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

T. Opriessnig,1 N. E. McKeown,2 E.-M. Zhou,1 X.-J. Meng2 and P. G. Halbur1

Correspondence
T. Opriessnig
tanjaopr@iastate.edu

1Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011-1250, USA
2Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0342, USA

There are marked differences in the 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 assigned randomly to three groups of 14 pigs each. At 7 weeks of age, group 1 pigs were mock-inoculated with saline, group 2 pigs were inoculated with PCV2-4838 (isolated from a pig with no evidence of PCV2-associated lymphoid lesions) and group 3 pigs were inoculated with PCV2-40895 (isolated from a pig with PCV2-associated lymphoid lesions and disease). The PCV2-4838 and PCV2-40895 isolates shared approximately 98–99% nucleotide sequence identity across the entire genome. A total of nine amino acid changes in ORF2 and two amino acid changes in ORF1 were identified between the two isolates. PCV2-4838-inoculated pigs had significantly more genomic copy numbers of PCV2 in their sera at 7 days post-inoculation (p.i.) (P<0.0001) and significantly fewer genomic copy numbers at 14, 21 and 28 days p.i. (P<0.05) compared with pigs inoculated with the PCV2-40895 isolate. Microscopic lesions in lymphoid tissues were significantly less severe (P<0.05) 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 suggest that PCV2 isolates from the USA differ in virulence in an SPF pig model.

INTRODUCTION

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

PMWS is characterized by 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) and 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).

Non-pathogenic PCV1 and 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 1758 to 1760 bp (Fenaux et al., 2000), whereas the genomes of PCV2 isolates range from 1767 to 1768 bp (Fenaux et al., 2000; Mankertz et al., 2000). PCV2 isolates from pigs in different countries share approximately 94–99.0% nucleotide sequence identity (De Boisséson et al., 2004; Fenaux et al., 2000).
The two main viral genes are encoded by open reading frame (ORF) 1 and ORF2 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 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). There are several other potential ORFs with coding capacities of >5 kDa (Meehan et al., 1998). The ORF3 protein has been characterized in vitro and shown not to be essential for PCV2 replication, but is involved in PCV2-induced apoptosis by activating the caspase-8 and caspase-3 pathways (Liu et al., 2005). PCV1 and PCV2 have been found to differ from each other in expression levels of NS- and Rep3c-associated RNAs (Cheung, 2003).

Mahé et al. (2000) identified four dominant immunoreactive epitopes in ORF2 by Pepscan analysis. Laroche et al. (2002) identified three major regions of amino acid heterogeneity among PCV2 isolates and two of these 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 (Laroche 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; Laroche 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, it has recently 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 whether 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 whether there were genetic changes in the PCV2 genome associated with the difference in virulence.

**METHODS**

**PCV2 isolates.** The PMWS-associated isolate PCV2-40895 was recovered from a 40 kg pig that was diagnosed with PMWS in 1998 on a farm in Iowa, USA. The pig had severe lymphoid depletion, moderate histiocytic replacement of follicles in lymphoid tissues and high levels of PCV2 antigen associated with the lesions, as determined by immunohistochemistry (IHC) (Sorden et al., 1999). PCV2-40895 has been well characterized genetically (Fenaux et al., 2000) and in the SPF pig model (Fenaux et al., 2002, 2003, 2004a, b; Oppiernann et al., 2003, 2004a, b, 2006a, b). The non-PMWS-associated isolate PCV2-4838 was recovered from a 45 kg pig that died from a respiratory disease in 2003 on 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 a routine fluorescent antibody 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 PCV2-4838 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 PCV2-4838.** DNA was extracted from pooled lymph node homogenate of pig 4838 using the QIAamp DNA Mini kit (Qiagen) following the manufacturer’s instructions. The complete genomic DNA of PCV2-4838 was amplified by PCR using the PCV2-specific primers F-PCV2SacII and R-PCV2SacII, as described previously (Fenaux et al., 2002), with the following modifications. The extracted DNA was amplified by PCR with Platinum PCR SuperMix High Fidelity (Invitrogen). The PCR consisted of an initial enzyme-activation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 2 min, with a final extension at 68°C for 30 min. The amplified PCR product of the PCV2-4838 genome was excised from the gel, purified and subsequently cloned into the vector pCR2.1 using the original TA Cloning kit (Invitrogen). The TA plasmid containing PCV2-4838 was subsequently sequenced using M13 forward and reverse primers, as well as several PCV2-specific primers including AORF2 (5′-GCCCTGAAATCTTCCATATGAAATATTATT-3′), BORF2 (5′-CCGAGTGGCTGTAAATCTTAC-3′), UP (5′-GTGAACCGCTCTTTGGATACGTCC-3′) and LP (5′-GCCGAAAAAGTGACAGTCC-3′). Sequences were assembled by using AssemblyLIGN (Accelrys). Percentage nucleotide and amino acid sequence identity among PCV2 isolates 4838 and 40895 was determined by using the MacVector computer program (Accelrys).

