one base pair (1767 bp) shorter than that of the PCV2a isolates (Cheung *et al.*, 2007). Phylogenetic analysis confirmed that isolates Can-17639 (EU340257) and NC-16845 (EU340258) grouped closely with other PCV2b isolates.

Experimental characterization and virulence comparison of PCV2a and PCV2b isolates

Clinical disease and macroscopic lesions. None of the pigs in any of the groups developed clinical disease. Macroscopic lesions were limited to enlarged lymph nodes and non-collapsed, mottled-tan lungs in individual pigs without statistical differences between groups (data not shown).

Effect of age on PCV2-infection. Analysis of the data indicated no effect of age of inoculation on the number of PCV2 DNA copies in serum ($P = 0.238$) or severity of microscopic lymphoid tissue lesions ($P = 0.215$). Therefore, the microscopic lesion and PCR data for the pigs inoculated at 4 and 9 weeks of age were combined during further evaluation.

There was a significant difference between age groups in anti-IgG antibody response: on 14 days p.i. the mean PCV2-IgG-S/P ratio ± standard error in pigs challenged at 9 weeks of age (0.242 ± 0.017) was significantly higher ($P < 0.001$) than in pigs challenged at 4 weeks of age (0.121 ± 0.016). No significant differences between groups inoculated with PCV2a and PCV2b ($P = 0.074$ and $P = 0.925$ for challenge at 4 and 9 weeks of age respectively) or individual isolates were noted.

Anti-PCV2-IgG antibody levels. Pigs in all inoculated groups seroconverted to PCV2 between 14 and 35 days p.i. Pigs in the PCV2a group had a significantly higher antibody response in comparison to PCV2b pigs at 14 and 21 days p.i. ($P = 0.019$ and 0.016 respectively). When analyzed by individual isolates, ISU-40895 had significantly ($P < 0.05$) lower S/P ratios at 14 days p.i. in comparison to ISU-4838. From 28 to 49 days p.i. this difference was reversed but remained significant ($P < 0.05$). Similarly, the anti-PCV2 IgG antibodies in Can-17639 pigs were lower than observed in the NC-16845 pigs on 35 and 49 days p.i. ($P = 0.020$ and 0.028 respectively).

Incidence and amounts of PCV2 DNA in serum samples. At the day of PCV2 inoculation, all pigs were free of PCV2 DNA. The amount of PCV2 DNA was significantly higher for pigs inoculated with PCV2a compared to PCV2b on 7 days p.i. ($P < 0.001$) but not on 14 days p.i. ($P = 0.07$). PCV2 DNA was detected on all days and in all pigs following inoculation with ISU-4838, ISU-40895, and NC-16845; however, the incidence of PCR-positive serum samples was lower in Can-17639 pigs (5/12 pigs on 7 days p.i.; 9/12 pigs on 14 days p.i., 5/6 pigs on 21 days p.i., 6/6 pigs on 28, 35, and 42 days p.i., and 5/6 pigs on 49 days p.i.). The amount of Can-17639 PCV2 DNA was significantly ($P < 0.001$) reduced on 7 (1.7±0.6) and 14 days p.i. (3.6±0.7) compared to all other groups.

Microscopic lesions and incidence of PCV2 antigen in tissues. Microscopic lesions were characterized by varying degrees of depletion of follicles in lymphoid tissues, histiocytic-to-granulomatous lymphadenitis, mild interstitial pneumonia, mild lymphohistiocytic myocarditis, and mild lymphohistiocytic perivascular cuffing in brain tissues. On 16 days p.i., when the data were combined for PCV2a and PCV2b inoculated pigs, there was no significant ($P = 0.716$) difference in overall PCV2-associated lymphoid lesions induced by the two clusters. When evaluated individually, ISU-40895 induced more severe lesions than ISU-4838 ($P = 0.003$) and NC-16845 induced more severe lesions than Can-17639 ($P < 0.001$) (Table 1). Additionally, the pigs inoculated with ISU-40895 and NC-16845 had significantly ($P < 0.05$) more severe lesions compared to the negative control pigs and Can-17639-inoculated pigs but were not different ($P > 0.05$) from each other. In pigs that were necropsied at 51 days p.i., there was mild-to-severe lymphohistiocytic interstitial nephritis present in individual PCV2a- and PCV2b-inoculated pigs. The overall PCV2-associated lymphoid lesions appeared to have resolved in all groups and were not different ($P = 0.533$) between clusters and isolates (Table 1).

