Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection

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Porcine circovirus type 2 (PCV2) is divided into two genetic clusters designated PCV2a and PCV2b. The objectives of this study were to determine whether isolates from different clusters vary in virulence and to determine whether infection with PCV2a isolates induces protective immunity against subsequent infection with a recent PCV2b isolate. One-hundred and thirteen conventional specific-pathogen-free (SPF) pigs were assigned randomly 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 uninoculated 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 with ISU-40895- and NC-16845-inoculated pigs. To evaluate cross-protection, six pigs within each group were challenged at 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 with singly 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 to be associated with PCV2b isolates in the field was not observed under the conditions of this study. Moreover, cross-protection between PCV2a and PCV2b exists.

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 aetiological 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 post-weaning multisystemic wasting syndrome (PMWS), is characterized clinically by wasting or decreased weight gain, enlarged lymph nodes and dyspnoea (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 the 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 have been made in Europe (Dupont *et al.*, 2008; Grau-Roma *et al.*, 2008).

It is still debatable whether differences in virulence between PCV2 clusters and isolates exist. PCV2 has been detected in both severely affected herds and clinically non-affected herds by PCR and ELISA. Interestingly, nearly identical PCV2 genomes have been found in healthy pigs and in diseased pigs from affected and unaffected 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 with 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 US 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 titre-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 geographical regions such as the UK (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, 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 with different genetic or clinical backgrounds. The second objective of this study was to determine whether infection with PCV2a isolates induces protective immunity against a recent PCV2b isolate.

METHODS

PCV2 isolates. Isolate ISU-40895 (cluster a; GenBank accession no. 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, b; Opriessnig *et al.*, 2003, 2004a, b, 2006a, b). 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, b, 2006a).

Isolate ISU-4838 (cluster a; GenBank accession no. 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 fram North Carolina 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 from North Carolina with a history of severe respiratory disease in 50 % of the pigs and ~20 % mortality in the group. The pigs had severe necrotizing lymphadenitis with lymphoid depletion of follicles and severe bronchointerstitial pneumonia and were positive for PCV2 (abundant staining) by IHC and for PRRSV by PCR.

Amplification and genetic and phylogenetic analyses of the complete genomic sequence of the PCV2 isolates. PCV2a isolates ISU-40895 and ISU-4838 have been 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). Primers F-PCV2SacII and R-PCV2ScacII were used as described previously (Opriessnig *et al.*, 2006c) to amplify the entire genome of both isolates. The PCR products, ~1.7 kb, were purified by gel extraction (QiaQuick gel extraction kit, Qiagen) and ligated into plasmid pSKII+ (Stratagene) after *Sac*II digestion of both plasmid and inserts. The pSKII+/PCV2b constructs were sequenced as described previously (Opriessnig *et al.*, 2006c). Sequence assembly was carried out using Lasergene6 (DNASTAR). Sequences were analysed and 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, DC; distributed by Sinauer Associates). 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 virus inocula. Construction of infectious DNA clones for the PCV2a isolates ISU-40895 and ISU-4838 has been reported previously (Fenaux *et al.*, 2002; Opriessnig *et al.*, 2006c). The infectious DNA clones of the PCV2b isolates NC-16845 and Can-17639 were constructed essentially as described by Fenaux *et al.* (2000) and Opriessnig *et al.* (2006c). The infectivity of all infectious DNA clones was confirmed by transfection of PK-15 cells followed by immunofluorescence detection of virus capsid antigen and the generation of infectious stock of the PCV2 isolates was carried out as described previously (Fenaux *et al.*, 2002, 2003, 2004a, b; Opriessnig *et al.*, 2006c) with a minor modification. DNA quantification for transfection was carried out using the Nanodrop spectrophotometer (Thermo Fisher Scientific). For comparison, the infectious titres of all four viral stocks were adjusted to the same titre, $10^{4.0}$ 50 % tissue culture infectious dose ($TCID_{50}$) per ml. Each pig received 5 ml of the respective PCV2 inoculum intranasally (3 ml) and intramuscularly (2 ml).

Animals and housing. One-hundred-and-thirteen crossbred, 21-day-old, conventional 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 13 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 fed a balanced, pelleted, complete feed ration free of animal proteins and antibiotics (Nature's Made) ad libitum.

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 9 weeks of age (Table 1). Six pigs per group were necropsied 16 days post-inoculation (p.i) and six to eight 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 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 with those of pigs singly inoculated with either PCV2a (ISU-40895 and ISU-4838) or PCV2b (NC-16845) at 9 weeks of age. The presence and amount of neutralizing PCV2-antibodies in heterologously challenged pigs were compared with those for pigs singly inoculated with either PCV2a or PCV2b at 4 weeks of age.