Isolate PCV2-40895 from a pig with PMWS has been sequenced in a previous study (Fenaux et al., 2000).

**Production of virus inocula.** The PCV2-4838 virus genome cloned in the TA vector was first digested with 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 a GENECLEAN Spin kit (Qbiogene). The concentration of viral genomic DNA was determined on a 1% agarose gel using the molecular mass ladder HyperLadder I (Bioline USA). The purified viral genomic DNA was concatenated using T4 DNA ligase (Stratagene) in an overnight ligation reaction at 4°C. To generate an infectious PCV2 stock, approximately 12 μg of the concatenated PCV2 genomic DNA was transfected into each of seven T-25 flasks of 70% confluent PK-15 cells using Lipofectamine Plus reagent (Invitrogen), essentially as described previously (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 titre was determined by inoculation of PK-15 cells with serially diluted virus stock, followed by an immunofluorescence assay (IFA) with PCV2-specific antibodies as described by Fenaux et al. (2002). The infectivity titre for PCV2-4838 virus stock was 0.5 x 10^6.5 TCID50 ml^-1.

For PCV2-40895, 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 concatenated and subsequently transfected into each of 15 T-25 flasks of 70% confluent PK-15 cells using Lipofectamine Plus reagent as described previously (Fenaux
et al., 2002). Three days after transfection, the virus stock was harvested and its infectivity titre was determined essentially as described for PCV2-4838 and as described previously (Fenaux et al., 2002). The infectivity titre of PCV2-40895 virus stock was 0.5 × 10^5.8 TCID₅₀ ml⁻¹.

**Experimental characterization of PCV2-4838 and PCV2-40895.** Forty-two colostrum-fed, crossbred, SPF pigs were purchased from a breeding 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 2 weeks of age and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, IA, USA. On arrival at the research facility, the majority of the pigs (35/42) had passively acquired PCV2 antibodies, which decayed over the following weeks as determined by a PCV2 ELISA based on the recombinant PCV2 capsid protein for PCV2-specific IgG antibodies (Nawagitgul et al., 2002). At the time of PCV2 inoculation at 7 weeks of age, seven of the 42 pigs still had evidence of low levels of passively acquired antibodies to PCV2. These pigs were assigned to the negative-control group, 1, to ensure that there was no influence on the outcome of experimental infection with PCV2 in groups 2 and 3. The remaining pigs were assigned randomly 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 7 weeks of age, group 1 pigs (n = 14) were mock-inoculated with 1 ml saline intramuscularly and 1 ml saline intranasally and served as the control group. Group 2 pigs (n = 14) were inoculated with PCV2-4838; each pig received 1 ml intramuscularly and 1 ml saline intranasally (~10^5.7 TCID₅₀ per pig). Group 3 pigs (n = 14) were inoculated with PCV2-40895; each pig received 1 ml virus and 0.7 ml saline intramuscularly and 1 ml virus and 0.8 ml saline intranasally (~10^5.7 TCID₅₀ per pig).