Prior exposure to PCV2 induces protective immunity against heterologous cluster challenge

Anti-PCV2-IgG antibody levels. After re-inoculation, the PCV2-antibody levels in PCV2a-b and PCV2b-a inoculated groups were not different ($P > 0.05$) compared to those in single infected pigs. A significant decrease or increase in anti-PCV2-IgG antibodies was not observed in dual-infected groups.

Neutralizing anti-PCV2-antibodies. Prior to inoculation, none of the pigs had neutralizing antibodies against PCV2a or PCV2b (data not shown). There were no differences in anti-PCV2a neutralizing antibody levels between singularly infected cluster PCV2a pigs (1.87±0.1, 2.41±0.2; mean ± std error for 35 and 49 days p.i. respectively) or cluster PCV2b pigs (2.03±0.1, 1.96±0.2) on 35 or 49 days p.i. ($P = 0.48$, 0.24; respectively). Similarly, there were no differences in anti-PCV2b neutralizing antibody levels between singularly infected cluster PCV2a (1.42±0.1, 1.8±0.1) or PCV2b (1.73±0.2, 1.58±0.1) inoculated pigs on 35 or 49 days p.i. ($P = 0.11$, 0.36; respectively). Pigs inoculated at 4 weeks of age with PCV2a or PCV2b and then re-inoculated at 9 weeks with a heterologous isolate did not have a statistically significant increase in anti-PCV2a or anti-PCV2b neutralizing antibody levels between 35 and 49 days p.i. ($P > 0.05$ for all comparisons).

Incidence and amount of PCV2 DNA in serum samples. PCV2 DNA levels in pigs inoculated with PCV2a and then PCV2b or PCV2b and then PCV2a were not different ($P > 0.05$) compared to those in single infected pigs (data not shown).

Sequencing and PCR typing of the PCV2 DNA present in re-inoculated (challenged) pigs. ORF2 sequencing of the DNA obtained from 1 randomly selected pig from each group on 49 day p.i. revealed the recovery of the PCV2 used for initial inoculation 49 days previously in all groups including re-inoculated groups. We were not able to detect the isolate used for re-inoculation/challenge in any of the serum samples analyzed. This finding was further confirmed by PCV2a/PCV2b specific quantitative real-time PCR. A total of 48 serum samples (24 on day 42 and 24 on day 49) were analyzed and the PCV2 cluster present in 47/48 serum samples was identified as the initial cluster which was used to inoculate the pigs 42 and 49 days previously, respectively. A mixed PCV2a and PCV2b population was detected in 1/48 samples on day 42. One week later, on day 49, the cluster used for re-inoculation was no longer detectible in serum from this pig.

Microscopic lesions and incidence of PCV2 antigen in tissues. Pigs inoculated at 4 weeks of age with PCV2a or PCV2b and re-inoculated at 9 weeks of age with the heterologous isolate had normal lymphoid tissues or mild PCV2-associated lesions similar to what is expected in the resolving stages of infection (Table 1). There was a reduction in the PCV2-associated lesions in pigs inoculated at 35 days p.i. with either PCV2a or

PCV2b if they had been exposed to the heterologous isolate 35 days previously and the reduction was significant ($P = 0.023$) for PCV2a-2b versus PCV2b.

Table 1: Group distribution of the 113 pigs, inoculation, and mean group PCV2-associated lymphoid score (group mean ± standard error) at 16 and 51 days post PCV2 inoculation (p.i).