Serology. Blood samples were collected upon the arrival of the pigs at the research facility and weekly thereafter until necropsy. For anti-PCV2 IgG antibodies, serum samples were tested by an ORF2-PCV2 IgG ELISA as described previously (Nawagitgul *et al.*, 2002). Samples were considered positive if the calculated sample-to-positive ($S:P$)

Table 1. Group distribution of the 113 pigs, inoculation details and PCV2-associated lymphoid lesion scores at 16 and 51 days p.i.

Lymphoid lesion scores are group means ± SEM. All pigs inoculated at 9 weeks of age were necropsied at 16 days p.i.

Group	n	Inoculation at 4 weeks of age		Inoculation at 9 weeks of age		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

NA, Not applicable.

ratio was 0.2 or greater. For neutralizing PCV2 antibodies a fluorescence focus neutralization assay was done on serum samples collected at 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. In addition, serum samples from one 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), PPV by haemagglutination 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 of Iowa State University.

Clinical evaluation. Following PCV2-inoculation, the pigs were evaluated daily for clinical signs including wasting, behavioural changes such as lethargy and inappetence.

PCV2 DNA quantification. DNA extraction from serum samples collected at 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 at 16 days p.i. and on the remainder at 51 days p.i. The total numbers of macroscopic lung lesions were estimated in a blinded fashion as described elsewhere (Opriessnig *et al.*, 2004b). Sections of lung, lymph nodes (superficial inguinal, mediastinal, tracheobronchial and mesenteric), tonsil, thymus, ileum, kidney, colon, spleen and liver were collected at necropsy and fixed in 10 % neutral-buffered formalin and processed routinely for histological examination.

Histopathology. Microscopic lesions in lungs, heart, liver, kidney, brain, ileum and colon were evaluated in a blinded fashion as described previously (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 (> 50 % of the lymphoid follicles containing cells with PCV2 antigen staining) (Opriessnig *et al.*, 2004b).

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

Sequencing. PCR products amplified from virus recovered from one randomly selected pig from each inoculation group at 49 days p.i were sequenced and compared with 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, Invitrogen Platinum PCR SuperMix High Fidelity was used. PCR products were run on a 1 % agarose gel and the expected 820 bp products were excised, purified and sequenced at the Virginia Bioinformatics Institute at Virginia

Tech using an Automated DNA Sequencer (Applied Biosystems). Sequences were analysed with the MacVector computer program and compared with the sequences of the original virus inocula.

Differential PCR. Serum samples obtained from singly inoculated and reinoculated pigs obtained at 42 and 49 days p.i. were tested for PCV2a and PCV2b by 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 ($P<0.05$), pairwise Wilcoxon tests were used to assess differences between groups. All data were analysed 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.

RESULTS

Genetic characterization of PCV2a and PCV2b isolates

Comparison of the entire genome sequences of the PCV2a and PCV2b isolates revealed an 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 protein amino acid sequences of the two PCV2b isolates were 100 % identical, although there was a single base 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 was replaced by 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. The replicase genes were less variable, with 98.5 % amino acid sequence identity. The genome length of the PCV2b isolates (1767 bp) was 1 bp shorter than that of the PCV2a isolates (Cheung *et al.*, 2007). Phylogenetic analysis confirmed that isolates Can-17639 and NC-16845 grouped closely with other PCV2b isolates (Fig. 1).

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 significant differences between groups (data not shown).
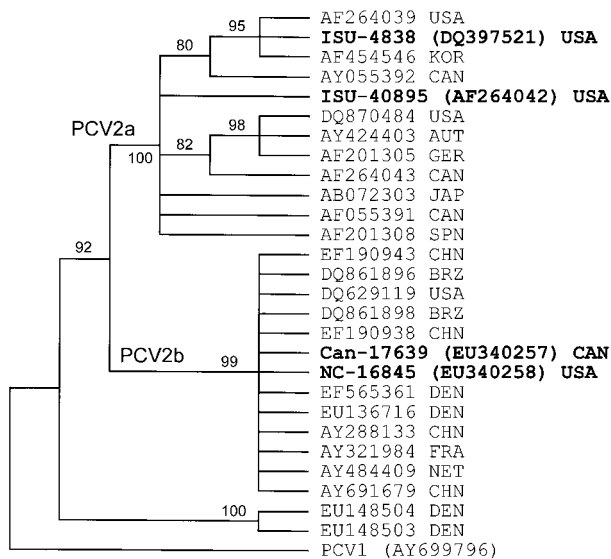
Fig. 1. Phylogenetic analysis of ORF2 genes of PCV2 isolates Can-17639 and NC-16845. A phylogenetic tree was constructed by the heuristic search method with 100 bootstrap replicates (maximum-parsimony algorithm). Bootstrap values (>75) are indicated above branches. The PCV2 isolates used in this study are in bold. A PCV1 isolate was used as the outgroup. Geographical locations of isolation are abbreviated as: AUT, Austria; BRZ, Brazil; CAN, Canada; CHN, China; DEN, Denmark; FRA, France; GER, Germany; JAP, Japan; KOR, Korea; NET, Netherlands; SPN, Spain.