**Serology.** Blood samples were collected on arrival of the pigs at the research facility, at 36, 27 and 5 days before PCV2 inoculation and mock inoculation and at 7, 14, 21 and 28 days post-inoculation (p.i.) 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 used the same ORF2 capsid antigen to detect PCV2-specific IgM antibodies. Corrected absorbance values were generated from the absorbance values against recombinant baculovirus carrying the ORF2 gene of PCV2 subtracted from that against wild-type baculovirus, which served as control antigen (Nawagitgul et al., 2002).

Serum samples from three randomly selected pigs in each group taken on arrival and at necropsy were tested for the presence of antibodies to PRRSV by PRRSV-ELISA (IDEXX Laboratories), to Porcine parvovirus (PPV) by haemagglutination-inhibition assay (Mengeling et al., 1988), to M. hyopneumoniae by ELISA as described previously (Bereiter et al., 1990) and to H1N1 SIV and H3N2 SIV by haemagglutination-inhibition assays according to the protocol used at the Veterinary Diagnostic Laboratory at Iowa State University.

**Clinical evaluation.** Pigs were monitored daily and scored for severity of clinical respiratory disease ranging from 0 (normal) to 6 (severe dyspnoea and abdominal breathing) (Halbur et al., 1995). 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 behavioural changes were recorded daily. Pigs were weighed on the day of PCV2 inoculation and at 7, 14, 21 and 28 days p.i.

**PCV2 DNA quantification.** DNA extracts were used for quantification of the copy number of PCV2 genomic DNA by real-time PCR (Opriessnig et al., 2003).

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

Lungs were insufflated with fixative (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, fixed in 10 % neutral-buffered formalin and processed for histological examination.

**Histopathology.** Microscopic lesions were evaluated in a blind fashion. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 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 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2004a).

**IHC.** IHC for the detection of PCV2-specific antigen was performed on formalin-fixed, paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial and mesenteric), tonsil, spleen and thymus using a rabbit polyclonal antiseraum (Sorden et al., 1999). PCV2 antigen scoring was done in a blind fashion and scores ranged from 0 (no signal) to 3 (more than 50 % of the lymphoid follicles contained 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 days p.i. in each inoculation group were sequenced and compared with the respective inoculum. A nested PCR was used to amplify the entire ORF2 gene for sequencing and sequence comparison. The PCR for a 50 μl reaction (first and second rounds) contained 1 × Taq buffer (Promega), 2 mM MgCl₂ (Promega), 0.2 mM dNTPs, 0.20 μM primer (outer or nested pair), 1×5 U Taq polymerase (Promega) and 8 μl extracted DNA template or first-round product. The external primer pair for the first-round PCR was N1ORF2 (5'-GGAACTGTACCTTTTTTG-GCCCG-3') and N2ORF2 (5'-GAGGATTATTCAGCGTGAAC-ACC-3') and the internal primer pair for the nested PCR was AORF2 and BORF2. 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, with a 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, with a 7 min final extension at 72 °C and a 4 °C hold. 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). Sequences were analysed with the MacVector computer program and compared with 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 analysed using analysis of variance (ANOVA). If an ANOVA
was significant \((P<0.05)\), pairwise testing using 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 a \(\chi^2\) test were used. The clinical scores for each pig were reduced to one weekly mean score and the resulting values were subjected to statistical analysis. Daily rectal temperature data were analysed with response feature analysis on mean 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 numbers. The complete genomic sequence of PCV2 isolate 4838 has been deposited in GenBank under accession number DQ397521. The sequence of PCV2-40895 has been reported previously (GenBank accession no. AF264042) (Fenaux et al., 2000).

**RESULTS**

Genetic characterization of PCV2 isolates 4838 and 40895

The complete genomes of both PCV2-4838 and PCV2-40895 were 1768 bp. The nucleotide sequence identity between the two isolates across the entire genome was 98.9\% with a total of 19 nucleotide differences between the two isolates. In ORF1, PCV2-40895 and PCV2-4838 shared approximately 99.5\% nucleotide and 99.4\% amino acid sequence identities. There was a total of five nucleotide differences between PMWS-associated PCV2-40895 and non-PMWS-associated PCV2-4838. Two of the five nucleotide differences were non-silent and resulted in amino acid changes: at position 280, PCV2-4838 had a serine compared with a phenylalanine in PCV2-40895, and at position 302, PCV2-4838 had a proline compared with a serine in PCV2-40895.