Group		n	Inoculation at 4 weeks of age		Inoculation at 9 weeks of age		Mean group PCV2-associated lymphoid lesion score	
			Isolate	*Cluster*	*Isolate*	*Cluster*	*16 days p.i.*	*51 days p.i.*
Control	1	13	-	-	-	-	0.0±0.02	0.6±0.2
PCV2a	2	14	ISU-40895	PCV2a	-	-	2.5±0.6	1.0±0.3
	3	6	-	-	ISU-40895	PCV2a	1.7±0.9	NA*
	4	12	ISU-4838	PCV2a	-	-	1.0±0.4	0.1±0.1
	5	6	-	-	ISU-4838	PCV2a	0.6±0.5	NA
PCV2b	6	12	Can-17639	PCV2b	-	-	0.1±0.1	0.0±0.0
	7	6	-	-	Can-17639	PCV2b	0.4±0.2	NA
	8	14	NC-16845	PCV2b			3.6±0.5	1.0±0.6
	9	6	-	-	NC-16845	PCV2b	4.0±1.2	NA
PCV2a-b	10	6	ISU-40895	PCV2a	NC-16845	PCV2b	0.8±0.3	NA
	11	6	ISU-4838	PCV2a	NC-16845	PCV2b	0.3±0.1	NA
PCV2b-a	12	6	NC-16845	PCV2b	ISU-40895	PCV2a	0.7±0.4	NA
	13	6	NC-16845	PCV2b	ISU-4838	PCV2a	0.8±0.4	NA

***** Not applicable. Pigs inoculated at 9 weeks of age were all necropsied at 16 days p.i.

VIII. Discussion

The recent increased isolation of PCV2b from severe PCVAD outbreaks in North America led to speculation over introduction of a PCV2 isolate with increased virulence. In 2005, 135 PCV2 isolates from PCVAD cases submitted to a lab in Canada were identified as type 2b compared to only one PCV2b isolate in 2004 (Carman *et al*., 2006). Gagnon *et al.* (2007) investigated 83 Canadian PCV2 cases submitted in 2005 and 2006 and found that 79.5% of the sequences were PCV2b. Twenty-four PCV2 sequences from isolates collected in 6 herds located in Iowa, Kansas, and North Carolina in 2005 were analyzed and 21 of the sequences were determined to be PCV2b (Cheung *et al.*, 2007). Similarly, Horlen *et al.* (2007) further characterized the PCV2 isolates involved in an outbreak of PCVAD in Kansas and found that all PCV2 genomic sequences recovered from affected animals belonged to the PCV2b cluster.

In the current study, cloned isolates from the PCV2a and the PCV2b cluster were compared side-by-side in the conventional SPF pig model. Clones were used instead of cell-culture propagated virus(es) to reduce the risk of inoculation of pigs with an unknown second virus(es) in the cell culture. Since the clone utilized may not necessarily be representative of the virulence of the original viral isolate, two PCV2 clones from each cluster were used.

Similar to our previous study (Opriessnig *et al.*, 2006c), these results confirm differences in virulence among PCV2 isolates. However, significant differences in virulence between isolates from the "old" PCV2a and the "new" PCV2b clusters were not found in our model. Neither the PCV2a isolates nor the recent PCV2b isolates recovered from field cases with high mortality induced clinical disease in singularly-inoculated conventional pigs. This outcome is different from what is usually seen under field conditions typical of

PCVAD. This could be in part explained by the limited stress under experimental conditions (unrestricted access to feed; optimized housing; reduced numbers of pen-mates) and the exposure to PCV2 only as opposed to repeated exposure to multiple pathogens under field conditions.

A previous study using the germ-free pig model and cell culture-derived PCV2a and PCV2b clones found that both, the PCV2a and the PCV2b isolate, induced severe disease in the gnotobiotic pig model (Lager *et al.*, 2007). In that study, 5/8 gnotobiotic pigs inoculated with PCV2 developed clinical PCVAD which is in contrast to our study where clinical disease was not observed. This further highlights the increased disease susceptibility of gnotobiotic pigs compared to conventional pigs; however, due to the small numbers of pigs used in the study by Lager *et al.* (2007) and the limited data presented on microscopic parameters, it could not be definitively concluded that differences in virulence existed between PCV2 clusters.