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 the 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 for further evaluation.

There was a significant difference between age groups in anti-IgG antibody response: at 14 days p.i., the mean PCV2 IgG S:P ratio in pigs challenged at 9 weeks of age (0.242 ± 0.017; mean$\pm$SEM) was significantly higher ($P<0.001$) than that 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 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 with PCV2b pigs at 14 and 21 days p.i. ($P=0.019$ and 0.016, respectively) (Fig. 2a). When analysed by individual isolates, ISU-40895 had significantly ($P<0.05$) lower S:P ratios at 14 days p.i. in comparison with ISU-4838. From 28 to 49 days p.i., this

difference was reversed but remained significant ($P<0.05$). Similarly, the levels of anti-PCV2 IgG antibodies in Can-17639-inoculated pigs were lower than those observed in the NC-16845-inoculated pigs at 35 and 49 days p.i. ($P=0.020$ and 0.028, respectively) (Fig. 2b).

Incidence and amounts of PCV2 DNA in serum samples. On 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 with those inoculated with PCV2b at 7 days p.i. ($P<0.001$) but not at 14 days p.i. ($P=0.07$) (Fig. 3a). 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-inoculated pigs (5/12 pigs at 7 days p.i., 9/12 pigs at 14 days p.i., 5/6 pigs at 21 days p.i., 6/6 pigs at 28, 35 and 42 days p.i. and 5/6 pigs at 49 days p.i.). The amount of Can-17639 PCV2 DNA was reduced significantly ($P<0.001$) at 7 (1.7 ± 0.6) and 14 days p.i. (3.6 ± 0.7) compared with all other groups (Fig. 3b).
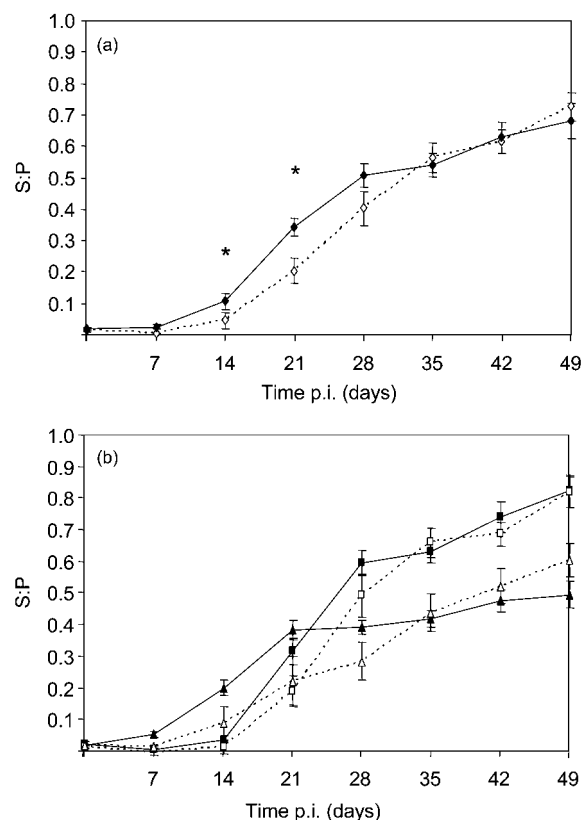