The ORF2 capsid gene of PCV2-40895 and PCV2-4838 shared 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 nine amino acid changes between PCV2-4838 and PCV2-40895 (Fig. 1). 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, PCV2-40895 and PCV2-4838 shared 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 ORF3, PCV2-4838 had a phenylalanine, whereas PCV2-40895 had a leucine.

Antibody responses of PCV2-4838 and PCV2-40895 infections in pigs

Both PCV2-infected groups had significantly higher PCV2 IgM-corrected absorbance values compared with negative-control pigs by 14 days p.i. \((P<0.001)\). PCV2-specific IgM antibodies peaked at 21 days p.i. in PCV2-40895-infected pigs (significantly higher than in the PCV2-4838-inoculated pigs, \(P<0.0001\)) and had waned by 28 days p.i. (Fig. 2).

In the PCV2-4838-inoculated pigs, four of 14 pigs had seroconverted by 14 days p.i., two of seven pigs were positive for PCV2-specific IgG at 21 days p.i. and three of seven pigs were positive for PCV2-specific IgG at 28 days p.i. In the PCV2-40895-inoculated pigs, one of 14 pigs, six of 14 pigs, three of seven pigs and four of seven pigs were positive for PCV2-specific IgG at 7, 14, 21 and 28 days p.i., respectively. There were no significant differences in mean anti-PCV2 IgG S:P ratios (Fig. 3).

Comparative pathogenicity of PCV2-4838 and PCV2-40895 in SPF pigs

Clinical disease. There was mild respiratory disease characterized by sporadic sneezing observed in both of the
PCV2-inoculated groups from 5 to 7 days p.i. None of the pigs developed rectal temperatures above 40°C and there was no difference in mean rectal temperatures. The mean daily weight gain was not different between groups.

**PCV2 viraemia length and genomic copy numbers.**

Viraemia was detected by PCR in 12/14, 10/14, five of seven and six of seven pigs inoculated with PCV2-4838 and in six of 14, 13/14, seven of seven and seven of seven pigs inoculated with PCV2-40895 at 7, 14, 21 and 28 days p.i., respectively. The mean numbers of PCV2 genomic copies in the serum are summarized in Fig. 4. At 7 days p.i., pigs inoculated with PCV2-4838 had significantly higher numbers of PCV2 genomic copies in their sera compared with the pigs inoculated with PCV2-40895 (P<0.001). At 14, 21 and 28 days p.i., the number of PCV2 genomic copies in the sera was significantly higher in the pigs inoculated with PCV2-40895 compared with

**Fig. 2.** Mean group IgM antibody response (corrected absorbance values) to PCV2-4838 and PCV2-40895 on the day of inoculation and at 7, 14, 21 and 28 days after PCV2 inoculation. *, Significant difference between groups (P<0.05).

**Fig. 4.** Mean group PCV2 genomic copy numbers obtained from pigs inoculated with PCV2-4838 (△) and from pigs inoculated with PCV2-40895 (■) at 7, 14, 21 and 28 days p.i. Error bars represent SEM. *, Significant difference between groups (P<0.05).

**Macroscopic lesions.** At 14 days p.i., 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 2.7±0.2 for the PCV2-40895-inoculated group, which was significantly different (P<0.01). At 28 days p.i., 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 mean enlargement of the lymph nodes at 28 days p.i. 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 significantly different (P<0.01).

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

**Fig. 3.** Mean group IgG antibody response (sample-to-positive ratios) to PCV2-4838 and PCV2-40895 on the day of inoculation and at 7, 14, 21 and 28 days after PCV2 inoculation. A, Residual passively acquired antibodies in seven of 14 control pigs; *, significant difference between groups (P<0.05).