The anti-PCV2-IgG response was reduced in pigs inoculated with ISU-4838 past 21 days post inoculation and isolate ISU-4838 induced less severe PCV2-associated microscopic lesions compared to isolate ISU-40895. Similar observations were found with the two PCV2b isolates used for the first time in the present study. Pigs inoculated with Can-17639 had significantly reduced PCV2 DNA copies in serum and significantly less severe microscopic lesions compared to NC-16845. These findings are quite interesting as the difference between the two PCV2b isolates was limited to one amino acid change located in the replicase gene which is not routinely used for sequencing in epidemiological investigations. However, when the data were combined for analysis of all PCV2a and PCV2b inoculated pigs, apart from the anti-PCV2-IgG antibody response at 14 and 21 days p.i., there were no significant differences observed on any of the other parameters evaluated (amount of anti-PCV2 neutralizing antibodies, amount of PCV2 DNA in serum, overall lymphoid lesion scores) between the PCV2a and PCV2b groups. The initial higher anti-PCV2-IgG levels in the PCV2a group could be explained by isolate variation rather than a truly better humoral response against cluster PCV2a. We therefore cannot confirm or rule out that there are significant differences in virulence between PCV2a cluster isolates and PCV2b cluster isolates in the conventional pig model.

In the second part of this study we evaluated cross protection between heterologous PCV2 clusters. Pigs were initially inoculated with PCV2a or PCV2b isolates and five weeks later inoculated with the heterologous isolate. Under the conditions of this study, the isolate used for the second challenge was not able to induce active infection or lesions in the pigs as determined by comparison to pigs without prior exposure. It needs to be pointed out that although a significant ($P = 0.046$) difference was observed in mean group lymphoid lesions between PCV2a and PCV2b inoculated pigs, cluster PCV2a included a total of 12 pigs inoculated with ISU-40895 and ISU 4838 whereas cluster PCV2b was represented by only 6 pigs inoculated with NC-16845. Pigs inoculated with Can-17639 at 9 weeks of age were not included in this part of the experiment.

Based on the results of sequencing and differential PCR, we showed that replication of the second challenge isolate was almost 100% blocked in re-inoculated pigs likely due to generation of an effective immune response generated after the first challenge. Neutralizing antibodies appear to be crucial in decreasing PCV2 replication and preventing clinical disease (Meerts *et al.*, 2005; 2006) and the amount of anti-PCV2a and anti-PCV2b neutralizing antibody levels was similar across all groups at the time of second challenge. The ability of PCV2a isolates to cross-protect against infection with PCV2b isolates is further supported by the success of the recently introduced commercial PCV2 vaccines used in the field (Opriessnig *et al.*, 2007; Fort *et al.*, 2008). All commercial vaccines available today are based on the PCV2a cluster and yet they are very successful in reducing morbidity and mortality in herds with PCVAD (Opriessnig *et al.*, 2007) from which PCV2b is now most commonly detected.

It still remains unclear why PCV2b apparently has emerged as the predominant cluster in the field since 2005. Based on serological investigations, the majority of the herds in North America were seropositive prior to the onset of the PCVAD outbreaks in 2005 (Larochelle *et al.*, 2003; Magar *et al.*, 2000; Opriessnig *et al.*, 2004c) and yet, the herds were apparently not protected and subsequently went through severe PCVAD. One

possible explanation for this is that protection induced by maternal antibodies versus active inoculation might be different since there is no cellular immunity afforded through passively-acquired antibodies. However, cellular immunity appears to play a major role in reducing PCV2 viremia and lesions as indicated by early work with PCV2 vaccines where pigs did not develop a detectable humoral immune response to PCV2, yet were protected against subsequent challenge (Fenaux *et al.*, 2004a). Another explanation for the recent devastating PCVAD outbreaks in North American would be the presence of an unknown triggering agent that specifically enhances replication of PCV2b and thereby increases disease severity.

References are available on Request.