Fig. 2. Mean group anti-PCV2-specific IgG response as determined by ELISA. (a) PCV2a (ISU-4838, ISU-40895) (◆) and PCV2b (Can-17639, NC-16845) (◇) clusters. (b) Individual groups ISU-40895 (■), ISU-4838 (▲), NC-16845 (□) and Can-17639 (△). An S:P ratio equal or greater than 0.2 is considered to be positive. Error bars represent SEM. *, Significant ($P<0.05$) differences between clusters.
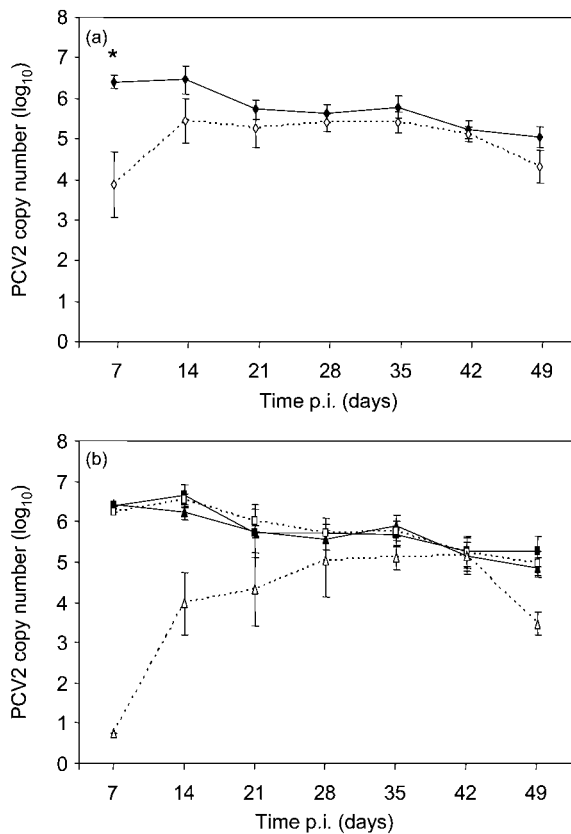
Fig. 3. Mean $\log_{10}$-transformed PCV2 genome copy number. (a) PCV2a (ISU-4838, ISU-40895) (◆) and PCV2b (Can-17639, NC-16845) (◇) clusters. (b) Individual groups ISU-40895 (■), ISU-4838 (▲), NC-16845 (□) and Can-17639 (△). Error bars represent SEM. *, Significant ($P<0.05$) differences between clusters.

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. At 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 with 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 reinoculation, the PCV2-antibody levels in PCV2a–b and PCV2b–a inoculated groups were not different ($P>0.05$) compared with those in singly infected pigs (Fig. 4). 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 pigs singly infected with cluster PCV2a (1.87 ± 0.1, 2.41 ± 0.2; mean$\pm$SEM for 35 and 49 days p.i., respectively) or with cluster PCV2b (2.03 ± 0.1, 1.96 ± 0.2) at 35 or 49 days p.i. ($P=0.48$ and 0.24, respectively). Similarly, there were no differences in anti-PCV2b neutralizing antibody levels between singly infected cluster PCV2a (1.42 ± 0.1, 1.8 ± 0.1) or PCV2b (1.73 ± 0.2, 1.58 ± 0.1) inoculated pigs at 35 or 49 days p.i. ($P=0.11$ and 0.36, respectively). Pigs inoculated at 4 weeks of age with PCV2a or PCV2b and then reinoculated 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).
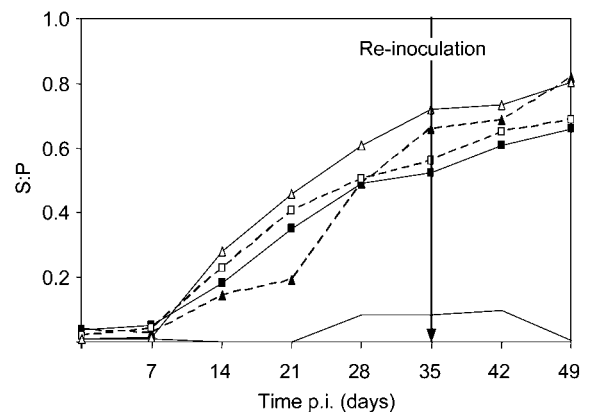

Fig. 4. Mean group anti-PCV2-specific IgG response as determined by ELISA for PCV2a- (ISU-4838 and ISU-40895; $n=12$) (■), PCV2b- (NC-16845; $n=6$) (▲) and dual-inoculated groups PCV2a–b (ISU-4838, ISU-40895, NC-16845; $n=12$) (□) and PCV2b–a (NC-16845, ISU-4838, ISU-40895; $n=12$) (△). Dual-inoculated groups were inoculated initially on day 0 with either PCV2a or PCV2b and were reinoculated with the heterologous strain on day 35 of the experiment. An S : P ratio equal or greater than 0.2 is considered to be positive. The solid line without symbols indicates the control group.