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Scores ranged from 0 (no lesions) to 3 (severe) for lymphoid depletion (LD) and histiocytic replacement (HR) of lymphoid follicles. Data are presented as incidence (mean group score ± SEM).

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*aSuperficial inguinal, external iliac, mediastinal and mesenteric lymph nodes.
†Different superscripts within columns (a, b, c) represent significantly different mean group scores within columns for each day p.i. (P<0.05).

p.i., there was severe lymphohistiocytic interstitial nephritis associated with PCV2 antigen in one of the PCV2-40895-inoculated pigs.

**Pathological lesions observed in the PCV2-infected pigs are solely attributable to the respective PCV2 inocula**

Several experiments were performed to confirm that the pathological lesions observed in inoculated pigs were indeed induced by the respective PCV2 inocula.

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

**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 until

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<table>
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<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>
<td>14 days p.i.</td>
<td></td>
<td></td>
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<tr>
<td>PCV2-4838 (n=7)</td>
<td>1/7</td>
<td>(0-1 ± 0-1)d†</td>
<td>1/7</td>
<td>(0-3 ± 0-3)a</td>
<td>2/7</td>
<td>(0-4 ± 0-3)a</td>
</tr>
<tr>
<td>PCV2-40895 (n=7)</td>
<td>6/7</td>
<td>(1-2 ± 0-4)b</td>
<td>7/7</td>
<td>(2-1 ± 0-3)b</td>
<td>7/7</td>
<td>(2-4 ± 0-3)b</td>
</tr>
<tr>
<td>Controls (n=7)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
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<tr>
<td>28 days p.i.</td>
<td></td>
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<tr>
<td>PCV2-4838 (n=7)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
<td>1/7</td>
<td>(0-1 ± 0-1)d</td>
<td>1/7</td>
<td>(0-1 ± 0-1)d</td>
</tr>
<tr>
<td>PCV2-40895 (n=7)</td>
<td>1/7</td>
<td>(0-3 ± 0-3)</td>
<td>6/7</td>
<td>(1-4 ± 0-4)b</td>
<td>7/7</td>
<td>(1-9 ± 0-3)b</td>
</tr>
<tr>
<td>Controls (n=7)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
<td>0/7</td>
<td>(0-0 ± 0-0)</td>
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</tbody>
</table>

*aSuperficial inguinal, external iliac, mediastinal and mesenteric lymph nodes.
†Different superscripts within columns (a, b) represent significantly different mean group scores within columns for each day p.i. (P<0.001 for all tissues, except thymus where P<0.05).
termination of the experiment. All negative-control pigs were negative for PCV2 DNA in their serum for the 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 (Allan & Ellis, 2000; Magar et al., 2000; Opriessnig et al., 2004c) and most pigs within herds are exposed to and seroconvert to PCV2 at some time during the growing period (Larochelle et al., 2003). However, many herds report no evidence of PCV2-associated disease, whilst others report high incidence and high mortality (Larochelle et al., 2003). Differences in the virulence of PCV2 isolates could be one logical explanation for this.

Molecular studies to determine the genetic variations of PCV2 have revealed that minor branches of PCV2 are associated with geographical origin rather than with differences in virulence (Fenaux et al., 2000; Hamel et al., 1998; Mankertz et al., 2000; Meehan et al., 2001) and it was concluded that differences in primary nucleotide sequence did not explain the differences in virulence seen in the field (De Boisséson et al., 2004; Grieson et al., 2004; Larochelle et al., 2002, 2003). However, when seven different PCV2 strains were compared in PK-15 cells, it was found that abortion-associated strains (GenBank accession numbers AJ293867 and AJ293868) had different replication kinetics compared with PMWS- or PDNS-associated PCV2 strains (Meerts et al., 2005). 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 acid differences compared with PCV2 isolates associated with PMWS.