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 with those in single-infected pigs (data
not shown).

**Sequencing and PCR typing of the PCV2 DNA present in
reinoculated (challenged) pigs.** ORF2 sequencing of the
DNA obtained from one randomly selected pig from each
group at 49 days p.i. revealed the recovery of the PCV2 used
for initial inoculation 49 days previously in all groups
including reinoculated groups. We were not able to detect
the isolate used for reinoculation/challenge in any of the
serum samples analysed. 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 analysed and the PCV2 cluster present in 47/48 serum
samples was identified as the initial cluster that was used to
inoculate the pigs 42 and 49 days previously, respectively. A
mixed PCV2a and PCV2b population was detected in one
out of 48 samples on day 42. One week later, on day 49, the
cluster used for reinoculation was no longer detectable 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 reinoculated 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 (Fig. 5), and the reduction was significant
($P = 0.023$) for PCV2a–b versus PCV2b.

DISCUSSION

The recent increase in isolation of PCV2b from severe
PCVAD outbreaks in North America led to speculation
regarding the introduction of a PCV2 isolate with increased
virulence. In 2005, 135 PCV2 isolates from PCVAD cases
submitted to a laboratory in Canada were identified as type
2b compared with 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 from
six herds located in Iowa, Kansas and North Carolina in
2005 were analysed 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
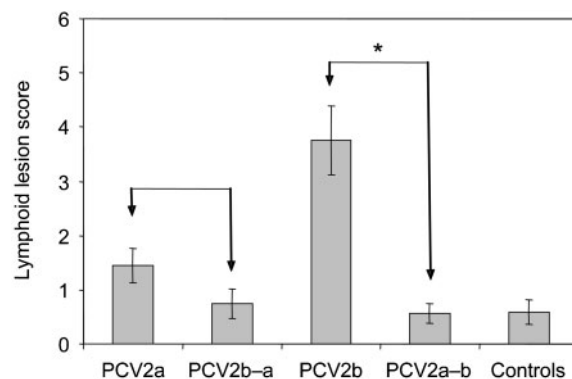PCV2b clusters were compared side by side in the

Fig. 5. Mean group PCV2-associated lymphoid lesions scores for
singly PCV2a- (ISU-4838 and ISU-40895; $n = 12$), PCV2b- (NC-
16845; $n = 6$) inoculated groups and dual-inoculated groups
PCV2a–b (ISU-4838, ISU-40895, NC-16845; $n = 12$) and
PCV2b–a (NC-16845, ISU-4838, ISU-40895; $n = 12$). Error bars
represent SEM. *, Significant ($P < 0.05$) reduction of lesions in the
dual-inoculated group compared with the singly inoculated groups.

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 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.

As in 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 singly inoculated
conventional pigs. This outcome is different from what is
usually seen under field conditions typical of PCVAD. This
could be explained in part by the limited stress under
experimental conditions (unrestricted access to feed, opti-
mized 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 PCV2b isolates induced severe disease in
the gnotobiotic pig model (Lager et al., 2007). In that
study, five out of eight gnotobiotic pigs inoculated with
PCV2 developed clinical PCVAD, which is in contrast to
our study, in which clinical disease was not observed. This
further highlights the increased disease susceptibility of
gnotobiotic pigs compared with conventional pigs; how-
ever, 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 concluded
definitively that differences in virulence existed between
PCV2 clusters.

The anti-PCV2 IgG response was reduced in pigs inoculated with ISU-4838 past 21 days p.i. and isolate ISU-4838 induced less severe PCV2-associated microscopic lesions compared with 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 numbers of PCV2 DNA copies in serum and significantly less severe microscopic lesions compared with 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 used routinely 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., no significant differences were observed for any of the other parameters evaluated (amount of anti-PCV2 neutralizing antibodies, amount of PCV2 DNA in serum and 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 (Fig. 2a) rather than a truly stronger 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 5 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 with pigs without prior exposure (Fig. 5). It needs to be pointed out that, although Fig. 5 shows a significant ($P=0.046$) difference 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 six 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 blocked by almost 100 % in reinoculated pigs, probably through generation of an effective immune response 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 amounts of anti-PCV2a and anti-PCV2b neutralizing antibody were 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 has apparently emerged as the predominant cluster in the field since 2005. Based on serological investigations, the majority of 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 viraemia 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 America would be the presence of an unknown triggering agent that enhances replication of PCV2b specifically and thereby increases disease severity.

ACKNOWLEDGEMENTS

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