The results of the present study indicate that PCV2-4838 and PCV2-40895 differ significantly in several parameters in vivo including serum antibody profiles, the amount of PCV2 in serum and in lymphoid tissues and the severity of PCV2-associated gross and microscopic lesions. PCV2-4838 was less virulent compared with PCV2-40895 in this study. The two PCV2 isolates had an overall nucleotide sequence identity of 98.9%. Within the ORF2 capsid gene, 11 non-silent nucleotide differences, resulting in nine amino acid differences, were identified between these two isolates. Larochelle et al. (2002) identified three immunogenic regions in ORF2 at residues 59–80, 121–136 and 180–191. Seven of the nine amino acid differences between PCV2-4838 and PCV2-40895 were located in these immunogenic regions (Fig. 1). The T134P change in PCV2-4838 was reported in a Canadian isolate recovered from a non-PMWS pig with generalized tremors (CAN-97V19; Larochelle et al., 2002). De Boisséson et al. (2004) compared French PCV2 isolates and determined the most frequent mutations in PCV2 genomes. Five of the nine amino acid differences in ORF2 between the two isolates observed in the present study (R59A, T63R, L185M, K206L and N232K) corresponded to these most frequent mutations (Fig. 1), whereas the two amino acid differences in ORF1, 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 GenBank did not result in any matches at position 302 (data not shown); thus, it appears that this amino acid in PCV2-4838 ORF1 is unique. A chimeric virus created by substitution of the PCV2 ORF1 with that of PCV1 in the genomic backbone of PCV2 has been shown to be attenuated in pigs (Fenaux et al., 2003).

The two PCV2 isolates in this study differed both in virulence and genetically, which was 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 variant in the population rather than the causative PCV2 was sequenced. This emphasizes the importance of in vivo comparison of isolates.

Differences in the virulence of attenuated Chicken anemia virus (CAV), another member of the family Circoviridae, 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 passage in vitro attenuated the virus in vivo (Fenaux et al., 2004b). Neither of the two previously described ORF2 amino acid mutations, P110A and R191S, was found in PCV2-4838 (Fig. 1). 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 those identified during serial passages in vitro. 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 difference in virulence. Further studies are warranted to map finely the critical mutation(s) responsible for virulence.
It is of interest that PCV2-4838 initially had significantly higher PCV2 DNA levels in serum compared with PCV2-40895. Mutations, especially those on the capsid protein, may affect virus capsid assembly, virus replication and binding capability in vivo. Thus, it is not surprising to see some differences in the kinetics and level of viraemia among different isolates. Determination of the threedimensional crystal structure of the PCV2-4838 capsid protein should be carried out and could provide further insight as to whether a particular mutation may affect virus binding or assembly and thus replication and virulence in the host.

It has been demonstrated that PCV2 replication is enhanced and PCV2-associated lesions are more severe in pigs co-infected with pathogens such as PPV (Allan et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000; Opriessnig et al., 2004b), PRRSV (Allan et al., 2000a; Harms et al., 2001; Rovira et al., 2002) and 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 enhance the severity of PCV2-associated disease and lesions. We knew that isolate PCV2-40895 was moderately to highly virulent (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 co-infections and the use of adjuvanted vaccines in the growing pigs. Our reasoning was that the number and combination of other co-factors were such that conditions were optimum for this isolate to have expressed its virulence.

Based on the parameters measured in this study, there were significant differences in virulence between the PCV2-40895 and PCV2-4838 isolates. 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-virulent or of low virulence. To confirm that these low-virulence PCV2 isolates are not a relevant problem would require demonstrating that known co-factors, such as concurrent infections, or the use of immune stimulants, such as adjuvanted vaccines, do not trigger progression of PCV2 infection, as has been demonstrated with the PCV2-40895 isolate (Opriessnig et al., 2004a, b). These low-virulence isolates such as PCV2-4838 should also be inoculated into pigs known to have an 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 that demonstrates a clear difference in virulence between two well-characterized PCV2 isolates in a controlled experimental setting using an SPF pig model. The availability of infectious PCV2 DNA clones and a well-defined pig model will enable scientists to understand further the molecular basis of virulence of PCV2-associated diseases and to develop effective vaccines and improve diagnostic assays.

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